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DEPARTMENT OF PLANT BIOTECHNOLOGY AND BIOINFORMATICS
VIB CENTER FOR PLANT SYSTEMS BIOLOGY

Rewiring the threads of the jasmonate interaction web

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Academic year 2016-2017

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Thesis submitted as partial fulfillment of the requirements for obtaining the degree of Doctor of Philosophy in Science: Biochemistry and Biotechnology

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
4'OMT	4'-O-METHYLTRANSFERASE
ABA	abscisic acid
ABD1	ABSCISIC ACID-HYPERSENSITIVE DAMAGED DNA BINDING PROTEIN1-CULLIN4–ASSOCIATED FACTOR1
ABF	ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTOR
ABI5	ABSCISIC ACID INSENSITIVE5
ABM	antibiotic biosynthesis monooxygenase
AFB2	AUXIN SIGNALING F-BOX PROTEIN2
AHA1	H(+)-ATPase
ALC	ALCATRAZ
ANAC019	NAC DOMAIN CONTAINING PROTEIN19
AOS	ALLENE OXIDE SYNTHASE
AROMA1	ACTIVATIONAL REGULATOR OF METHYL ANTHRANILATE1
ASK1	ARABIDOPSIS SKP1 HOMOLOGUE
AUX/IAA	AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE
AY-WB	Aster Yellows phytoplasma strain Witches' Broom
bHLH	basic helix-loop-helix
BiFC	bimolecular fluorescence complementation
BIS1	BHLH IRIDOID BIOSYNTHESIS1
BOS1	BOTRYTIS SUCEPTIBLE1
BS1	BIG SEEDS1
bZIP	basic leucine zipper
C5-SD	Δ(7)-STEROL-C5(6)-DESATURASE
CAB2	CHLOROPHYLL a/b-BINDING PROTEIN2
CAF1	CARBON CATABOLITE REPRESSOR4 ASSOCIATED FACTOR1
CaMV	cauliflower mosaic virus
CBF	C-REPEAT BINDING FACTOR
CCR4	CARBON CATABOLITE REPRESSOR4
CCT	CONSTANS, CO-like, and TOC1
CDF4	CYCLING DOF FACTOR4
CDK	CYCLIN-DEPENDENT KINASE
CDS	coding sequence
CHX	cycloheximide

CIN	CINCINNATA
CIPK26	CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE26
CKS1	CYCLIN-DEPENDENT KINASE SUBUNIT1
CMID	cryptic MYC2-interacting domain
CMV	cauliflower mosaic virus
CO	CONSTANS
COI1	CORONATINE INSENSITIVE1
COP1	CONSTITUTIVE PHOTOMORPHOGENIC1
COR	coronatine
COR-MO	coronatine-methyloxime
CPC	CAPRICE
CPS2	COPALYL DIPHOSPHATE SYNTHASE2
cRAP	common repository of adventitious proteins
CUL1	CULLIN1
CYS1	CYSTATIN1
DBR2	TRANSARTEMISINIC ALDEHYDE Δ11 (13) REDUCTASE2
DDB1	DAMAGED DNA BINDING PROTEIN1
DE	destruction element
DFL1	DWARF IN LIGHT1
DFR	DIHYDROFLAVONOL 4-REDUCTASE
DMSO	dimethyl sufloxide
DPF	DITERPENOID PHYTOALEXIN FACTOR
DWA1	DAMAGED DNA BINDING PROTEIN1-BINDING WD40 PROTEIN HYPERSENSITIVE TO ABSCISIC ACID1
EAR	ERF-associated amphiphilic repression
EDR1	ENHANCED DISEASE RESISTANCE1
EGL3	ENHANCER OF GL3
EIL1	EIN3-LIKE
EMSA	electrophoretic mobility shift assay
EIN3	ETHYLENE-INSENSITIVE3
EOT1	EXPRESSION OF TERPENOIDS1
ER	endoplasmic reticulum
ERF1	ETHYLENE RESPONSE FACTOR1
ET	ethylene
FAB2	FATTY ACID BIOSYNTHESIS2
FIL	FILAMENTOUS FLOWER
FIP1	FIN219-INTERACTING PROTEIN
FLS2	FLAGELLIN-SENSITIVE2

fLUC	firefly luciferase
FPKM	fragments per kilobase of transcript per million fragments mapped
FRc	continuous far-red
FT	FLOWERING LOCUS T
GA	gibberellic acid
GAME9	GLYCOALKALOID METABOLISM9
GFP	green fluorescent protein
GI	GIGANTEA
GL3	GLABRA3
GLR3.4	GLUTAMATE RECEPTOR3.4
GRX480	GLUTAREDOXIN480
GS	glucosinolate
GS	G/streptavidin-binding peptide
GUS	β-glucuronidase
HA	hemagglutinin
HFR1	LONG HYPOCOTYL IN FAR-RED1
HR	hypersensitive response
HSD	honestly significant difference
HY5	ELONGATED HYPOCOTYL5
IAA	indole-3-acetic acid
ICE1	INDUCER OF CBF EXPRESSION1
IQA	isoquinoline alkaloid
JA	jasmonate
JAM1	JASMONATE-ASSOCIATED MYC2-LIKE1
JAR1	JASMONATE RESISTANT1
Jas	jasmonate-associated
JAV1	JA-ASSOCIATED VQ MOTIF GENE1
JAZ	JASMONATE-ZIM DOMAIN
JPG	$JAZ10_{pro}$-$GUSPlus^{sec}$
JID	JAZ-interacting domain
KEG	KEEP ON GOING
KIX	KINASE-INDUCIBLE DOMAIN INTERACTING
LC	liquid chromatography
LHCA2	PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE2
LHW	LONESOME HIGHWAY
LOX3	LIPOXYGENASE3
LRR	leucine-rich repeat
LSU1	RESPONSE TO LOW SULFUR 1

LUMIER	luminescence-based mammalian interactome mapping
MAX2	MORE AXILLARY BRANCHES2
MED25	MEDIATOR25
MeJA	methyl jasmonate
MIA	monoterpene indole alkaloid
MiSSP7	mycorrhiza-induced small secreted protein7
MS	mass spectrometry
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
NAPPA	nucleic acid programmable protein array
NGAL2	NGATHA-LIKE PROTEIN2
NIG1	NACL-INDUCIBLE GENE1
NINJA	NOVEL INTERACTOR OF JAZ
NOT1	NEGATIVE ON TATA1
NPR1	NONEXPRESSOR OF PR GENES1
ONPG	o-nitrophenyl β-D-galactopyranoside
OPR3	OXOPHYTODIENOATE-REDUCTASE3
ORA59	OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59
ORCA3	OCTADECANOID DERIVATIVE-RESPONSIVE CATHARANTHUS APETALA2-DOMAIN3
ORF	open reading frame
PAL1	PHENYLALANINE AMMONIA LYASE1
PAP1	PRODUCTION OF ANTHOCYANIN PIGMENT1
pdb	protein data bank
PDF1	PROTODERMAL FACTOR1
PDF1.2	PLANT DEFENSIN1.2
PEG	polyethylene glycol
PhyA	PHYTOCHROME A
Phyre2	protein homology/analogy recognition engine version 2.0
PIF4	PHYTOCHROME INTERACTING FACTOR4
PLT	PLETHORA
PMT	PUTRESCINE N-METHYLTRANSFERASE
PP2AA3	PROTEIN PHOSPHATASE 2A SUBUNIT A3
PPD1	PEAPOD1
PUB10	PLANT U-BOX PROTEIN10
PVDF	polyvinylidene fluoride
PYL4	PYRABACTIN RESISTANCE-LIKE4
PYR1	PYRABACTIN RESISTANCE1

QC	quiescent center
qRT-OCR	quantitative realt-time polymerase chain reaction
RAB18	RESPONSIVE TO ABSCISIC ACID18
RAD23A	RADIATION SENSITIVE23A
RBX1	RING-BOX1
Rc	continuous red
RFP	red fluorescent protein
RGL3	RGA-LIKE PROTEIN3
RIN4	RPM1-INTERACTING4
RING	REALLY INTERESTING NEW GENE
rLUC	renilla luciferase
RSS3	RICE SALT SENSITIVE3
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	salicylic acid
SAP	STERILE APETALA
SAR	systemic acquired resistance
SAS	shade avoidance syndrome
SBP	streptavidin-binding peptide
SCF	Skp1/Cullin/F-box
SD	synthetic defined
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFR6	SENSITIVE TO FREEZING6
SGA	steroidal glycoalkaloid
SKP1	S PHASE KINASE-ASSOCIATED PROTEIN1
SLY1	SLEEPY1
SMR	siamese related
smY2H	small molecule yeast two-hybrid
SRDX	SUPERMAN repressive domain X
TAD	trans-activation domain
TAIR	the Arabidopsis information resource
TAP	tandem affinity purification
TAT3	TYROSINE AMINOTRANSFERASE3
TChAP	tandem chromatin affinity purification
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR
TEA	transient expression assay
TEV	tobacco etch virus
TGN	trans-Golgi network

TPS	terpene synthase
TF	transcription factor
TIC	TIME FOR COFFEE
TIR1	TRANSPORT INHIBITOR RESPONSE1
TGA2	TGACG SEQUENCE-SPECIFIC BINDING PROTEIN2
TOE1	TARGET OF EARLY ACTIVATION TAGGED1
TPL	TOPLESS
TPR	TOPLESS-RELATED
TRY	TRIPTYCHON
TSAR1	TRITERPENE SAPONIN BIOSYNTHESIS ACTIVATION REGULATOR1
TSB1	TRYPTOPHAN SYNTHASE BETA-SUBUNIT1
TT8	TRANSPARENT TESTA8
TTG1	TRANSPARENT TESTA GLABRA1
TYLCV	tomato yellow leaf curl virus
UBA2	ubiquitin-associated2
UBC21	UBIQUITIN CONJUGATING ENZYME21
VSP2	VEGETATITVE STORAGE PROTEIN2
VQ33	VQ MOTIF-CONTAINING PROTEIN33
WOX5B	WUSCHEL RELATED HOMEOBOX5B
WT	wild-type
WUS	WUSCHEL
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
YPDA	yeast extract peptone dextrose adenine
ZIM	zinc-finger protein expressed in inflorescence meristem
ZML	ZIM-LIKE

Summary

Plants are sessile organisms often living in a challenging environment that can continuously change. These external impulses trigger a genome-wide transcriptional reprogramming in the plant, controlled by hormones, to adapt to the changed conditions. Jasmonates (JAs) are lipid-derived phytohormones that are involved in many developmental and stress-related responses (**Chapter I.1**). In these processes, specialized metabolites are produced, which can possess interesting properties for potential use in pharmacological or industrial applications. The primary core of JA signalling is conserved among the plant kingdom, in contrast to the species-specific downstream biosynthetic metabolic pathways. Profound knowledge of the molecular mechanism of JA signalling is important if we want to tune the downstream responses, like the production of specialized metabolites in crops or medicinal plants (**Chapter I.2**). MYC transcription factors are the central proteins in the JA core complex and are repressed by a family of 13 JA-ZIM DOMAIN (JAZ) proteins in the model plant *Arabidopsis thaliana*. In triggered conditions, the bioactive conjugate JA-Ile is perceived by a co-receptor complex consisting of JAZ and CORONATINE INSENSITIVE1 (COI1), an F-box protein mediating the ubiquitination and subsequent proteasomal degradation of JAZ, releasing the MYC transcription factors.

In this research, we applied tandem affinity purification (**Chapter II.1**) in combination with yeast two-hybrid assays to further characterize the interactome of the JA core complex. The interaction behaviour of multiple components of this complex was modulated via site-directed mutagenesis. This approach and a forward genetic screen yielded two hyperactive versions of MYC2 that lost interaction with the majority of the JAZ repressors and resulted in an increased downstream response in Arabidopsis (**Chapters II.2 and II.3**). The JAZ proteins contain a C-terminal JA-associated (Jas) domain that mediates interaction with MYC2. The remaining interaction of one hyperactive MYC2 with JAZ1 and JAZ10 could be explained by the presence of an N-terminal cryptic MYC-interacting domain. This domain was observed in only some of the JAZ proteins and demonstrates specificity in the JAZ family. Specificity was also evidenced for JAZ12, which could uniquely interact with the E3 RING ligase, KEEP ON GOING (KEG), conferring stability to JAZ12. A specific motif in the Jas domain, only conserved among JAZ12 orthologues, was essential for this interaction (**Chapter II.4**). Besides the JAZ family, also the PEAPOD (PPD) proteins contain a Jas domain, which was unexplored until now. We showed that the Jas domain of the PPD proteins is important for interaction with transcription factors, such as JA-ASSOCIATED MYC2-LIKE2 (JAM2), a negative regulator of JA signalling (**Chapter II.5**). This led to a new structural model for PPD2, which resembles the molecular mechanism of the JAZ proteins, where PPD2 confers repression activity to JAM2.

Eventually, two protein interaction techniques were developed, a yeast two-hybrid system suitable for small compound-mediated interaction between F-box proteins and their targets (**Chapter II.6**) and tandem affinity purification in hairy roots of the model legume *Medicago truncatula* (**Chapter II.7**).

In conclusion, by rewiring the threads of the JA interaction web, we were able to bring a significant contribution to the understanding of the molecular mechanism of JA signalling. This knowledge, and in particular the hyperactive MYC2 constructs, can be used in the future to modulate JA responses, e.g. for metabolic engineering of medicinal plants.

Samenvatting

Planten zijn sessiele organismen die veelal in moeilijke en voortdurend veranderende omstandigheden leven. Deze externe impulsen induceren een genoomwijde transcriptionele herprogrammering, die gereguleerd wordt door plantenhormonen. Jasmijnzuur en zijn derivaten (JAs) zijn vetzuur-afgeleide plantenhormonen, die belangrijk zijn voor de ontwikkeling en verdediging van de plant (**Hoofdstuk I.1**). Dit gaat gepaard met de productie van gespecialiseerde metabolieten die vaak eigenschappen bezitten die interessant zijn voor de farmaceutische en industriële sector. De primaire JA-signaaltransductie is geconserveerd over het hele plantenrijk, in tegenstelling tot de lagergelegen species-specifieke metabolische takken. Een grondige kennis van het moleculaire mechanisme van de JA-signaalcascade is nodig als we de lagergelegen processen willen moduleren, zoals de productie van gespecialiseerde metabolieten in gewassen of medicinale planten (**Hoofdstuk I.2**). MYC transcriptiefactoren zijn de centrale eiwitten in het JA-eiwitcomplex en worden gerepresseerd door een familie van 13 JA-ZIM DOMAIN (JAZ) eiwitten in de model plant *Arabidopsis thaliana*. In geactiveerde omstandigheden wordt JA-Ile gebonden door een co-receptorcomplex, bestaande uit JAZ en CORONATINE INSENSITIVE1 (COI1). Deze laatste is een F-box eiwit die de ubiquitinatie en de daaropvolgende proteasomale degradatie van JAZ medieert, waardoor de MYC transcriptiefactoren vrijkomen.

In dit onderzoek pasten we tandem affiniteitszuivering (**Hoofdstuk II.1**) toe in combinatie met 'yeast two-hybrid' om het interactoom van het centrale JA-eiwitcomplex verder te karakteriseren. Het interactiegedrag van de verschillende eiwitten van dit complex werd aangepast via gerichte mutagenese. Dit leidde tot de ontwikkeling van twee hyperactieve MYC2 constructen, die interactie met het merendeel van de JAZ repressoren verloren, en hierdoor de lagergelegen responsen in Arabidopsis versterkten (**Hoofdstukken II.2 en II.3**). De JAZ eiwitten bezitten een C-terminaal JA-geassocieerd (Jas) domein die de interactie met MYC2 medieert. De aanhoudende interactie van een hyperactief MYC2 met JAZ1 en JAZ10 werd veroorzaakt door een N-terminaal cryptisch MYC-interagerend domein (CMID) gelijkaardig aan het Jas domein. Dit domein is aanwezig bij slechts enkele JAZ eiwitten en toont de specificiteit aan in de JAZ familie. Specificiteit treedt ook op bij JAZ12, die als enige JAZ eiwit kan interageren met het E3 RING ligase KEEP ON GOING (KEG), resulterend in een verhoogde stabiliteit van JAZ12. Een specifiek motief in het Jas domain, dat geconserveerd is tussen JAZ12 orthologen, was essentieel voor deze interactie (**Hoofdstuk II.4**). Behalve de JAZ familie, zijn er ook nog de PEAPOD (PPD) eiwitten die een Jas domein bezitten, hoewel dit domein tot nog toe niet gekarakteriseerd is. Wij toonden aan dat het Jas domein van de PPD eiwitten belangrijk is voor de interactie met transcriptiefactoren, zoals JA-ASSOCIATED

MYC2-LIKE2 (JAM2), een negatieve regulator van de JA-signaaltransductie (**Hoofdstuk II.5**). Deze resultaten leidden tot een nieuw structureel model voor PPD2, gelijkaardig aan het moleculaire mechanisme van JAZ, waarin PPD2 repressie verleent aan JAM2. Tenslotte hebben we twee eiwitinteractietechnieken ontwikkeld, een 'yeast-two hybrid' systeem geschikt voor kleine moleculen die interactie tussen F-box eiwitten en hun doelwitten mediëren (**Hoofdstuk II.6**) en tandem affiniteitszuivering in 'hairy roots' van de modelplant *Medicago truncatula* (**Hoofdstuk II.7**).

Als besluit kunnen we stellen dat we door karakterisering en herstructurering van het JA-interactieweb een significante bijdrage hebben geleverd aan de kennis inzake het moleculaire mechanisme van de JA-signaaltransductie. Deze kennis, met in het bijzonder de hyperactieve MYC2 constructen, kan in de toekomst gebruikt worden om de JA responsen te moduleren, zoals voor metabolische engineering van medicinale planten.

Scope of research

During their life cycle, plants are subjected to a diverse array of developmental and environmental impulses. When animals are in danger, they have the choice to fight or to flee. As plants are sessile organisms, they do not have the possibility to make this choice and rely completely on their defence strategies. A complex network of interwoven hormone-regulated pathways is the result of this life style. This network takes care of a constantly changing balance of energy consumption, shifting between growth, development and defence. The shifts from growth to development or growth to defence often go hand in hand with the production of specialized metabolites. Jasmonate (JA) is a phytohormone that plays an important role in several developmental and defence-related processes and induces the production of many specialized metabolites. The core JA signalling module arose during colonization of land by plants and is very conserved among land plants. In contrast, the downstream biosynthetic metabolic pathways, and their involved enzymes and products, are more species-specific. Metabolic engineering generally focuses on unravelling the path towards the final production of specialized metabolites, however this can be very cumbersome and time consuming.

Central transcription factors are conserved gates between JA primary signalling and specialized metabolism. These transcription factors steer the expression of other transcription factors and/or enzymes involved in the biosynthesis of bioactive compounds. Modulating the activity of these central transcription factors could allow us to manipulate a whole biosynthetic pathway or multiple downstream pathways. Conservation of JA primary signalling and the involved central transcription factors allows translation of basic research in *Arabidopsis thaliana* to other plants that produce interesting compounds. Our main research objective was to extend the current knowledge of JA-related central transcription factor complexes involved in the production of specialized metabolites.

To achieve this, we embraced two interaction techniques, tandem affinity purification and yeast two-hybrid and launched and contributed to multiple projects:

- Characterization of (potentially) JA-related transcription factor complexes via tandem affinity purification
- Generation of a hyperactive MYC2, a central transcription factor of the JA signalling machinery, by changing its interaction behaviour
- Amplification of JA responses by manipulation of multiple JA core components
- Study of the specific interaction between the JA repressor JA-ZIM DOMAIN12 (JAZ12), and KEEP ON GOING (KEG), a new player in the JA pathway

- Investigation of a potential role in JA signalling for the PEAPOD (PPD) proteins, which share multiple similarities with the JAZ repressors
- Optimization of a yeast two-hybrid system to check small molecule-dependent interactions between (JA-related) F-box proteins and their substrates
- Development of tandem affinity purification in the model legume *Medicago truncatula*

Understanding the regulatory mechanisms of transcription factor complexes, involved in the production of specialized metabolites, can lead to important breakthroughs in metabolic engineering, e.g. by the use of hyperactive transcription factors. Many plant specialized metabolites have properties that are beneficial for plant fitness and can be used in agriculture to optimize crop performance. Often, specialized metabolites are also of direct benefit for humans and can be health-promoting. However, these bioactive compounds are often produced in very low amounts. Worldwide, circa one billion people live in poor circumstances and are stuck to a monotonous diet, which results in a lack of essential nutrients. In addition, an insufficient supply of medicines exists in many countries and/or medicines are extremely expensive. Therefore, it is of general interest to aim for better performing crops or fortified crops with increased nutritional value and medicinal plants with enhanced production of pharmacological molecules.

I

Introduction

Chapter 1

Jasmonates: signal transduction components and their roles in environmental stress responses

Jonas Goossens[1,2], Patricia Fernández-Calvo[1], Fabian Schweizer[1], Alain Goossens

Adapted from:
Jonas Goossens[1], Patricia Fernández-Calvo[1], Fabian Schweizer[1] and Alain Goossens. ***Jasmonates: signal transduction components and their roles in environmental stress responses.*** *Plant Molecular Biology 2016, 91(6):673-689.*

[1]These authors contributed equally to this work

[2]Author Contributions: writing of the manuscript (Abstract, Introduction, The jasmonate receptor complex, Jasmonate signalling proteins are entry points in adaptation to environmental changes, Outlook: from model to field, Figure 2).

ABSTRACT

Jasmonates, oxylipin-type plant hormones, are implicated in diverse aspects of plant growth development and interaction with the environment. Following diverse developmental and environmental cues, jasmonate is produced, conjugated to the amino acid isoleucine and perceived by a co-receptor complex composed of the JASMONATE ZIM-DOMAIN (JAZ) repressor proteins and an E3 ubiquitin ligase complex containing the F-box CORONATINE INSENSITIVE1 (COI1). This event triggers the degradation of the JAZ proteins and the release of numerous transcription factors, including MYC2 and its homologues, which are otherwise bound and inhibited by the JAZ repressors. Here, we will review the role of the COI1, JAZ and MYC2 proteins in the interaction of the plant with its environment, illustrating the significance of jasmonate signalling, and of the proteins involved, for responses to both biotic stresses caused by insects and numerous microbial pathogens and abiotic stresses caused by adverse climatic conditions. It has also become evident that crosstalk with other hormone signals, as well as light and clock signals, plays an important role in the control and fine-tuning of these stress responses. Finally, we will discuss how several pathogens exploit the jasmonate perception and early signalling machinery to decoy the plants defence systems.

INTRODUCTION

Plant fitness and survival largely depend on the ability and plasticity to adapt developmental responses to a 24 h-changing environment (Valladares *et al.*, 2007) by extended and connected signalling networks, integrating the environmental inputs with the endogenous programmes (Thatcher *et al.*, 2009). Hormones are major players in the establishment and interconnection of the plant signalling networks and have traditionally been divided in two groups: growth-related hormones (auxin, gibberellic acid (GA), cytokinins, brassinosteroids and strigolactones) and stress-related hormones (abscisic acid (ABA), salicylic acid (SA), ethylene (ET) and jasmonic acid (JA)) (Bari & Jones, 2009; Depuydt & Hardtke, 2011). However, this classification is clearly artificial and both types of hormones regulate growth- and stress-triggered processes (Heil & Baldwin, 2002). A good example of such a paradox is JA, which has been discovered initially as a wound-related signal and later reported to also regulate several developmental processes (Wasternack & Hause, 2013; Campos *et al.*, 2014). In addition to hormones, other internal or external cues, such as the circadian clock and light, are key players in the plant's adaptation to environmental changes.

During evolution, plants have developed complex signalling networks to activate the most appropriate defence array against their enemies (Huot *et al.*, 2014; Vos *et al.*, 2015). Biotrophic pathogens, such as *Hyaloperonospora arabidopsidis*, require living cells to survive, multiply and spread. To cope with biotrophs, plants have established a sophisticated

immune system, regulated by SA. In the presence of such pathogens, the SA signalling cascade activates the expression of pathogenesis-related proteins followed by a local hypersensitive response (HR) and/or systemic acquired resistance (SAR). The HR leads to programmed cell death, a highly efficient strategy to deprive biotrophs of food sources, and restrict their growth and systemic spread (Jones & Dangl, 2006; Spoel & Dong, 2012; Xin & He, 2013). In contrast, necrotrophic pathogens, such as *Botrytis cinerea* and *Alternaria brassicicola*, kill their host cells and live on dead cells (Glazebrook, 2005; Mengiste, 2012). In this context, relying on an immune system based on programmed cell death would make plants an easy prey for necrotrophic pathogens. Likewise, the plant's survival would be compromised rapidly by insects that frequently change feeding location, if every feeding site would be programmed for cell death. To avoid such critical situations when attacked by necrotrophic pathogens or insects, plants possess additional immune strategies steered by other phytohormones that block the activation of the SA pathway. In general, immunity against herbivores is regulated by JA and to some extent by ABA, whereas immune responses against necrotrophic pathogens are controlled by JA and ET. Because they are mutually antagonistic, the negative interaction between the JA and the SA pathways is often referred to as negative crosstalk (Pieterse *et al.*, 2012). However, in some particular contexts, such as in thermotolerance, JA and SA have been shown to work in concert (Mur *et al.*, 2006; Clarke *et al.*, 2009). Recently, Liu *et al.* (2016) described the remarkable finding that JA can also be a positive regulator of SA-mediated defence against biotrophic pathogens.

JA and its derivatives, collectively called JAs, are lipid-derived compounds synthetized from α-linolenic acid (18:3) in the octadecanoid pathway in plant cells (Wasternack & Hause, 2013). Chemically, JAs are oxylipins with structures that resemble animal prostaglandins involved in inflammatory responses (Hamberg & Gardner, 1992). In 1962, Demole and colleagues reported the existence of methyl JA as a major component of the jasmine essence (Demole *et al.*, 1962). Since this early discovery, JA has been shown to regulate a broad range of stress-related responses and developmental processes in flowering plants (Wasternack & Hause, 2013; Kazan, 2015). During biotic stress JAs, not only positively regulate defence mechanisms against necrotrophic pathogens and insects with different lifestyles, but are also indispensable for successful symbiotic and mutualistic interactions with microbes and fungi (Wasternack & Hause, 2013; Wasternack, 2014). Furthermore, increasing evidence from several studies show a tight connection between JA signalling and developmental processes related to light, circadian signalling and resource allocation (Ballaré, 2014). Ultimately, crosstalk among virtually all known plant hormones increases the complexity of the above-mentioned processes and responses (Wasternack & Hause, 2013; Wasternack, 2014). Here, we present an overview of the role of JA in response to

environmental stresses, including a general update on the JA perception and early signalling modules. In addition, the crosstalk between JA and other hormones and cues from developmental processes or the environment will be discussed. Finally, we highlight some examples on how the JA signalling pathway is directly manipulated by a broad range of microbes.

THE JASMONATE RECEPTOR COMPLEX

The JA signalling pathway is triggered by a conjugate of JA with the amino acid isoleucine (JA-Ile), which is sensed by the co-receptor complex consisting of the F-box protein CORONATINE INSENSITIVE1 (COI1) and a JA ZIM-DOMAIN (JAZ) protein (Fig. 1A; Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007; Fonseca *et al.*, 2009). COI1 is stabilized by assembly into a Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex SCF^{COI1} (Yan *et al.*, 2013; Zhang F *et al.*, 2015). JA-Ile, together with inositol pyrophosphates, act as a molecular 'glue' that brings together the SCF^{COI1} complex and a JAZ protein (Yan *et al.*, 2009; Sheard *et al.*, 2010; Mosblech *et al.*, 2011; Laha *et al.*, 2015; Laha *et al.*, 2016). In *Arabidopsis thaliana*, the JAZ proteins were originally defined to constitute a family of 12 transcriptional repressors, but recently a 13th member has been added (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007; Thireault *et al.*, 2015). The structural signature of JAZ proteins is the C-terminal JA-associated (Jas) domain required for the interaction with COI1 and transcription factors (TFs), and the TIFY motif responsible for dimerization among JAZ proteins and for interaction with the NOVEL INTERACTOR OF JAZ (NINJA) (Katsir *et al.*, 2008; Chini *et al.*, 2009; Chung & Howe, 2009; Pauwels *et al.*, 2010; Pauwels & Goossens, 2011). The ERF-associated amphiphilic repression (EAR) motif-containing NINJA protein recruits the corepressor TOPLESS (TPL) and its related proteins (TPRs) to the JAZ-bound TFs (Pauwels *et al.*, 2010). In the presence of JA-Ile, JAZ are ubiquitinated by the SCF^{COI1} complex, followed by proteasomal degradation, which consequently releases the targeted TFs from repression. The primary function of JAZ proteins is to bind and repress the basic helix-loop-helix (bHLH) TF MYC2, a master regulator of JA signalling. Together with its homologues MYC3 and MYC4, MYC2 controls the activation of the majority of JA responses (Chini *et al.*, 2007; Kazan & Manners, 2009; Fernández-Calvo *et al.*, 2011).

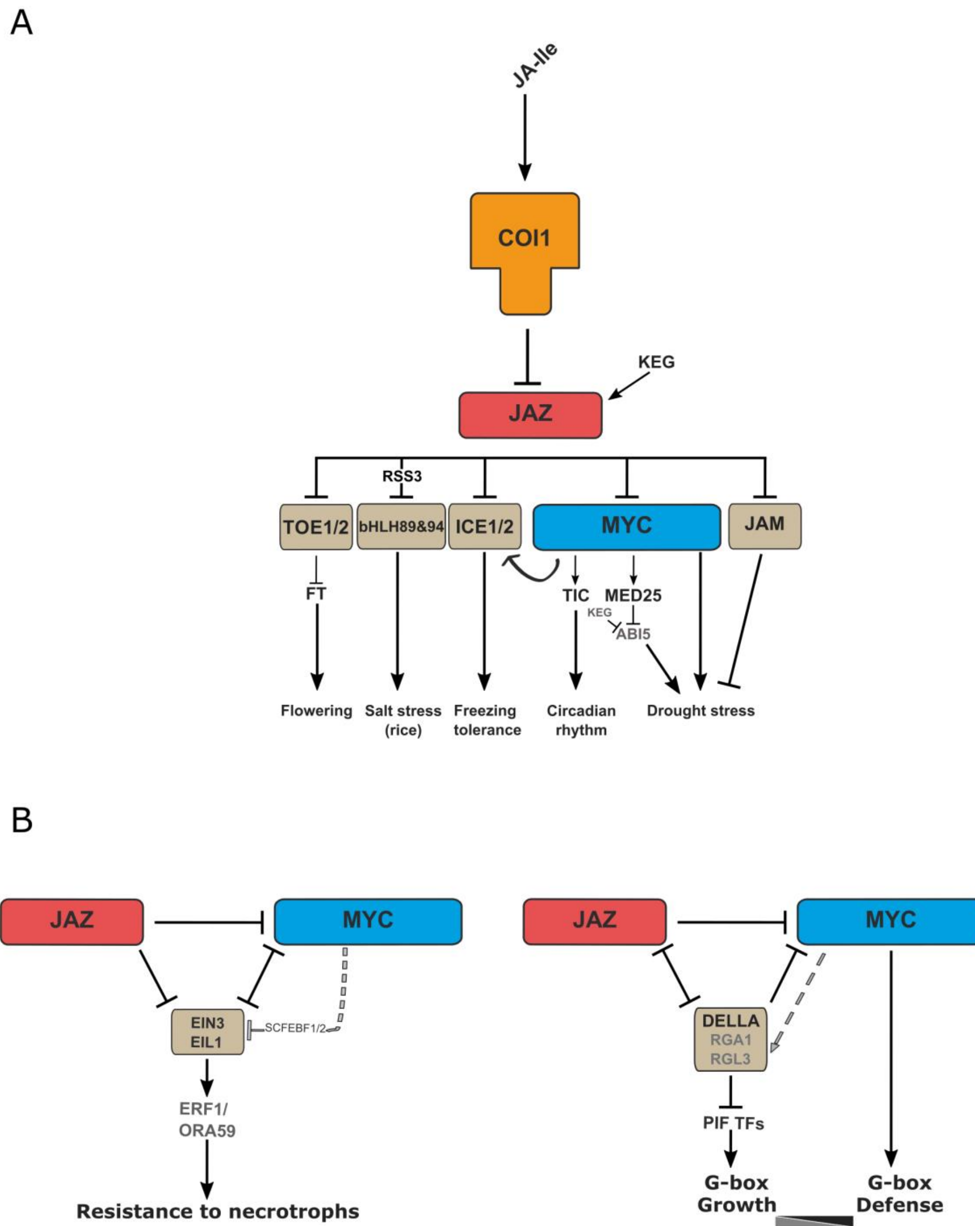

Figure 1: COI1, JAZ repressors and MYC2 are entry points for crosstalk in JA signalling. A) JA-Ile triggers SCF^{COI1}-mediated degradation of JAZ repressors leading to JA-specific responses. JA signalling is linked with abiotic stress, flowering and circadian rhythm by interaction of the JAZ repressors with the following TFs or other factors involved in these processes: TOE TFs in regulation of flowering time; RSS3 to inhibit bHLH TFs involved in salt stress responses in rice; ICE TFs in freezing tolerance; MYC and JAM TFs in drought stress. MYC2 can interact at its turn with TIC to modulate the circadian rhythm, and with MED25 to fine-tune the final outcome of ABA-mediated drought stress responses. KEG is a negative regulator of this drought response and stabilizes JAZ12. B) In the response to necrotrophic pathogens, the central ET TFs EIN3 and EIL1, are subjected to a two-level regulation executed by the JAZ repressors and by MYC2 via protein-protein interaction. MYC2 also induces transcription of $SCF^{EBF1/2}$ leading to degradation of EIN3/EIL1. C) The mechanistic relationship between JA and GA signalling represents the trade-off between growth and defence. JAZ and DELLA repressors interact with each other and have a mutual negative relationship. Furthermore, DELLAs can bind and repress MYC2 that, on its turn, can induce transcription of the DELLA repressor, *RGL3*.

The JAZ proteins were originally assumed to be functionally redundant, however, differential expression patterns and altered JA responses in several loss- and gain-of-function mutants suggest specific functions (Yan *et al.*, 2007; Chung & Howe, 2009; Grunewald *et al.*, 2009; Sehr *et al.*, 2010; Demianski *et al.*, 2012; Yang *et al.*, 2012; de Torres Zabala *et al.*, 2016; Yu *et al.*, 2016). Functional specificity among JAZ proteins is further supported by alternative splicing and modular specificity at the protein level (Pauwels & Goossens, 2011). Examples of the latter are: (1) occurrence of an EAR domain in JAZ5, JAZ6, JAZ7, JAZ8 and JAZ13, allowing direct interaction with TPL even in the absence of the NINJA adaptor (Kagale *et al.*, 2010; Shyu *et al.*, 2012; Thireault *et al.*, 2015); (2) the presence of an N-terminal cryptic MYC2-interacting domain, like in JAZ1 and JAZ10 (Chapter II.2; Moreno *et al.*, 2013; Goossens *et al.*, 2015); and (3) the absence of a canonical TIFY motif in JAZ13 (Thireault *et al.*, 2015). Finally, post-translational modifications, such as phosphorylation of JAZ12 and JAZ13, may add an additional layer of specificity (Durek *et al.*, 2010; Thireault *et al.*, 2015).

In the next sections, we will mainly focus on the central elements of the JA signalling module (COI1, MYC2 and JAZ proteins) and their role in response to abiotic and biotic stresses (Figs. 1 and 2). It should be noted though that numerous other TFs or other regulatory proteins have been found to interact with the JAZ proteins, either through the Jas or other domains, and have been shown to be involved in specific JA-modulated processes (Pauwels & Goossens, 2011; Kazan & Manners, 2012), which will be mentioned here where relevant. Noteworthy, the composition of the core JA receptor and the primary signalling module resembles that of other hormones, such as auxin, and even shares elements like the SCF complex (Cuéllar Pérez & Goossens, 2013). These shared components not only allow crosstalk between JA and other hormonal signalling cascades, but also create additional opportunities for pathogens to molecularly manipulate the plant defence mechanism.

JASMONATE SIGNALLING PROTEINS ARE ENTRY POINTS IN ADAPTATION TO ENVIRONMENTAL CHANGES

COI1

Loss-of-function *coi1* alleles were described for the first time in a screen for plants insensitive to coronatine (COR), a toxin produced by virulent strains of the pathogen *Pseudomonas syringae pv. tomato* DC3000 (Feys *et al.*, 1994; Xie *et al.*, 1998). COR is a 3D-mimic, and thereby an agonist, of JA-Ile that can activate the JA pathway by binding to the COI1-JAZ co-receptor complex. Activation of the JA pathway represses the SA pathway through negative crosstalk, an astute strategy of this *P. syringae* strain to increase its virulence. Accordingly, mutants of *COI1* are resistant to this strain (Xin & He, 2013; see also below). Likewise, increased resistance of *Nicotiana tabacum coi1* mutants to the tobacco mosaic virus can be explained by impeding the JA-mediated downregulation of the SA

pathway (Oka *et al.*, 2013). In contrast, loss of COI1 function has led to compromised resistance to necrotrophic fungi such as *B. cinerea* in Arabidopsis and tomato (Thomma *et al.*, 1998), *Sclerotinia* spp. in Arabidopsis and sunflower (Guo & Stotz, 2007; Talukder *et al.*, 2014), and *Fusarium* spp. in Arabidopsis (Berrocal-Lobo & Molina, 2004; Trusov *et al.*, 2009); to herbivorous insects such as the American serpentine leafminer (Abe *et al.*, 2013), phloem-feeding insects like the whitefly *Bemisia tabaci* B and aphids (*Myzus persicae*, *Brevicoryne brassicae*) (Mewis *et al.*, 2005; Zarate *et al.*, 2007), caterpillars (*Spodoptera littoralis, Pieris rapae*) (Reymond *et al.*, 2004; Bodenhausen & Reymond, 2007), and thrips (*Frankliniella occidentalis*) in Arabidopsis (Abe *et al.*, 2008), caterpillars in rice (Ye *et al.*, 2012), and caterpillars (*Manduca sexta*) in tobacco (Paschold *et al.*, 2007) and even to tortoises (Mafli *et al.*, 2012). Moreover, COI1 regulates resistance to the vascular pathogen *Verticillium longisporum*, but interestingly in a JA-Ile-independent manner (Ralhan *et al.*, 2012).

In addition to its well-reported importance for biotic stress responses, COI1 is involved in multiple developmental processes. Mutants of *coi1* have constrained stamen and pollen development (Xie *et al.*, 1998), are defective in JA-mediated root growth inhibiton (Xie *et al.*, 1998), in leaf senescence (He *et al.*, 2002) and in trichome initiation (Li *et al.*, 2004). COI1 also modulates signal transduction in light-driven developmental processes, for instance in the shade avoidance response (Fig. 2B). Under conditions of competition with neighbours, hence at a low ratio between red (R, perceived from the sunlight) and far-red (FR, reflected by the canopy) light, hypocotyls of *coi1* mutants elongate more than those of wild-type (WT) plants. Moreover, mutants of the FR light receptor-encoding gene *PHYTOCHROME A* (*PhyA*) contain higher levels of the JA precursor *cis*-(+)-12-oxophytodienoic acid (Robson *et al.*, 2010). In addition, light-mediated PhyA degradation has been shown to be dependent on JA (Riemann *et al.*, 2009). In response to low R/FR ratios, PhyA induces the expression of *JA RESISTANT1* (*JAR1*) in a COI1-dependent manner. Expression of *JAR1*, encoding the enzyme conjugating Ile to JA, leads to JA-Ile biosynthesis and to nuclear exclusion of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) during seedling development (Hsieh *et al.*, 2000; Wang *et al.*, 2011; Hsieh & Okamoto, 2014). Furthermore, JAR1 interacts with FIN219-INTERACTING PROTEIN (FIP1) under FR light conditions, leading to increased activity of JAR1 and enhanced JA signalling (Chen *et al.*, 2017). Taken together, these evidences suggest a complex crosstalk between JA and light signalling (Fig. 2B). To date, COI1/PhyA crosstalk was only analysed in a developmental context in seedlings, but given the important role of light signalling in plant immunity (de Wit *et al.*, 2013), it would be interesting to study the interplay between COI1 and PhyA in a stress-related context.

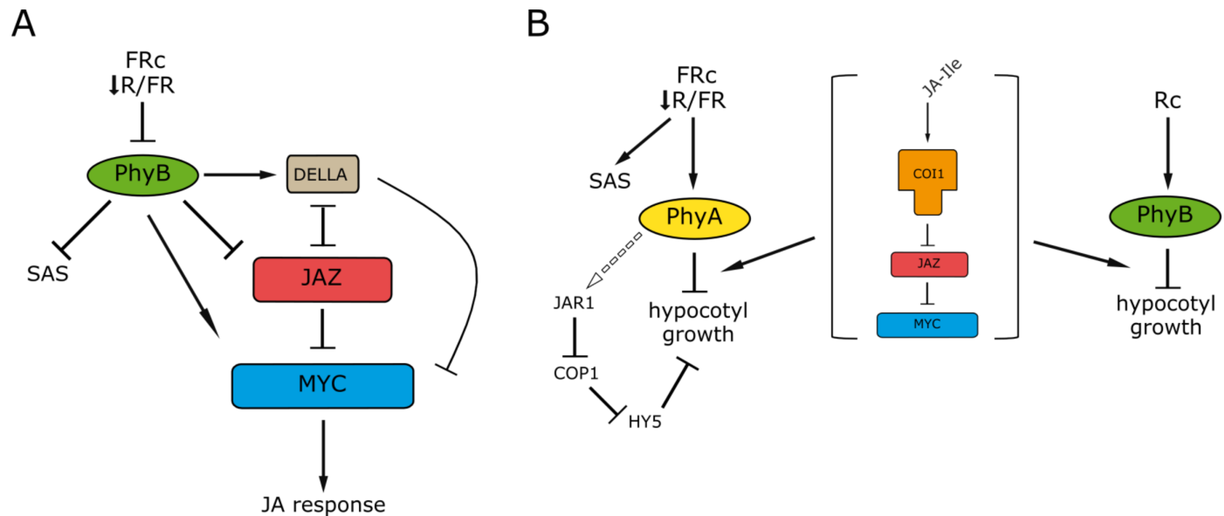

Figure 2: The JA and light signalling machineries are interlinked. A) JA signalling depends on light via PhyB signalling. In low R/FR or continuous FR (FRc) light conditions, FR-mediated photoconversion of PhyB to its inactive form leads to promotion of the shade avoidance syndrome (SAS). Inactivation of PhyB also leads to suppression of the JA response via stabilization of the JAZ proteins and degradation of the MYC TFs. In addition, the DELLA repressors of the GA pathway are less abundant in these conditions, resulting in the release of JAZ proteins to allow inhibition of MYC transcriptional activity, and, hence, inhibition of the JA response. B) PhyA signalling depends on the JA machinery. In low R/FR or FRc light conditions, PhyA signalling leads to suppression of (excessive) SAS. Hypocotyl growth is one of the responses of SAS and PhyA-mediated inhibition of hypocotyl growth depends on the different components of the JA machinery: COI1, JAZ and MYC TFs. Conversely, PhyA is required for the JA-mediated degradation of JAZ and some of the JA responses. Furthermore, PhyA leads to an increased expression of *JAR1*, resulting in the nuclear exclusion of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), which can no longer mediate the degradation of the ELONGATED HYPOCOTYL5 (HY5) protein leading to decreased hypocotyl length. In continuous R (Rc) light, the JA machinery is positively involved in the PhyB-mediated suppression of hypocotyl growth.

Finally, the early flowering phenotype of *coi1* mutants has been known for long time, but has been investigated only recently (Zhai *et al.*, 2015). Levels of FLOWERING LOCUS T (FT) and GIGANTEA (GI), modulators of flowering time (Sawa & Kay, 2011; Romera-Branchat *et al.*, 2014) were altered in *coi1* mutants (Hu *et al.*, 2013; Zhai *et al.*, 2015), indicating that JA negatively regulates flowering time in *Arabidopsis*. The above-mentioned study shed light on the molecular mechanism behind the crosstalk between JA signalling and flowering time. However, the implication of environmental stress responses in this crosstalk needs further investigation.

MYC2

The TF MYC2 was simultaneously identified by several groups in Arabidopsis and tomato (Abe *et al.*, 2003; Boter *et al.*, 2004; Lorenzo *et al.*, 2004). In both species, MYC2 is induced by JA and mechanical wounding in a COI1-dependent manner. In comparison with WT plants, *myc2* mutant plants are partially insensitive to JA-dependent root growth inhibition and, when treated with exogenous JA, *myc2* mutant plants show reduced induction of wounding and insect marker genes, such as *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), but upregulation of pathogen marker genes, such as *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59* (*ORA59*) and *PLANT DEFENSIN1.2* (*PDF1.2*) (Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). Accordingly, *myc2* plants are more resistant to necrotrophic pathogens like *B. cinerea* and *Plestosphaerella cucumerina* (Kazan & Manners, 2013). Furthermore, triple mutant *myc2myc3myc4* plants are defective in JA-triggered defence responses against herbivory by *Spodoptera littoralis* (Fernández-Calvo *et al.*, 2011; Schweizer *et al.*, 2013). These plants show no basal and inducible expression of genes of the toxic glucosinolate (GS) biosynthetic pathway and are almost devoid of GSs whereas *coi1-1* mutants still express basal levels of GS pathway genes and accumulate basal levels of GSs (Fernández-Calvo *et al.*, 2011; Schweizer *et al.*, 2013). MYC2, MYC3 and MYC4 directly interact with the MYB TFs MYB28, MYB29, MYB34, MYB51, MYB76, and MYB121 to form a transcriptional complex that regulates the GS pathway in a concerted manner (Schweizer *et al.*, 2013; Frerigmann *et al.*, 2014).

The MYC2 branch within the JA signalling pathway accounts not only for the activation of defence genes against insects, but also prevents the induction of ET-responsive defence genes against necrotrophic pathogens (through the ETHYLENE RESPONSE FACTOR1 (ERF1)/ORA59 branch; Fig. 1B). Mechanistically, this de-activation of the ERF branch can be explained by a dual role of MYC2, which in the presence of JA triggers the degradation of the ET-INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1) TFs and interferes with the DNA binding of the residual EIN3 protein available in the nucleus (Song *et al.*, 2014; Zhang *et al.*, 2014). Although the effect of ET on insect feeding is not fully understood, several studies have shown that plants mutated in ET signalling are less attractive and thereby more resistant to insects (Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011).

Similar to *coi1* mutants, *myc2myc3myc4* plants are more resistant to the COR-producing bacteria *P. syringae* than WT plants, indicating that the three MYC TFs act as negative regulators of SA signalling (Fernández-Calvo *et al.*, 2011). Indeed, MYC2 itself indirectly controls the JA/SA antagonism favouring the JA pathway. MYC2 induces the transcription of the DELLA protein RGA-LIKE PROTEIN3 (RGL3, one of the five repressors in GA signalling; Fig. 1C), which sequesters JAZ proteins that subsequently become less available to bind and

repress MYC TFs. In this particular scenario, JA signalling branch is potentiated and while the SA branch is attenuated, promoting resistance to necrotrophic pathogens, but increasing susceptibility to biotrophic organisms (Wild *et al.*, 2012). Because the JA/SA antagonism is bidirectional, the SA-mediated suppression of JA signalling has been extensively documented as well (Pieterse *et al.*, 2012). SA suppresses JA signalling, downstream of the COI-JAZ complex and involves two different types of TFs, i.e. TGACG SEQUENCE-SPECIFIC BINDING proteins (TGA2, TGA3, TGA5 and TGA6) and ORA59 (Fig. 1B).

On the one hand, SA recruits GLUTAREDOXIN480 (GRX480) to the proximity of TGA TFs, blocking the expression of JA-induced genes. On the other hand, SA reduces the accumulation of ORA59 and its capacity to promote expression of JA-dependent genes (Zander *et al.*, 2010; Van der Does *et al.*, 2013; Zander *et al.*, 2014). Several members of the WRKY TF family together with the SA master regulator, NONEXPRESSOR OF PR GENES1 (NPR1), have also been reported to play a role in the JA/SA antagonism. Unfortunately, their mechanism of action is still not fully understood (Spoel *et al.*, 2003; Romera-Branchat *et al.*, 2014; Caarls *et al.*, 2015; Gimenez-Ibanez *et al.*, 2015). The type of stress that challenges the plant in a given context will determine the mode of action of JA/SA antagonism, favouring either JA-mediated suppression of SA signalling or conversely, SA-dependent suppression of the JA pathway (Wild *et al.*, 2012). Remarkably, a recent study has shown that insect egg-triggered SA signalling mediates destabilization of the MYC TFs (Schmiesing *et al.*, 2016).

In medicinal plant species, such as *Catharantus roseus* and *Nicotiana* spp., MYC2 orthologues steer the biosynthesis of terpenoid indole alkaloids and nicotine, respectively (Todd *et al.*, 2010; Shoji & Hashimoto, 2011; Zhang *et al.*, 2011; De Geyter *et al.*, 2012; Zhang *et al.*, 2012). Both specialized metabolites function as insect toxins or deterrents to avoid feeding. In Arabidopsis, MYC2 interacts with the DELLA protein REPRESSOR OF GA1 (RGA1) to fine-tune the production of volatile sesquiterpenoids in flowers (Hong *et al.*, 2012), reflecting a possible role in pollinator attraction to ensure plant reproduction. Interestingly, it has also been reported that plants synchronize JA-mediated metabolite production with insect behaviour in a circadian-dependent manner (Goodspeed *et al.*, 2012). Accordingly, the circadian clock controls the expression of *MYC2*. Furthermore, MYC2 itself can interact with the circadian clock component TIME FOR COFFEE (TIC), leading to destabilization of MYC2 and inhibition of JA-dependent gene induction (Shin *et al.*, 2012). Therefore, MYC2 acts as a regulator to coordinate JA responses with the internal plant clock.

The PhyA-PhyE family of R and FR light photoreceptors is involved in the regulation of plant development and immunity (Hsieh & Okamoto, 2014; Moreno & Ballaré, 2014). The ratio between R and FR light in canopies directly affects the allocation of plant resources between growth and defence (Moreno & Ballaré, 2014). Correspondingly, activation of JA responses is

controlled by light quality. Indeed, in low R/FR ratios or monochromatic FR light, MYC2, MYC3 and MYC4 are destabilized in a PhyB-dependent manner (Fig. 2A). Conversely, FR-enriched environments led to a stabilization of JAZ repressors (discussed below). Hence, the low R/FR-triggered regulation of MYC/JAZ protein stability could molecularly and mechanistically explain the repression of JA responses in conditions of shade avoidance (Moreno *et al.*, 2009; Chico *et al.*, 2014).

In addition to numerous *myc2* loss-of-function alleles, gain-of-function mutants of *MYC2* and *MYC3* have been characterized recently. These mutants bear point mutations in the JID that prevents interaction with and therefore repression by JAZ proteins, rendering them hypersensitive to JA and ABA (Chapters II.2 and II.3; Smolen *et al.*, 2002; Frerigmann *et al.*, 2014; Gasperini *et al.*, 2015; Goossens *et al.*, 2015). A functional COI1-MYC2 signalling module is a prerequisite for the perception of the ABA burst leading to downstream signals in response to root colonization by oomycetes and fungi (Anderson *et al.*, 2004; Adie *et al.*, 2007). Similarly to MYC2, the JA-ASSOCIATED MYC2-LIKE1 (JAM1) TF is induced by ABA (Li *et al.*, 2007; Babitha *et al.*, 2013). However, JAM1 and its homologues, JAM2 and JAM3, negatively regulate JA signalling, most probably by interfering with or impeding the binding of MYC2, MYC3 and MYC4 to the G-box elements in JA-modulated promoters of JA-responsive genes (Nakata *et al.*, 2013; Sasaki-Sekimoto *et al.*, 2013; Fonseca *et al.*, 2014a).

The transcriptional activity of MYC2 requires interaction with MEDIATOR25 (MED25), a subunit of the Mediator complex known to pre-initiate the RNA polymerase complex assembly (Çevik *et al.*, 2012; Chen *et al.*, 2012). In contrast to MYC2, the ABA INSENSITIVE5 (ABI5) TF is degraded when associated with MED25, thereby repressing the expression of ABA-regulated genes (Kidd *et al.*, 2009; Chen *et al.*, 2012). Hence, MED25 plays a central role in the JA/ABA crosstalk, steering developmental and environmental responses. Moreover, SENSITIVE TO FREEZING6 (SFR6)/MED16, another subunit of the mediator complex, has also been reported to play a role in JA signalling (Wathugala *et al.*, 2012; Zhang *et al.*, 2012). Mutations in *sfr6/med16* impair induction of JA/ET marker genes and compromise resistance to necrotrophic fungal pathogens (Zhang *et al.*, 2012). Furthermore, SFR6/MED16 acts downstream of INDUCER OF CBF EXPRESSION (ICE) bHLH TFs and activates the expression of cold and freezing tolerance genes. Finally, a recent study in banana has reported that MYC2 interacts physically with ICE1 to putatively regulate JA-mediated chilling tolerance in fruit (Zhao *et al.*, 2013).

JAZ

The JAZ proteins were discovered in 2007 as the missing link between COI1 and MYC2 (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007). By identifying direct interactors of JAZ repressors, it was possible to disclose mechanistic insights in plant-environment responses

for which JA signalling is crucial (Fig. 1). In agreement with the above-mentioned positive role of JA in freezing tolerance, JAZ proteins were shown to bind and inhibit ICE1 and ICE2 (Hu *et al.*, 2013). Likewise, JAZ proteins are directly involved in the modulation of root growth inhibition during salt stress in rice. Rice JAZ9 and JAZ11 physically interact with the nuclear factor RICE SALT SENSITIVE3 (RSS3). Together with the JAZ proteins, RSS3 blocks the transcriptional activity of class-C bHLH TFs, which leads to the suppression of JA-mediated root growth inhibition during salt stress (Toda *et al.*, 2013). Correspondingly, the *rss3* loss-of-function mutant shows severe root growth inhibition in saline conditions and increased expression of JA-responsive genes, which is in agreement with the observations that overexpression of *JAZ9* in rice alleviates growth inhibition under salt stress (Wu *et al.*, 2015).

In relation to the functional specificity among JAZ proteins, our lab has recently shown that JAZ12 stability is increased through interaction with KEEP ON GOING (KEG), which specifically acts only with this JAZ isoform (Chapter II.4; Pauwels *et al.*, 2015). KEG is a RING-type E3 ubiquitin ligase that negatively regulates ABA signalling (Stone *et al.*, 2006; Liu & Stone, 2010). Accordingly, ABA treatment leads to the degradation of both KEG and JAZ12. KEG is also required for the secretion of apoplastic defence proteins against powdery mildew and plays therefore an important role in biotic defence. Moreover, a missense mutation in *KEG* suppresses resistance to powdery mildew in mutants of *ENHANCED DISEASE RESISTANCE1* (*EDR1*) (Wawrzynska *et al.*, 2008). Conversely, powdery mildew causes the degradation of KEG, thereby interfering with the plant defence responses (Gu & Innes, 2011).

Mutant alleles of *EIN3* or *EIL1*, the central TFs of the ET signalling pathway, show typical JA-deficient phenotypes such as increased susceptibility to *B. cinerea* and partial insensitivity to JA (Zhu *et al.*, 2011). In addition, EIN3/EIL1 and JAZ repressors have the ability to interact physically, suggesting that JAZ repressors control the activation of JA- and ET-mediated defence responses against necrotrophs (Fig. 1B). By interacting and repressing either MYC2 or EIN3/EIL1 TFs, JAZ proteins have therefore a balancing function in the activation of MYC2 and/or ERF branches during infection (Zhu *et al.*, 2011; Zhu & Lee, 2015). In addition, JAZ proteins have been shown to modulate susceptibility to *B. cinerea* in a circadian-dependent manner. Arabidopsis WT plants are more susceptible at dawn, which is not observed in *jaz6* knock-out plants (Ingle *et al.*, 2015). Recently, Boter and coworkers reported that the YABBY TF FILAMENTOUS FLOWER (FIL) is a direct target of JAZ3. Upon JA perception, FIL is released from JAZ3 repression, leading to transcriptional activation of the TF MYB75 involved in anthocyanin accumulation in leaves during defence responses (Boter *et al.*, 2015).

The JAZ-DELLA interaction plays a central function to control resource allocation between growth and defence. This interaction releases MYC2 from repression and leads to the expression of JA-regulated defence genes (Hou *et al.*, 2010). DELLA proteins can therefore be seen as positive regulators of the JA pathway. Accordingly, they were shown to promote resistance to necrotrophic pathogens and susceptibility to biotrophic pathogens (Navarro *et al.*, 2008; Wild *et al.*, 2012). Reciprocally, when binding to DELLA, JAZ repressors interfere with the DELLA-mediated repression of PHYTOCHROME INTERACTING FACTORS (PIFs), central TFs involved in GA signalling and growth regulation (Fig. 1C). In agreement with this hypothesis, it has been reported that *jaz9* loss-of-function and *JAZ9* overexpression lines show GA-hyposensitive and GA-hypersensitive phenotypes, respectively (Yang *et al.*, 2012).

The allocation of resources between growth and defence influences the timing of flowering and, consequently of seed production. The molecular relation between JAZ proteins and flowering time has recently been addressed (Zhai *et al.*, 2015). JAZ repressors can bind and inhibit ERF/AP2-type TFs TARGET OF EARLY ACTIVATION TAGGED1 (TOE1) and TOE2. These TFs are key modulators of flowering time because they indirectly repress the transcription of *FT*. Mechanistically, TOE TFs interfere with protein activity and stability of CONSTANS (CO), which is the direct activator of *FT* expression (Zhang B *et al.*, 2015). During the JA burst, the degradation of JAZ repressors releases TOE1 and TOE2, which in turn mediates *FT* repression and leads to a delay in flowering time. In this situation the plant prioritizes defence over reproduction.

Finally, similar to COI1, JAZ proteins have also been shown to be important components in light responses. A recent study reported that the increased stability of JAZ10 proteins in conditions of low R/FR ratios is dependent on PhyB (Leone *et al.*, 2014). In an FR-enriched environment, GA signalling is potentiated and DELLA proteins are degraded, which releases the JAZ proteins and leads to repression of MYC TFs (Leone *et al.*, 2014). In such environmental conditions, the JAZ protein level is increased, whereas MYC TFs are destabilized (Fig. 2A; Chico *et al.*, 2014). Additionally, overexpression of *JAZ1* in Arabidopsis seedlings leads to exaggerated shade responses in low R/FR conditions, as observed with *phyA* mutants. Furthermore, PhyA is required for wound- or JA-triggered SCF^{COI1}-mediated JAZ1 degradation in the shoot. It appears thus that, in conditions of competition with neighbours, the inactivation of JA signalling helps the plant to efficiently reallocate resources to rapid growth (Robson *et al.*, 2010).

HOW ATTACKERS EXPLOIT THE JA SIGNALLING COMPLEX TO FOOL THE PLANT

Over the last years, studies on defence responses in mutants have provided genetic evidence on how important the JA, SA and ET signalling pathways are in biotic stress responses. Interestingly, in some cases, there seems to be a discrepancy between the observed gene

activation and the relevance of the respective defence hormones. For instance, aphids and whiteflies activate the SA pathway, but do not perform better on SA-deficient or -insensitive plants (Mewis *et al.*, 2005; Mewis *et al.*, 2006; Zarate *et al.*, 2007). Likewise, some strains of *P. syringae* activate the JA pathway, but do not perform better on JA-insensitive *coi1* mutants (Feys *et al.*, 1994; Kloek *et al.*, 2001). The field of plant biology has made tremendous advances in the understanding of these highly complex interactions by discovering the molecular origin of most of these manipulations (Fig. 3).

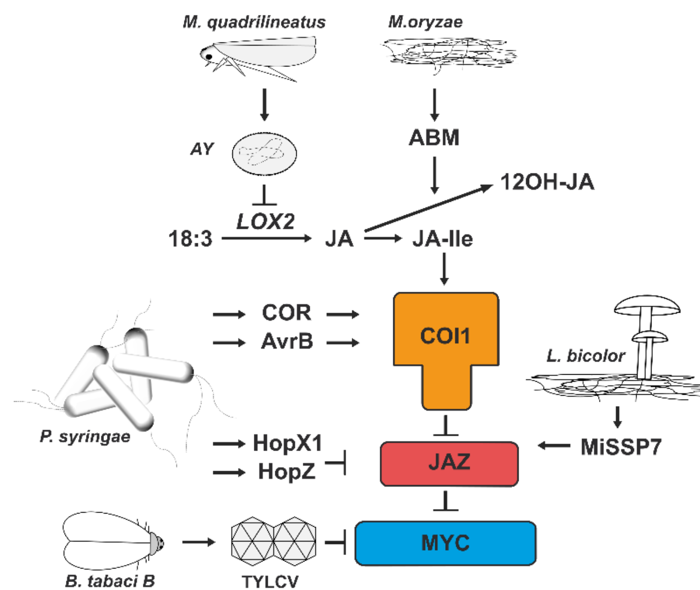

Figure 3: Insects and pathogens hijack the JA machinery. Various strategies are used by plant pathogens and insects to interfere directly with the JA signalling pathway. *M. quadrilineatus* is the vector of Aster Yellows phytoplasma, which injects the SAP11 effector to block *LOX2* expression necessary for JA production. ABM of the fungal pathogen *M. oryzae* converts the JA-Ile precursor JA into inactive 12OH-JA. *P. syringae* activates the JA pathway by producing the JA-Ile 3D-mimic COR and the AvrB effector, both promoting increased binding of the COI1 co-receptor, and the HopX1 and HopZ effectors contributing to the degradation of JAZ proteins. MiSSP7 of the mutualistic fungus *L. bicolor* stabilizes JAZ proteins to block the JA pathway. The whitefly *B. tabaci* B infects host plants with the Tomato yellow leaf curl virus (TYLCV) virus, which codes for the bC1 effector capable of blocking the transcriptional activity of MYC TFs.

The best-known example is *P. syringae pv. tomato* DC3000, which produces the previously mentioned JA-Ile mimic COR (Katsir *et al.*, 2008; Melotto *et al.*, 2008; Xin & He, 2013). In general, plants are capable of recognizing the *Pseudomonas*-derived flagellin through the FLAGELLIN-SENSITIVE2 (FLS2) receptor, resulting in stomatal closure, hence hindering the pathogen to penetrate the leaf (Gómez-Gómez & Boller, 2000; Zipfel *et al.*, 2004; Chinchilla *et al.*, 2006). However, the highly virulent *P. syringae pv. tomato* DC3000 strain produces COR, which, when bound to the COI1-JAZ co-receptor complex, activates stomatal reopening

and SA repression through activation of NAC DOMAIN CONTAINING PROTEIN19 (ANAC019), ANAC055 and ANAC072 TFs (Melotto *et al.*, 2006; Zheng *et al.*, 2012). Until now, COR has only been observed in *Pseudomonas*, but it is very likely that among the wide range of bacterial pathogens that plants face in nature, other strains or species produce JA mimics. For instance cinnacidin, a phytotoxin produced by the fungus *Nectria* sp. DA060097, contains a cyclopentalenone ring system with an isoleucine subunit linked by an amide bond. Biological characterization suggested a herbicidal activity equivalent to that of COR (Irvine *et al.*, 2008).

Like animals and plants, fungi are able to synthetize oxylipins (Brodhun & Feussner, 2011). Various studies have shown that JA production is a common feature among many plant-interacting pathogenic or mutualistic fungi (Miersch *et al.*, 1987; Miersch *et al.*, 1993; Tsukada *et al.*, 2010). Recently, a study has shown that *Fusarium oxysporum* f. sp. *conglutinans* and *F. oxysporum* f. sp. *matthioli*, which produce JA, JA-Ile and JA-Leu, are unable to infect JA-insensitive Arabidopsis mutants. In contrast, *F. oxysporum* f. sp. *raphani,* which produces JA, but no detectable levels of JA-Ile and JA-Leu, does not require a functional JA signalling pathway to be infectious (Cole *et al.*, 2014). Noteworthy is that both JA-Ile and JA-Leu, but not JA, have been shown to promote COI1-JAZ interaction (Thines *et al.*, 2007; Katsir *et al.*, 2008; Fonseca *et al.*, 2009). Although not known to produce JAs or any JA-mimics themselves, some fungi have the ability to metabolize JAs, which is another strategy to interfere with the JA machinery. For example, *Aspergillus niger* and *Pisolithus tinctorius* can hydroxylate JA (Miersch *et al.*, 1999b; Miersch *et al.*, 1999c). Besides the above-mentioned *Fusarium oxysporum* strains, many other strains have been shown to produce various JAs, including hydroxylated JAs (Miersch *et al.*, 1999a). Similarly, the rice blast fungus *Magnaporthe oryzae* possesses an antibiotic biosynthesis monooxygenase (ABM) that converts endogenous fungal JA into 12-OH-JA. Furthermore, loss of ABM function in *M. oryzae* leads to JA accumulation in rice, which induces host defence and blocks invasive growth (Patkar *et al.*, 2015). Recent advances in JA catabolism have shown that *in planta*, cytochrome P450 oxidases hydroxylate the bioactive form JA-Ile into inactive 12-OH-JA-Ile and 12-COOH-JA-Ile, which indicates that fungi use the same mechanisms as plants to inactivate the JA pathway (Miersch *et al.*, 2008; Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Heitz *et al.*, 2012; Koo *et al.*, 2014).

To date, no study has reported on the production of JAs in non-plant-interacting fungi, which strongly suggests that fungal JAs have evolved to enable interference with the host plant's JA signalling machinery. Further efforts are needed to shed light on the production of certain JAs in fungi and how they contribute to pathogen virulence. Inactivation of JA through hydroxylation is not solely linked to pathogenic fungi, as shown by the above-mentioned example of the ectomycorrhizal fungi *P. tinctorius*, but might even be a prerequisite for

successful mycorrhizal colonization (Miersch *et al.*, 2008). Additional evidence comes from a recent study showing that during poplar colonization, the mutualistic fungus *Laccaria bicolor* secretes a fungal effector mycorrhiza-induced small secreted protein7 (MiSSP7), which stabilizes JAZ6, leading to the repression of JA responses (Plett *et al.*, 2011; Plett *et al.*, 2014).

Effectors targeting JAZ proteins are not restricted to fungi, but have recently been described in several *P. syringae* strains as well. In contrast to the fungal effector of *L. bicolor*, these effectors promote JAZ degradation to activate JA signalling, like COR. HopZ1a acetylates host JAZ proteins, which promotes their COI1-mediated degradation (Jiang *et al.*, 2013). Similarly, *HopX1* codes for a Cys protease that directly degrades JAZ proteins in a COI1-independent manner (Gimenez-Ibanez *et al.*, 2014). In both cases, JAZ degradation leads to the activation of the JA pathway, thereby promoting bacterial infection. Finally, AvrB2 promotes the activation of the Arabidopsis plasma membrane H(+)-ATPase (AHA1), which is RPM1-INTERACTING4 (RIN4)-dependent and leads to an increased binding of the COI/JAZ complex and subsequent activation of downstream JA responses (Zhou *et al.*, 2015).

Although JA signalling confers resistance to herbivorous insects, only few examples of insects directly interfering with the pathway are known. The whitefly *B. tabaci* B infects Arabidopsis plants with the Tomato yellow leaf curl virus (TYLCV), which releases an effector protein bC1 that inactivates MYC2, leading to the downregulation of *TERPENE SYNTHASE (TPS)* genes, which reduces plant terpenoid production and leads to enhanced susceptibility to *B. tabaci* B (Li *et al.*, 2014). The leafhopper *Macrosteles quadrilineatus* is a vector of the Aster Yellows phytoplasma strain Witches' Broom (AY-WB), which secretes the SAP11 effector that binds and destabilizes CINCINNATA (CIN)-type TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR (TCP) TFs, which are activators of the expression of LIPOXYGENASE (*LOX*) genes involved in JA synthesis. AY-WB infection therefore blocks JA biosynthesis of their host plants, rendering them highly susceptible to leafhoppers (Sugio *et al.*, 2011).

As the list of organisms capable of manipulating the host signalling machinery is constantly growing, we principally discussed manipulation strategies involving well-characterized molecular effectors and targets. Pathogens fooling indirectly the JA defence through crosstalk manipulation have not been considered here, because this is out of the scope of this review. For a more comprehensive view of the host manipulation topic, we refer to previously published reviews (Kazan & Lyons, 2014; Gimenez-Ibanez *et al.*, 2016).

OUTLOOK: FROM MODEL TO FIELD

The discovery of JAZ repressors together with the application of "omics" technologies were two major milestones that greatly facilitated subsequent findings in the JA field. Considering the signalling network and crosstalk described in this review, it may be the right time for the JA community to adopt a systems biology approach that will allow building an interactive model of JA responses. Computational modelling, which has proven its value in the auxin and circadian rhythm fields (Swarup *et al.*, 2005; Domagalska & Leyser, 2011; Chew *et al.*, 2014), will integrate all knowledge available at a certain moment, allowing better prediction and planning of future research. Likewise, it will help to understand the extremely complex mechanisms plants have developed to cope with environmental stresses and to identify network hotspots, such as novel key modulators of plant responses. Chemical biology can also help to explore and dig further into diverse biological processes. Identification of new bioactive compounds or, alternatively, of novel functions for natural and synthetic metabolites can contribute to the characterization of hormone signalling pathways and might provide new targets for agronomic research (Staswick, 2009; Fonseca *et al.*, 2014b; Rigal *et al.*, 2014; Floková *et al.*, 2016).

Remarkably, being a master regulator of JA responses, the co-receptor complex COI-JAZ seems to be a preferential target of bacterial effectors and pathogen-derived small molecules such as COR. Monte *et al.* (2014) have described a synthetic antagonist of COR, COR-O-methyloxime (COR-MO), that is able to block the binding pocket of COI1, thereby preventing its binding to JAZ proteins and blocking the JA response. As already employed in fundamental research, COR-MO could potentially be used as a biotechnological tool for more sustainable and persistent agriculture (Monte *et al.*, 2014; Böhm *et al.*, 2016). Further investigation of JA-derivatives or analogues produced by plant-interacting fungi and microbes, could ameliorate the identification of antagonists or agonists of JA signalling. A possible example of such a molecule is cinnacidin (Irvine *et al.*, 2008). A very interesting strategy to render plants resistant to COR-producing *P. syringae* strains has recently been forwarded by the group of Sheng Yang He (Zhang L *et al.*, 2015). Guided by the crystal structure of COI1, Zhang and coworkers successfully modified the receptor to allow sufficient JA signalling while greatly reducing the sensitivity to COR. A similar approach has been described where an engineered version of the ABA receptor PYRABACTIN RESISTANCE 1 (PYR1) showed increased sensitivity to the agrochemical mandipropamid, leading to the induction of ABA-like responses including drought tolerance (Park *et al.*, 2015). Although very promising, these models are currently still in a proof of concept stage and require further testing, e.g. in field trials, to assess their applicability as a tool to improve strategies for crop protection. Most interestingly, the selectivity of the binding pockets of these receptors relies only on a few amino acids and could potentially be modified by potent and

booming genome editing technologies such as CRISPR/Cas9. Although public acceptance and legal status related to this technology remains to be determined, its impact on applied and fundamental research is immense. For instance, CRISPR/Cas9 allows the generation of multi-gene mutants, e.g. to encounter the elimination of redundant proteins such as the JAZ family. Furthermore, any gene of plant species with available genome or transcriptome sequence can be knocked out as far as transformation technologies are available (Bortesi & Fischer, 2015).

In the quest for sustainable agriculture and innovative crop care, it may become relevant to uncouple the "to grow or to defend dilemma" that plants experience when facing adverse environmental conditions. Recently, Campos and colleagues succeeded in uncoupling the trade-off between growth and defence by generation of the *jazQ phyB* mutant (Campos *et al.*, 2016). The *jaz* quintuple mutant showed a constitutive JA response translated in e.g. enhanced anti-insect defence and an attenuation in general growth. Loss of PhyB, however, suppresses this growth inhibition in the aerial organs while the defence response is unaltered. This could be explained by combined release of repression of the MYC and PIF TFs by respectively, JAZ proteins and PhyB. This new knowledge can be the trigger for researchers to take advantage of the crosstalk established between JA signalling and growth-triggering pathways, i.e. mainly gibberellin and light signalling, and engineer crops with more favourable resource allocation for agricultural purposes. Similarly, in phosphate-deficient conditions, Arabidopsis plant growth is strongly compromised. Rouached *et al.* (2011) have been successful in uncoupling growth inhibition from phosphate deficiency in transgenic Arabidopsis and rice plants, which resulted in normal growth in phosphate-deficient conditions (Rouached *et al.*, 2011). Likewise, since a role for JA signalling has been reported in nodulation and nitrogen fixation during competition with neighbours, it may be expected that adequately engineered crops will be also more efficient in the use of mineral resources from soils (Suzuki *et al.*, 2011; Nagata *et al.*, 2015). In line with this, and considering that JAZ repressors and/or MYC2 also interact with the ICE TFs and RSS3 and fine-tune ABA-responsive genes, engineering of JA signalling could lead to plants that tolerate salt, freezing and drought stresses (Fig. 1A). Due to complex hormonal networks and crosstalk, many uncertainties remain with regard to the outcome of an altered JA signalling. Integration of 'omics' research and bioinformatics could model and predict the outcome of engineered JA pathways and become implemented in applied research for improved agriculture (Mittler & Shulaev, 2013).

Finally, JA signalling is tightly connected with the production of bioactive specialized metabolites. Such small compounds are diverse in chemical nature and function and can act either as insect repellents for herbivores, or attractors for pollinators and parasites (Schoonhoven *et al.*, 1998; Howe & Jander, 2008). In this sense, JA-derived compounds can

be used as biologicals for crop protection and fertilization. Alternatively, metabolic engineering of defence pathways coupled with the JA pathway could represent a valuable approach for crop protection (Jirschitzka *et al.*, 2013). In addition, numerous plant-derived secondary metabolites such as the monoterpenoid indole alkaloids (e.g. vincristine, quinine), benzylisoquinoline alkaloids (e.g. morphine, codeine) or terpenoids (e.g. artemisinin, paclitaxel) have unique pharmaceutical properties to treat cancer or malaria and are included in the World Health Organization's List of Essential Medicines (World Health Organization, 2013). Their biosynthetic pathways are often specific to certain plant species or taxa, but are nonetheless generally controlled by a limited number of central TFs, which are usually conserved among different species from different families (Memelink, 2009; De Geyter *et al.*, 2012; Van Moerkercke *et al.*, 2015; Mertens *et al.*, 2016). Further research in the transcriptional regulation of these pathways will help to elucidate missing enzymatic and regulatory steps and will result in knowledge that can potentially be used for metabolic engineering (Chapters II.2 and II.3; De Boer *et al.*, 2011; Zhang *et al.*, 2011; Yu *et al.*, 2012; Sears *et al.*, 2014; Gasperini *et al.*, 2015; Goossens *et al.*, 2015; Van Moerkercke *et al.*, 2015; Cárdenas *et al.*, 2016).

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Chapter 2

Role and functioning of bHLH transcription factors in jasmonate signalling

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Published in:

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Role and functioning of bHLH transcription factors in jasmonate signaling. *Journal of Experimental Botany 2016, 10.1093/jxb/erw440.*

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[2]Author Contributions: writing of the manuscript (Abstract, Introduction, bHLH transcription factors involved in JA signalling in Arabidopsis, Perspectives, Figure 1).

ABSTRACT

Plant growth, development and interaction with the environment involve the action of multiple phytohormones. Transcription factors (TFs) of diverse families play essential roles in the signalling cascades triggered by the perception of a particular hormone. TFs may act alone or in a combinatorial fashion with other TFs, and may act specifically in a single hormonal signalling cascade or as signalling hubs for multiple hormones. In the signalling cascades triggered by the phytohormone jasmonate (JA), which modulates a diverse, but specific, range of aspects of plant growth, development and defence, the TFs of the basic helix-loop-helix (bHLH) family play an essential and often conserved role in the plant kingdom. Here, we first discuss the bHLH TFs involved in all kinds of JA-modulated processes in the model plant *Arabidopsis thaliana*. Secondly, we elaborate on the identity and role of bHLH TFs in the conserved JA-mediated elicitation of specialized metabolism of medicinal and crop species. Finally, we discuss which directions future fundamental research on the functioning of bHLH TFs in JA signalling may head for and how this research can be translated from model plants into crop and medicinal plant species to engineer traits of agronomical and industrial interest.

INTRODUCTION

Plants experience many changes throughout their life cycle, steered by different developmental or environmental cues, including exposure to abiotic and biotic stresses. Such impulses lead to genome-wide transcriptional reprogramming mediated by the, often combinatorial, action of a plethora of transcription factors (TFs), which are in turn regulated by signalling molecules such as the phytohormone jasmonate (JA). Stress-related and/or developmental impulses can induce the production of bioactive JAs in the plant, resulting in JA-modulated phenotypes that generally reflect a 'shift' in energy consumption, i.e. from growth to defence and/or growth to development, accompanied in many cases with the production of specialized (bioactive) metabolites (Huot *et al.*, 2014). Typical and best-characterized JA-modulated processes are inhibition of root growth, stamen development, leaf senescence and defence against herbivores and necrotrophic pathogens (Reinbothe *et al.*, 2009; Wasternack & Hause, 2013; Yan & Xie, 2015; Yuan & Zhang, 2015), but important roles in other processes, such as abiotic stress tolerance, are also emerging (Kazan, 2015).

JA-mediated reprogramming of each of these processes usually involves often tissue- or cell-specific signalling cascades with specific signal transduction components. However, it all starts with the perception by and induction of the JA core signalling machinery that can modulate the activity of central regulatory TFs that eventually connect to specific developmental or defence programmes. Indeed, changing the activity of these central TFs triggers a complex network of TF cascades, regulating the expression of downstream JA-

modulated genes, e.g. enzymes involved in the biosynthesis of specialized metabolites (Chapter I.1; Pauwels *et al.*, 2009; De Geyter *et al.*, 2012; Wasternack & Hause, 2013; Chini *et al.*, 2016; Goossens *et al.*, 2016). The central TFs of the JA core signalling complex interact with the JA ZIM DOMAIN (JAZ) repressors and are maintained in a repressed state in the absence of JAs, and hence in the absence of a triggering impulse. Repression by JAZ proteins is executed by inhibition of TFs from binding DNA or by recruiting the co-repressor proteins, i.e. TOPLESS (TPL) and the TPL-related proteins (TPRs), either directly or via interaction with the NOVEL INTERACTOR OF JAZ (NINJA) protein (Pauwels *et al.*, 2010; Pauwels & Goossens, 2011; Chini *et al.*, 2016). Particular developmental and environmental cues result in the production of an isoleucine conjugate of JA, JA-Ile, which is the bioactive form of JA (Fonseca *et al.*, 2009) that is sensed by the F-box protein CORONATINE INSENSITIVE 1 (COI1; At2g39940), which is assembled into the SKP1-CULLIN-F-box (SCF) E3 ubiquitin ligase complex SCF^{COI1}. Following JA-Ile perception, SCF^{COI1} forms a co-receptor complex with JAZ, after which the JAZ repressor is degraded via COI1-mediated ubiquitination and 26S proteasomal degradation (Chapter I.1; Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2009; Sheard *et al.*, 2010; Wasternack & Hause, 2013; Chini *et al.*, 2016; Goossens *et al.*, 2016; Nagels Durand *et al.*, 2016). This releases the central TFs, leading to a modulated expression of specific downstream target genes.

Many TFs implicated in JA signalling have been identified and functionally characterized, including many basic helix-loop-helix (bHLH)-type TFs (Pauwels & Goossens, 2011; De Geyter *et al.*, 2012; Zhou & Memelink, 2016). The family of bHLH TFs is very widespread among eukaryotes and exists in plants, animals and fungi. It has expanded in animals and land plants, following independent evolutionary events (Feller *et al.*, 2011). The bHLH domain consists of an N-terminal stretch of basic amino acid residues responsible for DNA binding and an HLH domain to form homo- or heterodimers. bHLH TFs typically bind E-box sequences (CANNTG), such as the G-box (CACGTG), in the promoter of their target genes, although binding specificity variation occurs (Carretero-Paulet *et al.*, 2010; Fernández-Calvo *et al.*, 2011). Arabidopsis contains more than 150 bHLH TFs, which have been characterized phylogenetically based on the sequence similarity of the bHLH domain (Heim *et al.*, 2003; Toledo-Ortiz *et al.*, 2003; Carretero-Paulet *et al.*, 2010; Pires & Dolan, 2010). Here, we will refer to the classification by Heim *et al.* (2003), which represents a comprehensive division of the bHLH TFs into multiple clades and subclades and, additionally, incorporates the conservation of other motifs next to the DNA-binding bHLH domain. These conserved motifs and the bHLH domain represent a modular organization of bHLH-type TFs, reflecting the possible occurrence of multiple protein-protein interactions, and hence the capacity to assemble into distinct TF complexes to modulate and/or empower their activity and/or specific function. Based on this modular organization of plant bHLHs, we will discuss the

Arabidopsis bHLH TFs related to JA signalling in the first part of this review. In general, JA signalling elicits primarily the biosynthesis of, often species-specific, bioactive, specialized metabolites that help plants to survive in a changing environment but that often also have interesting properties for humans (De Geyter *et al.*, 2012; Wasternack, 2014). In the second part of this review, we will therefore discuss the bHLH TFs that are involved in the regulation of specialized metabolism of medicinal and crop species and show that genericity among bHLH TFs enables translational research from model plants into species of agronomical and industrial interest. Finally, we give our perspectives on the future of research in the field of JA signalling.

bHLH TRANSCRIPTION FACTORS INVOLVED IN JA SIGNALLING IN ARABIDOPSIS

To date, four subclades of the bHLH TF family have been shown to be implicated in JA signalling in Arabidopsis, each with a different contribution to the JA response. Best characterized is the bHLH IIIe subclade, the members of which positively contribute to the general JA response. In contrast, the recently characterized bHLH IIId subclade TFs seem to exert a negative role in many JA-related responses. Finally, bHLH IIIf subclade TFs play a major role in JA-mediated anthocyanin accumulation and trichome initiation and two members of the bHLH IIIb subclade are involved in JA-induced freezing tolerance.

bHLH IIIe: the ‘positive’ JA subclade

In the core primary JA signalling pathway, MYC2 is known to be a central TF that is repressed by the JAZ proteins in the absence of JAs (Fig. 1A; Pauwels & Goossens, 2011; Chini *et al.*, 2016). Interaction between MYC2 and JAZ occurs via the JAZ-interacting domain (JID) located at the N-terminus of MYC2 and the C-terminal JA-associated (Jas) domain of the JAZ proteins. MYC2 is often seen as the central regulator of JA signalling (Kazan & Manners, 2013), because it is involved in the majority of JA-related processes, e.g. root growth inhibition (Chen *et al.*, 2011), production of flavonoids (Dombrecht *et al.*, 2007) and glucosinolates (Schweizer *et al.*, 2013; Frerigmann *et al.*, 2014), and defence against herbivores and pathogens (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). Typically, the activity and the function of a TF are determined by its ‘interaction state’ and any consequential post-translational modification.

In addition to the interaction with the JAZ proteins, MYC2 has several other binding partners, each affecting its function, activity and/or stability. For instance, interaction with TIME FOR COFFEE (TIC) regulates MYC2 protein accumulation in a circadian clock-dependent manner (Shin *et al.*, 2012) and phosphorylation by an unknown kinase facilitates MYC2 proteolysis, but, surprisingly, is also necessary to stimulate its activity (Zhai *et al.*, 2013). Furthermore, the E3 ubiquitin ligase PLANT U-BOX PROTEIN10 (PUB10) binds MYC2, resulting in

ubiquitination and subsequent 26S proteasomal degradation (Jung *et al.*, 2015). The stability of MYC2 is also regulated in light signalling and plant defence, though no direct mediators of degradation were identified in these processes. Light induces MYC2 stability depending on the presence of PHYTOCHROME B (PhyB), whereas CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) is required for the decrease of MYC2 levels in the dark (Chico *et al.*, 2014). Finally, herbivores can manipulate plant defence by egg deposition leading to activation of salicylic acid signalling and a decrease of MYC2, MYC3 and MYC4 levels, hence reducing JA-mediated insect defence (Bruessow *et al.*, 2010; Schmiesing *et al.*, 2016).

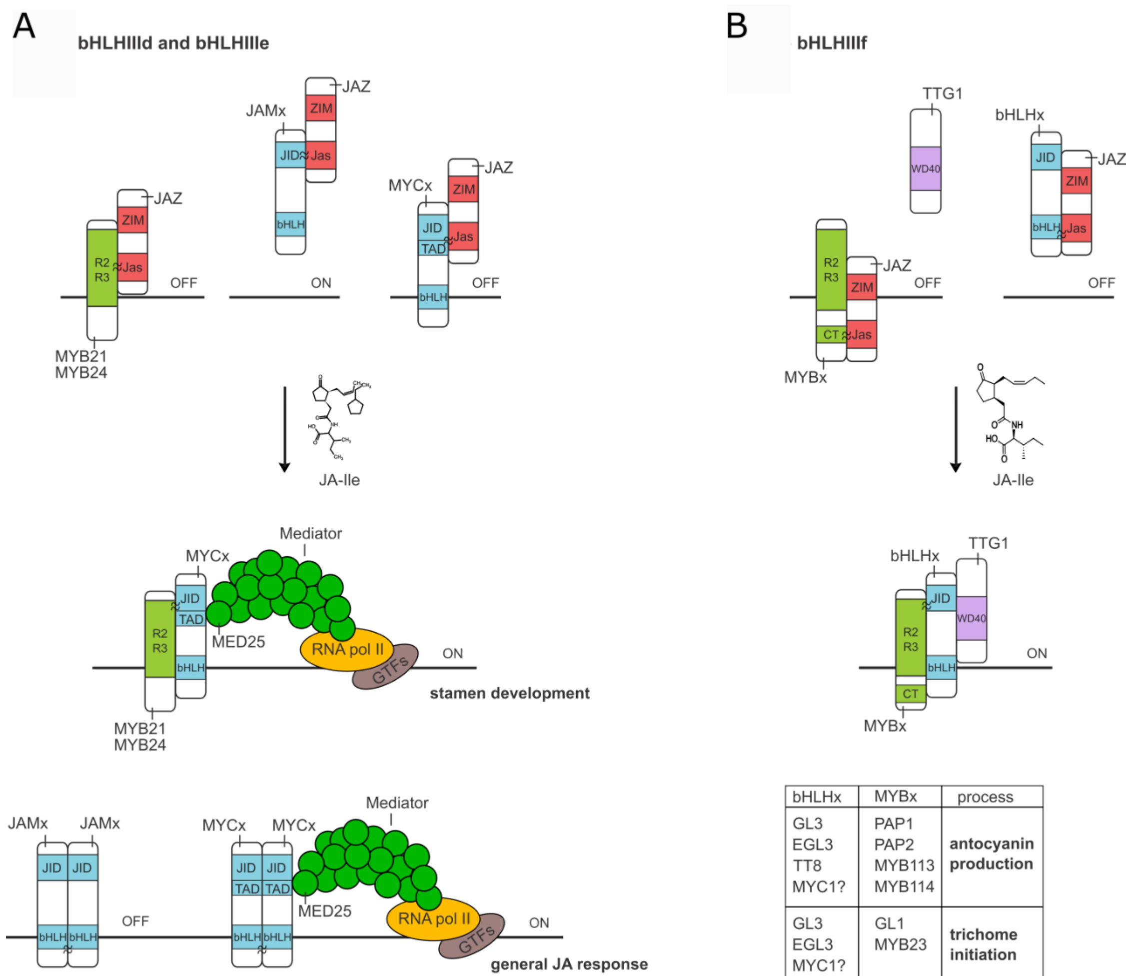

Figure 1: Modus operandi of bHLH-type TF complexes in JA signalling in Arabidopsis. A) bHLH IIId and bHLH IIIe subclades. JAZ proteins repress the activity of the MYC TFs, the JAM TFs and the R2R3 MYB proteins MYB21 and MYB24. In the presence of JA-Ile, JAZ proteins are degraded and heterodimers of MYB21 or MYB24 with MYC TFs regulate stamen development. Dimers of the JAM TFs and the MYC TFs play, respectively, a negative and a positive role in the general JA response. The MYC TFs interact with the MED25 protein of the Mediator complex to recruit the RNA polymerase II transcriptional machinery. B) bHLH IIIf subclade: The trimeric WD-repeat (TTG1)-bHLH-MYB complex is not assembled in the absence of JAs because of the interaction of JAZ repressors with both the R2R3 MYB TFs and the bHLH IIIf-type TFs. JA-Ile provokes degradation of the JAZ proteins, leading to the assembly of the trimeric complex. The bHLH and MYB TFs implicated in the corresponding specific JA-modulated processes are depicted in the box. Abbreviations: GTF, general transcription factors.

MYC2 acts together with two close homologues, MYC3 and MYC4, which are to some extent functionally redundant. However, they have a different expression pattern, and, as a consequence, different contributions to a given response (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011; Frerigmann *et al.*, 2014; Gasperini *et al.*, 2015). MYC2 is expressed particularly in the roots and is essential for root phenotypes, whereas MYC3 and MYC4 show low expression in (young) roots and rather contribute to phenotypes of aerial tissues, such as plant defence and biosynthesis of glucosinolates. The MYC TFs can homo- and heterodimerize, which is in agreement with their involvement in shared processes (Fernández-Calvo *et al.*, 2011). For instance, MYC2, MYC3 and MYC4 together control the expression of biosynthetic enzymes leading to the production of glucosinolates (Schweizer *et al.*, 2013; Frerigmann *et al.*, 2014) by means of interacting with specific MYB TFs. It is remarkable that the JID of the MYC TFs, essential for interaction with the JAZ proteins, is also essential for interaction with these MYB TFs. It is postulated that JAZ interaction leads to inhibition of heterodimerization between these MYB and MYC TFs. Adjacent to the JID is a trans-activation domain (TAD), which is responsible for interaction with MEDIATOR25 (MED25), a subunit of the Mediator complex, which leads to recruitment of the RNA polymerase II transcriptional machinery, hence enabling MYC TF activity (Fig. 1A; Çevik *et al.*, 2012; Chen R *et al.*, 2012). Notably, the stretch of acidic amino acids in the TAD also functions as a degron of MYC2, because deletion of this element diminishes ubiquitin-mediated proteasomal degradation of MYC2. Nonetheless, as indicated above, turnover of MYC2 is required for its proper transcriptional activity (Zhai *et al.*, 2013).

The crystal structure of the N-terminal part of MYC3 in complex with the Jas peptide of JAZ9 designated the residues that are essential for this interaction, which include amino acid residues not only in the JID of MYC3 but also in the TAD (Zhang *et al.*, 2015). Moreover, JAZ proteins compete with MED25 for MYC interaction, implying that they repress MYC TFs not only by interfering with DNA binding and/or recruitment of the co-repressors TPL and TPRs, but also by inhibiting MED25 interaction (Zhang *et al.*, 2015). In agreement with these findings, point mutations in the JID and TAD have been shown to have a tremendously positive effect on the activity of MYC2 and MYC3 caused by the loss of interaction with JAZ repressors (Chapters II.2 and II.3; Gasperini *et al.*, 2015; Goossens *et al.*, 2015). Notably, a point mutation converting a conserved Asp to an Asn in the JID of MYC3 not only causes less interaction with JAZ repressors (Chapter II.2; Goossens *et al.*, 2015), but also a stronger interaction with MYB34, a main transcriptional regulator of biosynthesis of indole glucosinolates (Frerigmann *et al.*, 2014).

MYC5/NACL-INDUCIBLE GENE1 (NIG1), another MYC2 homologue belonging to the bHLH IIIe subclade, is a positive regulator of salt stress signalling. Recently, using plants overexpressing MYC5 fused to a SUPERMAN repressive domain X (SRDX), MYC5 was shown to be involved in a set of typical JA responses, i.e. root growth inhibition and the development of stamen and pollen (Figueroa & Browse, 2015; Qi *et al.*, 2015a). In the latter process, MYC2, MYC3, MYC4 and MYC5 function redundantly to mediate the JA-inducible expression of MYB TFs involved in stamen development, i.e. MYB21, MYB24, MYB57 and MYB108. Among these MYB TFs, MYB21 and MYB24 can interact, via their N-terminal R2R3 DNA-binding domain, with the N-terminal domain of the MYC TFs, containing the JID and the TAD (Fig. 1A; Qi *et al.*, 2015a). Moreover, MYB21 and MYB24 themselves can interact with some of the JAZ repressors, also via their N-terminal R2R3 DNA-binding domain (Song *et al.*, 2011), leading to the hypothesis that the MYC TFs function in a cooperative manner with MYB21 and MYB24 in a bHLH-MYB complex to regulate stamen development. This complex can then be inactivated by repression of both the MYB and the MYC TFs by the JAZ proteins. Furthermore, as the domains responsible for bHLH-MYB interaction are also essential for interaction with the JAZ proteins, assembly of the complex and interaction with/repression by JAZ might be mutually exclusive. To conclude, the bHLH IIIe subclade consists of four MYC TFs that are, together, positively involved in the majority of JA responses.

bHLH IIId: the 'negative' JA subclade

The counterpart of the MYC subclade is the bHLH IIId subclade containing the JA-ASSOCIATED MYC2-LIKE (JAM) TFs. Like the MYC TFs, they also contain a JID capable of interaction with JAZ proteins; however, in contrast to the MYC TFs, the JAM TFs lack a canonical activation domain (Fig. 1A). JAM1 , JAM2, JAM3 and JAM4 act redundantly as negative regulators of many JA responses, including root growth inhibition, anthocyanin accumulation, insect defence and leaf senescence (Nakata *et al.*, 2013; Sasaki-Sekimoto *et al.*, 2013; Song *et al.*, 2013; Fonseca *et al.*, 2014a; Qi *et al.*, 2015a). Because JAM TFs do not contain a TAD, they fail to recruit the RNA polymerase II machinery to activate transcription. Similarly to the MYC TFs, JAM TFs can homo- and heterodimerize with each other but they are not able to interact with the MYC TFs (Nakata *et al.*, 2013; Fonseca *et al.*, 2014a). Most likely, they antagonize positive regulators of the JA pathway, e.g. the MYC TFs, by binding to the same target sequences and not by protein-protein interaction. For instance, competition of the JAM TFs with the MYC TFs for binding to cis-acting elements results in repression of MYC-activated genes, eventually leading to suppression of JA-mediated, MYC-dependent leaf senescence (Qi *et al.*, 2015b). The role of JAZ proteins in the mechanism of repression by the JAM TFs is not yet understood. Interestingly, using transient expression assays in protoplasts, Song *et al.* (2013) have shown that JAZ proteins cause a release of the repressing activity of the JAMs. This implies that the JAZ proteins do not recruit co-

repressors to increase repression as is the case for the MYC TFs, but that JAZ proteins inhibit the binding of the JAMs to the promoter of their target genes, leading to reduced repression. Notably, JAM1 has first been characterized as an abscisic acid-inducible TF playing a positive role in abscisic acid-mediated responses, e.g. drought resistance, and has been shown to possess transactivation activity in yeast (Li *et al.*, 2007). With these observations in mind, additional plant- or even pathway-specific proteins could be responsible for the repression carried out by the JAM TFs in the JA signalling pathway. Further research is necessary to gain a full understanding of the mechanism of repression by the JAM TFs.

bHLH IIIf: the trichome and anthocyanin subclade

Members of the bHLH IIIf subclade are involved in multiple processes, including seed coat differentiation, root hair initiation, anthocyanin production and trichome development, of which only the last two have been shown to be JA related. They all work in trimeric complexes consisting of the WD-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1), one of the bHLH IIIf-type TFs and an R2R3 MYB TF (Xu *et al.*, 2015; Brkljacic & Grotewold, 2017). The actual function of a so-called WD-repeat-bHLH-MYB complex is specified by the R2R3 MYB TF that is integrated in the complex (Fig. 1B). As such, anthocyanin production is regulated by the R2R3 MYB proteins PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1), PAP2 , MYB113 and MYB114 together in a complex with TTG1 and one of the bHLH IIIf-type TFs, i.e. TRANSPARANT TESTA8 (TT8), GLABRA3 (GL3) or ENHANCER OF GL3 (EGL3) (Zhang *et al.*, 2003; Zimmermann *et al.*, 2004; Gonzalez *et al.*, 2008; Qi *et al.*, 2011). Conversely, the R2R3 MYB proteins MYB23 and GL1 are responsible for trichome development and JA-induced trichome initiation and assemble in a complex with the bHLH IIIf-type TFs GL3 or EGL3 and the WD-repeat protein TTG1 (Zhang *et al.*, 2003; Zimmermann *et al.*, 2004; Kirik *et al.*, 2005; Maes *et al.*, 2008; Yoshida *et al.*, 2009). The trimeric TF complexes are negatively regulated by small R3 MYB proteins like CAPRICE (CPC), TRIPTYCHON (TRY) and MYBL2 that compete with R2R3 MYB proteins for binding with the bHLH IIIf-type TFs (Esch *et al.*, 2003; Dubos *et al.*, 2008; Matsui *et al.*, 2008; Wester *et al.*, 2009; Zhu *et al.*, 2009).

Similarly to the members of the bHLH IIId and bHLH IIIe subclades, the bHLH IIIf subclade members are characterized by the presence of an N-terminal JID that mediates interaction with the JAZ repressors in the former two subclades. However, although interactions with most of the JAZ proteins could also be observed for EGL3, TT8 and GL3 (Qi *et al.*, 2011), these interactions are not mediated via the JID but via the C-terminal part including the bHLH domain. Furthermore, the R2R3 MYB proteins PAP1 and GL1, but not the WD-repeat protein TTG1, could also interact with some of the JAZ proteins, at least in yeast two-hybrid (Y2H) assays (Qi *et al.*, 2011). It was postulated that interaction of the JAZ proteins with both

the bHLH IIIf TFs and the R2R3 MYB TFs inhibits the assembly of the WD-repeat-bHLH-MYB complex (Fig. 1B). As a consequence, expression of genes involved in anthocyanin biosynthesis and trichome initiation is repressed in the absence of bioactive JAs (Qi *et al.*, 2011). In the presence of JA-Ile, the JAZ proteins are degraded, leading to the assembly and activation of the WD-repeat-bHLH-MYB complex.

The fourth member of the bHLH IIIf subclade, MYC1, has been shown to interact with the WD-repeat protein TTG1 and the R2R3 MYB proteins PAP1, PAP2, MYB113, MYB114, GL1 and MYB23 (Zimmermann *et al.*, 2004; Symonds *et al.*, 2011; Zhao *et al.*, 2012). Furthermore, transient promoter activation assays have shown that co-expression of *MYC1* and *PAP1* or *PAP2* could induce transcription of *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), encoding an enzyme involved in anthocyanin biosynthesis (Zimmermann *et al.*, 2004). In addition, loss of MYC1 function leads to defects in trichome development (Symonds *et al.*, 2011; Zhao *et al.*, 2012). Altogether, this indicates that also MYC1 assembles in a trimeric WD-repeat-bHLH-MYB complex that is involved in anthocyanin production and/or trichome development; however, a direct link to JA signalling is still missing. Surprisingly, the basic region of the bHLH domain of MYC1 is divergent from the other members of the bHLH IIIf subclade and MYC1 has been predicted not to bind DNA in contrast to TT8, GL3 and EGL3 (Carretero-Paulet *et al.*, 2010). In addition, GL3 and EGL3 can form hetero- and homodimers via their bHLH domain, whereas MYC1 is not able to form homodimers or to bind GL3 (Zhao *et al.*, 2012). In line with these results, unlike standard TFs, MYC1 predominantly localizes in the cytoplasm, and nuclear translocation of MYC1 can be mediated by TRY or CPC, while MYC1 relocates GL1 to the cytoplasm (Pesch *et al.*, 2013). Further research is needed to elaborate the specific role of MYC1 in the formation of WD-repeat-bHLH-MYB complexes involved in anthocyanin accumulation and trichome initiation.

Other bHLH TFs involved in JA signalling

JA signalling has been shown to have a positive role in freezing tolerance and to lead to an increased expression of cold-induced genes. Conversely, cold stress induces endogenous JA biosynthesis. This could be explained mechanistically by JAZ-mediated repression of the TFs INDUCER OF CBF EXPRESSION1 (ICE1) and ICE2, key regulators of freezing tolerance (Hu *et al.*, 2013). The ICE TFs belong to the bHLH IIIb subclade and can trigger C-REPEAT BINDING FACTOR (CBF)-mediated induction of downstream genes leading to freezing stress responses (Chinnusamy *et al.*, 2003; Fursova *et al.*, 2009). Interestingly, ICE1 and ICE2 both do not contain a JID and it has been shown that the C-terminal tail of ICE1, excluding the bHLH domain, is essential for interaction with JAZ1 and JAZ4 (Hu *et al.*, 2013). As demonstrated for TT8 and EGL3, this again indicates JID-independent interaction of bHLH-type TFs with JAZ proteins.

bHLH TRANSCRIPTION FACTORS INVOLVED IN JA-MEDIATED ELICITATION OF SPECIALIZED METABOLISM IN OTHER PLANTS

Plants use a plethora of defence mechanisms to protect themselves from different kinds of stresses. Many of these defence mechanisms are species specific and involve the production of specific specialized metabolites, many of which also have important characteristics for potential use in pharmacological or industrial applications. JA signalling triggers the production of multiple specialized metabolites (De Geyter *et al.*, 2012; Wasternack, 2014). In contrast to the species specificity of the metabolic pathway and the biosynthetic enzymes catalysing the reactions, the controlling TFs are often conserved among different plant species (De Geyter *et al.*, 2012; Zhou & Memelink, 2016). We will elaborate here on conserved bHLH-type TFs involved in JA-mediated elicitation of specialized metabolism in other plant species. An overview of all bHLH TFs discussed in this review is given in Table 1. A neighbour-joining tree was constructed using the alignment of the full amino acid sequences of the bHLH TFs to assign each bHLH TF to the correct clade and to show the evolutionary relationship with the Arabidopsis bHLH TFs discussed in the previous section (Fig. 2). One of the best-characterized TF complexes involving bHLH TFs in the regulation of bioactive, specialized metabolites is that regulating the different branches of the monoterpene indole alkaloid (MIA) biosynthesis pathway in the medicinal plant *Catharanthus roseus*, as depicted in Fig. 3 to illustrate the combinatorial action of bHLH TFs and other TFs in this process.

Table 1. Overview of JA-steered bHLH TFs and the metabolite classes they control.

bHLH TF	**Organism**	**Metabolite class affected**	**Reference**
MYC2	Arabidopsis thaliana	Flavonoids, glucosinolates	Dombrecht *et al.* (2007) Schweizer *et al.* (2013)
MYC3	Arabidopsis thaliana	Flavonoids, glucosinolates	Schweizer *et al.* (2013)
MYC4	Arabidopsis thaliana	Flavonoids, glucosinolates	Schweizer *et al.* (2013)
JAM1	*Arabidopsis thaliana*	Anthocyanins	Sasaki-Sekimoto *et al.* (2013)
JAM2	*Arabidopsis thaliana*	Anthocyanins	Sasaki-Sekimoto *et al.* (2013)
JAM3	*Arabidopsis thaliana*	Anthocyanins	Sasaki-Sekimoto *et al.* (2013)
JAM4	*Arabidopsis thaliana*	Anthocyanins	Sasaki-Sekimoto *et al.* (2013)
TT8	*Arabidopsis thaliana*	Anthocyanins	Gonzalez *et al.* (2008)
GL3	*Arabidopsis thaliana*	Anthocyanins	Gonzalez *et al.* (2008)
EGL3	*Arabidopsis thaliana*	Anthocyanins	Gonzalez *et al.* (2008)
MYC1	*Arabidopsis thaliana*	Anthocyanins	Zimmermann *et al.* (2004)

MYC2	*Catharanthus roseus*	Monoterpene indole alkaloids	Zhang *et al.* (2011)
BIS1	*Catharanthus roseus*	Monoterpene indole alkaloids	Van Moerkercke *et al.* (2015)
BIS2	*Catharanthus roseus*	Monoterpene indole alkaloids	Van Moerkercke *et al.* (2016)
bHLH1	*Nicotiana benthamiana*	Pyridine alkaloids	Todd *et al.* (2010)
bHLH2	*Nicotiana benthamiana*	Pyridine alkaloids	Todd *et al.* (2010)
MYC1a	*Nicotiana tabacum*	Pyridine alkaloids	Shoji and Hashimoto (2011)
MYC1b	*Nicotiana tabacum*	Pyridine alkaloids	Shoji and Hashimoto (2011)
MYC2a	*Nicotiana tabacum*	Pyridine alkaloids	Shoji and Hashimoto (2011)
MYC2b	*Nicotiana tabacum*	Pyridine alkaloids	Shoji and Hashimoto (2011)
MYC2	*Artemisia annua*	Sesquiterpene lactones	Shen *et al.* (2016)
MYC1	*Solanum lycopersicum*	Terpenes	Spyropoulou *et al.* (2014)
MYC2	*Solanum lycopersicum*	Steroidal glycoalkaloids	Cárdenas *et al.* (2016)
TcJAMYC1	*Taxus cuspidata*	Taxanes	Lenka *et al.* (2015)
TcJAMYC2	*Taxus cuspidata*	Taxanes	Lenka *et al.* (2015)
TcJAMYC4	*Taxus cuspidata*	Taxanes	Lenka *et al.* (2015)
TSAR1	*Medicago truncatula*	Non-haemolytic triterpene saponins	Mertens *et al.* (2016a)
TSAR2	*Medicago truncatula*	Haemolytic triterpene saponins	Mertens *et al.* (2016a)
MYC2a	*Salvia miltiorrhiza*	Tanshinones and phenolic acids	Zhou *et al.* (2016)
MYC2b	*Salvia miltiorrhiza*	Tanshinones and phenolic acids	Zhou *et al.* (2016)
MYC2	*Malus x domestica*	Anthocyanins	An *et al.* (2016)
bHLH1	*Coptis japonica*	Isoquinoline alkaloids	Yamada *et al.* (2015)
bHLH1-1	*Eschscholzia californica*	Isoquinoline alkaloids	Yamada *et al.* (2015)
bHLH1-2	*Eschscholzia californica*	Isoquinoline alkaloids	Yamada *et al.* (2015)
DPF	*Oryza sativa*	Diterpene phytoalexins	Yamada *et al.* (2015)

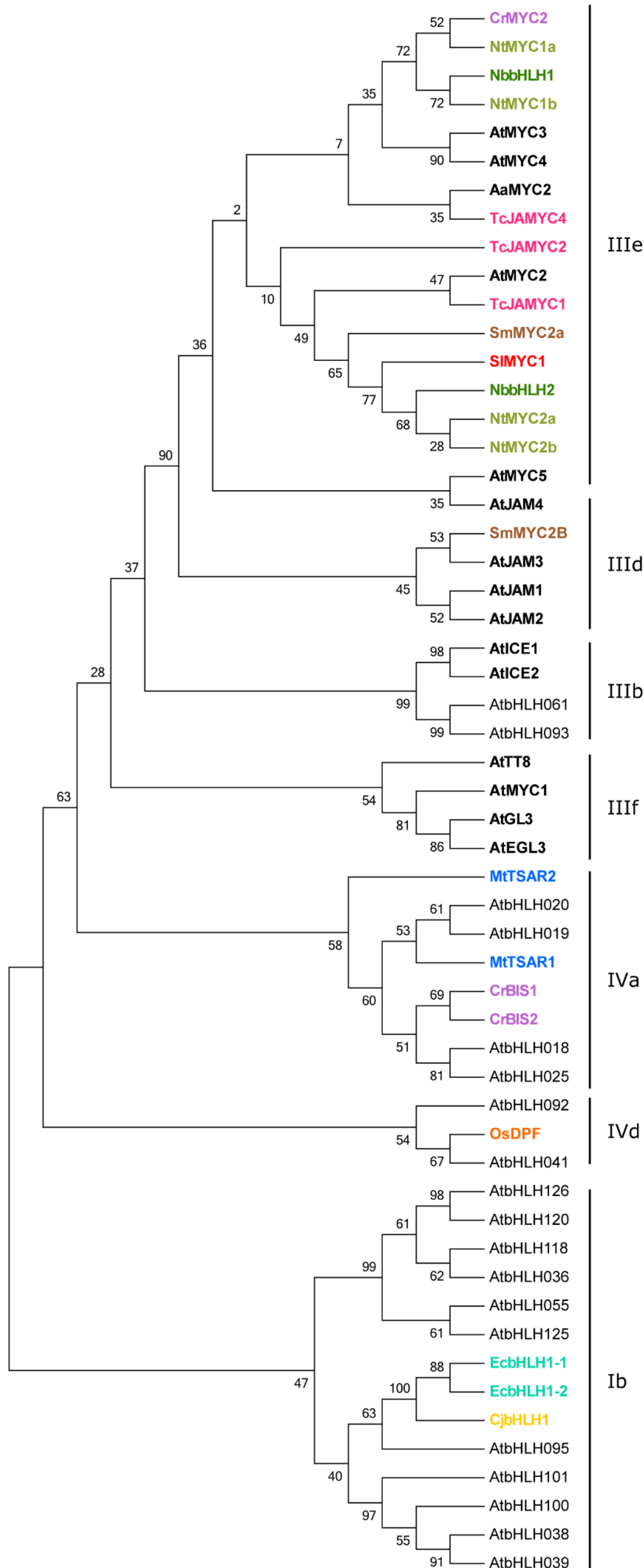
CrMYC2
NtMYC1a
NbbHLH1
NtMYC1b
AtMYC3
AtMYC4
AaMYC2
TcJAMYC4
TcJAMYC2
AtMYC2
TcJAMYC1
SmMYC2a
SlMYC1
NbbHLH2
NtMYC2a
NtMYC2b
AtMYC5
AtJAM4
SmMYC2B
AtJAM3
AtJAM1
AtJAM2
AtICE1
AtICE2
AtbHLH061
AtbHLH093
AtTT8
AtMYC1
AtGL3
AtEGL3
MtTSAR2
AtbHLH020
AtbHLH019
MtTSAR1
CrBIS1
CrBIS2
AtbHLH018
AtbHLH025
AtbHLH092
OsDPF
AtbHLH041
AtbHLH126
AtbHLH120
AtbHLH118
AtbHLH036
AtbHLH055
AtbHLH125
EcbHLH1-1
EcbHLH1-2
CjbHLH1
AtbHLH095
AtbHLH101
AtbHLH100
AtbHLH038
AtbHLH039
IIIe
IIId
IIIb
IIIf
IVa
IVd
Ib

Figure 2: Phylogenetic analysis of bHLH TFs with a function in JA signalling and/or the regulation of plant specialized metabolism. A neighbour-joining tree was constructed by aligning full amino acid sequences using the MEGA5 program (Tamura *et al.*, 2011). The Jones, Taylor and Thornton model was employed and the bootstrap analysis was carried out with 10 000 replicates (Jones *et al.*, 1992). The bHLH TFs with a proven function in JA-modulated processes are indicated in bold. Members from Arabidopsis (black) belonging to the subclades included in this tree with no proven role in JA signalling are indicated in normal font. Species other than Arabidopsis are shown in colour. Subclades are indicated on the right according to the classification system of Heim *et al.* (2003). Species abbreviations: Aa, *Artemisia annua*; At, *Arabidopsis thaliana*; Cj, *Coptis japonica*; Cr, *Catharanthus roseus*; Ec, *Eschscholzia californica*; Md, *Malus x domestica*; Mt, *Medicago truncatula*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; Sm, *Salvia miltiorrhiza*; Tc, *Taxus cuspidata*.

Subclade IIIe: the MYCs

Catharanthus roseus

Catharanthus roseus produces valuable MIAs such as the anti-cancer drugs vinblastine and vincristine (Almagro *et al.*, 2015; Dugé de Bernonville *et al.*, 2015). These are generated by the condensation of tryptamine with the monoterpene/iridoid compound secologanin to form strictosidine, which is further modified to give rise to several MIAs. All hitherto identified enzymes of the MIA biosynthesis pathway are induced by JA elicitation (van der Fits & Memelink, 2000; Van Moerkercke *et al.*, 2013). The subclade IIIe CrMYC2 is a JA-inducible TF that activates the expression of the ethylene response factor (ERF) *OCTADECANOID DERIVATIVE-RESPONSIVE CATHARANTHUS APETALA2-DOMAIN3* (*ORCA3*) and its homologue *ORCA2* (Fig. 3; Zhang *et al.*, 2011). ORCA3 has been identified as an activator of several enzyme-encoding genes needed to generate tryptamine, to perform the two-step conversion of loganic acid to secologanin and for various steps downstream of strictosidine (Fig. 3; van der Fits & Memelink, 2000). CrMYC2 activates the *ORCA3* promoter by binding a G-box-like sequence (Zhang *et al.*, 2011). RNAi silencing of *CrMYC2* in cell lines causes a reduction of *ORCA2* and *ORCA3* gene expression, which was even more dramatically pronounced when the lines were treated with JAs, and a decreased abundance of several MIA precursors. Overexpression of *CrMYC2* leads to the induction of *ORCA3* transcription and makes the lines hypersensitive to methyl jasmonate (MeJA). The effects on the abundance of MIA precursors were, however, not investigated (Zhang *et al.*, 2011).

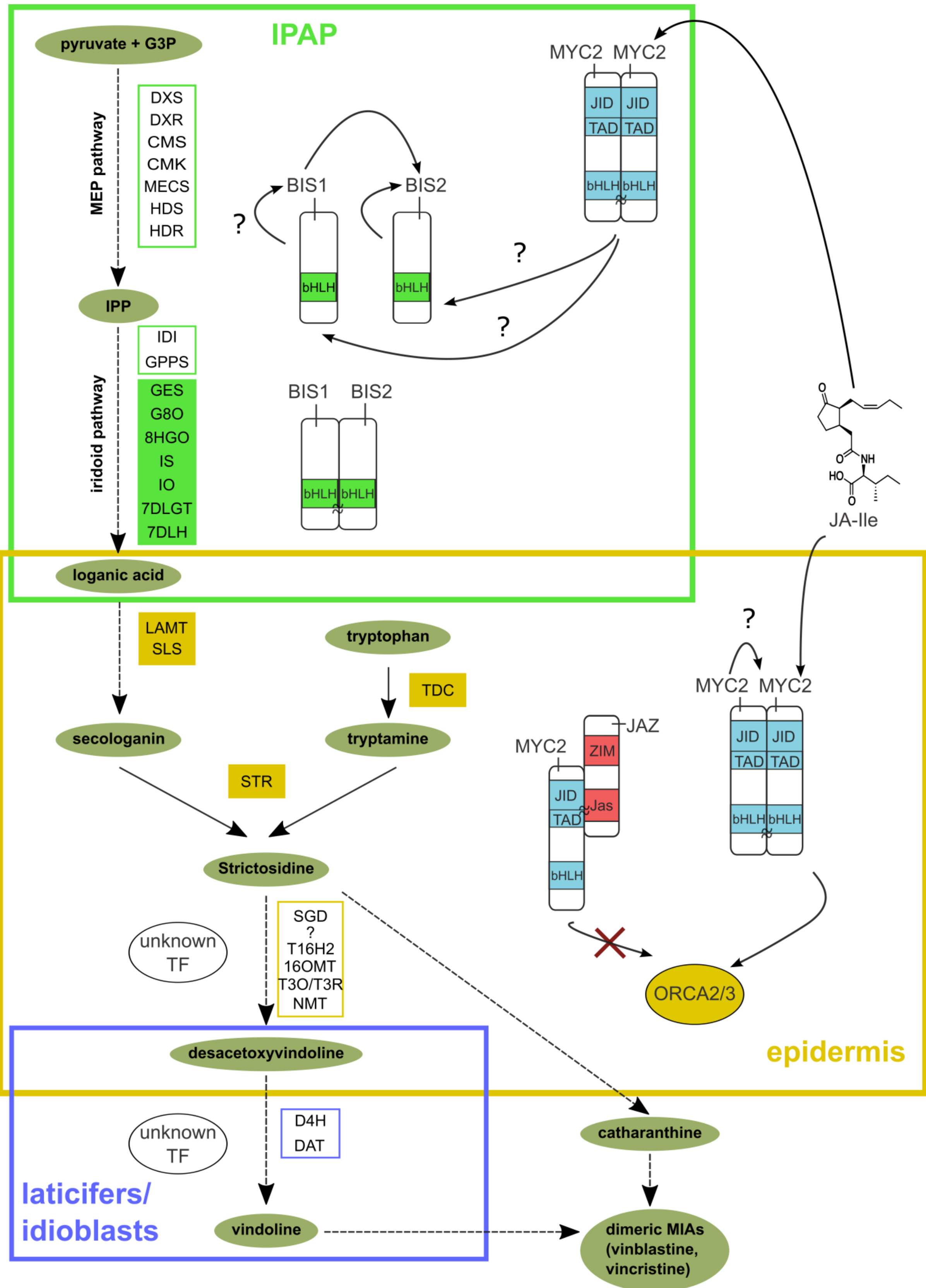
IPAP
pyruvate + G3P
MEP pathway
DXS
DXR
CMS
CMK
MECS
HDS
HDR
IPP
IDI
GPPS
iridoid pathway
GES
G8O
8HGO
IS
IO
7DLGT
7DLH
loganic acid
BIS1
BIS2
bHLH
?
MYC2
JID
TAD
JA-Ile
LAMT
SLS
tryptophan
TDC
secologanin
tryptamine
STR
Strictosidine
JAZ
ZIM
Jas
ORCA2/3
unknown TF
SGD
?
T16H2
16OMT
T3O/T3R
NMT
desacetoxyvindoline
epidermis
catharanthine
D4H
DAT
laticifers/ idioblasts
vindoline
dimeric MIAs (vinblastine, vincristine)

Figure 3: Combinatorial TF network that regulates specialized metabolism in the medicinal plant species *C. roseus*. The TF network is shown that regulates the different branches of the MIA biosynthesis pathway and that involves the bHLH TFs MYC2, BIS1 and BIS2 that genetically or physically interact with TFs from other families. Full and dashed straight arrows indicate enzymatic steps respectively. Curved arrows indicate regulatory activity of TFs. Question marks indicate possible (auto)-amplification loops. Enzymes marked in full green and yellow boxes are shown to be directly controlled by BIS and ORCA TFs, respectively. Enzymes boxed by green and yellow lines are expressed in internal phloem-associated parenchyma (IPAP) and epidermis cells, respectively, but whether they are also controlled by BIS and ORCA TFs remains to be determined. Abbreviations: CMK, 4-(CYTIDINE 50-DIPHOSPHO)-2C-METHYL-D-ERYTHRITOL KINASE; CMS, 4-(CYTIDINE 50-DIPHOSPHO)-2C-METHYL-D-ERYTHRITOL SYNTHASE; DAT, DEACETYLVINDOLINE 4-O-ACETYLTRANSFERASE; D4H, DESACETOXYVINDOLINE 4-HYDROXYLASE; 7DLGT, 7-DEOXYLOGANETIC ACID GLUCOSYL TRANSFERASE; 7DLH, 7-DEOXYLOGANIC ACID HYDROXYLASE; DXR, DXP REDUCTOISOMERASE; DXS, 1-DEOXY-DXYLULOSE-5-PHOSPHATE (DXP) SYNTHASE; GES, GERANIOL SYNTHASE; GPPS, GERANYL DIPHOSPHATE SYNTHASE; G8O, GERANIOL-8-OXIDASE; HDR, HYDROXYMETHYLBUTENYL 4-DIPHOSPHATE REDUCTASE; HDS, HYDROXYMETHYLBUTENYL 4-DIPHOSPHATE SYNTHASE; 8HGO, 8-HYDROXYGERANIOL OXIDOREDUCTASE; IO, IRIDOID OXIDASE; IDI, ISOPENTENYL DIPHOSPHATE ISOMERASE; IPP, isopentenyl diphosphate; IS, IRIDOID SYNTHASE; LAMT, LOGANIC ACID O-METHYLTRANSFERASE; MECS, 2C-METHYL-D-ERYTHRITOL-2,4-CYCLODIPHOSPHATE SYNTHASE; NMT, N-METHYLTRANSFERASE; 16OMT, 16-HYDROXYTABERSONINE O-METHYLTRANSFERASE; SGD, STRICTOSIDINE Β-GLUCOSIDASE; SLS, SECOLOGANIN SYNTHASE; TDC, TRYPTOPHAN DECARBOXYLASE; STR, STRICTOSIDINE SYNTHASE; T16H2 (CYP71D351), TABERSONINE 16-HYDROXYLASE 2; T3O, TABERSONINE 3-OXYGENASE; T3R, TABERSONINE 3-REDUCTASE.

Nicotiana species

Nicotiana species produce nicotine and related alkaloids, the biosynthesis of which is inducible by treatment of the roots with MeJA (Baldwin *et al.*, 1996; Goossens *et al.*, 2003). In general, production of tobacco alkaloids occurs exclusively in the roots after which they are transported to the leaves. The MYC2 homologues NbbHLH1 and NbbHLH2 have been shown to positively regulate nicotine production when their genes are overexpressed in *N. benthamiana*. Conversely, plants in which *NbbHLH1* or *NbbHLH2* were silenced only show a change in nicotine yields when they were treated with MeJA, suggesting that these bHLH TFs are only implicated in the defence-linked elicitation of the pathway and not in the regulation of the root-specific, 'default' activity (Todd *et al.*, 2010). NbbHLH1 and NbbHLH2 bind the G-boxes found in the *PUTRESCINE N-METHYLTRANSFERASE* (*PMT*) promoter, which steers expression of the first committed step in nicotine biosynthesis. The closely related species *N. tabacum* has a *NIC2* locus harbouring seven *ERF* genes that are all JA inducible and close homologues of the *C. roseus ORCA3*. Like ORCA3, these ERF TFs are also capable of activating the expression of multiple, in fact all, known nicotine biosynthesis genes (Shoji *et al.*, 2010). Furthermore, in tobacco, four bHLH IIIe subclade homologues could be retrieved: NtMYC1a and NtMYC1b group with NbbHLH1, and NtMYC2a and NtMYC2b group with NbbHLH2 (Shoji & Hashimoto, 2011). Also here, the NtMYC2s have been shown to bind the G-boxes in the promoters of various nicotine biosynthesis genes (Shoji & Hashimoto, 2011; Zhang *et al.*, 2012). Accordingly, transient expression assays have shown that NtMYC2b and *NIC2* locus

ERF TFs act synergistically in the transactivation of several pyridine alkaloid promoters (De Boer *et al.*, 2011; Shoji & Hashimoto, 2011). Simultaneous knock-down of both *NtMYC2s* mediates a strongly decreased expression of the nicotine biosynthesis genes and accordingly a dramatic reduction of pyridine alkaloids (Shoji & Hashimoto, 2011; Zhang *et al.*, 2012). In addition, all *NIC2* locus ERF TFs are downregulated in *NtMYC2* RNAi lines, which indicates that NtMYC2 plays a pivotal role in activating these *ERF* genes, just like CrMYC2 is paramount for the induction of *ORCA3* expression in *C. roseus*. In contrast, suppression of the *NIC2* locus ERF TFs does not affect *NtMYC2* gene expression levels. Like in Arabidopsis, NtMYC2 interacts with several JAZ repressor proteins. Without bioactive JAs, NtMYC2s are repressed by JAZ repressors (De Boer *et al.*, 2011; Shoji & Hashimoto, 2011; Zhang *et al.*, 2012). When *COI1* is silenced in tobacco plants, nicotine biosynthesis can no longer be elicited by MeJA, because the JAZ proteins cannot be degraded anymore, thereby illustrating the preservation of the core JA signalling machinery in *Nicotiana* species (Shoji *et al.*, 2008).

Artemisia annua

Artemisia annua or sweet wormwood constitutes the sole natural source of the broadly used anti-malaria drug artemisinin, a sesquiterpene lactone that is exclusively produced in the trichomes covering the aerial surfaces of the plant (Duffy & Mutabingwa, 2006; Covello, 2008; Maes *et al.*, 2011; Soetaert *et al.*, 2013; Muangphrom *et al.*, 2016). The *A. annua* MYC2 homologue AaMYC2 binds the G-box-like elements within the *TRANSARTEMISINIC ALDEHYDE Δ11 (13) REDUCTASE2* (*DBR2*) and *CYP71AV1* promoters, driving expression of these two genes encoding specific enzymes of the artemisinin pathway (Shen *et al.*, 2016). Accordingly, overexpression of *AaMYC2* leads to higher transcript levels of several biosynthesis genes and an increase in artemisinin levels. Silencing of *AaMYC2* causes the opposite effect and makes the plants also less sensitive to MeJA treatment (Shen *et al.*, 2016), pointing to the essential role of AaMYC2 in the JA signalling cascade, as in Arabidopsis. Furthermore, *AaMYC2* overexpression and silencing mediates also higher and lower stem anthocyanin accumulation, respectively, which further corresponds to its role Arabidopsis. Likewise, evidence for assembly of the conserved JA regulatory module has been obtained through binary interaction assays in yeast and tobacco, in which AaMYC2 was shown to interact with *A. annua* JAZ repressor proteins (Shen *et al.*, 2016).

Solanum lycopersicum

Two JA-responsive MYC2 homologues have been described in tomato (Boter *et al.*, 2004; Spyropoulou *et al.*, 2014). SlMYC1 has been shown to transactivate *Solanum lycopersicum* terpene synthase promoters in transient expression assays in *N. benthamiana* leaves. Furthermore, SlMYC1 has been found to act synergistically in combination with an earlier

identified zinc finger-like TF EXPRESSION OF TERPENOIDS1 (SlEOT1) in the expression of these promoters (Spyropoulou *et al.*, 2014).

Recently, an ORCA3 homologue has also been identified in tomato, called GLYCOALKALOID METABOLISM 9 (GAME9), which was shown to play an activating role in the regulation of the production of steroidal glycoalkaloids (SGA) in tomato organs (Cardenas *et al.*, 2016). GAME9 could for instance transactivate the *Δ(7)-STEROL-C5(6)-DESATURASE* (*C5-SD*) promoter in tobacco protoplasts. Interestingly, a synergistic transactivation effect of the *C5-SD* promoter was observed when GAME9 was combined with the MYC2 homologue SlMYC2. Transient expression assays and electrophoretic mobility shift assays (EMSAs) have shown that SlMYC2 binds the G-box in the *C5-SD* promoter (Cardenas *et al.*, 2016). Together, this indicates that genetic networks composed of bHLH IIIe subclade and ERF TFs play a conserved role in the JA-modulated activation of specialized metabolite pathways and employ conserved mechanistic routes to do so.

Salvia miltiorrhiza

Salvia miltiorrhiza or danshen is a widely used herb in Chinese medicine with proven health-promoting effects on patients suffering from cardiovascular and cerebrovascular diseases (Zhou *et al.*, 2005). The bioactive compounds of *S. miltiorrhiza* are tanshinone diterpenes and phenolic acids, such as tanshinone IIA and salvianolic acid B, the biosynthesis of which is inducible by MeJA in danshen hairy root cultures (Xiao *et al.*, 2009). Two MYC homologues, SmMYC2a and SmMYC2b, have been shown to be involved in this elicitation process (Zhou *et al.*, 2016). When either of them is silenced, gene expression levels of most tanshinone and phenolic pathway genes diminish and the total accumulation of tanshinones and phenolic acids decreases. Again, as in the species discussed above, SmMYC2a and SmMYC2b could recognize E-boxes in the promoters of pathway genes in EMSA studies and SmMYC2-SmJAZ repressor interaction could be demonstrated by Y2H (Zhou *et al.*, 2016).

Malus x domestica

In apples, the MdMYC2 was identified as a JA-inducible TF (An *et al.*, 2016). Y2H and pulldown assays have illustrated that MdMYC2 forms homodimers and complexes with MdJAZ2. An EMSA has demonstrated that MdMYC2 binds the G-box of the *AtJAZ3* promoter. Moreover, overexpression or silencing of *MdMYC2* in apple calli leads to an increased or decreased accumulation, respectively, of anthocyanin biosynthetic gene transcripts and anthocyanins, a process known to be regulated by JAs (An *et al.*, 2016).

Taxus cuspidata

In all of the above-mentioned plant species, MYC2 TFs execute a 'positive' regulatory role in the elicitation of specialized metabolism, as in Arabidopsis. In this regard, a recent publication reporting on the discovery of MYC2 homologues in the gymnosperm *Taxus cuspidata*, which is a source of the anti-cancer diterpene drug taxol, is somewhat discrepant (Lenka *et al.*, 2015). Taxol biosynthesis is also elicited by JAs and accordingly numerous reports demonstrate JA-responsiveness of the catalytic genes involved (Cusido *et al.*, 2014). In agreement with this, Lenka *et al.* (2015) have shown that promoters of seven taxol pathway genes are able to drive MeJA-inducible expression of a *β-glucuronidase* (*GUS*) reporter construct in transiently transformed *Taxus* cells, and again in line with the existing models, these promoters contain a large number of E-box sites. Unexpectedly however, the three MeJA-inducible MYC2-like bHLH TFs that were subsequently identified in *Taxus* display a negative rather than a positive regulatory role in taxol biosynthetic gene expression, at least when transiently overexpressed in *Taxus* cells (Lenka *et al.*, 2015). Whether this points to a divergent role of MYC2 TFs in *Taxus* and eventually gymnosperm species in general remains to be determined. It should be taken into account, though, that the TAD is only present in TcJAMYC1 and not in TcJAMYC2 and TcJAMYC4.

Subclade IVa

Catharanthus roseus

In addition to the JA-inducible IIIe subclade TF CrMYC2, two other JA-inducible bHLH TFs, but of subclade IVa, i.e. BHLH IRIDOID SYNTHESIS1 (BIS1) and BIS2, have been shown to be involved in the regulation of MIA biosynthesis in *Catharanthus roseus* (Van Moerkercke *et al.*, 2015; Van Moerkercke *et al.*, 2016). BIS1 and BIS2 act specifically on the iridoid branch of the MIA pathway (Fig. 3), which is localized specifically in the so-called internal phloem-associated parenchyma cells, as indicated by transient expression assays in tobacco protoplasts and *C. roseus* cells in which both TFs could specifically transactivate promoters of biosynthesis genes from the iridoid but not from other branches of the MIA pathway. BIS1 and BIS2 can form homo- and heterodimers, as shown in infiltrated *N. benthamiana* leaves, and in some cases act synergistically in transactivation assays (Van Moerkercke *et al.*, 2016). Accordingly, overexpression of *BIS1* or *BIS2* in stably transformed *C. roseus* cell cultures, as well as transient expression of *BIS2* in *C. roseus* flowers, leads to an elevated gene expression of the iridoid genes as well as of the methylerythritol-4-phosphate precursor pathway genes. Ultimately, this also boosts the accumulation of MIAs and several MIA intermediates (Van Moerkercke *et al.*, 2015; Van Moerkercke *et al.*, 2016). Conversely, silencing of *BIS1* in *C. roseus* hairy roots leads to decreased levels of MIAs, whereas *BIS2* silencing completely aborts the JA-inducible activation of the iridoid pathway genes and MIA

accumulation in transformed cell cultures, pointing to their essential role in the JA signalling cascade.
Furthermore, it has been shown that *BIS2* gene expression is induced by *BIS1* and *BIS2* overexpression, pointing to the existence of an amplification loop (Van Moerkercke *et al.*, 2016). Whether CrMYC2 may act in such a loop, as shown for *ORCA3* expression (Zhang *et al.*, 2011), remains to be determined. It should be noted that subclade IVa TFs can be clearly distinguished from MYC-type TFs, because they are about half their size, lack a JID and therefore cannot interact with the JAZ proteins, as shown in Y2H assays (Van Moerkercke *et al.*, 2015; Van Moerkercke *et al.*, 2016). Hence, an autoactivation mechanism, as shown for MYC2 in Arabidopsis, which can activate its own expression following release from JAZ repression (Pauwels *et al.*, 2008), is unlikely the cause for the JA-inducibility of the *C. roseus BIS* genes.

Medicago truncatula

The model legume *Medicago truncatula* or barrel medic produces a rich compendium of triterpene saponins, also in a JA-inducible fashion (Suzuki *et al.*, 2002; Pollier *et al.*, 2013). Two JA-inducible subclade IVa TFs, i.e. TRITERPENE SAPONIN BIOSYNTHESIS ACTIVATING REGULATOR1 (TSAR1) and TSAR2, specifically mediate the production of non-haemolytic and haemolytic saponins, respectively (Mertens *et al.*, 2016a). Overexpression of *TSAR1* and *TSAR2* in *M. truncatul*a hairy roots has shown increased accumulation of the respective saponin types and demonstrated that the mevalonate precursor pathway genes as well as the saponin-specific pathway genes were upregulated. In addition, transient expression assays in tobacco protoplasts and protein-binding micro-arrays have shown that both TSAR1 and TSAR2 recognize N-boxes (CACGAG, an E-box-like motif) in the saponin gene promoters.

The reported roles of the BIS and TSAR TFs put forward a recurrence of subclade IVa TFs in the regulation of terpene biosynthesis and suggest that they may occupy orthologous positions in the JA signalling cascade in different plant species, although activating different metabolite pathways. The only other bHLH subclade IVa TFs that have been characterized to some extent are bHLH20/NAI1 and bHLH25 from Arabidopsis. The latter is involved in the protection against nematodes (Jin *et al.*, 2011), whereas NAI1 is essential for the formation of endoplasmatic reticulum (ER) bodies, which are JA-induced ER-derived organelles that have only been described in the plant order of Brassicales, where they are ubiquitous in roots and seedlings (Matsushima *et al.*, 2004; Ahn *et al.*, 2010). A link between Arabidopsis terpene biosynthesis and Arabidopsis bHLH subclade IVa TFs, the expression of which is also JA-inducible, has not yet been investigated.

Subclade IVd and Ib

Oryza sativa

Rice produces a variety of diterpene phytoalexins, which are positively regulated by a JA-inducible bHLH IVd subclade TF, i.e. DITERPENOID PHYTOALEXIN FACTOR (DPF) (Yamamura *et al.*, 2015). Overexpression or knockdown of *DPF*, respectively, confers an increased or decreased diterpene accumulation and expression of many biosynthetic genes. DPF transactivates the promoters of *COPALYL DIPHOSPHATE SYNTHASE2* (*CPS2*) and C*YTOCHROME P450 MONOOXYGENASE 99A2* (*CYP99A2*) in transient expression assays, which are at least partly regulated through the presence of an N-box in the promoter sequence (Yamamura *et al.*, 2015). A link between bHLH IVd subclade TFs and JA signalling in Arabidopsis or other plant species has not yet been reported, and hence it remains to be determined whether these TFs have a specific role in rice and/or other monocot species or have a conserved role in JA signalling in the plant kingdom.

Coptis japonica and *Eschscholzia californica*

Coptis japonica is a producer of the isoquinoline alkaloid (IQA) berberine. A JA-inducible bHLH TF belonging to subclade Ib, i.e. CjbHLH1, has been identified. When this gene is silenced in *C. japonica* protoplasts, gene expression of many berberine biosynthesis genes is decreased. Accordingly, CjbHLH1 transactivates the promoters of the *3'-HYDROXY-N-METHYLCOCLAURINE-4'-O-METHYLTRANSFERASE* (*4'OMT*) and *CYP719A1* genes and chromatin immunoprecipitation has shown binding to these promoters (Yamada *et al.*, 2011). Likewise, in *Eschscholzia californica* or California poppy, the EcbHLH1-1 and EcbHLH1-2 homologues of CjbHLH1 are also inducible by MeJA (Yamada *et al.*, 2015). California poppy produces sanguinarine and shares part of the pathway with berberine. RNAi-mediated silencing of *EcbHLH1-1* and *EcbHLH1-2* results in a decreased expression of some IQA biosynthesis genes, as well as a decreased sanguinarine production in stably transformed cell lines (Yamada *et al.*, 2015). Although a link between bHLH Ib subclade TFs and JA signalling has not yet been reported in Arabidopsis, the fact that these TFs have a role in the regulation of IQA biosynthesis in distinct species from the Ranunculaceae (*C. japonica*) and Papaveraceae (*E. californica*) may point to a broader and conserved role in JA signalling in the plant kingdom, like for the bHLH IVa subclade TFs.

PERSPECTIVES

The Arabidopsis MYC TFs belong to the bHLH IIIe subclade and are central regulators that connect the JA core machinery to the downstream JA-induced responses. Evidence is accumulating that orthologues of MYC TFs can interact with JAZ repressors and are involved in the JA-mediated elicitation of the biosynthesis of specialized metabolites across the plant

kingdom, pointing to the conserved role of this major bHLH hub in JA signalling. Likewise, a similar and conserved role for bHLH IIIf subclade TFs in the JA-steered regulation of anthocyanin biosynthesis is conceivable. Also members of the bHLH IVa and Ib subclades have been shown to be involved in the JA-modulated regulation of specialized metabolism in at least two distinct species. Whether these bHLHs have a similar, widespread and conserved role as for instance MYC2 in JA signalling remains to be determined, but it is plausible.

Together, these findings indicate that TFs regulating biosynthetic metabolic pathways are in many cases conserved among different species, in contrast to the downstream target genes encoding the enzymes of specialized metabolism, which are often species-specific. Generally, these, often themselves JA-inducible, TFs control the expression of multiple biosynthetic enzymes of one particular specialized pathway in a coordinated manner, guaranteeing an optimized fine-tuning of the flux through the pathway and reprogramming of general metabolism. Hence, these TFs can be considered as suitable candidates for metabolic engineering of plants. Given their conserved role, a TF from a particular plant species may be used in a different, not necessarily related, plant species (Mertens *et al.*, 2016b). Consequently, fundamental research in model plants may be translatable into other plant species that produce specialized metabolites of interest for plants or humans. A recent illustration is the functional equivalence of the clade IVa TFs BIS1 and BIS2 from *C. roseus* and TSAR1 and TSAR2 from *M. truncatula*. *BIS1* could switch on the production of non-haemolytic saponins when ectopically expressed in *M. truncatula* hairy roots, whereas, conversely, ectopic expression of *TSAR1* and *TSAR2* in *C. roseus* petals copied the effect of BIS1 on the MIA pathway. This was shown to be due to the recognition of the same cis-elements (Mertens *et al.*, 2016b). Hence, we believe that the implementation of specific cis-elements may be an evolutionary strategy to recruit conserved TFs to control species-specific specialized metabolism pathways. Therefore, findings from a particular crop or medicinal plant species may be translatable into other, not necessarily closely related plant species, as already evidenced from the research described in the previous sections of this review. Further elucidation of the mechanistic regulation and modus operandi of Arabidopsis bHLH TFs, for instance, may provide novel avenues for directed engineering of TFs that may allow tweaking the JA response, and, ultimately, growth- and defence-related processes, to increase the yield of high-value molecules.

Additionally, conservation of the JA signalling module and the related downstream TFs could allow the interspecific use of small molecules to activate or block particular responses (Fonseca *et al.*, 2014b). For instance, coronatine-*O*-methyloxime and jarin-1 are chemical inhibitors of respectively the JA receptor and a biosynthetic enzyme of JA in Arabidopsis (Meesters *et al.*, 2014; Monte *et al.*, 2014). Likely, the use of these compounds in other species will also lead to inhibition of multiple JA responses. Still, the challenge will be to only

target specific responses, e.g. production of a particular type of specialized metabolites, by compounds that interfere with the function of involved TFs. This principle has already been demonstrated in the human field, where the naturally occurring triterpenoid celastrol and its derivatives could inhibit DNA binding of the oncoprotein c-Myc and its heterodimerization partner, Max, by modulating the structure of the c-Myc-Max heterodimer (Wang *et al.*, 2015).

So, in addition to the mere gene discovery, i.e. identifying which specific bHLH TFs are involved in a particular JA-modulated process, where should fundamental research on bHLH TFs head for? Our current knowledge of bHLH TFs indicates already some interesting tracks. For instance, the formation of heterodimers seems to play an important role in the activity of the different bHLH TF complexes and the specificity of their function. Heterodimerization is not limited to TFs of the same bHLH subclade but also occurs beyond bHLH subclades and even beyond family borders. Although many examples of such protein interactions have been described, undoubtedly, many more remain to be discovered. For example, Arabidopsis MYC2 has been shown to interact with TFs from other families such as ETHYLENE INSENSITIVE3 (EIN3) (Song *et al.*, 2014) and MYB factors that regulate glucosinolate biosynthesis (Schweizer *et al.*, 2013), and has been predicted to interact with multiple other bHLH-type TFs from different subclades, such as ICE1, JAM2, ALCATRAZ (ALC) and PHYTOCHROME INTERACTING FACTOR4 (PIF4) (Chen YA *et al.*, 2012). This was supported by MaICE1 in banana (*Musa acuminate)* that interacts with MaMYC2a and MaMYC2b (Zhao *et al.*, 2013). Likewise, the bHLH IIIf subclade TFs can incorporate in trimeric complexes with the WD-repeat protein TTG1 and R2R3 MYB TFs (Xu *et al.*, 2015; Brkljacic & Grotewold, 2017). It stands beyond doubt that combinatorial action of related or distinct TFs in multi-protein complexes will account for much of the observed aspects of the regulation of plant specialized metabolism, including the tissue- or cell-specific localization of the biosynthetic pathways. Combinatorial action of TFs does not imply physical interaction between proteins only. The above-discussed evidence from tomato and tobacco for instance, indicates that promoters of pathway genes harbour multiple cis-elements that allow the simultaneous docking of multiple TFs that do not necessarily interact physically. Indeed, recent research has shown that human TFs can cooperatively bind cis-acting elements to regulate transcription (Jolma *et al.*, 2015). The majority of the TF pairs identified in this study could not be explained by simple protein-protein interaction but rather by DNA-mediated cooperativity. As such, cooperating TFs might be overlooked during investigation of JA-related bHLH TFs in Arabidopsis, because previous studies have relied mainly on JA inducibility and cooperating TFs, including JA-related bHLH TFs, would not necessarily need to be JA inducible. Likewise, the necessity for post-translational modifications might lead to loss of interaction between TFs in widely applied screening methods like Y2H. Further

research in Arabidopsis and other species with the focus on cooperating TFs could lead to the identification of new TFs involved in JA signalling and expand our current mechanistic understanding of JA signalling.

The advanced knowledge of the regulation of the activity of the trimeric complexes involving the bHLH IIIf subclade TFs may provide profound inspiration for further research in the JA field. Indeed, in Arabidopsis, there are only four bHLH IIIf subclade TFs, but they regulate many distinct processes through myriad additional regulatory elements and processes (Brkljacic & Grotewold, 2017). These include the already mentioned modular architecture of gene regulatory regions and formation of specific TF complexes that affect DNA-binding affinity and specificity. But more features are involved such as chromatin remodelling through histone post-translational modifications, chromatin looping, post-translational modifications of the TFs themselves, the occurrence of alternative splicing, and interaction with small interfering peptides or metabolites. Most of these aspects have hardly been touched in the JA field to date, leaving many unexplored research avenues for numerous research groups.

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II

Results

Chapter 1

Interaction analysis of JA-related TFs via tandem affinity purification

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[1]Author contributions: Molecular cloning, transformation cell suspension cultures, tandem affinity purification, Y2H assay, immunoblotting, data interpretation and writing (all text and figures).

ABSTRACT

Plants make a whole diverse array of specialized metabolites that are important for their development and defence. Jasmonate signalling is involved in many of these processes and triggers conserved central transcription factors (TFs) to induce the biosynthesis of these bioactive compounds, which have important properties that are of general interest for humanity. However, overexpression of these central TFs is often not sufficient to enhance the downstream response. Here, we introduce the concept of changing the interaction behaviour of JA-related TFs in *Arabidopsis thaliana*, in order to modulate their activity and thereby stimulate specialized metabolism in plants. The initial step in this process is the characterization of the interactome of TFs of interest. For this, we applied the tandem affinity purification technique on four TFs (putatively) involved in jasmonate signalling: the R2R3 MYB TFs MYB2 and MYB108/BOS1, and the bHLH TFs NAI1 and MYC2. The acquired knowledge then can be used to rationally modify interactions with regulatory proteins to increase the activity of the TFs, in order to mediate enhanced jasmonate-related responses. The fact that central TFs like MYC2 have a conserved function in the plant kingdom might allow these 'hyperactive' TFs to be used for metabolic engineering of medicinal plants In the future. Here, no interactors could be found for MYB108/BOS1, but we could confirm known interactors of MYC2. In addition, we identified PAL1 as interactor of MYB2, although this interaction could not be confirmed by yeast two-hybrid.

INTRODUCTION

Jasmonate (JA) signalling is important for many developmental and defence-related processes in plants and often goes hand in hand with the production of specialized metabolites (Chapter I.1; Wasternack & Hause, 2013; Goossens *et al.*, 2016a). Many of these bioactive compounds possess important characteristics for potential use in pharmacological or industrial applications (De Geyter *et al.*, 2012; Wasternack, 2014). The path towards JA-elicited production of specialized metabolites, consists on the one hand of a very conserved primary signalling pathway and on the other hand of a more species-specific specialized metabolism, involving biosynthetic enzymes. The central bridge between the primary and specialized pathway is generally regulated by transcription factors (TFs) which, directly or indirectly, steer expression of different enzymes involved in a particular metabolic biosynthetic branch (Chapter I.2; De Geyter *et al.*, 2012; Goossens *et al.*, 2016b; Zhou & Memelink, 2016). Therefore, directing the focus of metabolic engineering on conserved TFs can open avenues in the quest for increased production of specialized metabolites of human interest. As such, basic research can be performed on model plants, like *Arabidopsis thaliana* and *Medicago truncatula*, to translate the gathered knowledge into medicinal plants or crops. For instance, the *Catharanthus roseus* orthologue of Arabidopsis MYC2, the central TF

in JA signalling, is involved in the production of the bioactive monoterpene indole alkaloids (MIAs) (Zhang *et al.*, 2011), such as vinblastine and vincristine, two compounds with particular human interest for their anti-tumour properties (Almagro *et al.*, 2015). However, the natural accumulation of MIAs is very low (Almagro *et al.*, 2015) and their chemical synthesis is not very evident (Zhou *et al.*, 2009), which make them expensive. Therefore, knowledge of Arabidopsis MYC2 could ameliorate metabolic engineering of the alkaloid pathway in *C. roseus*.

TFs are the central regulators of the specialized metabolism in plants and targeting these TFs for metabolic engineering avoids the necessity to characterize all the different enzymatic steps of a biosynthetic pathway, which can be very time-consuming and complex (Gantet & Memelink, 2002). Nevertheless, overexpression of TFs is in general not sufficient to increase the production of specialized metabolites due to complex post-translational regulation. Regulatory mechanisms that can change the activity and stability of TFs are mainly executed by protein-protein interactions. Identification of such interacting regulatory proteins can help to characterize TF complexes in order to modulate their activity by changing the involved interactions. Tandem affinity purification (TAP) coupled to mass spectrometry (MS) in Arabidopsis cell suspension cultures is a convenient method to identify interactors of proteins and has proven ability to characterize whole interaction networks, e.g. the cell cycle interactome (Van Leene *et al.*, 2010). Moreover, novel regulatory mechanisms have been revealed using TAP-MS (Pauwels *et al.*, 2010). The interaction behaviour of TF complexes can then be manipulated, e.g. by the insertion of point mutations, in order to modulate their activity. As such, TFs with increased activity can be generated, leading to an enhanced downstream response. Taking into account the conservation of central TFs involved in the regulation of plant specialized metabolism, the generation of 'hyperactive' TF constructs in Arabidopsis can be an appropriate tool to be applied in medicinal plants or crops to aim for an increased production of specialized metabolites.

Here, we performed TAP-MS analysis on four JA-related TFs: the two R2R3 MYB proteins, MYB2 and MYB108/BOTRYTIS SUSCEPTIBLE1 (BOS1), and the two bHLH-type TFs, NAI1 and MYC2. MYC2 was included in this analysis to validate the TAP-MS analysis, as many interactors of MYC2 have already been identified before (Kazan & Manners, 2013).

RESULTS

For all baits analysed here, Arabidopsis cell cultures were transformed with two bait constructs, N- or C-terminally tagged with GSrhino (Van Leene *et al.*, 2015), brought under the strong *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter. Low protein levels in TAP cell cultures is a commonly recurring problem for unstable MYB TFs due to ubiquitin-mediated 26S proteasomal degradation. To anticipate to this problem, we also generated fusion

constructs of our MYB baits with a ubiquitin-associated2 (UBA2) domain (Jang *et al.*, 2012). This domain originates from RADIATION SENSITIVE23A (RAD23A), a ubiquitin receptor delivering ubiquitinated proteins to the 26S proteasome (Farmer *et al.*, 2010). Ubiquitin receptors themselves are protected from proteasomal degradation by the presence of such UBA domains (Heessen *et al.*, 2005). Jang *et al.* (2012) showed enhanced stability for two unstable TFs, LONG HYPOCOTYL IN FAR-RED1 (HFR1) and PHYTOCHROME INTERACTING FACTOR3 (PIF3), and the unstable JA-related repressor JA-ZIM DOMAIN10 (JAZ10), when they were fused to an UBA domain. The use of this technique increased stability of the fused proteins without affecting their biological activity and did not have any general effects on the proteome or the plant (Jang *et al.*, 2012), in contrast to alternative techniques to stabilize proteins such as MG132 treatment, which inhibits the activity of the 26S proteasome.

To verify the effect of JA on the interaction behaviour of the JA-related TFs, the cell cultures were treated with 50 µM JA or with DMSO. JA signalling, and the consequent degradation of the JAZ proteins, happens very fast after JA treatment (Chapter II.4; Pauwels *et al.*, 2015) and the baits are thought to function in JA primary signalling. To get an optimal 'snapshot' of the JA-induced interaction behaviour of the baits, treatments were performed for 30 minutes.

NAI1

NAI1 is a JA-inducible bHLH-type TF that is involved in the formation of endoplasmatic reticulum (ER) bodies (Matsushima *et al.*, 2004). These specialized organelles are ER-derived structures that are widespread in seedlings and occur in the roots of mature plants but can specifically be observed in rosette leaves after wounding or methyl JA (MeJA)-treatment (Matsushima *et al.*, 2002). ER bodies accumulate β-glucosidases, which can hydrolyse specialized metabolites to form active products during plant defence (Yamada *et al.*, 2011). In addition, NAI1 is a very close homologue of the *C. roseus* bHLH IRIDOID BIOSYNTHESIS1 (CrBIS1), recently characterized in our group as an important transcriptional regulator in the biosynthetic pathway of MIAs in *C. roseus* (Van Moerkercke *et al.*, 2015). Altogether, these observations suggest a role for NAI1 in the JA-modulated specialized metabolism in Arabidopsis and therefore NAI1 was included in our TAP-MS analysis.

Only the Arabidopsis cell cultures overexpressing the C-terminally tagged NAI1 construct led to detectable protein levels after immunoblotting (Fig. 1). Therefore, TAP-MS analysis was performed only on the cell cultures expressing this construct. Unfortunately, we could only identify the bait protein NAI1 (Table 1).

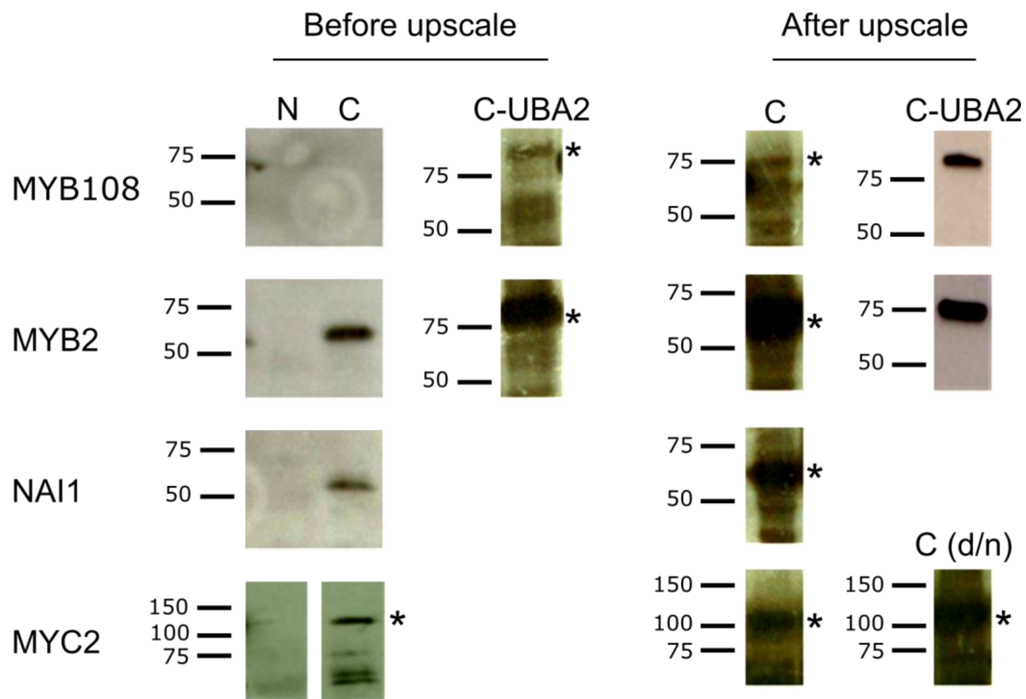

Figure 1: Protein expression analysis of TAP-tagged constructs. NAI1, MYB108, MYB2 and MYC2 were N- and C-terminally tagged with the GS^{rhino} or UBA2-GS^{rhino} TAP tag and overexpressed in Arabidopsis cell suspension cultures. All cultures were grown in the dark except for one MYC2-overexpressing culture that was grown in a day/night regime (indicated by d/n). Protein expression analysis was performed before upscale or after mock treatment of the upscaled cultures. Proteins were detected using the peroxidase anti-peroxidase immune complex during immunoblotting. If multiple protein bands are visible in one blot, the correct band is marked with an asterisk. Abbreviation: UBA2, ubiquitin-associated2.

The R2R3 MYB proteins MYB2 and MYB108/BOS1

MYB2 is an R2R3 MYB TF involved in several processes like drought stress, senescence in the late plant development and phosphate starvation (Abe *et al.*, 2003; Guo & Gan, 2011; Baek *et al.*, 2013). Furthermore, our group has shown that the expression of the *Medicago truncatula* orthologue of MYB2, ACTIVATIONAL REGULATOR OF METHYL ANTHRANILATE1 (AROMA1), was induced by MeJA in *M. truncatula* root cell suspension cultures (De Geyter, 2014). In *M. truncatula* hairy roots, AROMA1 induces the production of methyl anthranilate, which has a role in defence against pathogens and insects (Chambers *et al.*, 2013; Bernklau *et al.*, 2016). Furthermore, anthranilate is an intermediate in Trp biosynthesis, leading to the production of specialized metabolites (Maeda & Dudareva, 2012). MYB108/BOS1 is a close homologue of MYB2 and is known to play a role in multiple JA-regulated responses, such as stamen and pollen maturation (Mandaokar & Browse, 2009) and defence against abiotic and biotic stress (Mengiste *et al.*, 2003). Furthermore, in both processes, expression of *MYB108/BOS1* was dependent on JA signalling.

Table 1: Results of TAP-MS analysis of the different baits.

NAI1			MYB108			MYB108-UBA2			MYB2			MYB2-UBA2		
CTAP	# +JA	# mock	CTAP	# +JA	# mock	CTAP	# +JA	# mock	CTAP	# +JA	# mock	CTAP	# +JA	# mock
NAI1 (bait)	2	2	/	/	/	MYB108 (bait)	2	2	MYB2 (bait)	2	2	MYB2 (bait)	2	2
												PAL1	2	2

MYC2			MYC2-d/n cycle		
CTAP	# +JA	# mock	CTAP	# +JA	# mock
MYC2 (bait)	2	2	MYC2 (bait)	2	2
MYC3	2	1	MYC3	2	1
MYC4	1	1	MYC4	2	2
NINJA	1	1	NINJA	2	1
JAZ10	1	1	JAZ10	/	1
JAZ12	2	2	JAZ11	2	/
			JAZ12	2	2

[1]For each bait, cell cultures were treated for 30 min with JA or DMSO (mock) and TAP analysis was performed in duplicate representing technical repeats. The number of times a prey was recovered out of these two repeats is indicated (#). Abbreviation: d/n = day/night.

The protein levels of the cultures overexpressing the MYB proteins, tagged N- or C-terminally with the GS^{rhino} tag, were analysed. Immunoblotting could only detect C-terminally tagged MYB2 protein (Fig. 1). No MYB108/BOS1 proteins could be detected. However, a second expression analysis performed on upscaled cell cultures showed the presence of C-terminally tagged MYB108/BOS1 (Fig. 1). Only the bait protein MYB2 could be identified after TAP-MS analysis and no interactors were found for both MYB TFs. The MYB108/BOS1 bait protein could even not be detected in this TAP-MS analysis (Table 1).

In contrast to wild-type MYB108/BOS1, the UBA2-fusion construct of MYB108/BOS1 could be detected via immunoblotting before upscale of the cultures (Fig. 1). Moreover, MYB108/BOS1 bait protein could be retrieved during TAP-MS analysis, although no prey proteins could be identified (Table 1). Analysis of the cell cultures overexpressing MYB2-UBA2 led to the identification of one putative interactor, the enzyme PHENYLALANINE AMMONIA LYASE1 (PAL1) (Table 1). PAL1 is induced by various abiotic or biotic stresses and catalyzes the first step in the phenylpropanoid pathway leading to flavonoids, lignin and other specialized metabolites (Olsen *et al.*, 2008; Huang *et al.*, 2010). However, we could not demonstrate direct interaction between MYB2 and PAL1 by yeast two-hybrid (Y2H) analysis (Fig. 2).

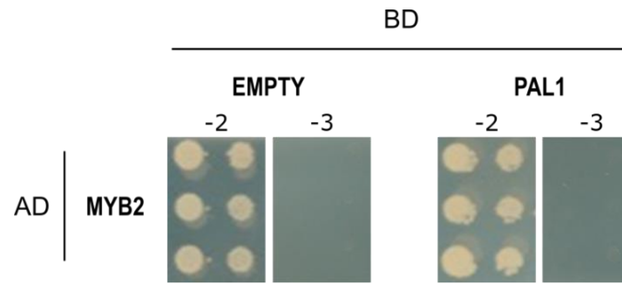

Figure 2: PAL1 and MYB2 do not interact in Y2H analysis. MYB2 fused to GAL4-AD was tested for interaction with PAL1 fused to GAL4-BD. Empty vector was used as negative control. Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium.

MYC2

MYC2 is the master TF of the JA signalling core and positively regulates the JA response (Chapter I.2; Kazan & Manners, 2013; Goossens *et al.*, 2016b). The interactome of MYC2 has been studied well over the last decade and TAP-MS analysis has been performed before on Arabidopsis cell suspension cultures overexpressing tagged MYC2 (Geerinck, 2010; Fernández-Calvo *et al.*, 2011). However, with the arrival of an improved TAP toolbox, i.e. a refined bait tag and optimized MS analysis (Van Leene *et al.*, 2015), TAP-MS analysis became more accurate and sensitive. Therefore, we re-analysed the interaction network of MYC2 using this improved TAP-MS method. MYC2 was tagged as described for NAI1 and transformed in Arabidopsis cell suspension cultures. Like the other tested baits, only the C-terminally tagged MYC2 construct could be detected via immunoblotting (Fig. 1) and TAP-MS analysis was only performed on these cell cultures. MYC2 protein stability is negatively regulated by dark and protein expression follows a circadian rhythm with highest levels during the day (Shin *et al.*, 2012; Chico *et al.*, 2014). However, TAP cell cultures are normally grown in continuous dark. Therefore, to reveal possible dynamic complex assembly, cell cultures expressing C-terminally tagged MYC2 were also grown in a day/night cycle and harvested during the day.

TAP-MS analysis led to the identification of the usual suspects; MYC3, MYC4, the JAZ proteins and NOVEL INTERACTOR OF JAZ (NINJA); although it was the first time MYC4 was detected in a TAP of MYC2. Furthermore, former TAP analysis could only detect JAZ10 after JA treatment whereas now JAZ10, JAZ11 and JAZ12 could be detected before and after JA treatment. In contrast to previous TAP experiments, JAZ2 could not be identified (Geerinck, 2010; Fernández-Calvo *et al.*, 2011; Table 1). All these confirmed interactors are part of the MYC2-related JA core machinery. MYC3 and MYC4 are close homologues of MYC2 and also positively regulate the JA response. Homo- and heterodimerization among MYC2, MYC3 and MYC4 have been suggested before via TAP and co-immunoprecipitation experiments (Fernández-Calvo *et al.*, 2011). Y2H analysis demonstrated that the MYC TFs each can

directly bind to the JAZ repressors (Fernández-Calvo *et al.*, 2011), which in turn can directly interact with NINJA (Pauwels *et al.*, 2010).

DISCUSSION

The activity of TFs is determined by multiple post-translational regulatory mechanisms, mostly conferred by protein-protein interaction. Hence, the characterization of the interaction network of TFs also provides information about the regulation of the concerned TFs. TAP-MS analysis is an appropriate technique for this as it performs a proteome-wide screen to detect interactors of a bait. In Arabidopsis cell cultures, this tool has been proven to perform well and the TAP toolbox is still being optimized to obtain even better results (Van Leene *et al.*, 2015). We extended this toolbox by introducing the UBA2-tag to decrease the turnover of unstable baits.

We performed TAP-MS analysis on four baits with a (potential) link to JA-involved defence and the related production of specialized metabolites: NAI1, MYB2, MYB108/BOS1 and MYC2. Unfortunately, no interactors were found for NAI1, MYB2 and MYB108/BOS1. Protein expression of MYB108/BOS1 could even not be detected via immunoblotting of the TAP cultures and TAP-MS analysis did not detect any MYB108/BOS1 bait protein. As instability of MYB proteins is a common problem during TAP experiments, we fused the UBA2 domain to MYB2 and MYB108/BOS1. The UBA2-fusion construct of MYB108/BOS1 in cell suspension cultures could readily be detected via immunoblotting and was also identified during TAP-MS analysis. In addition, PAL1 was picked up as interactor for MYB2 in the TAP-MS analysis of cell suspension cultures overexpressing the MYB2-UBA2 fusion construct.

Proteins identified during TAP-MS analysis are potential interactors of the bait proteins. To confirm these interactions and verify if the interactions are direct, a complementary method is necessary. Furthermore, in order to change the interaction behaviour of the bait TFs, and with this their activity, a suitable system is needed to easily and quickly assess interaction changes. Y2H is an appropriate method to detect direct interactors and allows identification of the domains responsible for the interaction between proteins using point mutations or deletion constructs. Unfortunately, interaction between PAL1 and MYB2 could not be confirmed via Y2H analysis.

Eventually, we endeavour to generate a 'hyperactive' TF by the change of its interaction behaviour. Therefore, MYC2 was included in our analysis as it is a well-described positive regulator of JA signalling. TAP-MS analysis of MYC2 led to the identification of known interactors, such as the JAZ repressors. Direct interaction between JAZ repressors and MYC2 has been shown before via Y2H (Chini *et al.*, 2007) and occurs via the JA-associated domain of JAZ (Melotto *et al.*, 2008) and the JAZ-interacting domain (JID) of MYC2 (Fernández-Calvo

et al., 2011). This knowledge can be used to generate a 'hyperactive' MYC2 by e.g. manipulating the JID of MYC2 to lose interaction with the JAZ repressors (Chapter II.2; Goossens *et al.*, 2015). Transformation of such a 'hyperactive' Arabidopsis construct into crops or medicinal plants could verify the genericity of 'hyperactive' TFs and could potentially mediate an increase in the production of specialized metabolites.

MATERIALS AND METHODS

Gene cloning

All cloning was carried out by Gateway® recombination (Thermo Fisher Scientific, Waltham, MA, USA). Baits were amplified from Arabidopsis cDNA using GoTaq® DNA polymerase (Promega, Fitchburg, WI, USA) and Gateway®-specific primers. PCR products were cloned in pDONR221 using BP Clonase® II enzyme mix (Thermo Fisher Scientific). The resulting entry vectors together with pEN-L4-NGS^{rhino}-R1 or pEN-L4-2-R1 and pEN-R2-GS^{rhino}-L3 (or pEN-R2-UBA2-GS^{rhino}-L3) were used to clone the baits into, respectively, the pK7m24GW2 (for N-terminal tags) or the pKCTAP (for C-terminal tags) destination vector (Van Leene *et al.*, 2015) using the MultiSite Gateway™ technology.

Transformation cell suspension cultures

Transformation was performed as described (Van Leene *et al.*, 2007). Wild-type *Arabidopsis thaliana* (Ler-0) cell suspension cultures (PSB-D) were maintained in 50 mL MSMO medium (4.43 g/L Murashige and Skoog basal salts with minimal organics (Sigma-Aldrich, Saint Louis, MO, USA), 30 g/L sucrose, 0.5 mg/L α-naphtaleneacetic acid, 0.05 mg/L kinetin, pH 5.7) at 25°C in the dark or in a light/dark (16h/8h) regime by gentle agitation (130 rpm). Every week, the cells were subcultured in fresh MSMO medium. Cell suspension cultures (PSB-D) were transformed via co-cultivation with *Agrobacterium* containing the tagged bait overexpressing TAP vectors. Three weeks after co-cultivation protein expression analysis was performed (see below). Cultures showing expression of the bait protein were upscaled for actual tap affinity purification.

Tandem affinity purification

TAP was performed as described with minor changes (Van Leene *et al.*, 2015). Plant material was harvested and total protein extract was prepared using extraction buffer (25 mM Tris-HCl, pH7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM *p*-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% (v/v) NP-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 µM E64, EDTA-free Ultra complete tablet (1/10 mL) and 5% (v/v) ethylene glycol). Protein expression analysis was performed (see below). GS^{rhino}-tagged bait was purified from 25 mg of total protein extract in two affinity purification steps. Total protein extract was incubated with IgG-Sepharose 6 Fast Flow beads (GE Healthcare, Chicago, IL, USA) for 1 h at 4°C and bound complexes were eluted by Rhinovirus 3C protease (GE Healthcare) for 1 h at 4°C. The eluted fraction was incubated with Streptavidin-Sepharose beads (GE Gealthcare) for 1 h at 4°C. Bound complexes were eluted by 1x NuPAGE sample buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1x NuPAGE reducing agent and 20 mM desthiobiotine (Sigma-Aldrich) for 10 min at room temperature.

LC-MS/MS and data analysis

LC-MS/MS and data analysis was performed as described (Van Leene *et al.*, 2015). Proteins were then separated via NuPAGE electrophoresis in a precast 4-12% (wt/vol) gradient NuPAGE Bis-Tris gel (Thermo Fischer Scientific) for 7 min at 200 V. Proteins were visualized by Coomassie brilliant blue G-250 (Sigma-Aldrich) staining. Protein digestion was performed via in-gel trypsin digestion using trypsin gold, mass spectrometry grade (Promega, Fitchburg, WI, USA). The obtained peptide mixtures were then analysed by LC-MS/MS using the UltiMate 3000 RSLC nano (Dionex, Sunnyvale, CA, USA) in-line connected to the LTQ Orbitrap Velos (Thermo Fisher Scientific). Data were analysed using the Mascot Distiller (version 2.4.1, MatrixScience) and the Mascot Daemon interface was used to search the data with the Mascot search engine (version 2.4.1, MatrixScience) against the TAIRplus database. Proteins with at least two matched high-confident peptides, of which at least one is unique to the protein, are retained. Specific binders are identified using a background list of nonspecific proteins.

Protein expression analysis

Protein expression analysis of the TAP cultures was performed via immunoblotting. 20 mL MSMO medium was added to 5 mL of 1-week-old transformed Arabidopsis cell suspension culture and grown for 3 d at 25°C in the dark by gentle agitation (130 rpm). These cultures or upscaled cultures were harvested and ground to homogeneity in liquid nitrogen. Total protein extract was prepared in an equal volume (w/v) of extraction buffer (see above). Soluble proteins were separated from cell debris by a double centrifugation at 36 900 x *g* for 20 min at 4°C. Proteins were separated by SDS-PAGE (4-15% Mini-PROTEAN® TGX™ Precast Gel; Bio-Rad, Hercules, CA, USA) and blotted on a polyvinylidene fluoride (PVDF) membrane (Trans-Blot® TurboTM Mini PVDF Transfer, Bio-Rad). After incubation with peroxidase anti-peroxidase immune complex, the signal was captured using detection substrate (Western Lightning® Plus-ECL; Perkin Elmer, Waltham, MA, USA) and X-ray films (Amersham Hyperfilm ECL; GE Healthcare). Total protein was visualized using Coomassie Brilliant Blue (Thermo Fisher Scientific) staining of the PVDF membrane.

Yeast two-hybrid

Y2H analysis was performed as described (Cuéllar Pérez *et al.*, 2013). MYB2 and PAL1 were fused to GAL4-AD or GAL4-BD via cloning into, respectively, pGAL424gate or pGBT9gate. The Saccharomyces cerevisiae PJ69-4A yeast strain (James *et al.*, 1996) was co-transformed with both using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) media lacking Leu and Trp (Clontech, Saint-Germain-en-Laye, France). Three individual colonies were grown overnight in liquid cultures at 30°C and 10- or 100-fold dilutions were dropped on control media (SD-Leu-Trp) and selective media lacking Leu, Trp and His (Clontech).

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Chapter 2

Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity

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Published in:
Jonas Goossens, Gwen Swinnen, Robin Vanden Bossche, Laurens Pauwels and Alain Goossens. ***Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity.*** *New Phytologist 2015, 206(4):1229-1237.*

[1]Author Contributions: molecular cloning, qRT-PCR, Y2H assays (qualitative and quantitative), immunoblotting (yeast and tobacco protoplasts), transient expression assays, data interpretation and writing (all text and figures).

ABSTRACT

- The bHLH transcription factor MYC2, together with its paralogues MYC3 and MYC4, is a master regulator of the response to the jasmonate (JA) hormone in *Arabidopsis thaliana*. In the absence of JA, JASMONATE ZIM (JAZ) proteins interact with the MYC proteins to block their activity. Understanding of the mechanism and specificity of this interaction is key to unravel JA signalling.
- We generated mutant MYC proteins and assessed their activity and the specificity of their interaction with the 13 Arabidopsis JAZ proteins.
- We show that the D94N mutation present in the *atr2D* allele of *MYC3* abolishes the interaction between MYC3 and most JAZ proteins. The same effect is observed when the corresponding conserved Asp (D105) was mutated in MYC2. Accordingly, MYC2^{D105N} activated target genes in the presence of JAZ proteins, in contrast to wild-type MYC2. JAZ1 and JAZ10 were the only JAZ proteins still showing interaction with the mutant MYC proteins, due to a second MYC interaction domain, besides the classical Jas domain.
- Our results visualize the divergence among JAZ proteins in their interaction with MYC proteins. Ultimately, the transferability of the Asp-to-Asn amino acid change might facilitate the design of hyperactive transcription factors for plant engineering.

INTRODUCTION

Jasmonates (JAs) are phytohormones involved in abiotic and biotic stress responses, and several developmental processes (Kazan & Manners, 2008; Pauwels *et al.*, 2008; Browse, 2009; Pauwels *et al.*, 2009; Kazan & Manners, 2011; Pauwels & Goossens, 2011; Wasternack & Hause, 2013). Although the perception of the bioactive hormone jasmonoyl-isoleucine (JA-Ile) and the core JA signalling complex are very conserved for different JA-controlled processes, specificity in downstream responses is mediated by (combinations of) transcription factors (TFs) (De Geyter *et al.*, 2012), among which the best studied and arguably the most important are the basic Helix-Loop-Helix (bHLH) TFs MYC2, MYC3 and MYC4 from *Arabidopsis thaliana* (Fernandez-Calvo *et al.*, 2011; Niu *et al.*, 2011; Kazan & Manners, 2013).

In the absence of bioactive JAs, the switch to downstream processes is blocked due to repression of the MYC TFs by the JASMONATE ZIM domain (JAZ) proteins (Chini *et al.*, 2007; Chini *et al.*, 2009; Chung & Howe, 2009). When JA-Ile is present, a co-receptor complex is assembled, consisting of CORONATINE INSENSITIVE 1 (COI1), JA-Ile and a JAZ protein. COI1 is an F-box protein, functioning as a substrate-specific adaptor of an Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex, that mediates ubiquitin-dependent degradation of the JAZ protein by the 26S proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008; Fonseca *et*

al., 2009; Sheard *et al.*, 2010), which releases the MYC TFs from repression. The three MYC TFs show very high sequence similarity, which is manifested in their functional redundancy (Cheng *et al.*, 2011; Fernandez-Calvo *et al.*, 2011; Niu *et al.*, 2011; Frerigmann *et al.*, 2014). Nonetheless, they each have a certain specific input in the JA response, which is likely constituted by their tissue-specific expression patterns (Fernandez-Calvo *et al.*, 2011; Niu *et al.*, 2011).

The MYC TFs have a nearly identical C-terminal bHLH domain responsible for DNA binding and a transcriptional activation domain responsible for the interaction with MEDIATOR25 (MED25), a subunit of the Mediator complex connecting them to the RNA polymerase (Cevik *et al.*, 2012; Chen *et al.*, 2012). The JAZ-Interacting Domain (JID) is essential for the interaction with the JAZ repressors (Fernandez-Calvo *et al.*, 2011) and R2R3-MYB proteins involved in the biosynthesis of glucosinolates (Schweizer *et al.*, 2013).

The JAZ family consists of 13 members, all with a similar modular build-up (Pauwels & Goossens, 2011; Thireault *et al.*, 2015). JAZ proteins are capable of repressing gene expression due to the interaction of their central ZINC-FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM (ZIM) domain with the NOVEL INTERACTOR OF JAZ (NINJA), an adaptor protein bridging JAZ with the co-repressors TOPLESS (TPL) and TPL-related proteins (TPRs) through its ERF-associated amphiphilic repression (EAR) domain (Pauwels *et al.*, 2010). This ZIM domain is also responsible for homo- and heterodimerization among the JAZ proteins. A JA-associated (Jas) domain, localized at the C-terminus, is responsible for their interaction with COI1, the MYC TFs and several other bHLH and R2R3-MYB TFs (Pauwels & Goossens, 2011).

Here, we show that a single amino acid change in the *atr2D* allele of *MYC3* abolishes binding with most, but not all, Arabidopsis JAZ proteins and leads to activation of JA responses. This amino acid residue is conserved in JAZ-interacting MYC TFs in Arabidopsis and other plant species. We show that the corresponding amino acid change in the paralogue MYC2 also leads to loss of JAZ repression and an increased MYC2 activity. Furthermore, we demonstrate that different JAZ proteins have multiple ways and capacities of binding MYC proteins; in particular, two JAZ proteins have evolved to have two MYC interaction domains.

RESULTS

The *atr2D* mutation releases MYC3 from JAZ interaction

The *atr2D* allele of *MYC3* has been described by Smolen *et al*. (2002) as a dominant mutant salvaging plants from the toxic effect of 5-methyl-Trp, an inhibitor of Trp biosynthesis, by increased expression of *Trp* genes. Furthermore, altered expression of stress-related genes has been observed in the *atr2D* mutant plants. The phenotype of *atr2D* is caused by a point

mutation of a conserved Asp (D94N) in the JID of MYC3 (Smolen *et al.*, 2002; Fernandez-Calvo *et al.*, 2011). Therefore, we performed a Y2H analysis between ATR2D, MYC3 and JAZ proteins to investigate the possibility that this mutation abolishes the interaction with the JAZ repressors. Indeed, ATR2D lost the capacity to interact with the majority of the JAZ repressors; only interaction with JAZ1, JAZ2, JAZ5 and JAZ10 remained (Fig. 1A). Immunoblot analysis demonstrated that MYC3 and ATR2D were present at similar levels in transformed yeast cells (Fig. S1), indicating that MYC3 protein stability was not affected by the D94N mutation. The transformed yeast in the Y2H assay contain two reporter constructs, *HIS3*, which allows growth on medium lacking His (classical Y2H), and *LacZ*, which allows quantification of the interaction. Quantitative Y2H revealed that the interaction of ATR2D was significantly diminished with all JAZ proteins (Fig. 1B), even with those that still showed interaction with MYC3 under selective pressure in the 'classical' Y2H (Fig. 1A). In the quantitative assay, only interaction of ATR2D with JAZ1 and JAZ10 was significantly different from the negative control. Together, our results indicate that the *atr2D* mutation abolishes or attenuates the interaction between MYC3 and the JAZ repressors.

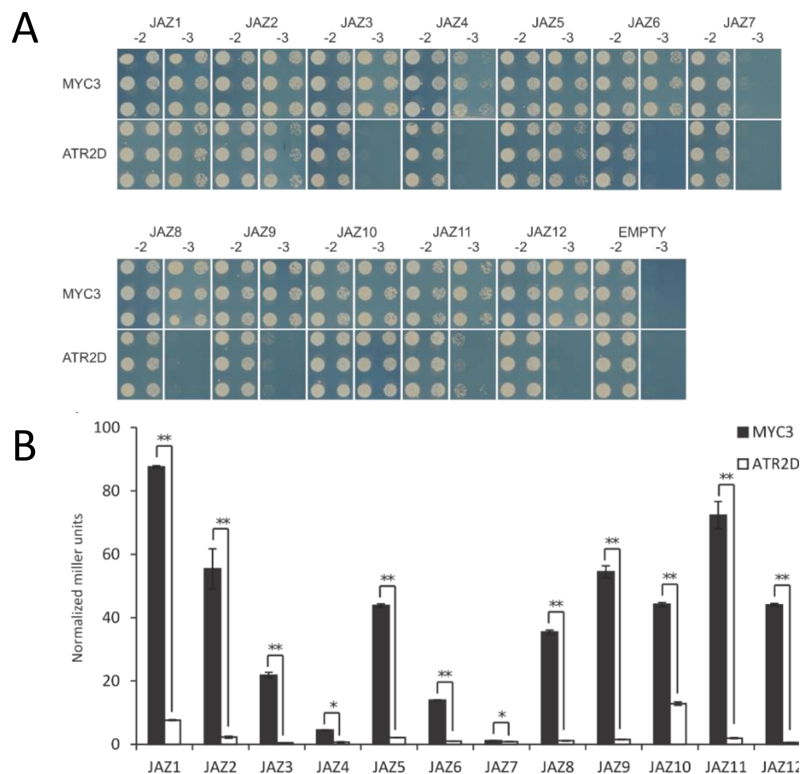

Figure 1: The *atr2D* mutation abolishes interaction with most JAZ repressors. Through Y2H, MYC3 and the mutant version ATR2D fused to GAL4-AD were tested for interaction with the 12 JAZ proteins fused to GAL4-BD. Empty vectors were used as negative control. A) Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium, respectively, control and selective medium. B) A liquid culture β-galactosidase assay was performed on the transformed yeasts. The activity of β-galactosidase was measured in Miller units and normalized to the negative control. Values are the mean (± SE) of three biological repeats. Significant differences (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$.

The D105N mutation increases MYC2 activity

MYC3 belongs to the clade of bHLH IIIe TFs, all containing a conserved N-terminal JID responsible for the interaction with the family of JAZ repressors (Heim *et al.*, 2003). Nearly all members of the subgroups bHLH IIId and bHLH IIIf also have this JID (Fig. S2) containing the conserved Asp residue of MYC3. Only TRANSPARANT TESTA 8 and MYC1 possess instead an Asn and a Glu residue, respectively. Furthermore, this conserved Asp was also conserved among MYC orthologues (Fig. S2). We hypothesized that the conserved Asp is essential for JAZ interaction in each homologue containing a JID. To test this hypothesis, we generated the $MYC2^{D105N}$ mutant by introducing a point mutation in MYC2. 'Classical' Y2H analysis indicated that $MYC2^{D105N}$ lost interaction with most of the JAZ proteins that were able to interact with MYC2 in this assay, except JAZ1 and JAZ10 (Fig. S3), which is similar to the Y2H analysis with ATR2D. Nevertheless, quantitative Y2H revealed that the strength of interaction between $MYC2^{D105N}$ and JAZ1 or JAZ10 was also severely reduced compared to their interaction with MYC2, as was also observed for ATR2D (Fig. 2A).

JAZ proteins exert their function as repressors by interaction with the adaptor NINJA that recruits co-repressors like TPL and TPRs (Pauwels *et al.*, 2010). Therefore, we would expect $MYC2^{D105N}$ to have a greater activity than MYC2 because of the loss of interaction with most of the JAZ proteins, hence with NINJA and the co-repressors. To assess this, we compared the transactivation potential of $MYC2^{D105N}$ and MYC2 by a transient expression assay in tobacco protoplasts with the MYC2-inducible *pLOX3::fLUC* construct as read-out (Pauwels *et al.*, 2008). In the absence of co-transfected JAZ, both MYC2 and $MYC2^{D105N}$ were able to transactivate the *LIPOXYGENASE3 (LOX3)* promoter to a similar extent (Fig. 2B). When co-expressed with JAZ proteins, complete inhibition of MYC2 transactivation activity was observed with all JAZ members tested (Fig. 2B). By contrast, depending on the JAZ member, a reduced to nearly no attenuation of transactivation activity was observed with $MYC2^{D105N}$ (Fig. 2B). To exclude the possibility that the D105N mutation increases the stability of MYC2, we transfected tobacco protoplasts with tagged versions of MYC2 and $MYC2^{D105N}$. Immunoblot analysis of the protein extracts revealed that MYC2 and $MYC2^{D105N}$ were present at similar levels in transfected tobacco protoplasts (Fig. S4), proving that the hyperactivity of $MYC2^{D105N}$ is not caused by increased protein stability. Hence, our data demonstrate that loss of binding to the JAZ repressors results in an increased transactivation potential of MYC2.

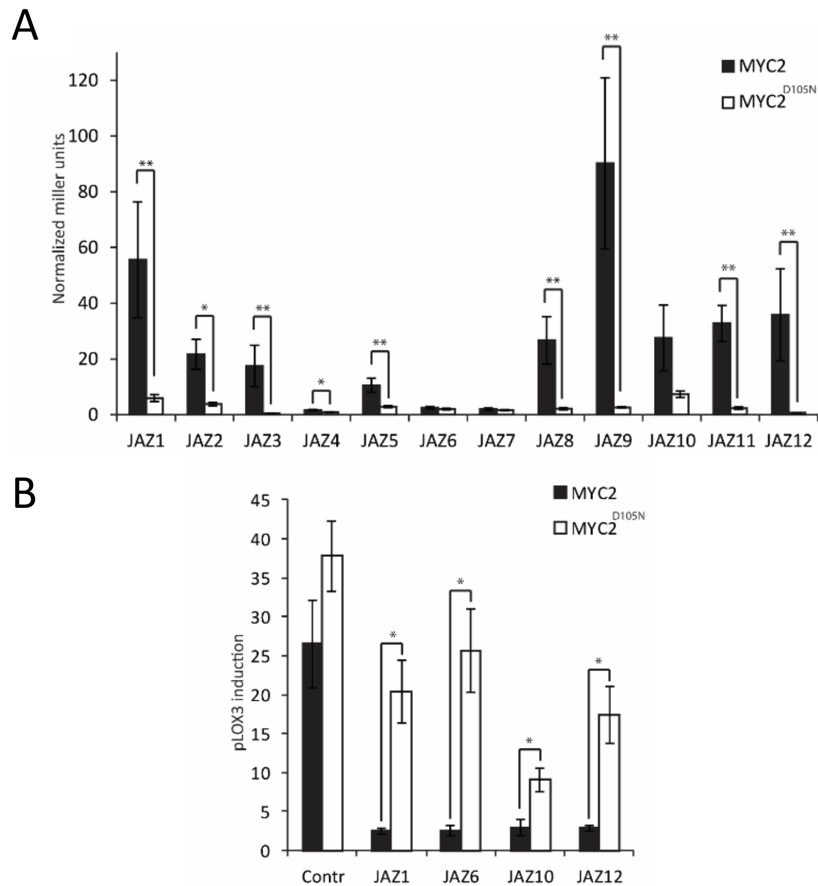

Figure 2: MYC2^{D105N} loses interaction with most of the JAZ proteins, resulting in the preservation of the transactivation potential in the presence of JAZ proteins. A) Quantitative Y2H analysis. MYC2 and MYC2^{D105N} fused to GAL4-AD were tested for interaction with the 12 JAZ proteins fused to GAL4-BD. Empty vectors were used as negative control. A liquid culture β-galactosidase assay was performed on the transformed yeasts shown in Fig. S3. The activity of β-galactosidase was measured in Miller units and normalized to the negative control. Values are the mean (± SE) of three biological repeats. Significant differences (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$. B) Transactivation assay by transient expression in tobacco protoplasts. The MYC2-inducible *pLOX3::fLUC* reporter construct was co-transfected with effector constructs overexpressing *MYC2*, *MYC2^{D105N}*, and/or *JAZ* genes and an *rLUC* construct for normalization. 'Contr' indicates a control assay with *MYC2* effector constructs co-transfected with a *GUS* expression construct instead of a *JAZ* effector construct. Values are fold-changes relative to protoplasts transfected only with a *GUS* expression construct instead of *MYC2* effector constructs and are the mean (± SE) of eight biological repeats. Significant differences (Student's *t*-test): *, $P < 0.05$.

An N-terminal cryptic MYC2-interacting domain is responsible for the interaction of JAZ1 and JAZ10 with MYC2^{D105N}

The Y2H analysis indicated that JAZ1 and JAZ10 are the main JAZ proteins able to maintain proper interaction with the mutated MYC constructs. Previously, Moreno *et al*. (2013) reported that JAZ10 contains an additional, N-terminal domain that was able to interact with MYC2. This cryptic MYC2-interacting domain (CMID) lacks conserved residues that have been shown to be essential for COI1 interaction and can thus be considered more specific for interaction with MYC TFs (Fig. 3A; Melotto *et al.*, 2008; Chung & Howe, 2009; Chung *et al.*,

2010; Sheard *et al.*, 2010; Withers *et al.*, 2012). To assess which domain is responsible for the remaining interaction with MYC2^{D105N}, truncated versions of JAZ10, that is JAZ10-N and JAZ10-C containing the CMID and the Jas domain, respectively, were generated and tested via Y2H. Both JAZ10 fragments were able to bind MYC2, but whereas the JAZ10-C fragment lost interaction with MYC2^{D105N}, the JAZ10-N fragment containing the CMID did not (Fig. 3B).

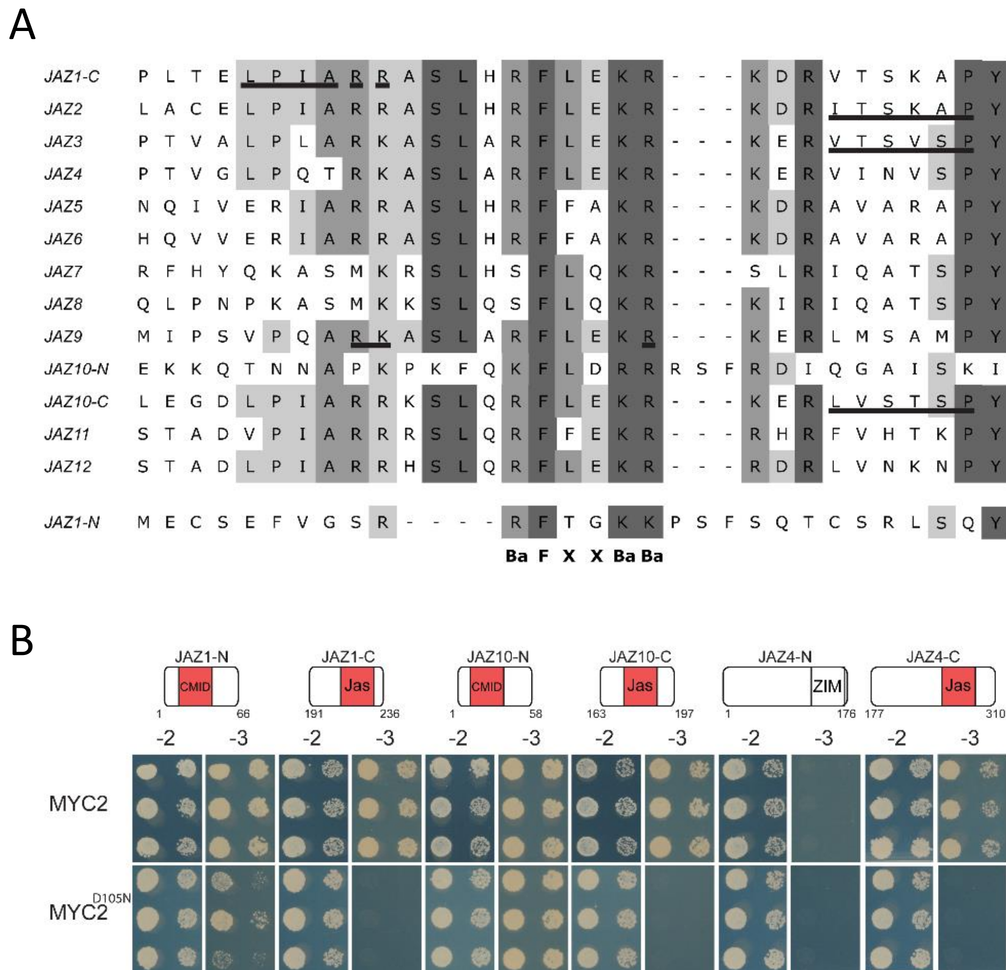

Figure 3: An N-terminal Jas-like domain enables JAZ1 and JAZ10 to interact with MYC2^{D105N}. A) Sequence alignment of the cryptic MYC2-interacting domain (CMID) and Jas domain of the JAZ proteins. The putative N-terminal CMID of JAZ1 and JAZ10 are aligned to the Jas domains of all JAZ proteins. Shading of the residues represents conservation among the different proteins. The sequences were aligned by MUSCLE and edited with UGene. Underlined residues are proven to be essential for interaction with COI1. The putative core sequence of the Jas motif is represented in bold underneath the alignment. Ba, basic amino acid residue; X, any amino acid residue; F, phenylalanine. B) MYC2 and MYC2^{D105N} fused to GAL4-AD were tested for interaction with N-terminal and C-terminal constructs of JAZ1 and JAZ10 fused to GAL4-BD. Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium. N-terminal and C-terminal parts of JAZ4 were included as negative and positive controls, respectively.

Because JAZ1 was also able to interact with MYC2^{D105N} (Figs. 2A and S3), we reasoned that a similar CMID motif might be present at the N-terminus of JAZ1. Alignment of JAZ1 with Jas domains of the JAZ proteins revealed a Jas-like motif in the N-terminus (Fig. 3A). Considering

this motif to be a true 'Jas motif', we postulate that the conserved core sequence of the Jas motif responsible for MYC2 interaction would be Ba-F-X-X-Ba-Ba, in which Ba refers to a basic residue (Arg or Lys). To test this hypothesis, we performed Y2H with truncated versions of JAZ1, which demonstrated that the N-terminal part of JAZ1 could indeed bind both MYC2 and MYC2^{D105N}, whereas the C-terminal Jas domain of JAZ1 could bind MYC2, but not MYC2^{D105N} (Fig. 3B). Hence, both JAZ1 and JAZ10 have two MYC interaction domains. Shorter constructs of the N-terminal domain or deletion constructs missing the N-terminal part of JAZ1 were not able to bind MYC2, indicating that amino acids residing somewhere in the first 66 amino acids of the protein sequence are essential for interaction of JAZ1ΔJas with MYC2 (Fig. S5).

Hyperactive MYC transcription factors cause a JA-related phenotype in Arabidopsis

It has been reported that the *atr2D* mutant shows an increased expression of *Trp* and stress-related genes (Smolen *et al.*, 2002). However, because the role of MYC TFs in JA signalling was still unsuspected at that time, a direct correlation with the JA signalling response was not investigated. To explore the effect of the Asp-to-Asn mutation in the MYC TFs on JA signalling *in planta*, we analysed the expression of JA response genes in the *atr2D* mutant and in *MYC2^{D105N}* overexpressing plants. As expected, qRT-PCR analysis of the *atr2D* mutant revealed increased transcript levels of *OXOPHYTODIENOATE-REDUCTASE3* (*OPR3*)*, LOX2* and *ALLENE OXIDE SYNTHASE* (*AOS*), involved in JA biosynthesis, and of the JA marker genes *JAZ10*, *TYROSINE AMINOTRANSFERASE3* (*TAT3*), *VEGETATITVE STORAGE PROTEIN2* (*VSP2*) and *PLANT DEFENSIN1.2* (*PDF1.2*), compared to those in wild-type plants (Fig. 4A). We also confirmed the increased expression of genes described by Smolen *et al*. (2002), such as *TRYPTOPHAN SYNTHASE BETA-SUBUNIT1* (*TSB1*). Notably, the *PDF1.2* gene showed a markedly higher induction (> 20-fold) in *atr2D* plants, as compared to the other tested genes that showed a rather moderate increase of *c*. two-fold only.

Induction of most JA marker genes was also observed in plants overexpressing the hyperactive *MYC2^{D105N}*, as compared to plants overexpressing *MYC2* or wild-type plants (Fig. 4B), which showed no altered expression of these target genes. Contrasting with the *atr2D* plants was the absence of *PDF1.2* induction in the *MYC2^{D105N}* overexpressing plants. Conversely, an extreme increase in *TAT3* transcript levels (by > 250-fold) was observed in plants overexpressing *MYC2^{D105N}*, whereas this was not the case in the *atr2D* plants. It should be noted here that we selected overexpression lines with comparable transcript levels of *MYC2* and *MYC2^{D105N}*, to allow comparative analysis of their activity *in planta*. The fact that *MYC2* overexpression was even slightly higher in this line compared to that of *MYC2^{D105N}* in the presented lines supports the proposition that MYC2^{D105N} contains a higher transactivation activity than MYC2 *in planta*. Nonetheless, as

illustrated in Fig. 4D, altered expression of, for example, the *TAT3* gene could also be observed in the extreme *MYC2* overexpressing line that we generated.

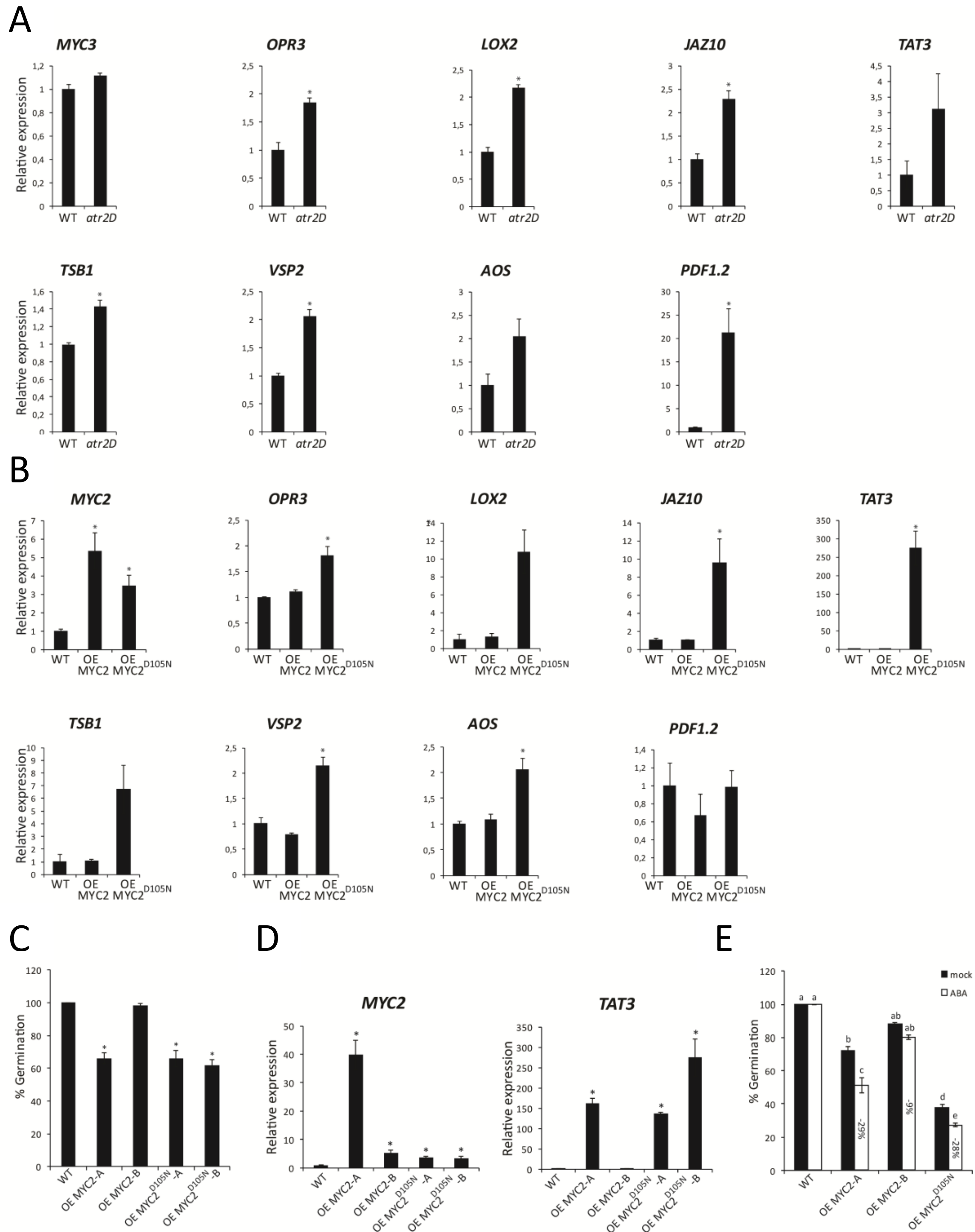

Figure 4. The *atr2D* mutant and *MYC2^{D105N}* overexpressing plants show activation of the jasmonate (JA) and abscisic acid (ABA) response. A) and B) qRT-PCR analysis of Arabidopsis seedlings from A) *atr2D* plants and B) plants overexpressing (OE) *MYC2* or *MYC2^{D105N}*. Values represent the expression ratios plotted relative to those in control wild-type (WT) plants and are the mean (± SE) of three biological repeats. Significant differences with respect to the WT (Student's *t*-test): *, $P < 0.05$. C) Germination rate of 7-d-old Arabidopsis seedlings from WT plants and two different *MYC2* or *MYC2^{D105N}* OE lines (A and B). Values represent the mean (± SE) of three

biological repeats. Significant differences with respect to the WT (Student's *t*-test): *, $P < 0.05$. The experiment was performed twice, obtaining similar results. D) *MYC2* and *TAT3* transcript levels in the germinating seedlings from (C). Values represent the expression ratios plotted relative to those in control WT plants and are the mean (± SE) of three biological repeats. Significant differences with respect to the WT (Student's *t*-test): *, $P < 0.05$. D) Germination rate of WT plants, two different *MYC2* OE lines (A and B) and one *MYC2^{D105N}* OE line grown for 7 d in ethanol (mock) or 0.1 µM ABA. The experiment was performed twice, obtaining similar results. Values represent the mean (± SE) of three biological repeats. Letters represent significant differences according to a Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$). Percentages in white bars depict the difference in germination rate between the mock and ABA condition of each line.

Finally, we investigated whether the activation of JA-related transcription affected JA sensitivity by assessing inhibition of root growth, a typical JA response (Staswick *et al.*, 1992). In comparison to wild-type plants, root growth was significantly lower both for *MYC2* and *MYC2^{D105N}* overexpressing plants in mock conditions, although this effect was more pronounced in the *MYC2^{D105N}* plants (Fig. S6). The same trend was observed in plants grown in the presence of MeJA; however, no JA-hypersensitivity in *MYC2* and *MYC2^{D105N}* overexpressing plants could be distinguished (Fig. S6).

Hyperactive MYC transcription factors cause an ABA-related phenotype in Arabidopsis

MYC2 is also a known positive regulator of abscisic acid (ABA) signalling and has been shown to be correlated with ABA-mediated inhibition of germination. Overexpression of *MYC2* results in hypersensitivity to ABA (Abe *et al.*, 2003; Lorenzo *et al.*, 2004) and *myc2* knock-outs show a decreased sensitivity to ABA (Abe *et al.*, 2003; Yadav *et al.*, 2005; Gangappa *et al.*, 2010). Accordingly, it was interesting to observe inhibition of germination without ABA treatment in *MYC2^{D105N}* overexpressing plant lines but not in plant lines overexpressing *MYC2* at a similar level (*c*. five-fold; OE *MYC2*-B; Figs. 4C, D and S7). However, in a line overexpressing *MYC2* by *c*. 40-fold (OE *MYC2*-A), a similar phenotype as for the *MYC2^{D105N}* overexpressing plants was observed (Figs. 4C, D and S7). Using *TAT3* as a marker of MYC2 activity (Fig. 4D; Chini *et al.*, 2007; Dombrecht *et al.*, 2007; Niu *et al.*, 2011), we can conclude that either through higher expression or through loss of JAZ binding and repression, MYC2 activity is correlated with a decreased germination efficiency and smaller germinated seedlings.

Lastly, we investigated the sensitivity to ABA by comparing germination efficiency between plants grown in the absence or presence of ABA. We used a small dose of ABA (0.1 µM) that did not inhibit the germination of wild-type plants (Fig. 4E). Nonetheless, this low dose of ABA led to decreased germination efficiency in plants overexpressing *MYC2* or *MYC2^{D105N}*, with plants overexpressing *MYC2^{D105N}* showing a higher sensitivity than those overexpressing *MYC2* at a similar level (OE *MYC2*-B). Comparable ABA-hypersensitivity was only achieved in the plants overexpressing *MYC2* by *c*. 40-fold (OE *MYC2*-B). Together, these

results indicate that the presence of the Asp-to-Asn mutation in MYC2 leads to an increased inhibition of germination associated with ABA-hypersensitivity.

DISCUSSION

Interaction between JAZ repressors and the MYC TFs is key in the JA signalling cascade. The Arabidopsis JAZ family consists of 13 members and the expansion of this protein family might point to functional specialization of individual JAZ members. In 2002, Smolen *et al*. described an allele of *MYC3* – that is, *ATR2D* – which caused a dominant stress-responsive phenotype. It has been noted before that the mutation in this allele caused an amino acid change (D94N) in the JID, possibly leading to loss of JAZ (Fernandez-Calvo *et al.*, 2011; Frerigmann *et al.*, 2014). Here, we show that this single amino acid change was sufficient to cause loss of interaction with most of the JAZ proteins. Interaction with ATR2D was detected only with JAZ1 and JAZ10, although the strength of this interaction was also diminished compared to the interaction with MYC3. Similar results were obtained for the artificial allele *MYC2^{D105N}*, containing the same conserved residue change (D105N), which confirms the importance of the Asp residue for JAZ interaction and the transferability of the *atr2D* mutation to paralogues and presumably orthologues that have a conserved JID.

Previously, interaction analysis with truncated JAZ proteins has established the presence of an extra MYC interaction domain in the N-terminus of JAZ10, described as CMID by Moreno *et al.* (2013). Here, we reveal the presence of an additional MYC interaction domain in JAZ1 as well. Importantly, these CMIDs were essential for the remaining interaction of JAZ1 and JAZ10 with ATR2D and MYC2^{D105N}. We can infer that JAZ1 and JAZ10 proteins have a similar domain organization, which is different from those found in other JAZ proteins. A rationale for the functional relevance of this extra MYC interaction domain in JAZ10 has been forwarded by Moreno *et al.* (2013) and Chung and Howe (2009). For *JAZ10*, an alternative splice variant, *jaz10.4*, has been described, which lacks the Jas domain, making it resistant to COI1 interaction and JA-Ile-mediated degradation. However, the CMID still allows interaction with MYC2 and repression of JA responses, possibly attenuating JA-signalling. Our results might point to a similar specialization of JAZ1. Correspondingly, overexpression of *JAZ1ΔJas* but not of *JAZ9ΔJas* led to JA-insensitivity (Withers *et al.*, 2012) and phenotypic changes were observed only for loss-of-function lines for *JAZ1* and *JAZ10*, but not for other *JAZ* genes (Grunewald *et al.*, 2009; Demianski *et al.*, 2012; Moreno *et al.*, 2013). However, *JAZ1* lacks the Jas intron responsible for alternative splicing present in most *JAZ* genes (Chung *et al.*, 2010), hence the biological relevance of the JAZ1 CMID needs further investigation.

Our results demonstrate that different JAZ domains (CMID and Jas), and hence different JAZ proteins, have different ways and capacities of binding the MYC proteins. This is consistent with the fact that JAZ proteins show several subtle but important differences in their modular domain sequence and architecture. For instance, JAZ8 lacks a COI1-interacting degron (Shyu *et al.*, 2012) and contains an EAR domain that is responsible for direct interaction with TPL without the need for the adaptor protein NINJA. The latter property is also observed for JAZ5 and JAZ6 (Causier *et al.*, 2012; Shyu *et al.*, 2012). Combining this with tissue-, cell- and organelle-specific expression of the different *JAZ* genes, this provides the plant with a robust and pleiotropic control machinery that allows appropriate fine-tuning of the JA response across the plant body.

MYC2^{D105N} was still capable of promoter transactivation in the presence of JAZ repressors, in contrast to MYC2. The consequence for JA signalling was further analysed *in planta* by analysis of JA marker gene expression in the *atr2D* mutant, and in *MYC2* and *MYC2^{D105N}* overexpressing Arabidopsis lines. All tested genes have previously been linked to MYC2 and/or MYC3 activity (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007; Hou *et al.*, 2010; Fernandez-Calvo *et al.*, 2011; Niu *et al.*, 2011; Sasaki-Sekimoto *et al.*, 2013; Schweizer *et al.*, 2013). Induction of these genes in *atr2D* mutant plants with respect to wild-type plants, establishes the direct correlation of the Asp-to-Asn amino acid change with JA signalling. Correspondingly, MYC2^{D105N}, but not MYC2 at comparable expression levels, was able to trigger expression of a common set of target genes. In agreement with previous reports (Niu *et al.*, 2011) , some specificity was observed when comparing the *ATR2D* and *MYC2^{D105N}* effects on the degree of induction of some genes, with a marked increased level of *TAT3* transcripts in the *MYC2^{D105N}* overexpressing plants, and of *PDF1.2* in the *atr2D* mutant.

In conclusion, a change of the conserved Asp to Asn in the JID results in hyperactive TFs due to loss of interaction with JAZ repressors, which is translated into activation of (part of) the JA response and other MYC-controlled processes. Our results suggest that the Asp-to-Asn mutation is transferable to paralogues and orthologues of the MYC TFs in other plant species. Thereby, this mutation might ultimately become a useful concept for the design of hyperactive TFs for plant engineering.

MATERIALS AND METHODS

Gene cloning

All cloning was carried out by Gateway® recombination (Life Technologies). The point mutation in MYC2^{D105N} was generated with the GeneTailor™ Site-Directed Mutagenesis system (Life Technologies, Carlsbad, CA, USA).

Plant material and growth conditions

The *Arabidopsis thaliana* (L.) Heynh ecotype Col-0 was used as wild-type for all experiments. For overexpression, the full-length open reading frame (ORF) of *MYC2* or *MYC2^{D105N}* was put under control of the CaMV35S promoter in the binary vector pK8m34GW-FAST (Vanholme *et al.*, 2013), which was introduced into the *Agrobacterium tumefaciens* strain C58C1 (pMP90) for transformation of Arabidopsis by the floral dip method (Clough & Bent, 1998). Plants were propagated to the T_3 generation for selection of homozygous plants for analysis. The *atr2D* mutant line is described in Smolen *et al*. (2002). Arabidopsis seeds were sterilized by the chlorine gas method, sown on plates containing MS medium (10 g l^{-1} sucrose, 8 g l^{-1} agarose, pH 5.7), supplemented or not with dimethylsulfoxide (DMSO), methyl jasmonate (MeJA; Duchefa-Biochemie, Haarlem, the Netherlands), ethanol or abscisic acid (ABA; Sigma-Aldrich), incubated in the dark at 4°C for 3 d, and transferred to a growth room with controlled conditions (21°C, 16 h : 8 h, light : dark regime).

Quantitative real-time PCR (qRT-PCR)

For each biological repeat, 20–80 seedlings were grown as described above for 8–10 d, pooled and snap-frozen. The material was ground in a Retsch MM300 mixer and total RNA was extracted using the Qiagen RNeasy kit (Qiagen). One microgram of RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed in the LightCycler 480 system (Roche) using the Fast Start SYBR Green I PCR mix (Roche) via the following program: pre-incubation (95°C, 10 s), 45 amplification cycles (incubation 95°C, 10 s; annealing 65°C, 15 s; elongation 72°C, 15 s). Transcript levels were normalized relative to the housekeeping genes *UBC10* (*AT5G53300*) and *PP2A* (*AT1G13320*). Primer sequences are presented in Table S1.

Yeast two-hybrid (Y2H) analysis

Y2H analysis was performed as described (Cuellar *et al.*, 2013). Bait and prey were fused to the GAL4-AD or GAL4-BD via cloning into pGAL424gate or pGBT9gate, respectively (Cuellar *et al.*, 2013). The *Saccharomyces cerevisiae* PJ69-4A yeast strain (James *et al.*, 1996) was co-transformed with bait and prey using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) media lacking Leu and Trp (−2) (Clontech, Saint-Germain-en-Laye, France). Three individual colonies were grown overnight in liquid cultures (−2) at 30°C and 10- or 100-fold dilutions were dropped on control media (−2) and selective media lacking Leu, Trp and His (−3) (Clontech).

In order to allow quantitative Y2H analysis, we adapted the above classical Y2H set-up as follows. Three individual colonies were grown overnight in liquid cultures (−2) at 30°C. A five-fold dilution in yeast extract peptone dextrose adenine (YPDA) medium (Clontech) was grown for another 2–3 h until an OD_{600} of 1.0–1.5 was reached. Lysis of pelleted cells was performed in Z buffer (0.06 M Na_2HPO_4; 0.04 M NaH_2PO_4; 0.01 M KCl; 0.001 M $MgSO_4$; pH 7) containing 0.15% 2-mercaptoethanol, 18% chloroform and 0.1% SDS. β-Galactosidase activity was quantified via spectrophotometry using the substrate o-nitrophenyl B-D-galactopyranoside (ONPG) and presented in Miller units.

In order to assess the protein stability of the different MYC2 versions in the transformed yeasts, immunoblot analysis was carried out. To this end, a pool of three individual transformant colonies was grown overnight in liquid selective medium at 30°C. The culture was diluted to an OD_{600} of 0.1 in 15 ml of YPD medium (Clontech) and grown for another 4–5 h. Total protein was extracted and separated by SDS-PAGE (4–15% Mini-PROTEAN® TGX™ Precast Gel, Bio-Rad) and blotted on a polyvinylidene fluoride (PVDF) membrane (Trans-Blot® Turbo™ Mini PVDF Transfer; Bio-Rad). After incubation with anti-GAL4-AD (Sigma-Aldrich) and anti-rabbit-horseradish peroxidase (GE Healthcare, Freiburg, Germany), the signal was captured using detection substrate (Western Lightning® Plus-ECL; Perkin Elmer) and X-ray films (Amersham Hyperfilm ECL; GE Healthcare). Total protein loading was visualized using Coomassie Brilliant Blue (Thermo Scientific, Waltham, MA, USA) staining of the PVDF membrane.

Transient expression assays

Transient expression assays in protoplast cells prepared from Bright Yellow-2 (BY-2) *Nicotiana tabacum* suspension cultured cells were performed as described (Vanden Bossche *et al.*, 2013). The reporter plasmid contained a *firefly luciferase* (*fLUC*) gene under control of the MYC2-inducible *LOX3* promoter. *GUS*, *MYC2*, *MYC2*^{D105N} and *JAZ* genes were expressed under control of the pCaMV35S promoter in effector plasmids. Plasmids with the *Renilla luciferase* (*rLUC*) expressed under control of the pCaMV35S promoter serve for normalization for transfection efficiency. Protoplast cells were transfected with 2 µg of each plasmid using the PEG/Ca^{2+} method and grown overnight in the dark at room temperature with gentle agitation. After lysis of the cells, the luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega).

In order to assess the protein stability of the different MYC2 versions in the tobacco protoplasts, *MYC2* and *MYC2*^{D105N} ORFs were fused to a 3xFlag-6xHis-tag and expressed under control of the pCaMV35S promoter in the pm43GW7 plasmid (Pauwels *et al.*, 2010). The resulting vectors were used for protoplast transfection as described above. Transfected protoplasts were pooled and total protein was extracted. Immunoblot analysis was performed as described above but with anti-Flag (Sigma-Aldrich) and anti-mouse-horseradish peroxidase (GE Healthcare) antibodies.

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Chapter 3

Multilayered organization of jasmonate signalling in the regulation of root growth

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Adapted from:
Debora Gasperini, Aurore Chételat, Ivan F. Acosta, Jonas Goossens, Laurens Pauwels, Alain Goossens, René Dreos, Esteban Alfonso and Edward E. Farmer. ***Multilayered Organization of Jasmonate Signalling in the Regulation of Root Growth.*** *Plos Genetics 2015, 11(6):e1005300*

Note: According to my own contributions and to the relevance of the paragraphs for my thesis, I did not include following topics: repeated cotyledon wounding response, the specific expression patterns of the MYC TFs and NINJA in the root, the contribution of the MYC TFs to the ninja root phenotype and the transcriptome analysis of ninja-1 roots. We merely focused on myc2-322B in combination with NINJA.

[1]Author contributions: Molecular cloning, Y2H assays, transient expression assays, data interpretation and participation to writing of the manuscript (Figures 3 and 4 and conclusions from these data).

ABSTRACT

Physical damage can strongly affect plant growth, reducing the biomass of developing organs situated at a distance from wounds. These effects, require the activation of jasmonate (JA) signalling. Regulatory JA signalling components were manipulated to delineate their relative impacts on root growth. The new transcription factor mutant *myc2-322B* was isolated. *In vitro* transcription assays and whole-plant approaches revealed that *myc2-322B* is a dosage-dependent gain-of-function mutant that can amplify JA growth responses. Moreover, *myc2-322B* displayed extreme hypersensitivity to JA that totally suppressed root elongation. The mutation weakly reduced root growth in undamaged plants but, when the upstream negative regulator NINJA was genetically removed, *myc2-322B* powerfully repressed root growth through its effects on cell division and cell elongation. Furthermore, in a JA-deficient mutant background, *ninja1 myc2-322B* still repressed root elongation, indicating that it is possible to generate JA responses in the absence of JA. In nature, growing roots are likely subjected to constant mechanical stress during soil penetration that could lead to JA production and subsequent detrimental effects on growth. Our data reveal how distinct negative regulatory layers, including both NINJA-dependent and -independent mechanisms, restrain JA responses to allow normal root growth.

INTRODUCTION

The development, architecture and mass of nascent plant organs are plastic and can be strongly influenced by injury to pre-existing tissues. Wounding reduces plant biomass and damage to young tissues can strongly reduce growth rates, (e.g. Poveda *et al.*, 2003). In the case of above ground tissues, most of the growth restriction that occurs subsequently to physical damage depends on the activation of the jasmonate (JA) pathway (Yan *et al.*, 2007; Zhang & Turner, 2008; Yang *et al.*, 2012), which has a pivotal role in controlling herbivore-inducible gene expression and coordinating resource allocation between defence and growth (Browse, 2009a; Wasternack & Hause, 2013). In contrast to the observation of JA-mediated growth restriction in leaves, root growth responses following damage to aerial organs are so far, not clearly understood. What has emerged to date, however, is that the same basic JA signalling components operate in shoots and roots, although the genetic architecture of the JA pathway appears to be simpler in roots (Acosta *et al.*, 2013).

JA signalling, whether for defence or organ growth restriction, requires the production and perception of low molecular mass lipidic regulators of the JA family, including the biologically active form jasmonoyl-L-isoleucine (JA-Ile) (Staswick & Tiryaki, 2004; Fonseca *et al.*, 2009). The transcriptional changes resulting from JA-Ile perception enable plants to modulate the allocation of resources in defence at the expense of growth (Yang *et al.*, 2012). In the

absence of JA-Ile, JASMONATE ZIM-DOMAIN (JAZ) proteins bind and repress JA-dependent transcription factors (TFs) by recruiting the general co-repressors TOPLESS (TPL) and TPL-Related (TPR) proteins through an interaction with the adaptor protein Novel Interactor of JAZ (NINJA) (Pauwels *et al.*, 2010), or directly as in the case of JAZ8 (Shyu *et al.*, 2012b). TPL in turn recruits histone deacetylases and histone methyltransferases to inhibit transcription (Wang *et al.*, 2013). Upon stimulation, JA-Ile accumulates and promotes the binding of JAZ proteins to the F-box protein CORONATINE INSENSITIVE 1 (COI1) (Chini *et al.*, 2007; Thines *et al.*, 2007). This interaction mediates the ubiquitination and degradation of JAZs (Chini *et al.*, 2007; Thines *et al.*, 2007), liberating TFs to interact with the MED25 subunit of the Mediator complex and recruit RNA polymerase II to JA-responsive genes (Çevik *et al.*, 2012; Chen *et al.*, 2012).

Currently, the basic helix-loop-helix (bHLH) MYC2 TF is considered a master regulator of most JA responses and a convergence node with other signalling pathways (Kazan & Manners, 2013). It can act as both activator and repressor to regulate diverse aspects of JA-mediated gene expression (Dombrecht *et al.*, 2007). Two MYC2-like bHLH proteins (MYC3 and MYC4) also interact with JAZs and act additively with MYC2 in mediating a subset of JA-regulated responses (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011). A simplified scheme for JA signalling is shown in Fig. 1. All components shown in the figure can be manipulated to affect the output (defence/growth) of the pathway and, in addition, JA responses can be powerfully activated with exogenous JA. Whatever the upstream manipulation of JA levels or pathway components, much of their activity converges on MYCs. This leads to the theoretical possibility, shown in the dashed box in Fig. 1, that strong gain-of-function mutants of MYC2 might be able to recapitulate JA responses in the absence of JA itself. Furthermore, such effects should be facilitated if negative regulatory layers (like that imposed by NINJA) could be removed. Herein, using *Arabidopsis thaliana* seedlings we investigated this possibility.

Although JA-induced root growth inhibition assays have been widely employed to identify JA mutants in Arabidopsis, reviewed in (Browse, 2009b; Wasternack & Hause, 2013), the basal root length of those mutants does not differ from wild-type (WT) (Staswick *et al.*, 1992; Feys *et al.*, 1994; Lorenzo *et al.*, 2004). To date, mutants in *NINJA* represent the sole example of a JA signalling component known to affect normal root growth (Acosta *et al.*, 2013). However, unlike plants grown in medium supplied with exogenous JA where root growth is inhibited as a consequence of reduced meristem cell number and cell elongation (Chen *et al.*, 2011), *ninja* mutants display de-repressed JA signalling and shorter roots in the absence of JA due to a defect in cell elongation only (Acosta *et al.*, 2013). Thus, *ninja* mutants suggest the existence of additional organ- and cell-specific mechanisms of negative regulation that restrict JA signalling responses in the root. A novel gain-of-function allele of *MYC2*, either

alone or in combination with *ninja* and JA biosynthesis mutants, revealed the existence of several layers of negative regulation that keep JA responses at bay to allow normal root growth. These results provide a basis for the future engineering of damage- or JA-controlled organ growth, an area of potential importance in agriculture.

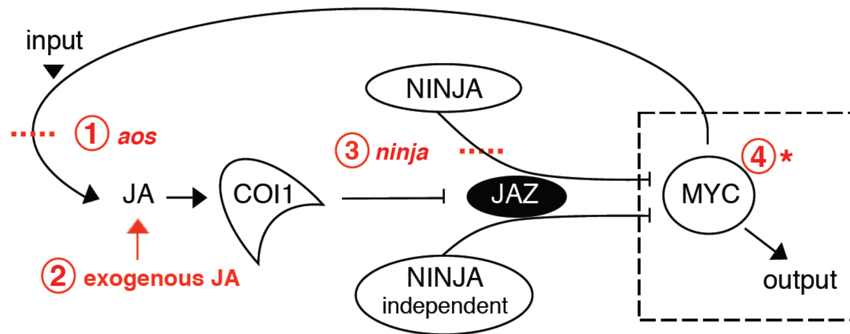

Figure 1: Interventions used in this study to manipulate the jasmonate pathway. Shown are: 1. loss-of-function mutation of the JA synthesis gene *allene oxide synthase* (*aos*); 2. treatment with exogenous JA; 3. loss-of-function mutations in the negative regulator *NINJA*; 4. gain-of-function mutation of MYC transcription factors. The dashed box surrounding MYC indicates that it is conceptually possible to use an overactive MYC to drive JA responses in the absence of JA synthesis (step 1) and of negative regulators like NINJA (steps 1 and 3 combined). This was achieved using a novel *myc2* mutant that amplifies JA responses.

RESULTS

myc2-322B: A novel *MYC2* allele

MYC2, MYC3 and MYC4 are expressed basally in the root meristem but insertion alleles in these genes do not show root growth alterations (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011). However, based on the scheme in Fig. 1 it is possible that gain-of-function alleles of MYCs constitutively activate JA signalling. One such allele in *MYC3* is already known (Smolen *et al.*, 2002). We extended the genetic screen used by Acosta *et al.* (2013) to search for new mutants that, under basal conditions, display ectopic expression of a secretable $JAZ10_{pro}$-$GUSPlus^{sec}$ (*JGP*) reporter, which is JA responsive (Acosta *et al.*, 2013). Unlike the weak *JGP* activity observed in the WT (Fig. 2A; Acosta *et al.*, 2013), one such mutant displayed basal *JGP* activity in the early differentiation zone of the primary root tip without reaching mature parts of the upper root (Fig. 2B). The mutant segregated recessively from WT *MYC2* (Fig. S8) and was mapped by whole-genome sequencing to a G to A transition in position 493 of the *MYC2* gene causing a Glu-to-Lys (E165K) substitution. This is in agreement with the reported function of MYC2 in JA-mediated *JAZ10* induction (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011).

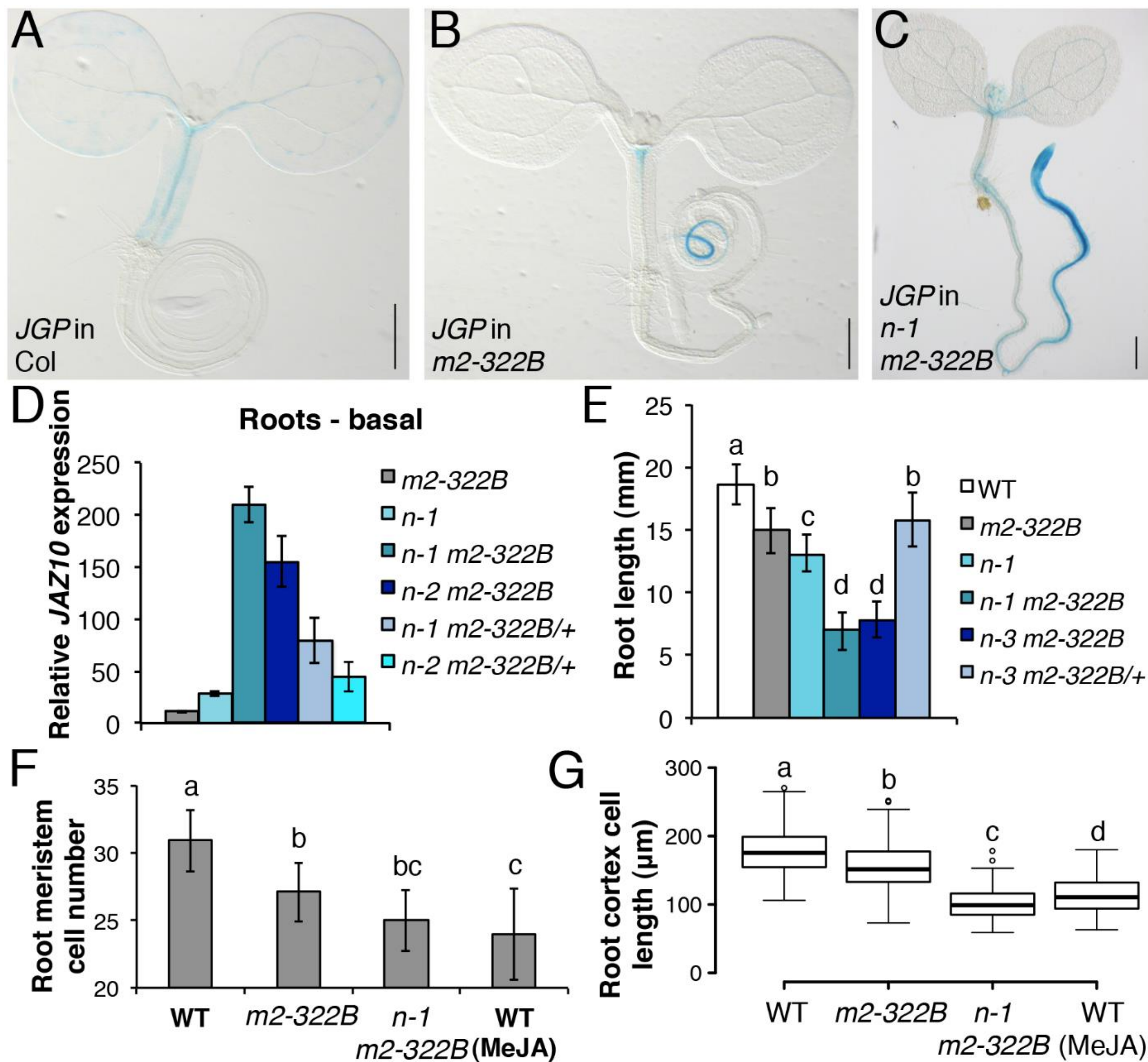

Figure 2: *myc2-322B* exhibits enhanced JA responses in a NINJA-dependent manner. *JGP* expression in 5-do seedlings of WT (A), *myc2-322B* (B, *m2-322B*) and *ninja-1 myc2-322B* (C, *n-1 m2-322B*). Note the constitutive reporter activity stained in blue (Scale bars = 0.5 mm). (D) qRT-PCR of basal *JAZ10* expression in 5-do roots of *myc2-322B*, *ninja-1* (*n-1*), *ninja-1 myc2-322B* (*n-1 m2-322B*), *ninja-2 myc2-322B* (*n-2 m2-322B*), *ninja-1 myc2-322B/+* (*n-1 m2-322B/+*) and *ninja-2 myc2-322B/+* (*n-2 m2-322B/+*). *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of unwounded WT samples, which are set to 1. Bars represent the means of three biological replicates (±SD), each containing a pool of ~60 roots. (E) Root length quantification of WT and 7-do mutant lines. Data are the means (±SD) from 20–48 plants. Primary root meristem cell number (F) and box plot summary of cortex-cell length (G) in 5-do seedlings of WT and mutant lines grown in control conditions, and of WT grown in presence of 25 µM MeJA (n = 10). Letters above bars and box plots (E-G) indicate statistically significant differences between samples as determined by Tukey's HSD tests ($P < 0.01$).

The relatively confined *JGP* expression of *myc2-322B* (Fig. 2B) led us to hypothesize that a transcriptional repression mechanism is still able to inhibit the putative excessive MYC2^{E165K} activity in *myc2-322B*. Indeed, by removing the NINJA-dependent repression mechanism in

ninja-1 myc2-322B double mutants, the effects on *JGP* activity became more remarkable, extending to a much wider domain in the primary root including the meristem (Fig. 2C). To further assess the functionality of this novel *myc2* allele we tested its influence on JA-mediated gene expression. *myc2-322B* showed ~10 fold higher-than-WT *JAZ10* expression in 5-do roots (Fig. 2D). By contrast, neither basal *JGP* nor *JAZ10* expression differed from WT in aerial tissues (Figs. 2B and S9). Consistent with our hypothesis that a NINJA-dependent repression mechanism blocks excessive JA responses in *myc2-322B*, basal *JAZ10* expression reached ~200 higher-than-WT levels in roots of *ninja myc2-322B* double mutants and intermediate *JAZ10* and *JGP* levels in double mutants homozygous for *ninja* and heterozygous for *myc2-322B* (*ninja myc2-322B/+*) (Figs. 2D and S10). Although *myc2-322B* segregated recessively in the WT background, we found that it is a dosage-dependent, gain-of-function allele once it is released from NINJA-dependent repression mechanisms in specific zones of the primary root.

When grown in control conditions, *myc2-322B* had up to 20% shorter roots than WT (Fig. 2E), associated with decreased meristem cell number and cell elongation in the differentiation zone (Fig. 2F-G). Root growth was more strongly affected in *ninja myc2-322B* double mutants: primary root length in control conditions reached only 50% of the WT length, and both meristem cell number and cell elongation in the differentiation zone were markedly reduced, and were similar to those of WT treated with JA (Fig. 2F-G).

We extended our studies to the *atr2D* allele of *MYC3* in which a D94N missense mutation in the JAZ-interacting domain (JID) causes released repression from most JAZ proteins and activation of stress-responsive genes (Chapter II.2; Smolen *et al.*, 2002; Fernández-Calvo *et al.*, 2011; Goossens *et al.*, 2015). The *atr2D* mutant accumulated ~3 times higher-than-WT *JAZ10* transcript levels in roots of seedlings (Fig. S11) without altering root length (Fig. S12). Conversely, roots of *ninja-1 atr2D* double mutants accumulated ~100 fold higher *JAZ10* levels than WT but their length was similar to *ninja* mutants. *JAZ10* transcripts were ~30 fold higher-than-WT in roots of *myc2-322B atr2D* double mutants (Fig. S11) but this did not reduce root length beyond that in *myc2-322B* alone (Fig. S12).

MYC2^{E165K} results in enhanced transcriptional activity and partial release from JAZ repression

The *myc2-322B* mutant presumably produces a MYC2 protein affected in the transcriptional activation domain (TAD, residues 149–188) responsible for recruiting the Mediator transcription initiation complex (Çevik *et al.*, 2012; Zhai *et al.*, 2013). Embedded in the TAD of MYC2, a stretch of acidic amino acids (MYC2$^{154–165}$) constitutes the destruction element (DE) required for both proteasome-dependent degradation and MYC2 functionality (Zhai *et al.*, 2013). Deleting the entire DE resulted in a more stable MYC2 protein that could no

longer induce the transcription of MYC2 regulated genes, such as *LIPOXYGENASE2* (*LOX2*) (Zhai *et al.*, 2013). The putative MYC2^{E165K} variant found in *myc2-322B* is altered in the last amino acid of the DE and could result in defective proteolysis and/or transcriptional activity. A functional MYC2^{E165K}-CITRINE transgene driven by the endogenous *MYC2*$_{pro}$ was expressed in the same root cells as WT MYC2-CITRINE but with a much stronger reporter signal (Figs. S13 and S14). This apparently higher MYC2^{E165K} protein accumulation was not the result of increased transcripts as *MYC2* levels were the same between WT and *myc2-322B* mutant roots (Fig. S15). Furthermore, after inhibition of *de novo* protein synthesis with cycloheximide (CHX), MYC2^{E165K}-CITRINE levels decreased over time, suggesting that the mutant protein is subjected to degradation just as WT MYC2-CITRINE (Fig. S16). Probably due to higher initial levels, MYC2^{E165K}-CITRINE was still visible 60 min after CHX treatment, while MYC2-CITRINE had almost disappeared (Fig. S16). Concomitantly, we could also not detect aberrations in *LOX2* accumulation 1 h after wounding in *myc2-322B* (Fig. S17), implying that MYC2^{E165K} is functional.

We then assessed the transactivation capacity of MYC2^{E165K} and its regulation by JAZ repressors. In transient expression assays in tobacco protoplasts, MYC2^{E165K} had higher than WT activity in inducing the MYC2-responsive promoter of *LOX3* (*LOX3*$_{pro}$) driving the expression of a *FIREFLY LUCIFERASE* (*fLUC*) reporter (Fig. 3). The transactivation of *LOX3*$_{pro}$ by WT MYC2 was counteracted by co-expression with all 7 JAZ proteins tested (Fig. 3). However, the transactivation of *LOX3*$_{pro}$ by the MYC2^{E165K} mutant protein was inhibited by co-expression with JAZ1 only, while it was less repressed by JAZ8, JAZ9 and JAZ10, and it failed to be inhibited by JAZ4, JAZ6 and JAZ12 (Fig. 3). We further compared the ability of WT MYC2 and MYC2^{E165K} to interact with 12 JAZ proteins in yeast two-hybrid (Y2H) assays. As reported previously (Chapter II.2; Fernández-Calvo *et al.*, 2011; Goossens *et al.*, 2015), WT MYC2 was able to interact with all JAZs, except with JAZ7 (Fig. 4). On the other hand, MYC2^{E165K} was able to strongly interact only with JAZ1 in Y2H (Fig. 4). Thus, MYC2^{E165K} promotes the expression of early JA-responsive genes as a consequence of both increased transactivation capacity and reduced inhibition by JAZ proteins. The gain-of-function behaviour of MYC2^{E165K} was then tested using an *in planta* genetic approach.

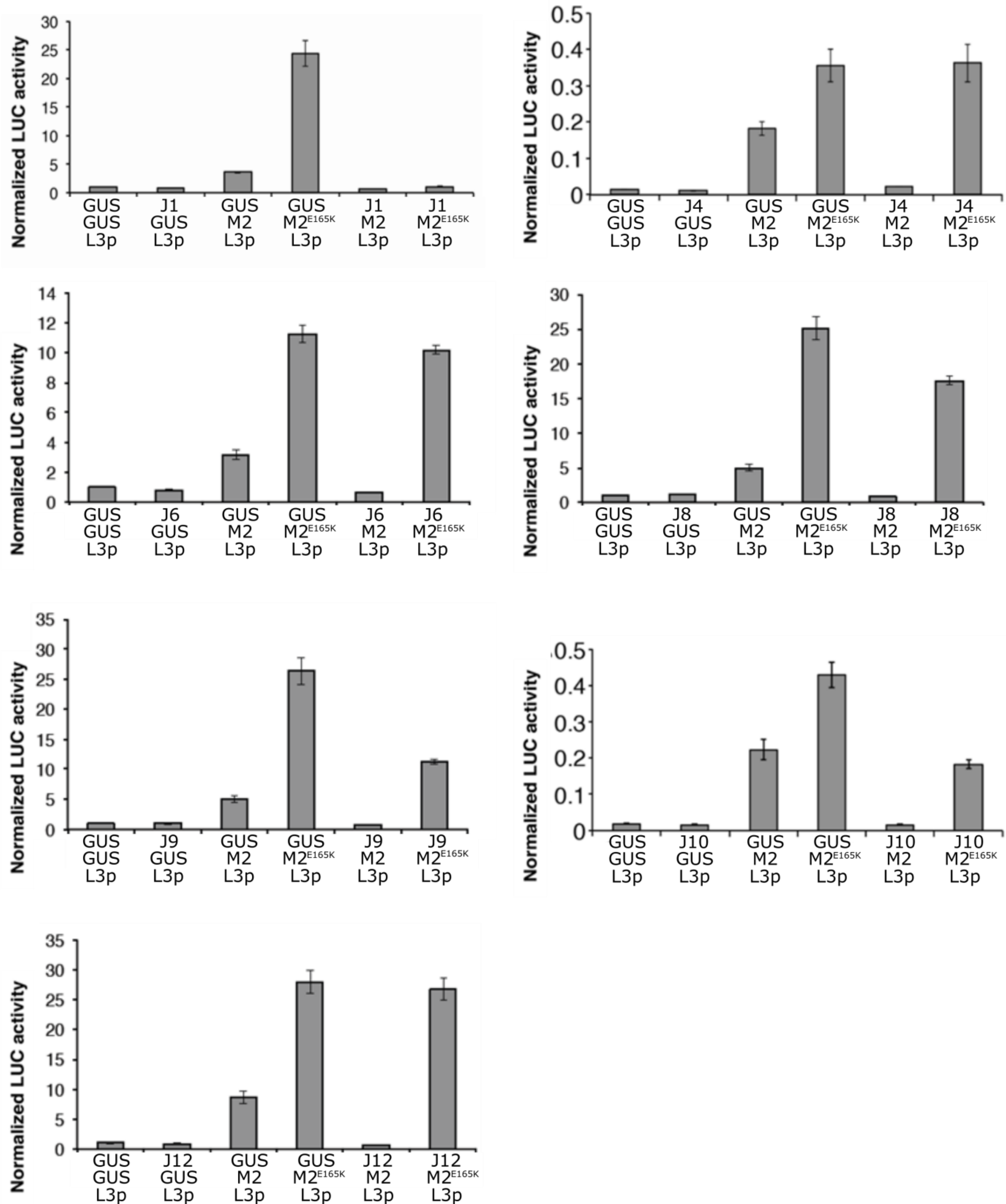

Figure 3: MYC2^{E165K} has increased transactivation capacity. Transactivation of the *LOX3* promoter by transient expression of MYC2 or MYC2^{E165K} in the presence or absence of JAZ1, JAZ4, JAZ6, JAZ8, JAZ9, JAZ10 and JAZ12 repressors. Tobacco protoplasts were transfected with a $LOX3_{pro}$-*fLUC* (L3p) reporter construct, a $35S_{pro}$-*MYC2* (M2) or $35S_{pro}$-$MYC2^{E165K}$ (M2^{E165K}) effector constructs in the presence or absence of $35S_{pro}$-*JAZ1* (J1), $35S_{pro}$-*JAZ4* (J4), $35S_{pro}$-*JAZ6* (J6), $35S_{pro}$-*JAZ8* (J8), $35S_{pro}$-*JAZ9* (J9), $35S_{pro}$-*JAZ10* (J10) or $35S_{pro}$-*JAZ12* (J12) constructs, and a $35S_{pro}$-*rLUC* normalization construct. The $35S_{pro}$-*GUS* (GUS) was used as control. Bars represent the means of 8 biological replicates (±SE) of normalized fLUC:rLUC activities.

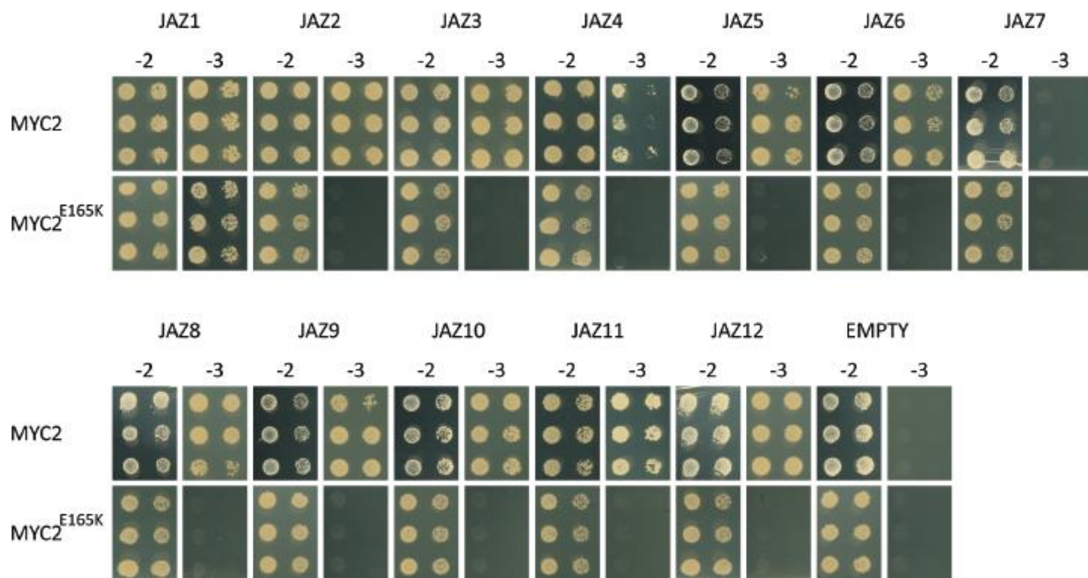

Figure 4: MYC2^{E165K} lost interaction with most JAZ repressors except with JAZ1. Yeast cells co-transformed with prey (MYC2 or MYC2^{E165K}), fused to GAL4-AD, and baits (JAZ1-12), fused to GAL4-BD, were selected and grown on synthetic defined media lacking Leu and Trp (-2) as transformation control and on selective media lacking Leu, Trp and His (-3) to test protein interactions.

The *ninja-1* and *myc2-322B* mutations were introgressed into backgrounds that are fully male-sterile as a consequence of abolished JA production (*aos*) or signalling (*coi1-1*). Remarkably, when MYC2^{E165K} was liberated from NINJA-dependent repression, it was able to restore fertility of *aos* and of *coi1-1* mutants in *ninja-1 myc2-322B aos* and *ninja-1 myc2-322B coi1-1* combinations, whereas the WT copy of MYC2 failed to do so (Figs. 5 and S18). *MYC2* transcript levels in stage 12 flowers were similar between WT and *aos*, whereas they were increased in the *ninja-1 myc2-322B aos* triple mutant (Fig. S19). Furthermore, we tested whether the restored fertility was a consequence of *MYB21* and *MYB24* induction, two TFs essential for male fertility whose expression is impaired in *aos* flowers (Reeves *et al.*, 2012). In the *ninja-1 myc2-322B aos* triple mutant the expression of *MYB21* rose to WT levels and that of *MYB24* was intermediate between that of *aos* and WT. Conversely, the expression of both TFs was lower-than-WT in *ninja-1 aos* mutants with WT MYC2 (Fig. S19). The growth effects of MYC2^{E165K} are therefore not seedling specific, but extend into reproductive organs and adult-phase rosettes (Fig. S20). To consolidate this, we used a repetitive leaf-wounding assay that is known to cause a JA-dependent reduction in WT rosette growth (Yan *et al.*, 2007; Zhang & Turner, 2008). In this assay, the *myc2-322B* mutant was more sensitive than the WT to wound-induced growth reduction (Fig. S21). Finally, *myc2-322B* was more susceptible than WT when challenged with the generalist herbivore *Spodoptera littoralis* (Fig. S22).

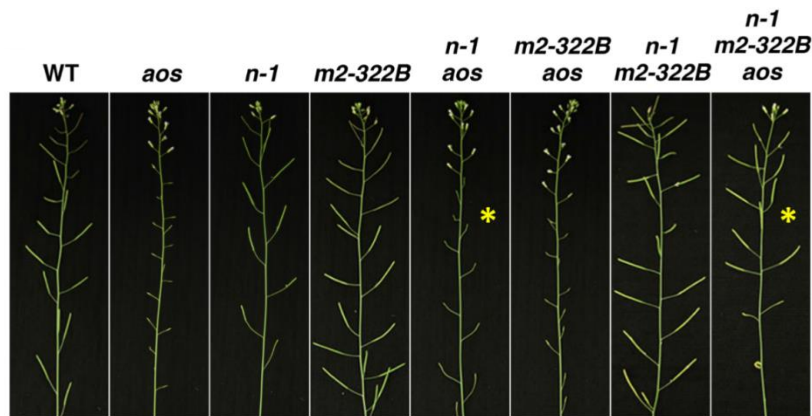

Figure 5: MYC2^{E165K} is a MYC2 gain-of-function allele. Main inflorescences from 5 week-old plants of WT,*aos*, *ninja-1* (*n-1*), *myc2-322B* (*m2-322B*), *ninja-1 aos* (*n-1 aos*), *myc2-322B aos* (*m2-322B aos*), *ninja-1 myc2-322B* (*n-1 m2-322B*) and *ninja-1 myc2-322B aos* (*n-1 m2-322B aos*). Note the restored fertility in the *ninja-1 myc2-322B aos* triple mutant compared to the sterility of *ninja-1 aos* with a WT MYC2 protein (yellow asterisks).

MYC2^{E165K} renders roots hypersensitive to exogenous JA

Loss-of-function *myc2* alleles are partly insensitive to JA-mediated root growth inhibition while the overexpression of *MYC2* causes mild hypersensitivity (Lorenzo *et al.*, 2004) with 75% reduction in root length compared to the 50% reduction observed for the WT (Niu *et al.*, 2011). The *myc2-322B* mutant responded far more strongly to exogenous JA: its root length was up to 99% shorter in media supplemented with methyl JA (MeJA) compared to control conditions (Fig. 6A-C). The JA-mediated hypersensitivity phenotype was specific to *myc2-322B* and did not extend to the *atr2D* allele of MYC3 (Fig. S12) or to plants overexpressing a *MYC^{D105N}* variant with diminished JAZ binding ability (Chapter II.2; Goossens *et al.*, 2015). Moreover, all mutant combinations with *myc2-322B* displayed a hypersensitive phenotype in JA-mediated root growth inhibition assays (Fig. 6C), with almost no measurable root meristem (Fig. 6B). Triple mutant combinations of *ninja-1 myc2-322B aos* and *ninja-1 myc2 coi1-1* showed that the extreme root growth reduction constitutively observed in *ninja-1 myc2-322B* double mutants partly relies on de novo JA synthesis and signalling as triple mutants had intermediate root lengths between the *ninja- 1 myc2-322B* and *myc2-322B* mutants (Fig. 6C). Consistently, *ninja-1 myc2-322B aos* was hypersensitive to MeJA treatment, while *ninja-1 myc2-322B coi1-1* was completely insensitive (Fig. 6C).

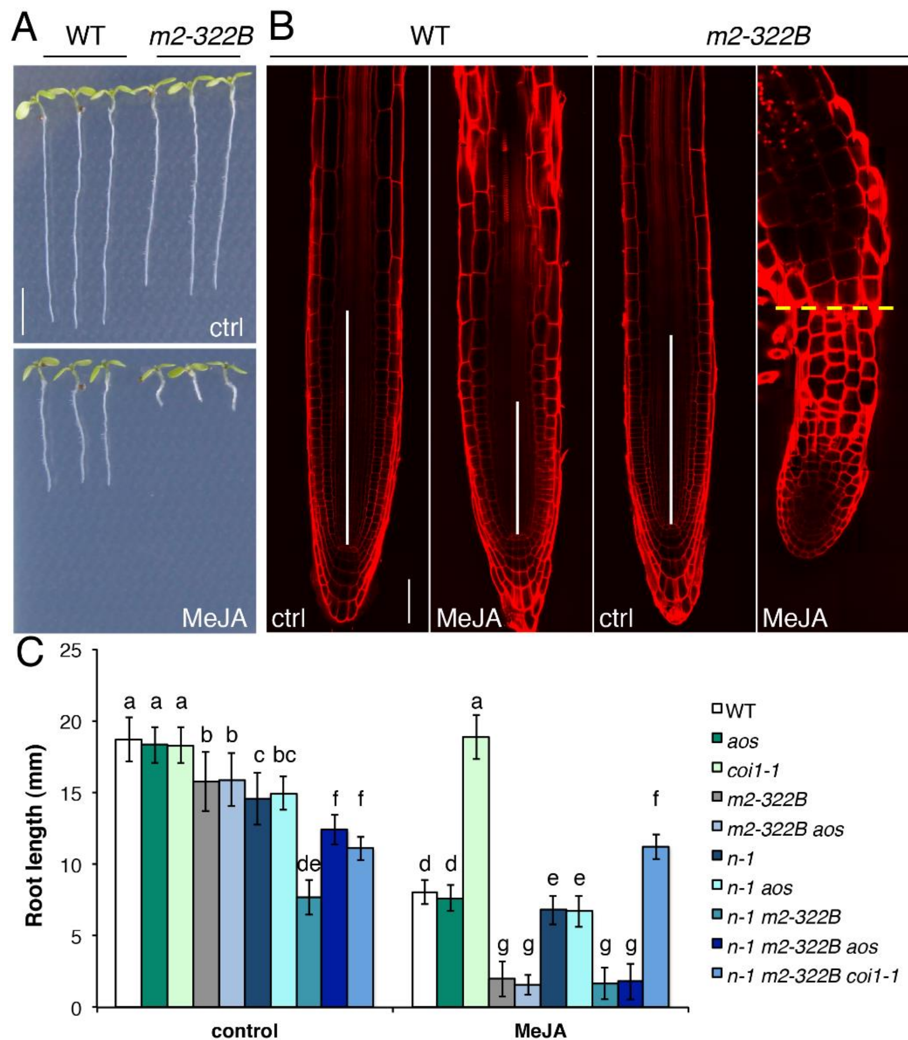

Figure 6: MYC2 ^{E165K} confers extreme hypersensitivity to exogenous JA. (A) Representative 7-do seedlings of WT and *myc2-322B* (*m2-322B*) mutants grown in control conditions (ctrl) or on media supplemented with 25 µM MeJA. Scale bar = 0.5 cm (B) Confocal microscopy images of propidium iodide stained primary root meristems of WT and *myc2-322B* 5-do seedlings grown in the absence (ctrl) or presence of 25 µM MeJA. Scale bar = 50 µm. Vertical white bars represent the root division zone and the horizontal yellow dashed line marks the root—hypocotyl boundary of *myc2-322B* grown in the presence of MeJA. (C) Root length of 7-do seedlings of the indicated genotype grown in the absence (control) or presence of 25 µM MeJA. *n-1* refers to *ninja-1*. Data shown are means (± SD) from 20–49 plants. Letters above bars indicate statistically significant differences between samples as determined by Tukey's HSD test (P < 0.01)

DISCUSSION

myc2-322B is a gain-of-function *MYC2* mutant

Increased *JAZ10* expression in the root of *myc2-322B* is associated with reduced root growth. While *JGP* reporter expression coincides with reduced cell elongation in the root differentiation zone of *myc2-322B*, the *JGP* expression does not correlate with the reduced cell proliferation observed in the root division zone. JA-induced MYC2 inhibits cell proliferation in the root division zone by directly repressing the expression of *PLETHORA*

(*PLT*) genes that mediate auxin regulation of stem cell niche maintenance in the root division zone (Chen *et al.*, 2011). Because the regulatory function of TFs may depend on the cell-type specific network of interactions, the transcriptional outputs of MYC2 might differ in different cell-types and root areas: repression of *PLT* genes in the root division zone without activating *JAZ10* transcription and *JAZ10* activation in the root differentiation zone leading to compromised cell elongation.

Although MYC2^{E165K} is mutated in the transcriptional activation domain (TAD), it lost the ability to interact with JAZ repressors with the exception of JAZ1, as indicated by Y2H assays. This suggests that, in addition to the previously defined JAZ-interacting domain (JID) of MYC2 (Chini *et al.*, 2007; Thines *et al.*, 2007), the destruction element (DE) within the TAD of MYC2 (Zhai *et al.*, 2013) might influence JAZ binding. Accordingly, JAZ1 was able to fully repress MYC2^{E165K} transcriptional activity in protoplast transient expression assays; while JAZ8/-9/-10 were able to only partly repress MYC2^{E165K} transcriptional activity, and JAZ4/-6/-12 did not show repressor capacity on MYC2^{E165K}. The observed repressor activity of JAZ8/-9/-10 on MYC2^{E165K} in plant protoplasts is likely due to the ability of JAZ proteins to form hetero-dimers among each other (Pauwels & Goossens, 2011). Specifically, JAZ1 was shown to interact with JAZ8, JAZ9 and JAZ10 (Chini *et al.*, 2009; Chung & Howe, 2009), suggesting that the repressor activity of JAZ8/-9/-10 observed in transient expression assays may rely on hetero-dimerization with a tobacco JAZ1 orthologue and consequent binding to MYC2^{E165K}. The results emphasize the diversity among JAZ proteins in interacting with MYC2 and with one another (Pauwels & Goossens, 2011). Similarly to MYC2^{E165K}, a recently identified MYC2^{D105N} allele mutated in the JID of MYC2 causes impaired protein interactions with most JAZ repressors (Chapter II.2; Goossens *et al.*, 2015). However, the transactivation potential of MYC2^{D105N} did not differ from WT MYC2 in inducing *pLOX3-fLUC* in tobacco protoplasts (Chapter II.2; Goossens *et al.*, 2015), while that of MYC2^{E165K} from the *myc2-322B* mutant was much greater than WT MYC2. Since the TAD of MYC2 is also necessary for MED25 binding (Çevik *et al.*, 2012; Chen *et al.*, 2012), it also remains possible that the MYC2^{E165K}-MED25 interaction is altered in *myc2-322B*, favouring a more efficient docking of the Mediator complex to recruit RNA polymerase II and initiate gene transcription.

An alternative scenario is provided by our finding that MYC2^{E165K} seems to accumulate at higher levels than WT MYC2 in basal conditions. It is possible that MYC2^{E165K} is translated more rapidly than WT MYC2, as we did not detect differences in *MYC2* transcript levels between WT and *myc2-322B*. If this was the case, JAZ repression might be relieved if a higher number of MYC2^{E165K} molecules outcompetes the available number of JAZ repressors. This supports the control of MYC2 levels within strict limits as a regulatory layer of JA signalling. Such a mechanism is responsible for the strong effects of shade and light signalling in JA-regulated responses (Chico *et al.*, 2014).

Finally, at the reproductive stage, *myc2-322B* in the appropriate background (*ninja-1 myc2-322B aos* or *ninja-1 myc2-322B coi1-1*) revealed that it is possible to recapitulate archetype hormone response phenotypes (in this case male fertility) in the absence of the hormone (JA) or of its receptor (COI1). These results also imply a putative role of MYC2 in male fertility as we found that basal *MYC2* expression in flowers is *aos*-independent. Thus, the gain-of-function *myc2-322B* allele released from NINJA-dependent repression could induce the expression of *MYB21* and *MYB24*, while WT MYC2 was unable to do so.

Myc2-322B represents a novel allele that may find many uses, for example in amplifying JA responses after mild stimulation. Moreover, this mutant will be useful for dissecting JA signalling events in both the adult and reproductive phases. In fact, the mutant rendered rosette leaves hypersensitive to wounding, although it displayed decreased resistance to a chewing herbivore relative to the WT. It is possible that the herbivore susceptibility phenotype of *myc2-322B* is a consequence of increased MYC2^{E165K} repressor activity on some defence genes (e.g. *PDF1.2*) (Lorenzo *et al.*, 2004). Increasing JA signalling may not always lead to enhanced defence against herbivores.

Layers of root growth regulation by JA signalling

The constitutive *JAZ10* expression and root length phenotypes in *myc2-322B* were relatively mild due to NINJA-dependent and -independent repression mechanisms, such as direct recruitment of TPL by one or more NINJA-independent JAZs (Zhu *et al.*, 2011; Shyu *et al.*, 2012a); direct recruitment of HISTONE DEACETYLASE 6 (HDA6) by JAZ1 to inhibit transcription (Zhu *et al.*, 2011); MYC2 stability, turnover and phosphorylation (Zhai *et al.*, 2013); and regulation of MYC-MED interactions to promote transcription. Indeed, removing NINJA-dependent repression from MYC2^{E165K} in *ninja myc2-322B* double mutants led to much stronger phenotypes: extended *JGP* reporter activity along the whole root, 200 fold higher-than-WT root *JAZ10* expression and root length and cellular phenotypes analogous to those of the WT treated with JA. NINJA-dependent repression mechanisms probably inhibit the basal activity of MYC TFs expressed in the root, explaining the lack of morphological phenotypes of *myc* KO mutants. This was further confirmed in heterozygous *myc2-322B*/+ mutants that showed no defects in *JGP* reporter activity or root length phenotypes in the presence of functional NINJA, but that displayed increased *JAZ10* levels and reduced growth in a *ninja* background. A synergistic effect on *JAZ10* expression was also observed in a double mutant between *ninja-1* and a gain-of-function allele of MYC3, *atr2D*. However, the *ninja-1 atr2D* morphological root phenotypes did not differ from those of *ninja*, indicating that this mutant version of MYC3 induces JA signalling without affecting root growth.

It is likely that the activity of MYC2 is tightly controlled by yet additional NINJA-independent repression mechanisms because, except in *coi1-1* backgrounds, all mutant combinations with *myc2-322B* were hypersensitive to JA in root growth assays. These findings imply that such repression mechanisms are still able to partly repress basal JA responses in the *ninja myc2-322B* double mutant to allow root growth, and that they rely on JAZ repressors, particularly JAZ1, which are readily degraded in the presence of JA (Chini *et al.*, 2007; Thines *et al.*, 2007). NINJA-dependent and -independent mechanisms, together with tight controls on MYC2 levels or activity, represent multiple regulatory levels adopted to repress JA responses and guarantee normal root development in the absence of JA. However, MYCs are ready to activate JA responses in the event of a JA stimulus. Such a multilayered organization of JA signalling repression mechanisms also explains the lack of a JA-hypersensitive phenotype in *ninja* mutants. The NINJA-dependent repression mechanism requires docking onto JAZ repressors, thus JAZ stability is epistatic to NINJA-dependent repression and lack of a functional NINJA protein cannot render the roots more sensitive than WT in JA-mediated root growth inhibition assays.

CONCLUSION

JA signalling in roots is constitutively restrained by multiple negative regulatory layers in basal conditions. However, damage to plant tissues through herbivory and environmental insult is common if not omnipresent in nature and stimulates the JA pathway. The resulting growth restriction can strongly impact plant productivity and is therefore of both fundamental and agronomic importance. Taking roots as a model, and using the simplified scheme for canonical JA signalling shown in Fig. 1, we show that it is possible to manipulate regulatory layers in the JA pathway such that cell division and cell elongation can be constrained and that general JA responses can be generated without the need for JA treatment or wounding. This led to the characterization of a gain-of-function *MYC2* mutant that could uncouple JA biosynthesis from JA response. This type of approach may lead to future strategies to alter organ growth and, potentially, uncouple it from defence responses that occur when JA signalling is initiated.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana accession Columbia (Col) was the WT line as well as the genetic background of previously described mutants and transgenic lines used in this study: *aos* (Park *et al.*, 2002), *coi1-1* (Feys *et al.*, 1994), *JGP*, *ninja-1*, *ninja-2 and ninja-3* (Acosta *et al.*, 2013), *myc2* (*jin1-2*), *jin1-7* (Lorenzo et al., 2004) and *atr2D* (Smolen *et al.*, 2002). The *myc2-322B* mutant allele was identified in a forward genetic screen for ectopic expression of a secretable *JAZ10*$_{pro}$*-GUSPlus*^{sec} (*JGP*) reporter and was identified with whole-genome sequencing of bulk segregants, as described (Acosta *et al.*, 2013).

For experiments with *myc2-322B*, the WT control was the *JGP* reporter line shown to have the same root length, basal and wound induced *JAZ10* expression as Col-0 (Acosta *et al.*, 2013).

After seed stratification for 2 d at 4°C, plants were grown at 21°C under 100 µE m^{-2} s^{-1} of light with photoperiod depending on the application (seedlings: 14 h light, 10 h dark; soil-grown plants for herbivory assays: 10 h light, 14 h dark; soil-grown plants for crosses and phenotyping: 24 h light). For seedling growth, seeds were surfaced-sterilized and grown on half-strength Murashige and Skoog solid medium (0.5X MS, 0.5 g/L MES hydrate, pH 5.7) supplemented with 0.7% agar (for horizontally-grown seedlings sawn on 200 µm pore size nylon mesh) or 0.85% agar (for vertically-grown seedlings), as described previously (Acosta *et al.*, 2013).

Plant treatments

For repetitive wounding, cotyledons of vertically grown seedlings were pierced with a micro-needle (36 gauge beveled needle) in aseptic conditions under a stereomicroscope. Wounding started in the morning (7–8 am) of the third day after transfer to the phytotron (3-do seedlings) and was repeated every 12 h on alternate cotyledons, for a total of 5 wounds per seedling.

Single cotyledon wounding of seedling, MeJA treatments, root phenotypic measurements (total length of primary root and cellular measurements), herbivory assays with *S. littoralis* were performed as described (Acosta *et al.*, 2013).

Histochemical detection of GUS activity in the primary root

GUS staining and histology of horizontally grown entire seedlings were performed as in (Acosta *et al.*, 2013). For GUS staining and sectioning of the primary root, vertically grown 5-do seedlings were carefully transferred to GUS staining solution (50 mM sodium phosphate buffer pH 7.0, 0.1% Triton X-100, 3 mM $K4Fe(CN)_6$, 3 mM $K3Fe(CN)_6$, 0.5 mg/ml X-Gluc) and incubated at 37°C in the dark for 2–4 h. For imaging the primary root tip, the reaction was stopped by replacing the staining solution with 50 mM sodium phosphate buffer pH 7.0. Roots were then immediately mounted in freshly prepared chloral hydrate: glycerol: water solution (8:2:1). For cross-sections of GUSPlus reporter lines, the staining solution was replaced with 15% EtOH in water for 30 min, followed by 30 min incubation in 30% EtOH at RT. Seedling were then transferred to a 1% agarose support, bunches of 10–15 roots were closely aligned at the root tip and submerged with 1% warm agarose. Hardened agarose blocks containing the aligned roots were excised in ~0.4 cm^3 cubes that were dehydrated through an ethanol series (30%, 50%, 70%, 90% and twice absolute) under agitation for 30 min each at 4°C. Samples were embedded in Technovit 7100 resin (Haslab GmbH, Ostermundigen, Switzerland) according to the manufacturer's instructions. Briefly, samples were vacuum infiltrated with infiltration solution: absolute EtOH (1: 1) for 2 h and with infiltration solution for 3 h. Finally, samples were hardened with embedding solution and hardened agarose blocks were aligned at the root tip, arranged in sectioning moulds and covered with additional embedding solution under anaerobic conditions. Samples were sectioned on a Leica RM2255 microtome using disposable Leica TC-65 blades into 6 µm sections and visualized with an upright Leica DM5500 microscope fitted with a DFC420 camera.

Gene expression analyses

For qRT-PCR experiments of *JAZ10*, *MYC2* and *LOX2*, 5-do seedlings were grown horizontally, separated in shoots and roots or kept intact and collected for basal and 1 h after cotyledon wounding expression analysis. To determine the expression levels of *MYB21* and *MYB24*, stage 12 flowers (largest closed buds) from plants grown in continuous light were separated from the rest of the inflorescence and frozen in liquid N_2. Each biological replica consisted of equivalent flower buds from 3–4 inflorescences of the same plant. For genotypes in which flower maturation was impaired (*aos*, *aos ninja-1*) or delayed (*aos myc2-322B ninja-1*), equivalent stage flowers were identified according to their position in the inflorescence stem. RNA and cDNA were prepared as in (Gfeller *et al.*, 2011). Quantitative RT-PCR was performed as described (Chauvin *et al.*, 2013). Primers for qRT-PCR have been previously reported: *JAZ10* (At5g13220) and *UBC21* (At5g25760) in (Gfeller *et al.*, 2011) and *LOX2* (At3g45140) in (Glauser *et al.*, 2009). To quantify *MYC2* (At1g32640) transcripts the following primers were used: gtgcgggattagctggtaaa and atgcatcccaaacactcctc.

Genotyping

For selection of multiple mutant combinations from segregating populations *ninja-1* was amplified with ggaggatgagtcacggaaag and gggagctggactggtgagta primers and digested with *AciI* (WT = 359, 142 bp; mutant = 501 bp); *ninja-2* was amplified with tggtggttcttcttccaacc and gcaacaggttgtttgccttc primers and digested with *Hpy188I* (WT = 284, 209 bp; mutant = 284, 108, 101 bp); *ninja-3* was amplified with caacgggagacaacagcaac and tggcttgagagtttgatccg primers and digested with *TspRI* (WT = 302, 132, 2 bp; mutant = 436 bp); *myc2-322B* was amplified with gtcatcgaaaccaagaaaaacgatt and gagacggagatcgagttcgc primers and digested with *HinfI* (WT = 143, 25 bp; mutant = 168 bp); *atr2D* was amplified with caccacaacaaccacctcag and tgaagcagagaggcagagaag and digested with *BccI* (WT = 269, 162 bp; mutant = 431 bp).

Transgenic lines

Promoter for *MYC2* was amplified from WT genomic DNA with oligonucleotides cggggtacctcgtgtatttgtgtctgcatgt and ttcccccccgggtccataaaccggtgaccggtaa, 2.1 kb and cloned by restriction with *XmaI* and *KpnI* into a modified pUC57 (Chauvin *et al.*, 2013) to create pEN-L4-promoter-R1 clones. Underlined sequences represent *XmaI* and *KpnI* sites. Coding DNA sequences (CDS) of *NINJA* (ggggacaagtttgtacaaaaaagcaggctgcatggacgatgataatgggctc and ggggaccactttgtacaagaaagctgggttggtgtgagctgacgctgcag), *MYC2* and *MYC2*^{E165K} (ggggacaagtttgtacaaaaaagcaggctgcatgactgattaccggctaca and ggggaccactttgtacaagaaagctgggttaccgatttttgaaatcaaacttgc) were amplified with oligonucleotides specified in parenthesis containing the appropriate *att* sites (underlined). CDSs of *NINJA* were amplified from WT cDNA, of *MYC2*^{E165K} from *myc2-322B* genomic DNA, and of *MYC2* from WT genomic DNA. Amplification products were recombined into pDONR221 (Invitrogen) to produce pEN-L1-gene-L2 clones. To generate protein fusions under the control of endogenous promoters, pEN-L4-promoter-R1- plasmids were recombined with pEN-L1-CDS-L2 and pEN-R2-*CITRINE*-L3 plasmids into pB7m34gw by Multisite Gateway Technology to obtain pDEST-B4-promoter-B1-CDS-B2-*CITRINE*-B3

clones. All constructs were introduced into Arabidopsis backgrounds by floral dip Agrobacterium-mediated transformation. For protein fusions, lines were selected on media containing DL-Phosphinothricin 40 µg/ml (Duchefa Biochemie B.V., Haarlem, The Netherlands). A minimum of two independent transgenic lines were used for each construct to perform experiments and verify reproducibility.

Confocal microscopy

Confocal laser scanning microscopy was performed on a Zeiss LSM 700 confocal microscope with vertically grown 5-do seedlings. Roots were mounted in 0.5X MS with or without 30 µg/ml propidium iodide (Sigma). Excitation and detection windows were set as follows: CITRINE 488 nm (dye Citr), 490–555 nm (BP 490–555 filter); propidium iodide 555 nm (dye PI), 615–700 nm (LP 615 filter). All images shown within one experiment were taken with identical settings. Image processing was done with FIJI (http://fiji.sc/Fiji).

Transient expression assay in *Nicotiana tabacum* protoplasts

Transient expression assays were performed as described (De Sutter *et al.*, 2005; Vanden Bossche *et al.*, 2013). Protoplasts were prepared from a Bright Yellow-2 tobacco cell culture and co-transfected with a reporter plasmid containing the *firefly luciferase* (*fLUC*) reporter gene driven by the *LOX3* promoter (Pauwels *et al.*, 2008), a normalization construct expressing *Renilla luciferase* (*rLUC*) under control of the *35S* promoter (De Sutter *et al.*, 2005) and effector constructs. Effector constructs were made by Gateway cloning of MYC2, MYC2^{E165K}, JAZ1, JAZ4, JAZ6, JAZ8, JAZ9, JAZ10 and JAZ12 into the destination vector p2GW7 under control of the *35S* promoter. The p2GW7-GUS effector plasmid was used as mock (Pauwels *et al.*, 2010). For each transfection, 2 µg of each plasmid was used. After transfection, protoplasts were incubated overnight and then lysed; fLUC and rLUC activities were determined with the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiency and technical error were corrected by normalization of fLUC by rLUC activities. All transactivation assays were conducted in an automated experimental set-up with 8 biological replicates for each effector combination.

Yeast two-hybrid analysis

Y2H analysis was performed as described (Cuéllar Pérez *et al.*, 2014). Bait and prey were fused to the GAL4-AD or GAL4-BD via cloning into pGAL424gate or pGBT9gate (PSB, Ghent), respectively. The *Saccharomyces cerevisiae* PJ69-4A yeast strain (James *et al.*, 1996) was co-transformed with bait and prey using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) media lacking Leu and Trp (-2) (Clontech). Three individual colonies were grown overnight in liquid cultures (-2) at 30°C and 10-fold or 100-fold dilutions were dropped on control media (-2) and selective media lacking Leu, Trp and His (-3) (Clontech).

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Chapter 4

The RING E3 ligase KEEP ON GOING modulates JASMONATE ZIM-DOMAIN12 stability

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Published in:

Laurens Pauwels, Andrés Ritter, Jonas Goossens, Astrid Nagels Durand, Hongxia Liu, Yangnan Gu, Jan Geerinck, Marta Boter, Robin Vanden Bossche, Rebecca De Clercq, Jelle Van Leene, Kris Gevaert, Geert De Jaeger, Roberto Solano, Sophia Stone, Roger W. Innes, Judy Callis and Alain Goossens. ***The RING E3 Ligase KEEP ON GOING Modulates JASMONATE ZIM-DOMAIN12 Stability.*** *Plant Physiology 2015, 169(2):1405-17.*

[1]Author contributions: Molecular cloning, Y2H assays, transient expression assays, degradation assays, immunoblotting, data interpretation, participation to writing of the manuscript (Figures 2C, 4A, 4B and 4C and conclusions from these data).

ABSTRACT

Jasmonate (JA) signalling in plants is mediated by the JASMONATE ZIM-DOMAIN (JAZ) proteins that repress the activity of several transcription factors regulating JA-inducible gene expression. The hormone JA-Ile triggers the interaction of JAZ repressor proteins with the F-box protein CORONATINE INSENSITIVE1 (COI1), part of an Skp1/Cullin/F-box protein (SCF) E3 ubiquitin ligase complex, and their degradation by the 26S proteasome. In *Arabidopsis thaliana*, the JAZ family consists of 13 members. The level of redundancy or specificity among these members is currently not well understood. Here, we characterized JAZ12, encoded by a highly expressed *JAZ* gene. JAZ12 interacted with the transcription factors MYC2, MYC3, and MYC4 *in vivo* and repressed MYC2 activity. Using tandem affinity purification, we found JAZ12 to interact with SCF^{COI1} components, matching with observed *in vivo* ubiquitination and with rapid degradation after treatment with JA. In contrast to the other JAZ proteins, JAZ12 also interacted directly with the E3 RING ligase KEEP ON GOING (KEG), a known repressor of the ABSCISIC ACID INSENSITIVE5 transcription factor in abscisic acid signalling. To study the functional role of this interaction, we circumvented the lethality of *keg* loss-of-function mutants by silencing *KEG* using an artificial microRNA approach. Abscisic acid treatment promoted JAZ12 degradation, and *KEG* knock-down led to a decrease in JAZ12 protein levels. Correspondingly, KEG overexpression was capable of partially inhibiting COI1-mediated JAZ12 degradation. Our results provide additional evidence for KEG as an important factor in plant hormone signalling and a positive regulator of JAZ12 stability.

INTRODUCTION

The JASMONATE ZIM DOMAIN (JAZ) proteins are central in the signal transduction cascade triggered by the plant hormone (+)-7-iso-jasmonoyl-l-isoleucine (JA-Ile). In the presence of JA-Ile, they form a co-receptor complex with the F-box protein CORONATINE INSENSITIVE1 (COI1) (Fonseca *et al.*, 2009, Sheard *et al.*, 2010). COI1 forms part of the E3 ubiquitin ligase complex Skp1/Cullin1/F-box $(SCF)^{COI1}$ that mediates the ubiquitination of the interacting JAZ, which then leads to degradation of the JAZ protein by the proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007). Within seconds after the perception of JA-Ile, JAZ proteins begin to degrade (Pauwels *et al.*, 2010).

JAZ proteins function as repressors of specific transcription factors in the absence of JA-Ile (Niu *et al.*, 2011; Pauwels and Goossens, 2011; Zhu *et al.*, 2011; Hu *et al.*, 2013; Nakata *et al.*, 2013; Sasaki-Sekimoto *et al.*, 2013; Song *et al.*, 2013; Fonseca *et al.*, 2014). The perception of jasmonates (JAs) thus leads to the derepression of these transcription factors, followed by a rapid reprogramming of gene expression (Pauwels *et al.*, 2008; Attaran *et al.*, 2014). One of the best characterized transcription factors repressed by JAZ proteins is the basic helix-loop-helix protein MYC2 (Lorenzo *et al.*, 2004; Chini *et al.*, 2007; Kazan and Manners, 2012).

MYC2-JAZ interactions are predominantly mediated by the C-terminal Jas domain on JAZ proteins, which is also the site of JA-Ile and COI1 interaction (Katsir *et al.*, 2008; Melotto *et al.*, 2008; Sheard *et al.*, 2010). The molecular mechanism by which JAZ proteins repress transcription factor activity includes recruitment of the co-repressor TOPLESS (TPL), either directly or through the adaptor protein NOVEL INTERACTOR OF JASMONATE ZIM DOMAIN (NINJA) (Pauwels *et al.*, 2010; Acosta *et al.*, 2013).

There are 13 JAZ proteins in *Arabidopsis thaliana* (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007; Thireault *et al.*, 2015). It is assumed that there is a high level of redundancy, as indicated by the fact that many single jaz loss-of-function mutants do not have a phenotype (Thines *et al.*, 2007). However, some distinct properties of JAZ proteins are being discovered. Whereas most of the JAZ proteins interact with NINJA for interaction with the co-repressor TPL, JAZ7 and JAZ8 do not (Pauwels *et al.*, 2010). JAZ7 and JAZ8 have an ethylene-responsive element binding factor-associated amphiphilic repression motif themselves, which mediates direct interaction with TPL (Shyu *et al.*, 2012). Moreover, JAZ8 lacks a canonical degron; therefore, it is unable to associate strongly with COI1 in the presence of JA-Ile and, thus, is resistant to JA-mediated degradation (Shyu *et al.*, 2012). Other examples are JAZ1 and JAZ10, which contain, besides the C-terminal Jas domain, an additional Jas-like domain at their N terminus called the cryptic MYC2-interacting domain, which also mediates interaction with MYC2 (Chapter II.2; Moreno *et al.*, 2013; Goossens *et al.*, 2015). Possibly, this domain explains the dominant JA-insensitive phenotype when overexpressing *JAZ10.3* and *JAZ10.4* splice variants. These variants lack part of the Jas domain and lose COI1 interaction, but they can still interact with MYC2 (Moreno *et al.*, 2013).

Several studies have localized GFP-fused JAZ proteins to the nucleus (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007; Chung and Howe, 2009; Grunewald *et al.*, 2009; Shyu *et al.*, 2012; Withers *et al.*, 2012). These observations are consistent with JAZ proteins functioning to repress transcription factors by promoting binding to the co-repressor TPL. Recently, it was proposed that the nuclear localization of JAZ proteins is dependent on their interaction with MYC2. In a *myc2* loss-of-function mutant, yellow fluorescent protein (YFP)-JAZ9 is partially mislocalized to the cytoplasm (Withers *et al.*, 2012). These results suggest that JAZ function is at least partially regulated by subcellular localization.

The pathway of JA signalling resembles in many ways that of ABSCISIC ACID-INSENSITIVE5 (ABI5) and related ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTOR (ABF) basic leucine zipper (bZIP)-type transcription factors. G-box elements in promoters of JA- and abscisic acid (ABA)-responsive genes are recognized by basic helix-loop-helix- and bZIP-type transcription factors, respectively. These transcription factors then interact indirectly or directly with NINJA or the NINJA-related ABI5-binding proteins (Garcia *et al.*, 2008). The

latter also function as TPL adaptor proteins (Pauwels *et al.*, 2010). Furthermore, several points of crosstalk between JA and ABA signalling have been reported, possibly underlying the complex interplay between these pathways in disease resistance (Anderson *et al.*, 2004). For instance, the genes encoding the ABA receptors PYRABACTIN RESISTANCE-LIKE4 (PYL4) and PYL5 are upregulated by JA, and their mutants show altered JA responses (Lackman *et al.*, 2011). Conversely, MYC2 also plays an important role in this crosstalk, as it had been identified as a positive regulator of ABA signalling (Abe *et al.*, 2003; Lorenzo *et al.*, 2004). Correspondingly, overexpression of a mutant MYC2 version that cannot be fully repressed by JAZ proteins leads to ABA hypersensitivity (Chapter II.2; Goossens *et al.*, 2015).

The protein KEEP ON GOING (KEG) is an additional regulator of the bZIP-type transcription factors ABI5, ABF1, and ABF3 (Stone *et al.*, 2006; Chen *et al.*, 2013). This RING-type ubiquitin E3 ligase interacts directly with ABI5 and is a negative regulator of ABA signalling (Liu and Stone, 2010, 2013). In the absence of ABA, KEG ubiquitinates ABI5, leading to its proteasomal degradation (Stone *et al.*, 2006; Liu and Stone, 2010). ABA treatment, however, promotes KEG self-ubiquitination and degradation, leading to an increase in ABI5 levels (Liu and Stone, 2010). Intriguingly, in contrast to the nuclear ABI5 subcellular localization, KEG is present in the trans-Golgi network (TGN) (Gu and Innes, 2011) and is proposed to be a regulator of post-Golgi trafficking, including the secretion of apoplastic defense proteins (Gu and Innes, 2012). However, when inactivating the RING domain of KEG or mutating a conserved Lys in ABI5, an interaction between KEG and ABI5 can be observed outside the nucleus in the cytoplasm and at the TGN (Liu and Stone, 2013). This leads to a model in which cytoplasmic KEG activity regulates nuclear ABI5 levels because the latter is transported between these compartments (Liu and Stone, 2013).

Here, we characterized JAZ12 as a representative JAZ protein that interacts with SCF^{COI1} in a JA-Ile-dependent manner and negatively regulates MYC2. JAZ12 was found to be ubiquitinated *in vivo* and rapidly degraded upon JA treatment. However, in contrast to other JAZ proteins, JAZ12 interacted through its Jas domain directly with the HERC2-like (for HECT and RCC1-like) repeat domain of KEG. Intriguingly, KEG acted as a positive regulator of JAZ12 protein levels. ABA treatment increased JAZ12 degradation, and knocking down *KEG* led to lower JAZ12 levels, whereas overexpression of *KEG* partially prevented the COI1-mediated degradation of JAZ12.

RESULTS

Isolation of COI1 and JAZ12 protein complexes

Previously, we reported that tandem affinity purification (TAP) of JAZ1 in Arabidopsis cell cultures copurified the F-box protein COI1 when cells were treated for 1 min with JA (Pauwels *et al.*, 2010). Here, we generated Arabidopsis cells expressing *COI1* tagged on its C terminus with the protein G/streptavidin-binding peptide (GS) tag. After mock treatment with ethanol, we could purify the SCF components ARABIDOPSIS SKP-LIKE1 (ASK1), ASK2, and CULLIN1 (CUL1) (Table 1). When treated with 50 µm JA for 1 min, JAZ12 and NINJA were found. No peptides of other JAZ proteins were detected. RNA sequencing (RNA-Seq) analysis revealed that *JAZ12* was an abundantly expressed *JAZ* gene both in this system and in Arabidopsis seedlings (Fig. 1A), likely explaining why it was recovered preferentially over other JAZ proteins.

Table 1: Overview of the proteins purified by TAP with JAZ12 and COI1.

AGI	**Protein**	**JAZ12**			**COI1**	
		Cells -JA	**Cells +JA**	**Seedlings -JA**	**Cells -JA**	**Cells +JA**
AT5G20900	JAZ12	4	4	1	-	2
AT5G13220	JAZ10	2	2	-	-	-
AT1G72450	JAZ6	-	-	1	-	-
AT1G74950	JAZ2	4	3	1	-	-
AT4G28910	NINJA	4	4	1	-	2
AT4G17880	MYC4	4	4	1	-	-
AT1G32640	MYC3	4	4	1	-	-
AT1G32640	MYC2	4	4	1	-	-
AT2G39940	COI1	-	4	1	2	2
AT1G75950	ASK1	-	4	1	2	2
AT5G42190	ASK2	-	3	1	2	2
AT4G02570	CUL1	-	2	1	2	2
AT5G12140	CYS1	2	2	-	-	-
AT5G13530	KEG	4	4	1	-	-

Proteins were identified using peptide-based homology analysis of mass spectrometry data. Background proteins were withdrawn based on the frequency of occurrence of copurified proteins in a large GS TAP data set (Van Leene *et al.*, 2015). Numbers indicate the times the prey was identified in two experiments per column (−/+JA) with COI1 and four experiments per column (−/+JA) with JAZ12 for cells. Only one experiment was performed for seedlings. Only preys identified in at least two experiments were retained. AGI, Arabidopsis Genome Initiative identifier. –, Prey was not identified in this experiment.

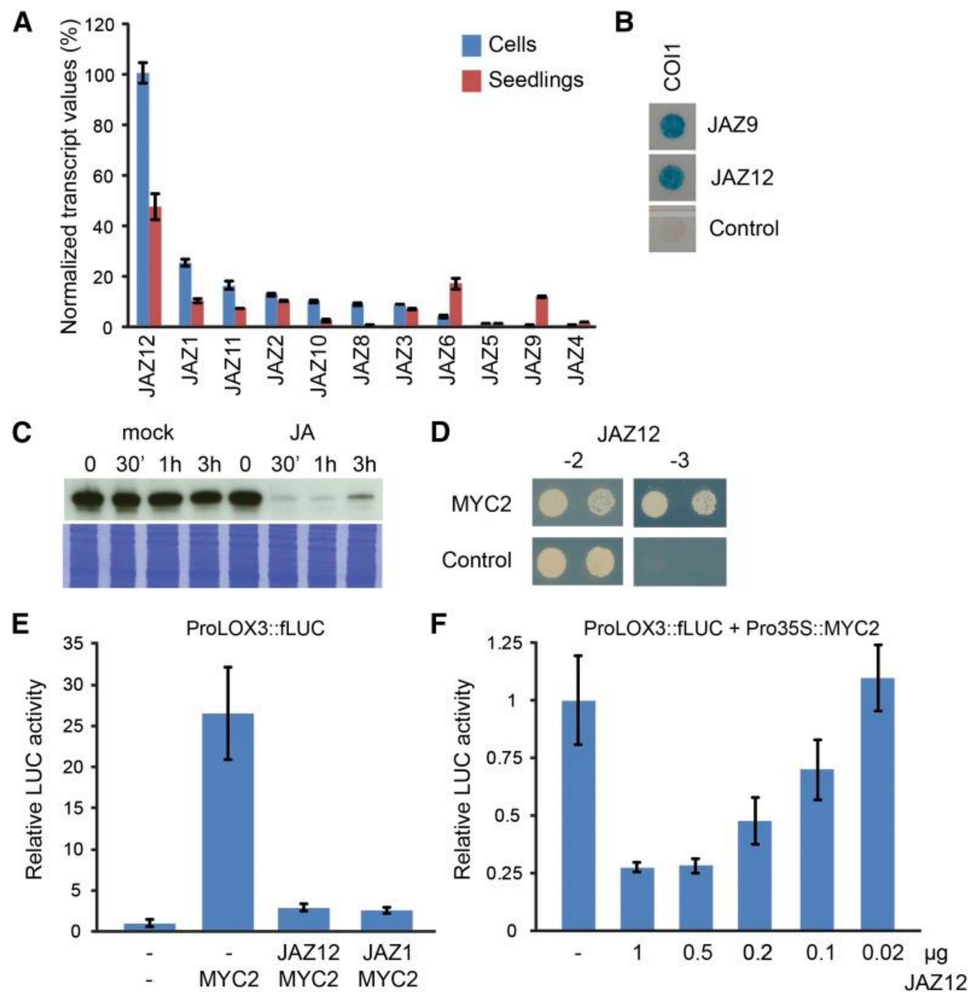

Figure 1: JAZ12 is a prototypal JAZ protein. A) *JAZ12* is a highly expressed JAZ gene in Arabidopsis suspension cultured cells and seedlings. Reads per kilobase per million mapped for each JAZ gene were plotted relative to *JAZ12* using RNA-Seq data from Arabidopsis suspension cultured cells (blue) and seedlings (red). *JAZ7* transcripts were not detected. B) COR mediates a direct interaction between COI1 and JAZ12. The EGY48 (p8opLacZ) yeast (*Saccharomyces cerevisiae*) strain was co-transformed with COI1 in pGILDA and JAZ9 and JAZ12 in pB42AD or pB42AD alone (control). Transformed yeasts were spotted on inducing medium containing Gal and raffinose supplemented with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside acid and 50 µm COR. C) JAZ12 is rapidly degraded by JA. Seven-day-old cell cultures expressing *JAZ12-GS* were treated with 50 µm JA or an equal volume of ethanol for the time points indicated. JAZ12-GS was detected by immunoblot using peroxidase anti-peroxidase (top), and the membrane was stained to inspect equal loading with Coomassie Blue (bottom). D) JAZ12 interacts directly with MYC2. The PJ69-4A yeast strain was co-transformed with JAZ12 in pGBKT7 and MYC2 in pGAD424gate or pGAD424gate as a control. The 10× and 100× dilutions of transformed yeasts were spotted on control medium lacking Leu and Trp (-2) or selective medium additionally lacking His (-3). E) JAZ12 is capable of repressing MYC2-mediated transactivation of the *LOX3* promoter. Tobacco protoplasts were transfected with a pLOX3-fLUC reporter construct and the indicated effector constructs in p2GW7, resulting in Pro-35S-mediated overexpression of *MYC2, JAZ12/JAZ1*, or both. A construct with the *Renilla luciferase* (rLUC) under control of Pro-35S was co-transfected for the normalization of fLUC activity. Error bars represent se of eight biological replicates. Two micrograms of each construct was transfected, and total DNA added was equalized with p2GW7-GUS plasmid. F) Dose-dependent inhibition of MYC2 activity by JAZ12. The experiment was as in (E) but p2GW7-JAZ12 was transfected at the indicated amounts of DNA, equalized for total plasmid DNA with p2GW7-GUS.

Next, we obtained *JAZ12-GS*-expressing cells and affinity-purified JAZ12-associated proteins from cells. In mock-treated cells, JAZ12 copurified with the JAZ proteins JAZ10 and JAZ2, NINJA, and the transcription factors MYC2, MYC3, and MYC4 (Table 1). When treated briefly with JA, we detected COI1, ASK1, ASK2 and CUL1 peptides (Table 1), indicating that the SCF^{COI1} complex associated with JAZ12. A Cys protease inhibitor, CYSTATIN1 (CYS1), was picked up in both mock- and JA-treated cells. CYS1 is induced by wounding and JA treatment and can block cell death induced by an avirulent *Pseudomonas syringae* pv. *Maculicola* strain (Belenghi *et al.*, 2003).

Using our TAP approach for Arabidopsis seedlings (Van Leene *et al.*, 2015), we could show that JAZ12 formed a similar complex as in the JA-treated suspension cells, thus associating with SCF^{COI1} even in the absence of exogenously added JA. Only one difference was noted: JAZ6 but not JAZ10 copurified. In the TAP performed with seedlings, a JAZ12 peptide modified with di-Gly could be found, pinpointing that Lys-169 is a putative *in vivo* modification site for ubiquitin or a ubiquitin-like protein site (Kirkpatrick *et al.*, 2005). Finally, in all JAZ12 TAP experiments, a previously unassociated protein was detected, the E3 ubiquitin ligase KEG, which has previously been linked to ABA signalling (Stone *et al.*, 2006).

JAZ12 is a typical JAZ protein

The protein sequence of JAZ12 is representative of the JAZ protein family. Besides the ZIM and Jas domains, no other conserved protein sequences have been reported for JAZ12 (Cuéllar Pérez *et al.*, 2014). The best-known protein-protein interactions of the JAZ proteins could be confirmed for JAZ12 by TAP (Table 1). To verify this further, we first tested direct interaction with COI1 using the LexA-based yeast two-hybrid (Y2H) system, which has been used previously to show interactions between COI1 and JAZ9 (Melotto *et al.*, 2008). Both JAZ9 and JAZ12 interacted with COI1 in the presence of 50 µm coronatine (COR), a JA-Ile mimic (Fig. 1B). Next, we tested the JA-mediated degradation of JAZ12. Cells expressing GS-tagged *JAZ12* were treated with 50 µm JA or ethanol for 30 min, 1 h, and 3 h. Already at the first time point, JAZ12-GS protein was nearly undetectable (Fig. 1C).

Y2H analyses also confirmed the direct interaction of JAZ12 with MYC2 (Fig. 1D). We have shown previously that a reporter construct with the JA-inducible *LIPOXYGENASE3* (*LOX3*) promoter driving a *firefly luciferase* (*fLUC*) reporter gene is transactivated by MYC2 in tobacco (*Nicotiana tabacum*) protoplasts (Pauwels *et al.*, 2008). Co-transfecting JAZ12 with MYC2 abolished this induction completely (Fig. 1E). This repressive effect of *JAZ12* co-expression was dose dependent, as illustrated by a titration curve using decreasing amounts of JAZ12 (Fig. 1F).

Finally, we isolated a transfer DNA (T-DNA) insertion line in *JAZ12*, which we called *jaz12-1*. The T-DNA is inserted in the Jas intron (Fig. S23A) (Chung and Howe, 2009). Therefore, we tested the expression of *JAZ12* with primers 5′ and 3′ of the T-DNA. While *JAZ12* expression 5′ of the T-DNA was only modestly (approximately 50%) reduced (Fig. S23B), very few transcript 3′ of the T-DNA (approximately 10%) could be found (Fig. S23B). JA-induced root growth inhibition in *jaz12-1* plants was similar to that in control plants (Fig. S23C). This suggests that either enough functional JAZ12 protein is present in this line or that redundancy exists with other JAZ proteins. The latter is in line with a previous report that single *jaz* loss-of-function mutants lack a phenotype (Thines *et al.*, 2007).

KEG interacts specifically with JAZ12

In the TAP analysis, we identified KEG as an interactor of JAZ12 (Table 1). In parallel, we performed a Y2H library screen using full-length KEG as bait. This screen identified JAZ12 as a direct interactor. Out of the 40 colonies with in-frame interactors, 35 corresponded to JAZ12. The other five interactors were FATTY ACID BIOSYNTHESIS2 (FAB2), CHLOROPHYLL a/b-BINDING PROTEIN2 (CAB2), GLUTAMATE RECEPTOR3.4 (GLR3.4), chlorophyll-binding PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE2 (LHCA2), and an oxidoreductase (At1g60710), all picked up only once in the Y2H screen. Subcloning of KEG revealed that the Y2H interaction with JAZ12 was mediated by the C-terminal HERC2-like repeat domain of KEG (Fig. 2A). Conversely, full-length KEG, as well as the fragment consisting of only the HERC2-like repeat domain, interacted specifically with the Jas domain of JAZ12 (Fig. 2B). MYC2 and NINJA were used as positive controls for interactions with the Jas and ZIM domains, respectively.

We also cloned the *JAZ12-1* variant from *jaz12-1* complementary DNA. The T-DNA insertion in the Jas intron in this line leads to the expression of an mRNA with the Jas intron retained. Because a stop codon is present in the intron, it results in a JAZ12 variant with a partial Jas domain, lacking C terminally the PY sequence (Fig. S23A). As expected, the COR-dependent interaction of JAZ12-1 with COI1 could no longer be observed (Fig. 2C). As controls, we included JAZ12(RR142/143AA) and JAZ12(F149A) constructs in which residues of the Jas motif that are essential for COI1 interaction were mutated. These mutant JAZ12 proteins, however, were still capable of KEG interaction (Fig. 2C; Sheard *et al.*, 2010). It has been shown previously that ΔPY variants of JAZ2.2 and JAZ10.3 can still interact with MYC2 (Chung and Howe, 2009). In contrast, JAZ12-1 did not interact with MYC2 or with KEG but still interacted with NINJA (Fig. 2B).

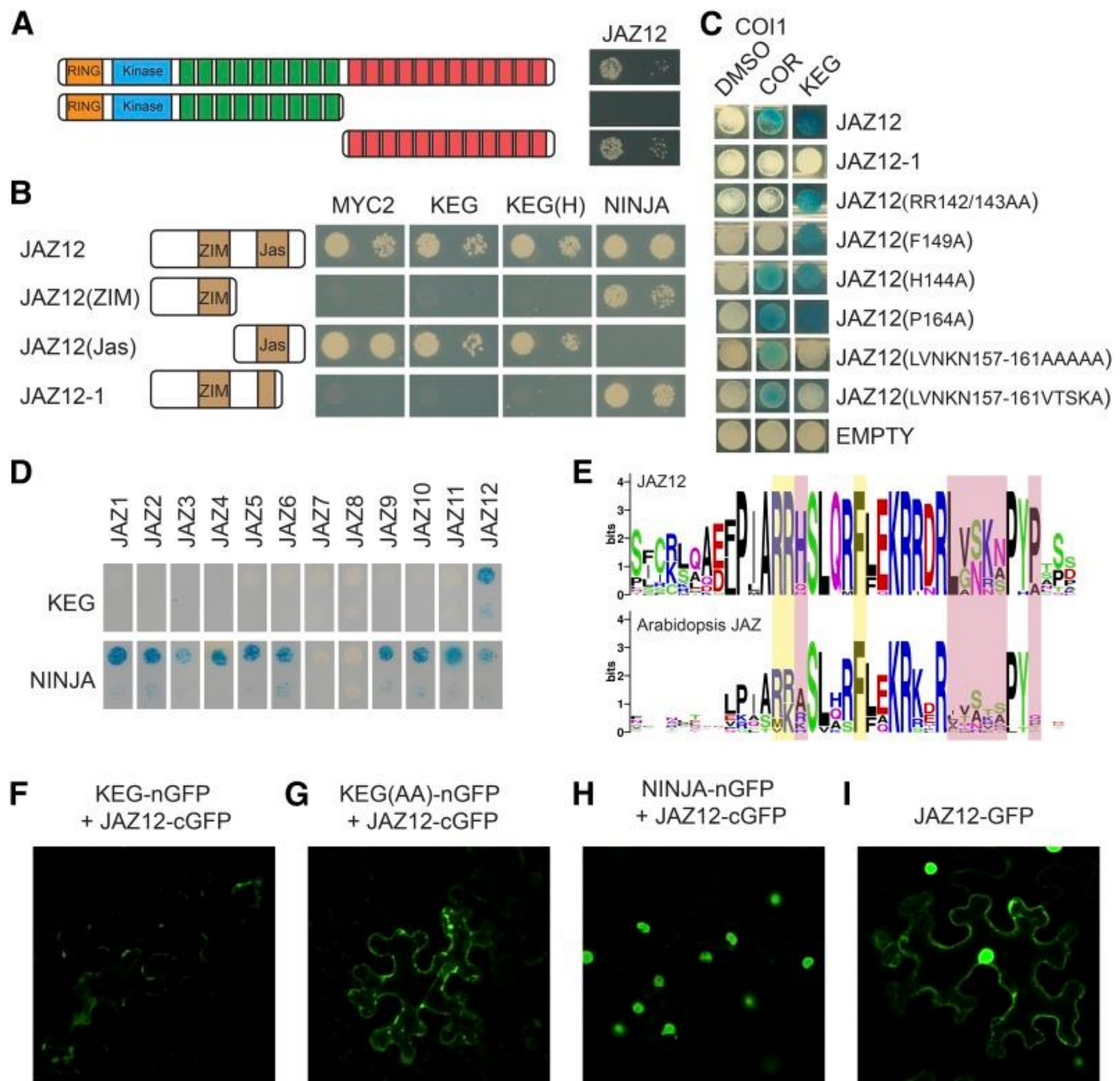

Figure 2: KEG interacts directly and specifically with JAZ12. A) JAZ12 interacts directly with the HERC2 repeats of KEG. The KEG protein structure is schematically represented with the RING domain in orange, kinase domain in blue, ankyrin repeats in green, and HERC2 repeats in red. B) The Jas domain of JAZ12 interacts with KEG. In A) and B) the PJ69-4A yeast strain was co-transformed with JAZ12 in pGBKT7 and MYC2, NINJA, KEG, or KEG fragments in pGADT7gate. The 10× and 100× dilutions of transformed yeasts were spotted on selective medium lacking His. C) Interaction between KEG or COI1 and JAZ12, JAZ12-1, or other JAZ12 mutants. D) JAZ12 is the only JAZ protein capable of interacting with KEG. In C) and D) the EGY48 (p8opLacZ) yeast strain was co-transformed with KEG or NINJA in pGILDA and JAZ in pB42AD. Transformed yeasts were spotted on inducing medium containing Gal and raffinose supplemented with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside acid and 50 µm COR or dimethyl sulfoxide (DMSO) as indicated. E) WebLogo consensus sequence of the Jas motif in JAZ12 orthologues (top) and Arabidopsis JAZ proteins (bottom). Amino acids essential for COI1 interaction are highlighted in yellow and those assayed for KEG interaction in pink. F) to I) KEG interacts extranuclearly with JAZ12. F) and G) N. benthamiana leaf epidermal cells were transiently transformed with JAZ12-cGFP and KEG-nGFP (F) or KEG(AA)-nGFP (G). H) NINJA-nGFP interacts in the nucleus with JAZ12-cGFP. I) JAZ12-GFP localizes to both the nucleus and the cytoplasm in transiently transformed *N. benthamiana* leaves.

As the Jas domain is conserved in all JAZ proteins, we next tested 12 JAZ proteins for interaction with KEG using Y2H assays. Interestingly, only JAZ12 was capable of interacting with KEG, both in the LexA-based (Fig. 2D) and the GAL4-based (Fig. S24A) systems. NINJA was used as a positive control in these assays, as it interacts with most JAZ proteins (Fig. 2D). As KEG is known to autoubiquitinate *in vitro*, causing its degradation (Liu and Stone, 2010), we hypothesized that this might prevent interactions in yeast. Therefore, we also tested the KEG(AA) variant, which has a disrupted RING domain (C29A, H31A, and C34A; Stone *et al.*, 2006) and can no longer autoubiquitinate, for interaction with all JAZ proteins. We again observed interaction only with JAZ12 (Fig. S24B).

To identify what makes JAZ12 unique, we identified JAZ12 orthologues in different plant species based on synteny (Fig. S24C). Several amino acids in the JAZ12 Jas domain were more conserved between JAZ12 orthologues as compared with other JAZ proteins in Arabidopsis (Fig. 2E and S24D). Based on this comparison, we changed His-144, Pro-164, or a stretch of five amino acids, LVNKN157-161, to Ala(s) and tested the interaction with COI1 and KEG. With the latter construct, KEG interaction was lost while the COR-mediated COI1 interaction was retained (Fig. 2C). Finally, we changed the sequence LVNKN with the corresponding sequence of JAZ1, VTSKA. Again, KEG interaction was lost, suggesting that one or more of these amino acids that are conserved between JAZ12 orthologues are necessary for KEG interaction.

Finally, we assessed the JAZ12-KEG interaction using bimolecular fluorescence complementation (BiFC) by fusing the proteins with N-terminal or C-terminal fragments of GFP (designated nGFP and cGFP, respectively). When wild-type *KEG-nGFP* and *JAZ12-cGFP* were transiently co-expressed together in *Nicotiana benthamiana* leaves, we observed only a weak GFP signal, which was detected only outside the nucleus (Fig. 2F). However, when we used KEG(AA)-nGFP, a strong signal in the cytoplasm was observed (Fig. 2G). This corresponds to the reported extranuclear localization of KEG-GFP (Gu and Innes, 2011) and the localization of the interaction between KEG and ENHANCED DISEASE RESISTANCE1 (EDR1) (Gu and Innes, 2012) and between KEG and ABI5 (Liu and Stone, 2013). Co-expression of *NINJA-nGFP* led to a nuclear signal (Fig. 2H). Correspondingly, the expression of *JAZ12-GFP* resulted in expression in both the nucleus and the cytoplasm in *N. benthamiana* leaves (Fig. 2I). However, the roots of a stable 35S cauliflower mosaic virus promoter-driven *JAZ12-GFP* Arabidopsis line only expressed detectable *GFP* in the nucleus (Fig. S24E-F).

TAP of KEG(AA)

To assess the KEG interactome further, we generated Arabidopsis cells expressing N-terminal GS-tagged *KEG(AA)* for TAP assays (Table 2). By using the *KEG(AA)* mutant, we hoped to stabilize the tagged KEG protein and its targets, because ubiquitination and subsequent degradation are prevented. We treated the cells with 50 µm JA for 1 min. Although we could not find peptides for JAZ12 or ABI5/ABFs, we did find peptides of the known KEG target protein CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE26 (CIPK26). This kinase is a positive regulator of ABA signalling and can interact and phosphorylate ABI5 (Lyzenga *et al.*, 2013).

Table 2: Overview of the proteins purified by TAP with GS-KEG(AA)

AGI	Protein	-JA	+JA
AT5G13530	KEG	2	2
AT1G05460	SDE3	-	2
AT3G55620	EIF6A	-	2
AT5G21326	CIPK26	-	2

Information is as in Table 1. Numbers indicate the times the prey was identified in two experiments per column. AGI, Arabidopsis Genome Initiative identifier; CIPK26, CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE26; EIF6A, EUKARYOTIC INITIATION FACTOR6A; SDE3, SILENCING DEFECTIVE3. –, Prey was not identified in this experiment.

KEG knock-down lines are ABA hypersensitive

All three isolated knock-out mutants of *KEG, keg1, keg2,* and *keg3*, are seedling lethal, leading to postgerminative growth arrest (Stone *et al.*, 2006). To allow physiological analysis of KEG function, we used the artificial microRNA (amiRNA) method (Schwab *et al.*, 2006) to generate a knock-down line of *KEG*. We obtained a single *KEG* amiRNA line (line 14) whereof heterozygous plants showed normal Mendelian inheritance and for which we could select a homozygous population. This population showed retarded growth (Fig. 3A), although it had only modestly (±50%) reduced *KEG* expression at the RNA level (Fig. 3D). The effect on KEG protein levels could be more dramatic because it has been reported that, in plants, amiRNAs often do not only change transcript levels but repress translation, leading to reduced protein levels (Li *et al.*, 2013). To evaluate the success of *KEG* knock-down, we analysed the reported ABA hypersensitivity of *KEG* knock-out mutants (Stone *et al.*, 2006). The amiRNA line had a stronger reduction of primary root growth than wild-type Columbia-0 (Col-0) when grown on 5 µm ABA (Fig. 3B-C) but not on 2.5 µm JA. Moreover, *KEG* amiRNA seedlings grown on control medium had a severely reduced number of lateral roots after 11 d (Fig. 3B). Inhibition of lateral root biogenesis is a well-known effect of exogenous ABA treatment (De Smet *et al.*, 2003). We then checked the expression of several ABA and JA marker genes in

this line (Fig. 3D). The ABA markers *RESPONSIVE TO ABSCISIC ACID18* (*RAB18*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) were clearly up-regulated. Although *VSP2* is a known JA marker, *VSP2* expression is known to be up-regulated by ABA as well (Anderson *et al.*, 2004). The JA markers *MYC2* and *JAZ12*, on the other hand, were not induced (Fig. 3D). Based on these data, we conclude that *KEG* amiRNA lines have a constitutive ABA response in the absence of exogenous ABA.

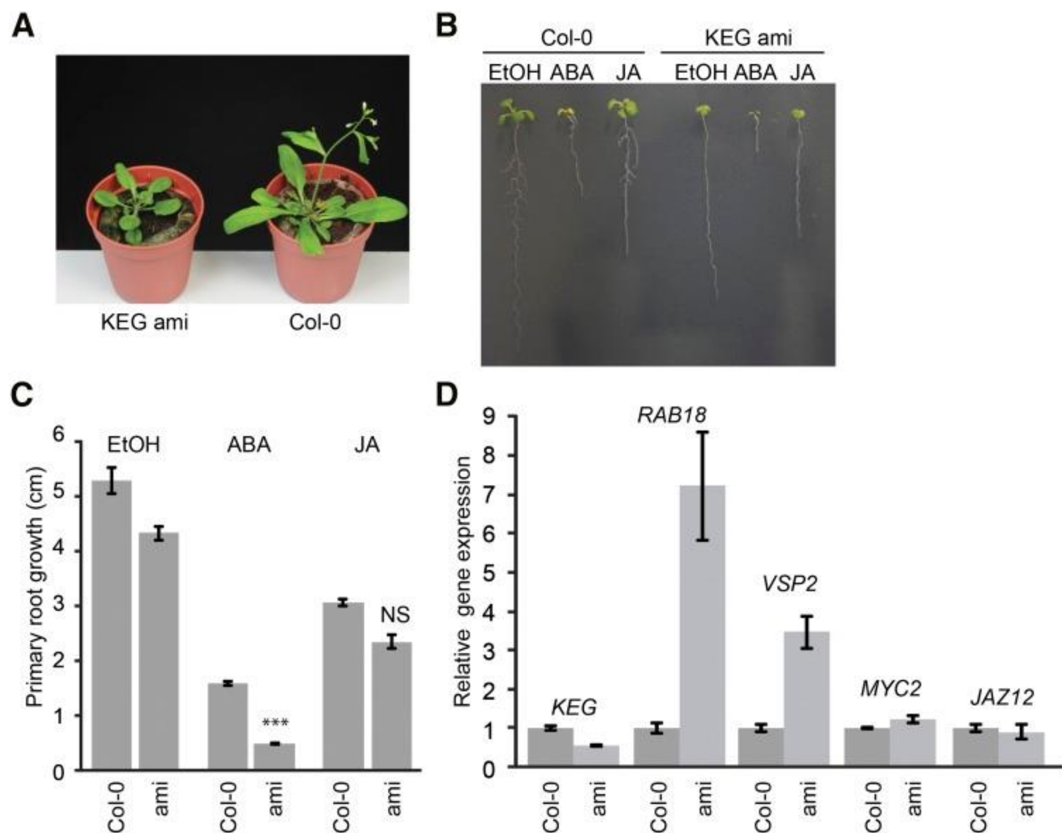

Figure 3: *KEG* knock-down lines are hypersensitive to ABA. A, Retarded growth of soil-grown *KEG* amiRNA line 14 plants. B and C, Root growth assays. Plants were grown for 4 d and transferred to medium containing ethanol (EtOH; control), 5 µm ABA, or 2.5 µm JA for 6 d. B, Representative seedlings. C, Average increase in primary root length after transfer. Values indicate means of three biological repeats (each consisting of eight seedlings) ± se. The treatment × genotype interaction effect (two-way ANOVA) is indicated (***, $P < 0.001$). NS, Not significant. There were also significant effects ($P < 0.001$) of each treatment and of the genotype. D, Expression of *KEG*, *RAB18*, *VSP2*, *MYC2*, and *JAZ12* in ethanol control seedlings from B. Error bars depict se (n = 3).

The amiRNA targeting *KEG* was expressed under the control of a 35S promoter using the pFAST-R02 vector (Shimada *et al.*, 2010). This vector harbours a red fluorescent protein (RFP) fusion expressed in the dormant dry seed, facilitating the selection of transformants. We used this property to confirm the phenotype of line 14 using a second independent transformant, line 2, which showed non-Mendelian inheritance and for which, even after several generations, no homozygous line was obtained. We used the presence of the RFP

marker to our advantage and compared RFP− with RFP+ plants in experiments with this line. RFP+ plants showed reduced *KEG* levels (Fig. S25A) and ABA hypersensitivity (Fig. S25B-C).

KEG is a positive regulator of JAZ12 stability

We used the *KEG* amiRNA lines also to test the relation between KEG activity and JAZ12 protein levels. Therefore, we crossed them with a *JAZ12-GFP* line and tested JAZ12-GFP levels by immunoblot analysis (Fig. 4A). To our surprise, JAZ12-GFP levels were decreased in the *KEG* knock-down lines (Fig. 4A; Fig. S26A), whereas the transcript levels of the *JAZ12-GFP* transgene were unaffected (Fig. S26B).

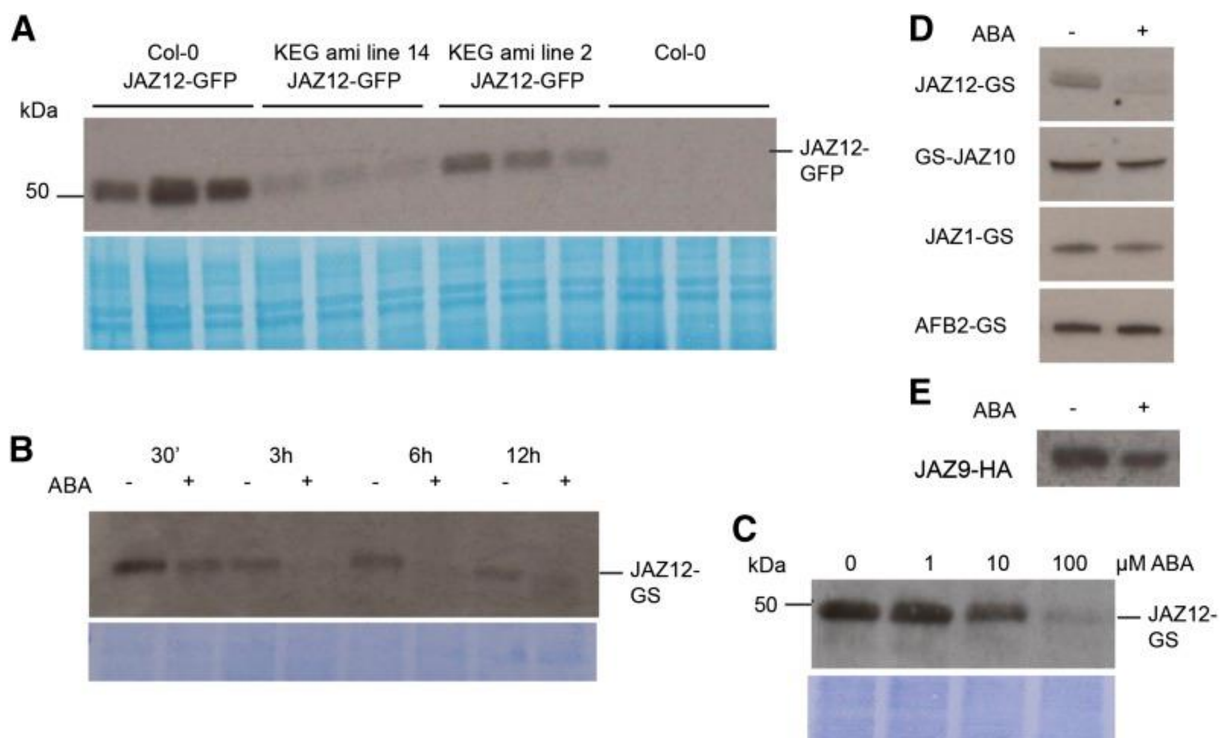

Figure 4: KEG is a positive regulator of JAZ12 stability. A) Immunoblot showing JAZ12-GFP levels in three biological repeats of a *JAZ12-GFP* line, its crosses with the *KEG* amiRNA lines, and the wild type (Col-0). Crosses were homozygous for JAZ12-GFP and RFP+, the latter indicative of *KEG* amiRNA expression. B) ABA enhances JAZ12-GS degradation. Seedlings were transferred from agar to liquid MS medium supplemented with 100 µm ABA (+) or ethanol (−), and protein levels were monitored over time. C) Dose response of ABA on JAZ12-GS protein levels 6 h after transfer. Membranes were stained with Coomassie Blue to inspect equal loading. D) and E) ABA-mediated degradation of other JAZ proteins and AFB2. Immunoblots show the expression of GS-tagged (D) or hemagglutinin (HA)-tagged (E) proteins treated with ethanol or 100 µm ABA for 6 h. A representative experiment of three biological repeats is shown. In all experiments, 6-d-old seedlings were used.

KEG is known to auto-ubiquitinate in the presence of ABA, leading to its degradation (Liu and Stone, 2010). Therefore, we treated seedlings producing GS-tagged JAZ12 with ABA by transferring them to liquid Murashige and Skoog (MS) medium containing ABA or ethanol. After transfer, JAZ12-GS is degraded over time in the control, and the addition of ABA results in increased JAZ12 degradation (Fig. 4B), in a dose-dependent manner (Fig. 4C). After 6 h, we

observed a nearly complete loss of JAZ12-GS, while GS-tagged JAZ1, JAZ10, or the unrelated AUXIN SIGNALLING F-BOX2 (AFB2) did not show alteration in protein levels compared with mock treatment (Fig. 4D). HA-tagged JAZ9 protein levels also showed some reduction after ABA treatment, but to a far lesser extent than JAZ12-GS (Fig. 4E).

To study the effect of *KEG* overexpression on JAZ12 stability further, we used transient expression in *N. benthamiana*. Whereas the expression of *JAZ12-GS* alone resulted in strong protein accumulation, co-expressing *COI1* abolished JAZ12-GS levels completely (Fig. 5A). When co-expressing *KEG* with *JAZ12-GS*, we did not observe changes in the JAZ12-GS levels, compared with the *JAZ12-GS* expressed in the absence of KEG (Fig. 5A). However, when co-expressing *KEG* together with *COI1* and *JAZ12-GS*, we could observe a protective effect of KEG on COI1-mediated JAZ12-GS degradation (Fig. 5A). Finally, we transiently expressed *JAZ12* fused to *GFP* together with *COI1* and/or *KEG* to study if COI1-mediated degradation and protection by KEG were specific for a subcellular compartment. This assay indicated that JAZ12-GFP was degraded both inside and outside the nucleus in the presence of COI1. Similarly, co-expression of *KEG* restored JAZ12 levels both intranuclearly and extranuclearly (Fig. 5B). Taken together, our data demonstrate that nucleus-localized COI1 and TGN-localized KEG both influence JAZ12 levels independently of JAZ12 subcellular localization.

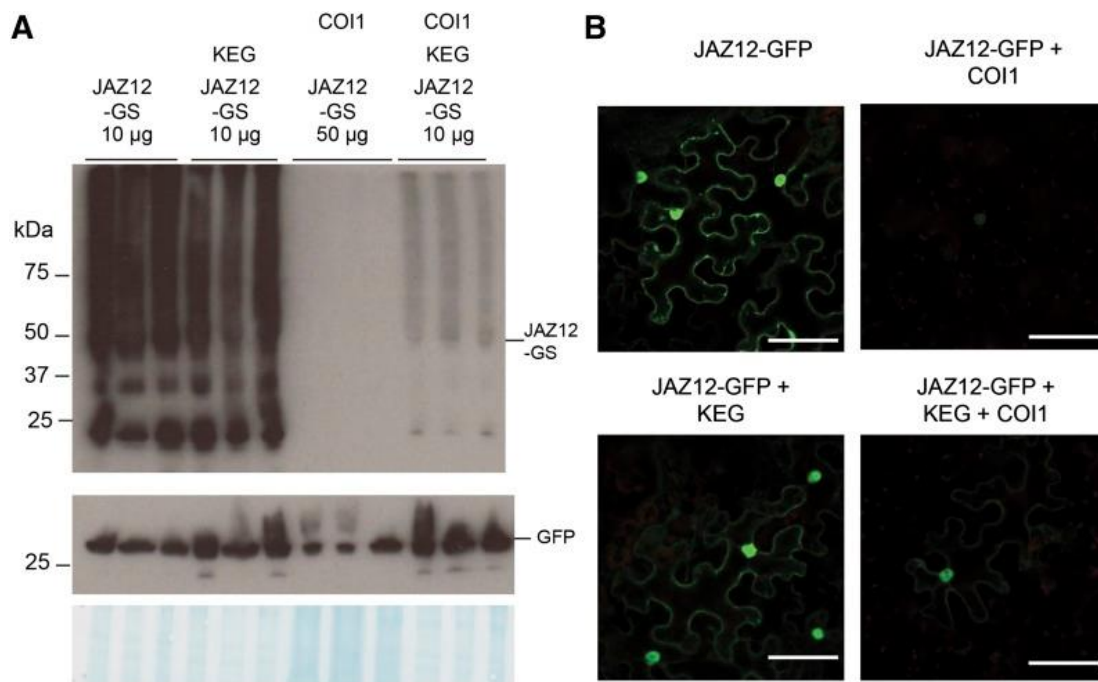

Figure 5: KEG and COI1 influence JAZ12-GFP levels independent of its subcellular localization. A) Immunoblot showing JAZ12-GS and GFP levels in *N. benthamiana* leaves co-infiltrated with *JAZ12-GS*, *KEG*, and/or *COI1*. *GFP* was co-transformed as a control. Each combination was co-infiltrated in triplicate. COI1-containing protein extracts were loaded in 5× excess (50 µg). The bottom gel shows Coomassie Blue staining of the blotted membrane. B) *N. benthamiana* leaves were transiently transformed with *JAZ12-GFP*, *COI1*, and/or *KEG* and imaged using a confocal microscope. Bars = 50 µm.

DISCUSSION

Use of TAP-mass spectrometry to study ubiquitin E3 ligases

Although the interaction between SCF^{COI1} and JAZ proteins is well established and has been studied using methods such as Y2H (Thines *et al.*, 2007; Melotto *et al.*, 2008) and pull-down (Thines *et al.*, 2007; Fonseca *et al.*, 2009) assays, we show here that the COI1-JAZ interaction can be determined *in vivo* using TAP-mass spectrometry using either the F-box protein or its target as bait. In our cell culture system, the interaction was dependent on the addition of JA, which presumably is converted to the bioactive JA-Ile form *in vivo*. In seedling cultures, however, the interaction was already observed without any exogenous JA treatment. Accordingly, in this system, we were able to detect tryptic peptides of JAZ12, which had the di-Gly mark of ubiquitination. Previously, direct proof of the ubiquitination of JAZ proteins was scarce. To our knowledge, such *in vivo* proof of ubiquitination has only been reported for JAZ6 via an untargeted proteomics screen for ubiquitinated proteins (Saracco *et al.*, 2009). Our TAP-mass spectrometry approach also allowed us to identify core components of the SCF complex (ASK1, ASK2, and CUL1) using either the F-box protein or its target JAZ12. This confirms the association of COI1 with an SCF complex (Xu *et al.*, 2002). Recently, it was shown that the association with ASK1 and CUL1 is essential for COI1 stability (Yan *et al.*, 2013).

Notably, we detected the interaction of JAZ12 only with MYC2, MYC3, and MYC4, both in cell cultures and in seedlings, although many other transcription factors have been reported to interact with the JAZ proteins, including JAZ12 (Pauwels and Goossens, 2011; Hu *et al.*, 2013; Nakata *et al.*, 2013; Sasaki-Sekimoto *et al.*, 2013; Song *et al.*, 2013; Fonseca *et al.*, 2014). Similarly, JAZ12 is the only JAZ for which we observed interaction using COI1 as bait. Negative results using TAP-mass spectrometry can be related to the expression levels of target proteins, and indeed, *JAZ12* was found to be a highly expressed *JAZ*, at least at the transcript level.

JAZ12 interacts with KEG in the cytoplasm

TAP with JAZ12 identified the E3 ligase KEG as an interactor of JAZ12. KEG is known to play a role in ABA signalling and interacts with the bZIP transcription factors ABI5, ABF1, and ABF3 through the ankyrin-repeat domain and with the kinase CIPK26 (Stone *et al.*, 2006; Chen *et al.*, 2013; Lyzenga *et al.*, 2013). KEG promotes the proteasome-mediated degradation of these proteins in order to repress ABA signalling.

KEG also plays a role in pathogen resistance. The kinase EDR1 interacts at the TGN with KEG through the HERC2-like repeat domain. The *keg4* allele carrying a missense mutation in this domain was identified to suppress the *edr1*-mediated increased resistance to *Golovinomyces*

cichoracearum (Wawrzynska *et al.*, 2008). The *keg4* mutation or deletion of the entire HERC2-like repeat domain led to a primarily cytoplasmic localization of KEG, while this was unobserved for wild-type KEG (Gu and Innes, 2011).

The localization of KEG to the TGN and the activity in the nucleus by association with transcription factors could be reconciled by the finding that, although ABI5 is observed to be exclusively nuclear, the interaction between KEG and ABI5 localized to the cytoplasm when the RING domain of KEG was inactivated or a conserved Lys in ABI5 was mutated (Liu and Stone, 2013). Cytoplasmic turnover of ABI5 by KEG is proposed to also control nuclear ABI5 levels and, hence, its activity (Liu and Stone, 2013).

Here, we found JAZ12-GFP to be exclusively in the nucleus in Arabidopsis seedling roots, but upon transient overexpression in *N. benthamiana* leaves, it was also seen in the cytoplasm. As with ABI5, we found that the interaction between KEG and JAZ12 occurs outside the nucleus. For JAZ9, it was shown that nuclear localization is dependent on the interaction with the MYC2 transcription factor, and YFP-tagged JAZ9 accumulated in the cytosol in a *myc2* mutant (Withers *et al.*, 2012). MYC2, MYC3, and MYC4 are known to be short-lived proteins regulated by proteasomal degradation (Zhai *et al.*, 2013; Chico *et al.*, 2014). GFP-tagged MYC2 is largely removed from the cell in the dark and far-red light (Chico *et al.*, 2014), and MYC2 protein levels oscillate over time under the control of the circadian clock (Shin *et al.*, 2012).

Determination of the detailed physiological and spatiotemporal circumstances in which JAZ12, possibly through (absence of) MYC2 interaction, favors interaction with KEG outside the nucleus will be the subject of further study.

Toward a functional role for the KEG-JAZ12 interaction

We show here that JAZ12 is ubiquitinated *in vivo*, is a target of the SCF^{COI1} complex, and is degraded upon a JA stimulus, thus behaving like a canonical JAZ protein. Signal transducers are commonly targeted by multiple ubiquitin E3 ligases. ABI5, for example, is also targeted by the CUL4-based E3 ligases ABSCISIC ACID-HYPERSENSITIVE DAMAGED DNA BINDING PROTEIN1 (DDB1)-CULLIN4–associated factor1 (ABD1) and DDB1 binding WD40 HYPERSENSITIVE TO ABSCISIC ACID2 (DWA1)/DWA2 besides KEG (Lee *et al.*, 2010; Seo *et al.*, 2014). Unexpectedly, however, our results did not support our intuitive hypothesis of KEG as an instigator of JAZ12 ubiquitination and subsequent degradation. On the contrary, several observations pointed to KEG as a positive regulator of JAZ12 stability: (1) a *KEG* amiRNA line showed reduced JAZ12-GFP levels; (2) *KEG* overexpression protected JAZ12 from COI1-mediated degradation; and (3) ABA promoted JAZ12 degradation.

We considered the possibility that these observations are independent of the interaction between KEG and JAZ12 and caused by the repression of ABA signalling by KEG. The *KEG* amiRNA lines presented here indeed showed ABA hypersensitivity at the seedling stage, consistent with earlier reports studying loss-of-function *keg* alleles (Stone *et al.*, 2006). ABA induces JA marker genes such as *VSP2* (Anderson *et al.*, 2004), which is also induced in the *KEG* amiRNA line. Moreover, ABA biosynthesis is known to be required for the increase in JA levels after *Pythium irregulare* infection (Adie *et al.*, 2007). Notwithstanding, we did not observe any induction of JA marker genes, such as *MYC2*, nor any hypersensitivity to JA in the *KEG* amiRNA line. Furthermore, ABA treatment only affected JAZ12 levels dramatically, while JAZ1 and JAZ10 were unaffected. JAZ9 levels also decreased, albeit far more modestly. The latter is in line with a recent report showing degradation of the Jas9-Venus reporter upon ABA treatment (Larrieu *et al.*, 2015). Hence, the reduced JAZ12 stability after ABA treatment and in the *KEG* amiRNA line is unlikely to be caused by a general elevated JA signalling. Nevertheless, the regulation of JAZ12 stability by KEG is linked to SCF^{COI1}: *KEG* overexpression protected JAZ12 from COI1-mediated degradation, which was not caused by a tethering of JAZ12 outside the nucleus. GFP-tagged JAZ12 was observed in increasing amounts in both the cytoplasm and the nucleus when *KEG* was co-expressed with *COI1*. Hence, this work highlights KEG as a novel point of crosstalk between the plant hormones ABA and JA.

MATERIALS AND METHODS

Molecular cloning

The open reading frames (ORFs) of AFB2 and COI1 lacking a stop codon and JAZ10 with a stop codon were PCR amplified with the primers listed in Table S2 and cloned in a Gateway-compatible entry clone. For KEG(AA), we used the entry clone described previously (Stone *et al.*, 2006). For TAP constructs, a MultiSite Gateway LR reaction was performed with destination vectors pKCTAP and pKNTAP for C- and N-terminal fusions, respectively. In both cases, ORFs were fused to the GS-TAP tag and put under the control of the 35S promoter. JAZ12-GFP was constructed by recombining a JAZ12 entry clone with pGWB5 (Mita *et al.*, 1995). The *KEG* amiRNA construct was designed with the Web MicroRNA Designer (www.weigelworld.org) and constructed by PCR amplification with the primers listed in Table S2 and pRS300 as template (Schwab *et al.*, 2006). The PCR product, to which attB sites were added, was recombined with pDONR221 (Invitrogen) and then pFAST-R02 (Shimada *et al.*, 2010) as the destination vector. BiFC constructs for Pro-35S:ORF-tag or Pro-35S:tag-ORF using the N- and C-terminal halves of enhanced GFP were constructed by multisite Gateway reactions using pK7m34GW, pH7m34GW (Karimi *et al.*, 2005), or pH7m24GW as described (Boruc *et al.*, 2010) combined with pDONR207 and pDONR221 entry clones of JAZ12, NINJA, KEG, and KEG(AA) (with or without stop codon).

Plant material

The *jaz12-1* (SALK_055032) allele was obtained from the Arabidopsis Biological Resource Center (Alonso *et al.*, 2003). Seedlings were PCR genotyped using a T-DNA and gene-specific primers (Table S2). Amplicons were sequenced to confirm the location of the T-DNA.

For stable transformation in Arabidopsis, the *Agrobacterium tumefaciens* strain C58C1 (pMP90) was used to transform Col-0 plants by floral dip (Clough and Bent, 1998). Transformants were selected based on kanamycin for JAZ12-GS and by ProOLE1:OLE1-RFP expression in seeds for *KEG* amiRNA lines.

***In vitro* plant growth**

For all the experiments described with plants grown *in vitro*, Arabidopsis seedlings were sterilized by the chlorine gas method and sown on sterile plates containing the corresponding growth medium. Plates were kept in the dark at 4°C for 2 d for stratification, after which they were transferred to a growth room with 21°C temperature and a 16-h-light/8-h-dark regime. The day of the transfer was considered as 0 d after stratification.

TAP

Arabidopsis cell suspension cultures (PSB-D) were transformed without callus selection as described previously (Van Leene *et al.*, 2008). For treatments, 50 µm JA (Duchefa) was added to the cell culture for 1 min before harvesting cells in liquid nitrogen. Affinity purification and liquid chromatography-tandem mass spectrometry analysis were as described (Van Leene *et al.*, 2015). For TAP of JAZ12-GS from seedlings, a homozygous line containing a single T-DNA locus was identified and grown in liquid MS medium as described (Van Leene *et al.*, 2015).

RNA-Seq

RNA was isolated from wild-type PSB-D cell cultures and 14-d-old Col-0 seedlings in three biological repeats using the RNeasy Plant Mini Kit (Qiagen) and DNase I treated (Promega). A Trueseq RNA-Seq library (Illumina) was compiled and sequenced as 50-bp single read using Illumina HiSeq 2000 technology at GATC Biotech. Read quality control, filtering, mapping to The Arabidopsis Information Resource 10 Arabidopsis genome, and read counting were carried out using the Galaxy portal running on an internal server (http://galaxyproject.org/). Sequences were filtered and trimmed with the Filter FASTQ v1 and FASTQ Quality Trimmer v1 tools, respectively, with default settings (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were subsequently mapped to The Arabidopsis Information Resource 10 version of the Arabidopsis genome using GSNAPv2 (Wu and Nacu, 2010), allowing a maximum of five mismatches. The reads that uniquely map to the genome were used for quantification on the gene level with htseq-count from the HTSeq python package (Anders *et al.*, 2015). Reads per kilobase per million values were calculated for each *JAZ* gene in each sample.

Y2H analysis

The Y2H screen was conducted using a custom library manufactured by Invitrogen using RNA isolated from a mixture of seedlings and rosette leaves from short-day-grown plants. The latter were collected from plants at multiple time points following infection with *Pseudomonas syringae* pv *tomato* strain DC3000. The library was cloned into the Invitrogen pDEST22 low-copy vector. A bait construct containing full-length *KEG* was cloned into pDEST32. Library screening was performed following the protocol provided by Invitrogen. For both GAL4- and LexA-based assays, cloning, yeast strains, culturing, transformation, and reporter assays were done as described (Cuéllar Pérez *et al.*, 2013), except for the COI1 interaction assays. In those, 50 µm COR (Sigma-Aldrich) was added to the medium or dimethyl sulfoxide as a control and 5-µL yeast suspensions were dropped manually on small petri plates. Entry clones for JAZ and MYC proteins were generated as described previously (Pauwels *et al.*, 2010; Fernández-Calvo *et al.*, 2011). Truncations and mutants of *JAZ12* were made by PCR amplification using the primers listed in Table S2. KEG truncations were cloned as described previously (Stone *et al.*, 2006).

Transient expression assays

Cloning, tobacco (*Nicotiana tabacum*) BY-2 protoplast preparation, automated transfection, lysis, and luciferase measurements were carried out as described previously (Vanden Bossche *et al.*, 2013). The pLOX3:fLUC and p35S:MYC2 constructs were generated as described (Pauwels *et al.*, 2008). *JAZ12* was cloned in the plasmid p2GW7 for overexpression (Vanden Bossche *et al.*, 2013).

JA degradation assay

Seven-day-old cells expressing *JAZ12-GS* were subcultured in individual flasks for 2 d and treated with 50 µm JA or ethanol at the different time points. Cells were harvested and snap frozen in liquid nitrogen.

ABA degradation assay

Seedlings expressing epitope-tagged proteins were grown *in vitro* in pools of 10 to 15 seedlings for 6 d after germination. Each pool was transferred to 1 mL of MS medium without agar on a 24-well plate containing the indicated concentration of ABA or an equal volume of ethanol. After incubation for the indicated time in the growth room, seedlings were harvested and snap frozen in liquid nitrogen.

Immunoblotting

Total protein was extracted using extraction buffer (25 mm Tris-HCl, pH 7.6, 15 mm MgCl2, 150 mm NaCl, 15 mm p-nitrophenyl phosphate, 60 mm β-glycerophosphate, 0.1% Nonidet P-40, 0.1 mm Na3VO4, 1 mm NaF, 1 mm phenylmethylsulfonyl fluoride, 1 µm E64, complete proteinase inhibitor [Roche], and 5% ethylene glycol), and the protein concentration was determined using the Bradford assay (Bio-Rad). Samples were denatured in Laemmli buffer, run on a 4% to 15% TGX gel (Bio-Rad) for 20 min at 300 V, and subsequently blotted on a 0.2-µm polyvinylidene difluoride membrane (Bio-

Rad). Antibodies used were peroxidase anti-peroxidase (P1291; Sigma-Aldrich), anti-HA (3F10; Roche), and anti-GFP (abcam290) antibodies. Chemiluminescent detection was performed with Western Bright ECL (Isogen; http://www.isogen-lifescience.com/).

Root growth assays

For *jaz12-1*, seedlings were grown on MS medium plates (10 g L−1 Suc and 8 g L−1 agar, pH 5.7) containing 2.5 or 10 µm JA (Duchefa) or ethanol. Seedlings were grown vertically under the conditions described above. Plates were scanned 11 d after stratification, and primary root length was measured by means of the EZ-Rhizo software (Armengaud *et al.*, 2009).

For *KEG* amiRNA lines, seedlings were grown as above on MS plates for 4 d and then transferred to new plates containing 2.5 µm JA (Duchefa), 5 µm ABA (Sigma-Aldrich), or ethanol. Primary root length was marked, and seedlings were grown for another 6 d before scanning. Primary root growth following transfer was measured by means of ImageJ software (http://imagej.nih.gov).

Gene expression analysis

Frozen plant material was ground in a Retsch MM300 mixer, and total RNA was extracted using the Qiagen RNeasy kit (http://www.qiagen.com/). An RNase-free DNase step was performed following the manufacturer's instructions for the preparation of RNA. Next, 1 µg of total RNA was used for complementary DNA synthesis with the iScript kit (Bio-Rad; http://www.bio-rad.com/). Quantitative reverse transcription-PCR was performed on a LightCycler 480 system (Roche; http://www.roche.com) using the Fast Start SYBR Green I PCR mix (Roche). At least three biological repeats and three technical repeats were used for each analysis. Expression data were normalized using two reference genes, UBIQUITIN-CONJUGATING ENZYME21 (UBC21; At5g25760) and PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3; At1g13320). The primer sequences are provided in Table S2.

Transient expression in *Nicotiana benthamiana*

Wild-type *N. benthamiana* plants (3–4 weeks old) were used as a transient protein expression system in the BiFC and JAZ12 stability experiments. Constructs were transiently expressed by A. tumefaciens-mediated transient transformation of lower epidermal leaf cells as described previously (Boruc *et al.*, 2010) using a modified buffer (10 mm MgCl2 [1 m stock solution; Merck], 10 mm MES [0.5 m stock solution; Duchefa], and 100 µm acetosyringone [100 mm stock solution; Sigma-Aldrich]) and the addition of a *P19*-expressing *A. tumefaciens* strain to boost protein expression (Voinnet *et al.*, 2003). All *A. tumefaciens* strains were grown for 2 d, diluted to an optical density of 1 in infiltration buffer, and incubated for 2 h at room temperature before mixing in a 1:1 ratio with other strains and injecting. For BiFC and JAZ12-GFP stability assays, lower epidermal cells were examined for fluorescence using confocal microscopy 3 d after infiltration. For immunoblots, infiltrated leaf tissue was harvested 3 d after infiltration and immediately frozen in liquid nitrogen.

Confocal microscopy

For subcellular localization of JAZ12-GFP in Arabidopsis seedlings, seedlings were briefly incubated in propidium iodide (3 mg L−1; Sigma-Aldrich) and subsequently washed and mounted in milliQ water. Fluorescence microscopy was performed with an Olympus FV10 ASW confocal microscope.

Bioinformatics

Orthologues of Arabidopsis JAZ12 were identified using the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication) based on synteny. Sequences were aligned using MUSCLE with default settings in JalView 2.8.2 (www.jalview.org). Sequence logos were created using WebLogo (http://weblogo.berkeley.edu/).

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Chapter 5

PEAPOD signalling : a copycat of JAZ?

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ABSTRACT

PEAPOD (PPD) proteins are negative regulators of meristemoid cell division in leaves of *Arabidopsis thaliana*. On a molecular level, these proteins have many similarities with the JASMONATE-ZIM DOMAIN (JAZ) proteins, negative regulators of jasmonate (JA) signalling. Both contain a C-terminal JA-associated (Jas) domain, which is important for interaction of the JAZ proteins with transcription factors. However, PPD proteins were thought to bind DNA directly and no interacting transcription factors have been found yet, until now. We changed a Tyr in the Jas domain of PPD2 to a 'JAZ-conserved' Phe in order to modify the interaction behaviour of PPD2. This mutation allowed interaction with bHLH-type transcription factors, including JA-ASSOCIATED MYC2-LIKE2 (JAM2), a transcription factor negatively regulating JA signalling. In addition, wild-type PPD2 conferred transcriptional repression capacity to JAM2 to inhibit expression of target genes. Finally, we showed that *PPD* pre-mRNAs undergo many different splicing events resulting in truncated constructs lacking, partially or fully, their Jas domain. Our results demonstrate the strength of using knowledge of JAZ proteins in the PPD research. We propose a model for the mode of action of PPD2, where PPD2 does not bind directly to DNA but interacts with JAM2 to mediate repression of specific target genes.

INTRODUCTION

Proteins are regulated at multiple levels, post-transcriptionally as well as post-translationally, and work in protein complexes wherein protein-protein interactions are crucial for the activity of the complex. Changing the interaction behaviour of proteins can serve to gain knowledge about the regulation of these proteins and can help to characterize the complexes where they work in. Site-directed mutagenesis combined with yeast two-hybrid (Y2H) allows to induce or abolish interactions between proteins and to easily assess changes in the interaction behaviour, respectively. Point mutations are preferred over deletion or fusion constructs because of the low impact on the integrity of the proteins as point mutations do not affect the length of the protein and have less chance to modify the protein structure and localisation. In the jasmonate (JA) signalling core complex, change of single or multiple amino acids has been shown to alter interactions among the proteins of the complex (Pauwels *et al.*, 2010; Sheard *et al.*, 2010; Moreno *et al.*, 2013).

The JA signalling core consists of the central transcription factors (TFs) MYC2, MYC3 and MYC4, which are repressed by the JA-ZIM DOMAIN (JAZ) proteins in the absence of jasmonates (JAs) (Chini *et al.*, 2007; Thines *et al.*, 2007; Fernández-Calvo *et al.*, 2011). The bioactive JA-Ile is perceived by a co-receptor complex consisting of a JAZ protein and the F-box protein CORONATINE INSENSITIVE1 (COI1) resulting in the ubiquitin-mediated 26S proteasomal degradation of JAZ (Chini *et al.*, 2007; Thines *et al.*, 2007; Fonseca *et al.*, 2009).

The family of JAZ repressors contains 13 members, all characterized by the presence of a conserved C-terminal JA-associated (Jas) domain that mediates interaction with both COI1 and the MYC TFs (Pauwels & Goossens, 2011; Thireault *et al.*, 2015). Nevertheless, specificity in this Jas domain exists. For instance, JAZ8 and JAZ13 do not contain a canonical degron in their Jas domain, responsible for COI1 interaction. Correspondingly, JAZ8 and JAZ13 have an increased stability in the presence of JAs (Shyu *et al.*, 2012; Thireault *et al.*, 2015).

More insights in the specificity of the Jas domain was shown by interruption of interaction between MYC2 and the JAZ proteins. Change of a conserved amino acid in the activation domain or JAZ-interacting domain (JID) of MYC2, resulted in the loss of interaction with most of the JAZ repressors, except for JAZ1 and/or JAZ10 (Chapters II.2 and II.3; Gasperini *et al.*, 2015; Goossens *et al.*, 2015). Further investigation showed that the presence of an N-terminal cryptic MYC2-interacting domain (CMID) in JAZ1 and JAZ10 is responsible for the remaining interaction with MYC2 (Chapter II.2; Moreno *et al.*, 2013; Goossens *et al.*, 2015). The occurrence of this domain is in agreement with JA-insensitive phenotypes of plants overexpressing JAZ10.3 and JAZ10.4, two splice variants of JAZ10 (Chung & Howe, 2009; Moreno *et al.*, 2013). These variants are stable dominant repressors of the JA pathway as they do not contain, or only partially, the C-terminal Jas domain resulting in less affinity for COI1, while interaction with MYC2 is retained. This alternative splicing event does not only occur for JAZ10 however. Alternative splicing of other *JAZ* pre-mRNAs also leads to truncated proteins lacking multiple amino acids of the Jas domain, showing less affinity for COI1 (Chung *et al.*, 2010).

Specificity among the JAZ proteins does not only occur due to differences in the Jas domain. Generally, JAZ proteins execute their repressing function by recruitment of the co-repressors TOPLESS (TPL) or the TPL-related proteins (TPRs), brought in the vicinity by the adaptor protein NEW INTERACTOR OF JAZ (NINJA) (Pauwels *et al.*, 2010). However, JAZ8 and JAZ13 are able to interact directly with TPL via an N-terminal ERF-associated amphiphilic repression (EAR) motif, without the need for NINJA (Shyu *et al.*, 2012; Thireault *et al.*, 2015). Similarly, JAZ5 and JAZ6 contain both two EAR motifs and can directly interact with TPL (Kagale *et al.*, 2010; Causier *et al.*, 2012).

JAZ proteins, with the exception of JAZ13 (Thireault *et al.*, 2015), contain a conserved TIF[F/Y]XG motif, which is essential for both homo- and heterodimerization (Chung & Howe, 2009) and interaction with NINJA (Pauwels *et al.*, 2010). This so-called TIFY motif is part of a larger zinc-finger protein expressed in inflorescence meristem (ZIM) domain. Besides the JAZ proteins, six other proteins contain a ZIM domain: ZIM, ZIM-LIKE1 (ZML1), ZML2, TIFY8, PEAPOD1 (PPD1) and PPD2. These are divided in group I and group II based on their domain architecture (Vanholme *et al.*, 2007). Group I constitutes of ZIM, ZML1 and ZML2,

characterized by the presence of a T[I/L]SFXG motif, a CONSTANS, CO-like, and TOC1 (CCT) domain and a C2C2-GATA zinc-finger domain. All other TIFY proteins make up group II, characterized by the presence of the TIF[F/Y]XG motif, able to interact with NINJA (Pauwels *et al.*, 2010). In addition, all members of group II, except for TIFY8, contain a C-terminal Jas domain (Cuéllar Pérez *et al.*, 2014).

Unique for PPD1 and PPD2 is the presence of an N-terminal PPD domain, responsible for interaction with KINASE-INDUCIBLE DOMAIN INTERACTING8 (KIX8) and KIX9 (Gonzalez *et al.*, 2015). Similar to NINJA, the KIX proteins contain an EAR motif and connect the PPD proteins to TPL, conferring repressor activity to PPD. Interestingly, the Jas domain of PPD1 and PPD2 is divergent from the canonical Jas domain of the JAZ proteins that mediates interaction with boh COI1 and the MYC TFs (Fig. 2; Pauwels & Goossens, 2011). However, no specific interactions have been linked yet to the Jas domain of the PPD proteins. Recently, PPD1 and PPD2 were shown to interact in the nucleus with the F-box protein STERILE APETALA (SAP) that assembles into an Skp1/Cullin/F-box (SCF) complex resulting in the degradation of the PPD proteins (Wang *et al.*, 2016). However, whether this occurs via the Jas domain of PPD has not been investigated yet.

The PPD proteins are involved in diverse growth- and development-related processes, such as hypocotyl growth, flowering and the control of seed size (Ge *et al.*, 2016; White, 2017). Furthermore, loss-of-function mutants targeting both PPD1 and PPD2 have an increased leaf area and dome-shaped leaves due to increased proliferation of meristemoid cells in the stomatal cell lineage (White, 2006; Gonzalez *et al.*, 2015). This was a consequence of increased expression of genes related to cell division, meristemoid cells and stomatal development (Gonzalez *et al.*, 2015). In agreement with SAP-mediated degradation of the PPD proteins, SAP is genetically linked to PPD-regulated proliferation of meristemoid cells (Wang *et al.*, 2016). Intriguingly, tandem chromatin affinity purification (TChAP) of Arabidopsis cell suspension cultures overexpressing an HBH-tagged PPD2 pointed to an overrepresentation of the G-box (CACGTG) or of a related sequence (CACGCG) in circa 50% of the found DNA sequences bound by PPD2 (Gonzalez *et al.*, 2015). Furthermore, PPD2 is able to bind a viral coat protein promoter during geminiviral infection (Lacatus & Sunter, 2009). However, PPD1 or PPD2 have never been shown to bind plant DNA directly.

Here, we will look deeper into the analogies between PPD and JAZ proteins on a molecular level, focusing on the yet uncharacterized Jas domain of the PPD proteins, in order to further characterize the PPD proteins.

RESULTS

Alternative splicing of *PPD* leads to truncations in the Jas domain

In most *JAZ* genes, the C-terminal Jas domain is encoded by two exons separated by one intron, the Jas intron. Retention of this intron by alternative splicing is observed for several *JAZ* transcripts leading to truncated JAZ proteins lacking the C-terminal part of the Jas domain (Chung *et al.*, 2010). Furthermore, an additional alternative splice variant lacking the complete Jas domain, JAZ10.4, is generated by an upstream alternative splicing event within the third exon of *JAZ10* (Chung & Howe, 2009). This demonstrates the existence of multiple alternative splicing strategies that lead to the generation of truncated JAZ constructs lacking, partially or fully, the Jas domain.

Similar to the gene architecture of the JAZ proteins, the Jas domain in the *PPD1* and *PPD2* genes is also encoded by two exons, exon 7 and exon 8, separated by a Jas intron (Fig. 1A). Retention of this intron would lead to a premature stop codon. Therefore, we verified the occurrence of retention of the Jas intron leading to truncated PPD constructs lacking the C-terminal part of the Jas domain. A model for an alternative PPD1 splice variant is already reported by the Arabidopsis information resource (TAIR) database. This splice variant shows retention of the Jas intron resulting in a premature stop codon, hence lacking the C-terminal part of the Jas domain. No such PPD2 alternative splice variants have been identified so far. To verify if retention of the Jas intron occurs in the *PPD1* and *PPD2* transcripts, RT-PCR analysis was performed on whole Arabidopsis seedlings using specific primers for the Jas intron (Fig. 1B). An alternative spliced transcript with inclusion of the Jas intron was detected for both *PPD1* and *PPD2*, though the abundance of the *PPD2* variant was low in whole seedlings. To exclude the possibility of genomic DNA contamination, truncated constructs were sequenced, confirming the presence of the Jas intron but not the other introns. Surprisingly, the *PPD1* alternative spliced mRNA is also missing part of exon 6 due to an alternative splice acceptor, leading to a premature stop codon and resulting in a truncated protein lacking the complete Jas domain (Fig. 2). These observations indicate that multiple alternative splicing strategies are employed for *PPD1* and *PPD2*, leading to truncated constructs with partial or complete loss of the Jas domain.

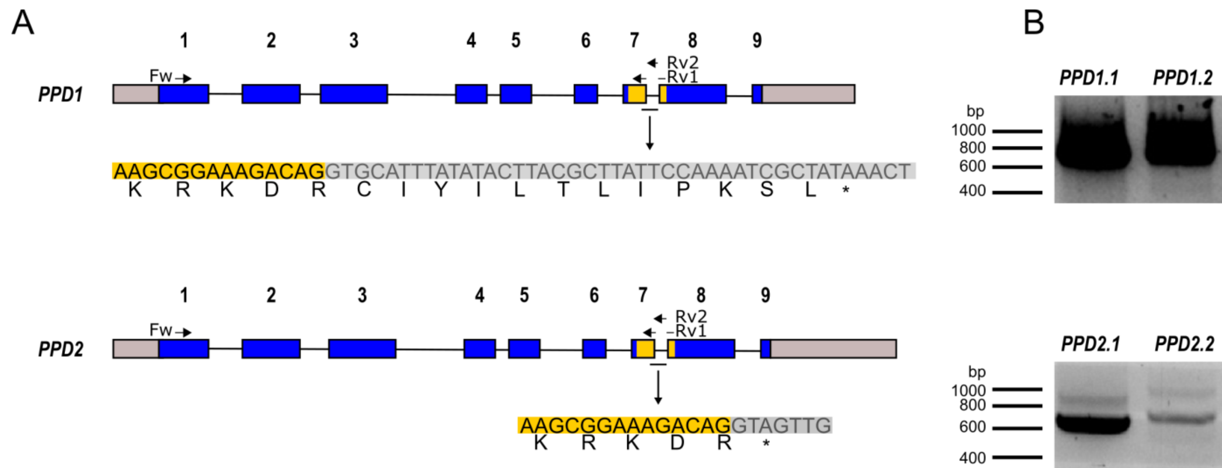
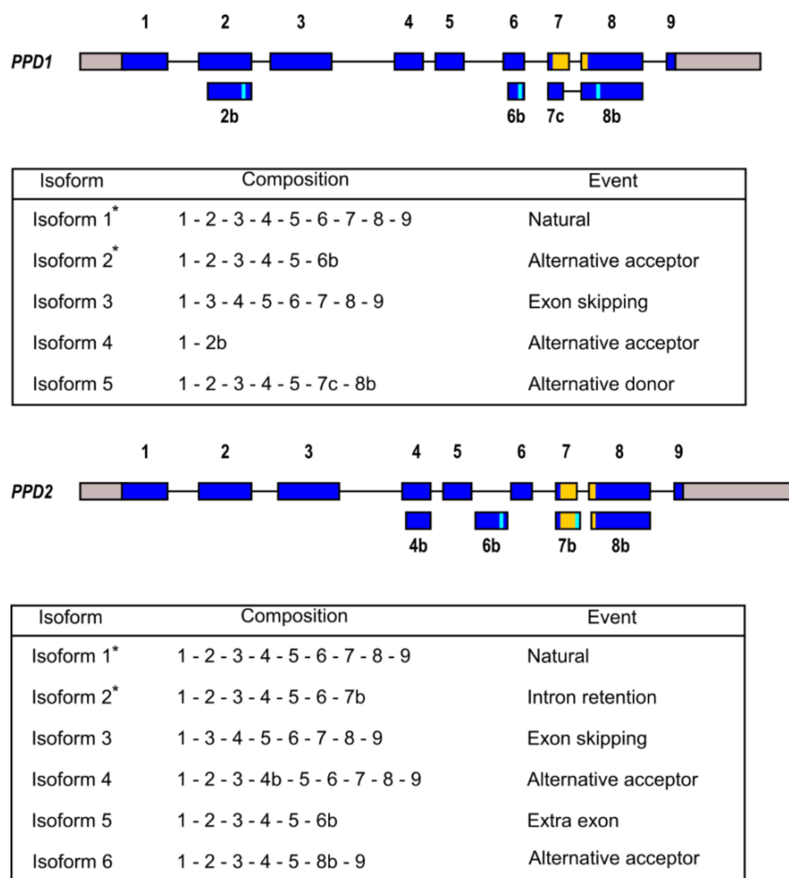


Figure 1: Alternative splicing of the pre-mRNAs of *PPD1* and *PPD2* leads to the retention of the Jas intron. A) Gene architecture of *PPD1* and *PPD2*. Grey and blue bars indicate respectively untranslated regions and open reading frames in the exons. The Jas domain is highlighted in yellow. The predicted amino acid sequence in case of Jas intron retention is indicated below each gene model. B) RT-PCR analysis of Arabidopsis seedlings shows amplification of full length *PPD* transcripts and of transcripts resulting from Jas intron retention. Transcript specific primers were used and are indicated in panel A.



Isoform	Composition	Event
Isoform 1*	1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9	Natural
Isoform 2*	1 - 2 - 3 - 4 - 5 - 6b	Alternative acceptor
Isoform 3	1 - 3 - 4 - 5 - 6 - 7 - 8 - 9	Exon skipping
Isoform 4	1 - 2b	Alternative acceptor
Isoform 5	1 - 2 - 3 - 4 - 5 - 7c - 8b	Alternative donor

Isoform	Composition	Event
Isoform 1*	1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9	Natural
Isoform 2*	1 - 2 - 3 - 4 - 5 - 6 - 7b	Intron retention
Isoform 3	1 - 3 - 4 - 5 - 6 - 7 - 8 - 9	Exon skipping
Isoform 4	1 - 2 - 3 - 4b - 5 - 6 - 7 - 8 - 9	Alternative acceptor
Isoform 5	1 - 2 - 3 - 4 - 5 - 6b	Extra exon
Isoform 6	1 - 2 - 3 - 4 - 5 - 8b - 9	Alternative acceptor

Figure 2: Overview of all reported alternative splicing events of the pre-mRNAs of *PPD1* and *PPD2*. Gene architecture is presented as indicated in Fig. 1. The alternative exons are also presented and a blue light line indicates the premature STOP codon. The different possible isoforms are given in the boxes where the asterisks indicate the isoforms that are shown in our results.

This statement is reinforced by a recent publication presenting high-resolution gene expression data from individual cell types of Arabidopsis roots (Li *et al.*, 2016). These data show multiple alternative splicing events for both *PPD1* and *PPD2*, most of them leading to a premature stop codon resulting in a truncated PPD protein lacking the Jas domain (Fig. 2).

JAZ proteins are degraded by SCF^{COI1} in the presence of JA-Ile (Chini *et al.*, 2007; Thines *et al.*, 2007; Fonseca *et al.*, 2009). Truncated JAZ constructs lacking partially or fully the Jas domain are stable versions that cannot be degraded by SCF^{COI1} in the presence of JAs (Chung & Howe, 2009; Chung *et al.*, 2010; Moreno *et al.*, 2013). PPD proteins also interact with an F-box protein, SAP, leading to their degradation (Wang *et al.*, 2016). We performed Y2H analysis to verify if truncated PPD proteins also lose interaction with SAP. However, no direct interaction between SAP and the PPD proteins could be observed with this method (Fig. 3).

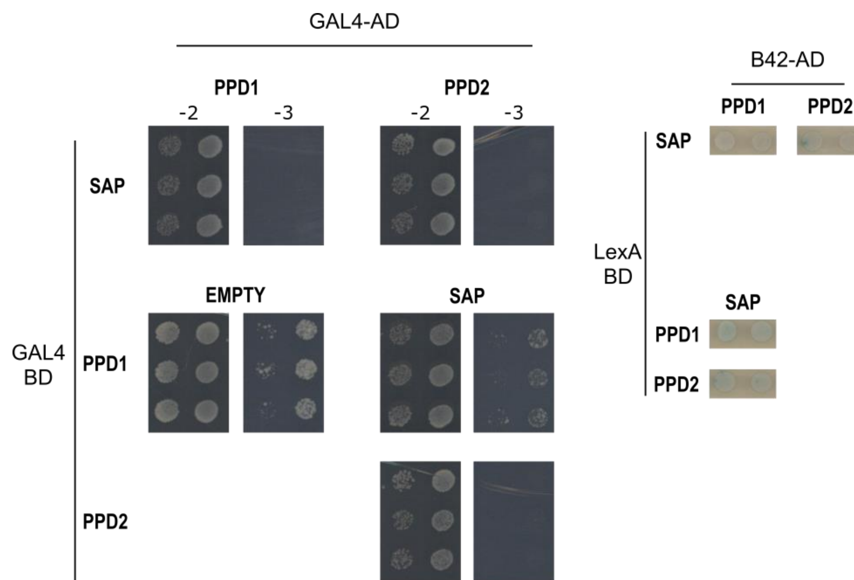


Figure 3: SAP does not show interaction with the PPD proteins via Y2H. Left: SAP and PPD (full length) constructs were fused to GAL4-BD and GAL4-AD, transformed in yeast and selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium. Right: SAP and PPD (full length) constructs were fused to LexA-BD and B42-AD, transformed in yeast, selected on SD-Ura-Trp-His+X-gal medium and tested for β-galactosidase activity (blue colour). Empty vector was used as negative control.

A modified Jas domain of PPD2 mediates interaction with JA-related transcription factors

Alignment of the Jas domain of all JAZ proteins with the divergent Jas domain of PPD1 and PPD2 demonstrates conservation of many amino acids essential for interaction of the JAZ proteins with COI1 and target TFs (Fig. 4).

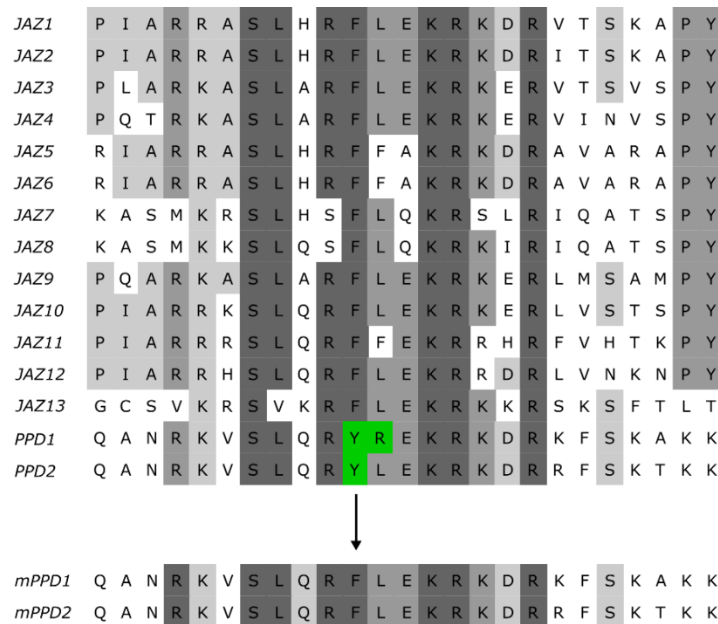


Figure 4: Alignment of the Jas domain of the JAZ proteins and PPD proteins. Indicated in green are the central amino acids that are conserved among most JAZ proteins but not in PPD1 and PPD2. These amino acids were changed into the respective amino acid of the canonical Jas domain to create mPPD1 and mPPD2.

Moretheless, a remarkable difference could be seen in the middle of the Jas domain, where a conserved Phe was occupied by a Tyr in the PPD sequences. Moreover, in PPD1 this Tyr is followed by an Arg, instead of a Leu, which is conserved in most JAZ proteins. We generated two new constructs, mutated PPD1 (mPPD1) and mPPD2, by changing these amino acids into the respective amino acids of the canonical Jas domain as indicated in Fig. 4. The conserved Phe is crucial for interaction of JAZ6 with COI1 in the presence of coronatine (COR), a mimic of JA-Ile (Sheard *et al.*, 2010). We confirmed this for JAZ9 in a Y2H assay showing loss of COR-mediated interaction between JAZ9(F230A) and COI1 (Fig. 5). However, the Tyr-to-Phe mutation in mPPD2 could not induce interaction with COI1 in the presence of COR.

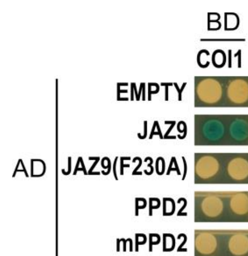


Figure 5: mPPD2 does not induce interaction with COI1. COI1 fused to LexA-BD was tested for interaction with JAZ and PPD2 constructs fused to the B42-AD. Yeasts transformed with both plasmids were selected on SD-Ura-Trp-His+X-gal medium supplemented with 50 µM COR and tested for β-galactosidase activity (blue colour). Empty vector was used as negative control.

The Jas domain of JAZ proteins is responsible for interaction with many JA-related bHLH-type TFs. Mostly, these TFs possess a JID responsible for the interaction with the JAZ proteins (Chapter I.2; Goossens *et al.*, 2016). As PPD1 and PPD2 contain a divergent Jas domain, we hypothesized that they are able to interact with a JID-containing bHLH-type TF. In agreement, TChAP analysis of Arabidopsis cell suspension cultures overexpressing HBH-tagged PPD2 resulted in the identification of the G-box sequence (CACGTG) or a similar sequence (CACGCG) as PPD2-bound DNA sequences (Gonzalez *et al.*, 2015), a characteristic feature of bHLH-type TFs. Based on sequence conservation, the JID was identified in all members of the bHLH subgroups IIId, IIIe and IIIf (Chapter II.2; Heim *et al.*, 2003; Goossens *et al.*, 2015). We tested all of them for interaction with PPD1, PPD2, mPPD1 and mPPD2 via Y2H (Fig. 6). Furthermore, the bHLH-type TF LONESOME HIGHWAY (LHW) was also included in the assay as it contains a structure similar to the 3D structure of the JID of MYC2 (Detected via Phyre2; Kelley *et al.*, 2015). Only JA-ASSOCIATED MYC2-LIKE1 (JAM1), a negative regulator of JA signalling (Sasaki-Sekimoto *et al.*, 2013; Song *et al.*, 2013; Fonseca *et al.*, 2014), could be detected as a direct interactor of PPD2. However, the mutated constructs mPPD1 and mPPD2 could interact with MYC2, MYC3, JAM1 and JAM2.

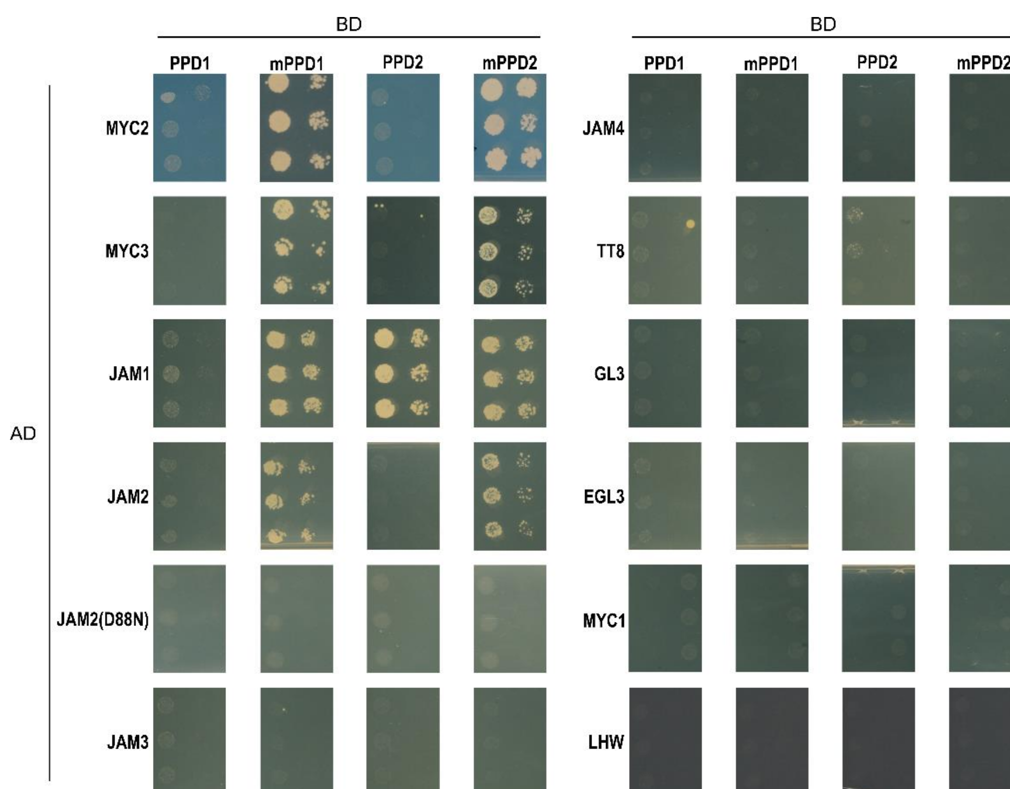


Figure 6: Mutated PPD1 and PPD2 induce interaction with JA-related transcription factors. PPD constructs fused to GAL4-BD were tested for interaction with bHLH-type TFs containing a JAZ-interacting domain fused to GAL4-AD. Yeasts transformed with both plasmids were selected on SD-Leu-Trp-His medium.

Transcriptome-wide screen for transcription factors interacting with PPD2

To find other candidate TFs putatively interacting with PPD2, we applied an unbiased transcriptome-wide Y2H screen followed by next generation sequencing (Y2H-Seq; Erffelinck, Ribeiro et al., unpublished). Herefore, we used a cDNA library prepared from Arabidopsis suspension cells. The yeast colonies that grew on selective media were pooled and prey plasmids were collected for next generation sequencing. The resulting fragments per kilobase of transcript per million fragments mapped (FPKM) values were standardized to the FPKM results of a Y2H-seq screen where NINJA was used as bait. A cut-off value of six was used to remove potential background proteins. The remaining genes were then ordered by highest FPKM value with a minimum value threshold of 50 (Table S3). Out of this list, we identified two TFs that were highly ranked and could putatively interact with PPD2: WUSCHEL RELATED HOMEOBOX5B (WOX5B) and NGATHA-LIKE PROTEIN2 (NGAL2). These candidates were still higher ranked in our list than NINJA and KIX9, two proteins known to interact with PPD2 in Y2H assays (Pauwels *et al.*, 2010; Gonzalez *et al.*, 2015). Lower in the list, more TFs were identified, e.g. TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR20 (TCP20), predicted to have a bHLH DNA binding domain, and bHLH106 (AT2G41130). RESPONSE TO LOW SULFUR 1 (LSU1) was one of the top hits in this screen and was shown to directly interact with JAZ proteins (*Arabidopsis* Interactome Mapping Consortium, 2011). PROTODERMAL FACTOR1 (PDF1), a target of PPD2 (Gonzalez *et al.*, 2015), was also picked up.

WOX5B is expressed in the root quiescent center (QC) and is important for the root stem cell maintenance. In particular, the columella stem cells remain undifferentiated due to WOX5B-mediated recruitment of TPL/TPR co-repressors to the promoter of *CYCLING DOF FACTOR4 (CDF4)*, an enhancer of columella cell differentiation (Pi *et al.*, 2015). WOX5B is related to WUSCHEL (WUS) (Haecker *et al.*, 2004), a homeodomain TF that is reported to have high affinity for the G-box sequence (Busch *et al.*, 2010). WOX5B itself binds to the promoter of *CYCD3;3* to inhibit its expression in the QC (Forzani *et al.*, 2014). Remarkably, *CYCD3;3* is also regulated by PPD2 and moreover, contains a G-box-like sequence in its promoter (CACGTG) (Gonzalez *et al.*, 2015). Finally, Li *et al.* (2016) compared the transcriptome across different root cell types and PPD2 was shown here to be expressed highest in the QC of the root. Our second candidate, NGAL2, is also a transcriptional repressor and represses the expression of the plant organ size regulator *KLU* by binding a G-box-like sequence in its promoter (CACTTG) (Zhang *et al.*, 2015). Altogether, this makes WOX5B and NGAL2 appropriate candidate interactors of PPD2. Unfortunately, we were not able to show direct interaction of WOX5B or NGAL2 with PPD2 in a binary Y2H assay (Fig. 7).

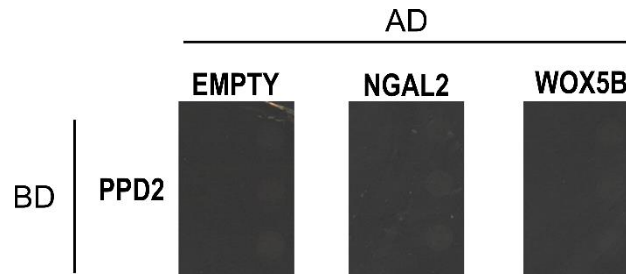


Figure 7: PPD2 does not show interaction with NGAL2 and WOX5B in a binary Y2H assay. PPD2 fused to GAL4-BD was tested for interaction with NGAL2 and WOX5B fused to GAL4-AD. Yeasts transformed with both plasmids were selected on SD-Leu-Trp-His medium.

PPD2 confers repression activity to JAM2

The biological relevance of the candidate interactors was tested via transient expression assays (TEAs) in tobacco protoplasts using a promoter fusion construct of the promoter of *DWARF IN LIGHT (DFL1)* and a *firefly luciferase* gene as read-out. The *DFL1* promoter is a direct target of PPD2 and expression of *DFL1* was increased in the *PPD* knock-down transgenic line, *ami-ppd* (Gonzalez *et al.*, 2015). Moreover, the *DFL1* promoter contains a G-box sequence, typically bound by bHLH-type TFs. We compared the effect of JAM1, JAM2, MYC2, WOX5B and NGAL2 on the promoter activity of *DFL1* (Fig. 8A and 8B). Only MYC2 was able to activate *pDFL1*. No significant effect was observed for JAM1, JAM2, WOX5B and NGAL2. It was shown before that PPD2 alone does not mediate repression during TEAs and that co-expression of KIX8 and/or KIX9 is needed to induce repression of target promoters (Gonzalez *et al.*, 2015). Here, expression of PPD2 and KIX9 together could not repress *pDFL1* (Fig. 8A). However, combined expression of PPD2 and KIX9 with JAM2 was able to mediate repression of *pDFL1*. In contrast, combined expression of PPD2 and KIX9 with JAM1 or MYC2 did not have any effect (Fig. 8A). When both KIX8 and KIX9 where co-expressed with PPD2, *pDFL1* was (slightly) repressed (Fig. 8B and 8C). Unfortunately, this repression was not affected by NGAL2 and only slightly, but not significantly, increased by co-expression of WOX5B (Fig. 8B). Interestingly, repression mediated by co-expression of JAM2 and PPD2/KIX8/KIX9 was higher than the individual PPD2/KIX8/KIX9 repression effect (Fig. 8C).

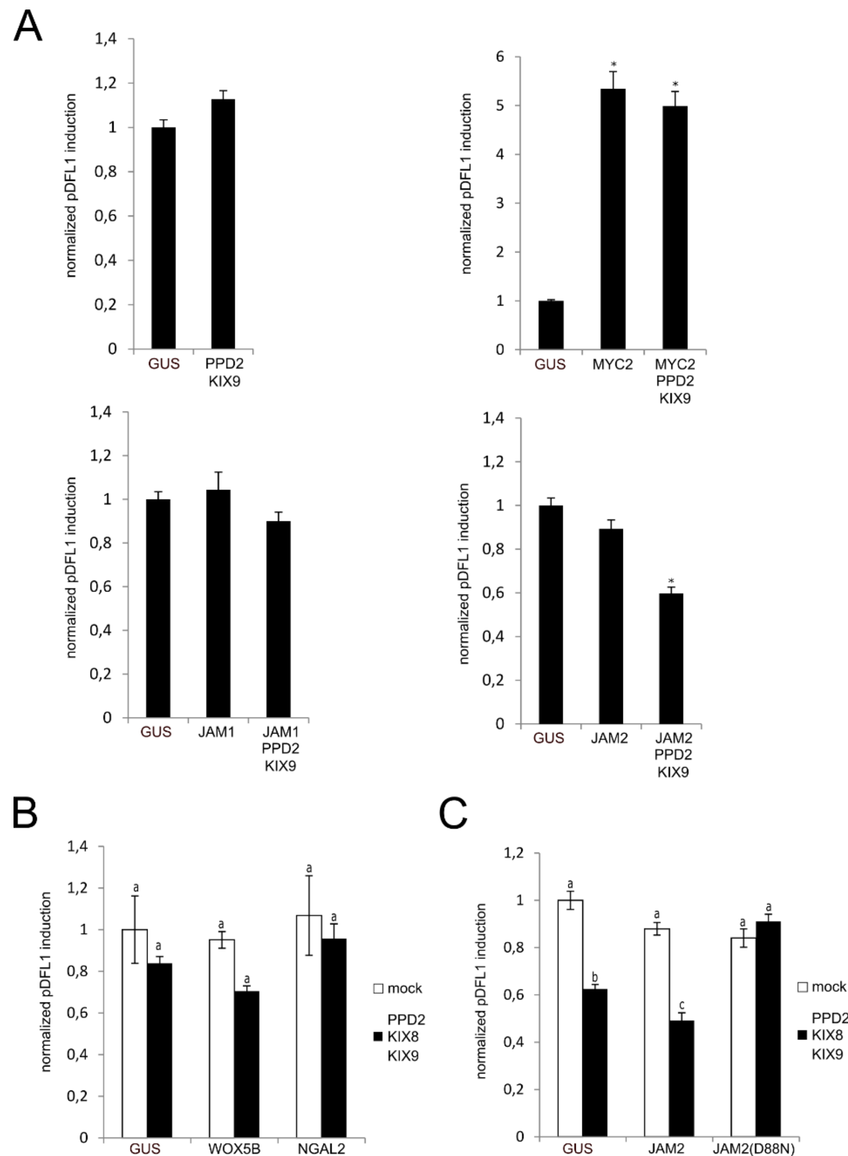


Figure 8: PPD2 confers repression capacity to JAM2 at the promoter of *DFL1*. Transactivation expression assays were performed in tobacco protoplasts. The pDFL1::fLUC reporter construct was co-transfected with effector constructs overexpressing PPD2, KIX8 and KIX9, and/or TFs, potentially interacting with PPD2: A) MYC2, JAM1 and JAM2; B) WOX5B and NGAL2; C) JAM2(D88N), the latter which does not interact with PPD2. GUS expression construct is transfected as control. Values are fold-changes relative to the controls and are the means (+/-SE) of eight biological repeats. Significant differences are marked with an asterisk (Student's t-test; P < 0.01; Panel A) or small letters (Tukey's Honestly Significant Difference test; P < 0.01; Panel B and C).

This combinatorial repression mechanism of JAM2 and PPD2/KIX8/KIX9 was tested on the promoters of *CYCD3;2* and *JAZ1*. *CYCD3;2* is a PPD2-regulated gene (Gonzalez *et al.*, 2015) that is specifically induced in a meristemoid-enriched background (Pillitteri *et al.*, 2011) and *JAZ1* is a typical JA-regulated gene (Thines *et al.*, 2007). Expression of PPD2/KIX8/KIX9 alone could repress *pCYCD3;2* in contrast to JAM2, which by itself did not have any repression effect (Fig. 9A). Again, co-expression of JAM2 and PPD2/KIX8/KIX9 caused repression exceeding the effect of PPD2/KIX8/KIX9 alone, although not statistically significant. On the

other hand, *pJAZ1* could be repressed by JAM2 alone and not by PPD2/KIX8/KIX9 (Fig. 9B). Combining JAM2 and PPD2/KIX8/KIX9 mediated an increase in the repression of *pJAZ1*, surpassing the repression effect of JAM2 alone.

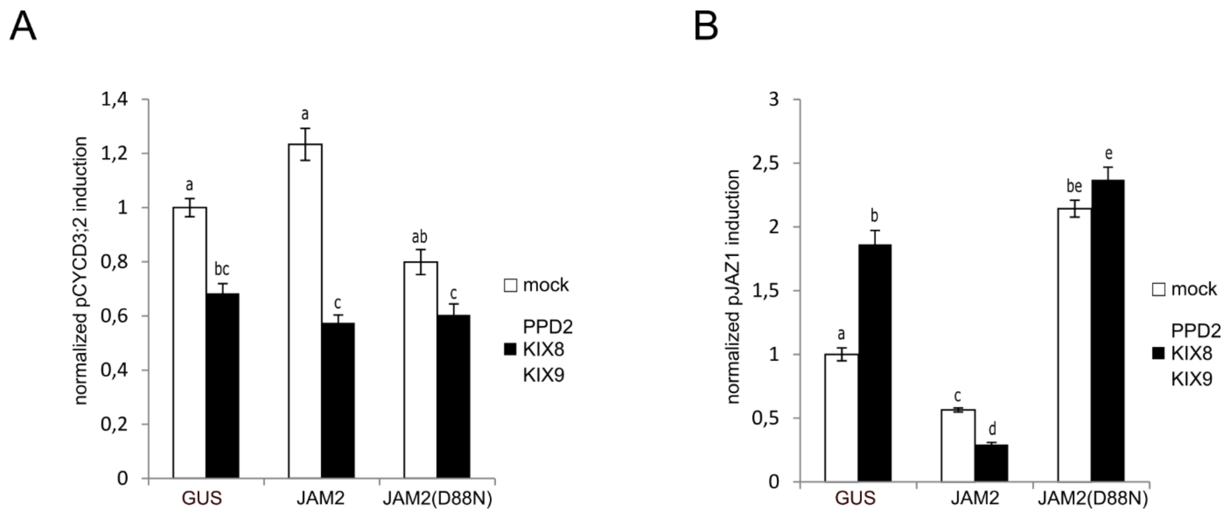


Figure 9: PPD2 confers repression capacity to JAM2 at the promoter of *JAZ1* but not *CYCD3;2*. Transactivation assays by transient expression in tobacco protoplasts transfected with A) the pCYCD3;2::fLUC reporter construct or B) pJAZ1::fLUC reporter construct. The reporter constructs were co-transfected with effector constructs overexpressing PPD2, KIX8 and KIX9, and/or JAM2 or JAM2(D88N), the latter which does not interact with PPD2. GUS expression construct is transfected as control. Values are fold-changes relative to the controls and are the mean (+/-SE) of eight biological repeats. Significant differences are marked with small letters (Tukey's Honestly Significant Difference test; $P < 0.01$).

The results of the TEAs suggest that PPD2 may confer repression activity to JAM2, although it can not be excluded that there could also be an additive effect of the repression effect of both JAM2 and PPD2/KIX8/KIX9. To exclude the latter, we changed a conserved Asp to Asn in the JID of JAM2, corresponding to the respective amino acid change in MYC2(D105N) that caused loss of interaction with almost all JAZ proteins (Chapter II.2; Goossens *et al.*, 2015). Similarly, JAM2(D88N) lost interaction with mPPD2 in a Y2H assay (Fig. 6) and reversed all repression of *pDFL1* and *pJAZ1* in the TEAs (Fig. 8C and 9B). This demonstrates the requisite of interaction between JAM2 and PPD2 in order to mediate repression of expression of *DFL1* and *JAZ1*, at least in tobacco protoplasts. In contrast, JAM2(D88N) did not interfere with the repression effect on *pCYCD3;2* mediated by JAM2 in combination with PPD2/KIX8/KIX9 (Fig. 9A).

DISCUSSION

PPD2 lacks a known DNA binding domain and was so far not shown to bind plant DNA. In addition, TChAP analysis of Arabidopsis cell suspension cultures overexpressing *PPD2* pointed to an overrepresentation of a G-box (CACGTG) or a similar motif (CACGCG) in the identified peak sequences (Gonzalez *et al.*, 2015). Based on these observations, we hypothesized that PPD2 may not bind DNA directly but that it may confer transcriptional repression properties to a G-box-binding TF. Via binary Y2H assays using mutated PPD constructs we identified multiple TFs putatively interacting with PPD2: MYC2, MYC3, JAM1 and JAM2. In addition, WOX5B and NGAL2 were picked up in a genome-wide Y2H screen, although, interaction could not be confirmed via binary Y2H. JAM2 was the only candidate found to show a statistically significant combinatorial transcriptional repression effect with PPD2, KIX8 and KIX9 in TEAs. This was demonstrated independently for the promoters of *DFL1* and *JAZ1*.

In the JA signalling pathway, JAM2 interacts with the JAZ proteins (Song *et al.*, 2013; Fonseca *et al.*, 2014). However, co-expression of *JAZ1* in Arabidopsis protoplasts reversed the repression capacity of JAM2 (Song *et al.*, 2013), hence JAZ proteins probably do not confer repression activity to JAM2. Therefore, we postulate that PPD2 confers repression activity to JAM2. This was verified using a mutated version of JAM2, JAM2(D88N), that lost interaction with PPD2. JAM2(D88N) reversed the repression effect of JAM2 and PPD2/KIX8/KIX9 on *DFL1* and *JAZ1* expression. This indicated that interaction of JAM2 with PPD2 is crucial for repression of these promoters. However, JAM2(D88N) was not able to reverse repression of the *CYCD3;2* promoter. Possibly, PPD2 binds another TF at the regulatory elements of *CYCD3;2*. These results allow us to generate a model where PPD2 is a general transcriptional repressor by binding the adaptors KIX8 and/or KIX9 and/or NINJA to bridge TPL co-repressors to PPD2 and JAM2 or another bound TF (Fig. 10). The biological process and the outcome of this PPD2-mediated regulation is then determined by the bound TF and the involved promoter.

Changing the Tyr to a JAZ-conserved Phe in the Jas domain in PPD2 caused interaction with JAM2 in Y2H. However, wild-type PPD2, together with KIX8 and KIX9, was able to confer transcriptional repression activity to JAM2 in tobacco protoplasts. Since this amino acid change does not occur in plants, this may suggest that this Tyr in the Jas domain of PPD2 is subjected to a post-translation modification in yeast, such as phosphorylation, that would block protein-protein interaction.

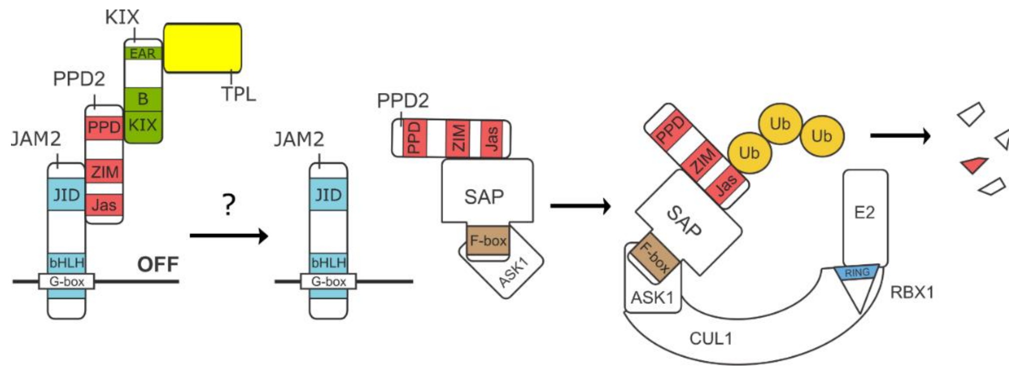


Figure 10: Proposed model of PPD2-involved signalling. The KIX proteins connect TPL co-repressors to PPD2. Interaction of PPD2 with JAM2, mediates repression of JAM2-bound target genes. An unknown signal induces interaction of the F-box protein SAP with PPD2 leading to proteasomal degradation of PPD2, releasing JAM2.

Our results demonstrate the existence of multiple different alternative splicing events that lead to truncated PPD1 or PPD2 proteins lacking partially or fully the C-terminal Jas domain. However, the biological relevance for this has not been revealed yet. Alternative splicing of *JAZ* pre-mRNAs also leads to truncated constructs lacking partially or completely the Jas domain. These JAZ splice variants possess less affinity for the F-box protein COI1 and are therefore stable versions of JAZ in the presence of JAs, dominantly repressing bound TFs (Chung & Howe, 2009; Chung *et al.*, 2010; Moreno *et al.*, 2013). Correspondingly, PPD alternative splice variants may possibly lead to loss of interaction with the F-box protein SAP resulting in stabilized PPD proteins. To investigate this, the determination of the interaction domain responsible for the interaction between SAP and PPD is an asset. An appropriate tool for this is the Y2H technique, however, we could not establish interaction between SAP and PPD in this way. Possibly, a natural compound could be necessary for the interaction of PPD with SAP. This was also observed for the interaction between COI1 and the JAZ proteins, where addition of JA-Ile or COR is essential to mediate interaction in Y2H (Thines *et al.*, 2007; Melotto *et al.*, 2008). Further investigation of the interaction between the PPD proteins and SAP is necessary to look deeper into the biological relevance of alternative splicing of PPD.

In conclusion, our results confirm that many molecular similarities exist between the JAZ proteins and the PPD proteins. No phenotypical link has been established yet between JA signalling and the PPD proteins. However, as was shown here, research on PPD-involved signalling can be tremendously accelerated if we take advantage of these similarities with JAZ proteins.

MATERIALS AND METHODS

Gene cloning

All cloning was carried out by Gateway® recombination (Thermo Fisher Scientific, Waltham, MA, USA). The point mutations in PPD1(Y242F and R243L), PPD2(Y243F) and JAM2(D88N) were generated with the GeneTailor™ Site-Directed Mutagenesis system (Thermo Fisher Scientific).

Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from *Arabidopsis thaliana* (L.) Heynh ecotype Col-0 seedlings via the Qiagen RNeasy kit (Qiagen, Hilden, Germany). One microgram of RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Specific primers (Table S4) and GoTaq® DNA polymerase (Promega, Fitchburg, WI, USA) were used for amplification of cDNA via the following program: initial denaturation (95°C, 5 min), 40 amplification cycles (denaturation 95°C, 30 s; annealing 60°C, 30 s; elongation 72°C, 60 s), final extension (72°C, 5 min). Amplified cDNA was visualized in an agarosegel by Invitrogen™ SYBR™ Safe (Thermo Fisher Scientific).

Yeast two-hybrid

Y2H analysis was performed as described (Cuéllar Pérez *et al.*, 2013). Two systems were used depending on the tested proteins. In the GAL4 system, bait and prey were fused to the GAL4-AD or GAL4-BD via cloning into pGAL424gate or pGBT9gate respectively. The *Saccharomyces cerevisiae* PJ69-4A yeast strain (James *et al.*, 1996) was co-transformed with bait and prey using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) media lacking Leu and Trp (Clontech, Saint-Germain-en-Laye, france). Three individual colonies were grown overnight in liquid cultures at 30°C and 10- or 100-fold dilutions were dropped on control media (SD-Leu-Trp) and selective media lacking Leu, Trp and His (Clontech). For the LexA system, the bait and prey were fused to the B42-AD or LexA-BD via cloning into pB42ADgate or pGILDAgate respectively. The *S.cerevisiae* EGY48 yeast strain (Estojak *et al.*, 1995) was used and transformants were selected on SD media lacking Ura, Trp and His. Two individual colonies were grown overnight in liquid cultures at 30°C and 10-fold dilutions were dropped on control media (SD-Ura-Trp-His) and selective media containing X-gal (Duchefa, Haarlem, The Netherlands). Coronatine (50 µM) was added to the medium to test for interaction with COI1.

Yeast two-hybrid screen – deep sequencing

Yeast transformation was performed based on Cuéllar Pérez *et al.* (2013). The bait, PPD2, was fused to the GAL4-BD via cloning into pDEST™32 (Thermo Fisher Scientific). The *S. cerevisiae* PJ69-4A yeast strain (James *et al.*, 1996) was transformed in two transformation rounds with 0.5 µg of bait plasmid DNA and 50 µg of cDNA library plasmid DNA using the PEG/lithium acetate method. The ProQuest two-hybrid cDNA library is generated by cDNA synthesis from RNA extracted from Arabidopsis At7 suspension cells and cloned into pDEST™22 (Thermo Fisher Scientific). At least 10^6 transformants were plated on control media (SD-Leu-Trp) and selective media lacking Leu, Trp and His (Clontech) supplemented with 5 mM 3-amino-1,2,4-triazole (Sigma-aldrich, Saint Louis, MO, USA). Colonies

were dissolved and pooled in 20 mL of purified water and plasmids were collected using the Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research, Irvine, CA, USA). Prey constructs were amplified via PCR using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and specific pDEST™22 primers (Table S4). The PCR mixture was purified using the CleanPCR kit (CleanNA, Alphen aan den Rijn, The Netherlands) and sent for Next Generation Sequencing (GATC Biotech, Constance, Germany). The processed sequencing reads were mapped to the Arabidopsis genome (TAIR10) using TopHat (Trapnell *et al.*, 2009) and reads were counted with Cufflinks (Trapnell *et al.*, 2010), providing the FPKM values per sequenced gene.

3D structure prediction via Phyre2

A 3D structure for the amino acid sequence occupying the JID of MYC2 (93-160; Fernández-Calvo *et al.*, 2011) was predicted via the protein homology/analogy recognition engine version 2.0 (Phyre2; Kelley *et al.*, 2015). The resulting protein data bank (.pdb) file was loaded into BackPhyre to search this structure against the Arabidopsis genome.

Transient expression assay

Transient expression assays in protoplast cells prepared from Bright Yellow-2 (BY-2) *Nicotiana tabacum* suspension cultured cells were performed as described (Vanden Bossche *et al.*, 2013). The reporter plasmid contained a firefly luciferase (fLUC) gene under control of the *DFL1*, *CYCD3;2* or *JAZ1* promoter. GUS, PPD2, KIX8, KIX9 and the tested transcription factors were expressed under control of the CaMV35S promoter in effector plasmids. Plasmids with the Renilla luciferase (rLUC) expressed under control of the CaMV35S promoter serve for normalization for transfection efficiency. Protoplast cells were transfected with 2 µg of each plasmid using the PEG/Ca^{2+} method and grown overnight in the dark at room temperature with gentle agitation. After lysis of the cells, the luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega).

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Chapter 6

Development of a small molecule yeast two-hybrid system

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[1]Author contributions: molecular cloning, Y2H assays, data interpretation and writing (all text and figures).

ABSTRACT

Plant chemical biology and genetics have been important for the current understanding of hormone signalling. Plant genetics, however, is confronted with limitations, i.e. gene redundancy and gene lethality, that can be overcome by chemical compounds, which can target individual proteins or protein families in a time- and tissue-controlled manner. Auxin perception is mediated by the F-box proteins TIR1/AFB and the AUX/IAA repressors. Identification of compounds, blocking or mediating interaction of these hormone receptor complexes, can contribute to the further unravelling of auxin signalling. Yeast two-hybrid (Y2H) is a quick and convenient method to evaluate hormone-mediated protein-protein interactions. Here, we show that perturbations in the first helix of the F-box domain of TIR1, AFB1, AFB2 and AFB5 increase the auxin-mediated interaction with AUX/IAA proteins during Y2H. A similar approach can be applied to F-box proteins in co-receptor complexes of other hormones, such as jasmonate. These mutated constructs can be implemented in a Y2H system leading to a better sensitivity for small molecule-mediated interactions between F-box proteins and their targets. This small molecule Y2H system could serve to screen chemical libraries for new bioactive molecules and/or to screen for new F-box protein substrates using a cDNA library. Finally, compounds that can modulate hormone signalling can potentially be applied in the agrochemical field to improve the performance of crops.

INTRODUCTION

Plant chemical biology has been important during the exploration of plant hormone perception and signalling. Generation or identification of bioactive compounds resulted in a lot of knowledge about the molecular organization and biological function of several signalling pathways. In particular, binding studies of agonists or antagonists of hormones to the respective receptor complexes had a tremendous impact on our current understanding of hormone signalling. Nevertheless, plant genetics has been very important for the current understanding of plant hormone biology but is now confronted with limitations, such as gene redundancy and gene lethality. This can be overcome by plant chemical biology as small compounds can target several conserved sites of redundant protein families and gene lethality can be addressed by the application of compounds in a spatiotemporal controlled manner. For comprehensive views on plant chemical biology, we refer to recent reviews (Fonseca *et al.*, 2014; Rigal *et al.*, 2014).

Frequently, large chemical libraries are used to screen for compounds being active in hormone signalling. In addition, thanks to the revelation of crystal structures of many receptor complexes, rational design of active molecules has opened doors for plant chemical biology and their applications in the agrochemical field. Auxinole and coronatine-methyloxime (COR-MO) are perfect examples to demonstrate the rational design of

antagonists of indole-3-acetic acid (IAA) and jasmonate (JA)-Ile, the endogenous bioactive compounds for auxin and JA signalling, respectively. COR-MO blocks the formation of the JA co-receptor complex consisting of the F-box protein CORONATINE INSENSITIVE1 (COI1) and a JA-ZIM DOMAIN (JAZ) repressor. Thereby, COR-MO inhibits Skp1/Cullin/F-box $(SCF)^{COI1}$-mediated degradation of JAZ resulting in repression of JAZ-bound TFs and suppression of JA-mediated responses in *Arabidopsis thaliana* and *Nicotiana benthamiana* (Monte *et al.*, 2014). Auxin is perceived by a similar co-receptor complex (Fig. 1) formed by the F-box proteins TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOX PROTEIN (AFB) and the AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE (AUX/IAA) repressors (Salehin *et al.*, 2015). Auxinole blocks auxin-mediated assembly of the co-receptor complex leading to inhibition of the auxin response in Arabidopsis, tomato and moss (Hayashi *et al.*, 2012).

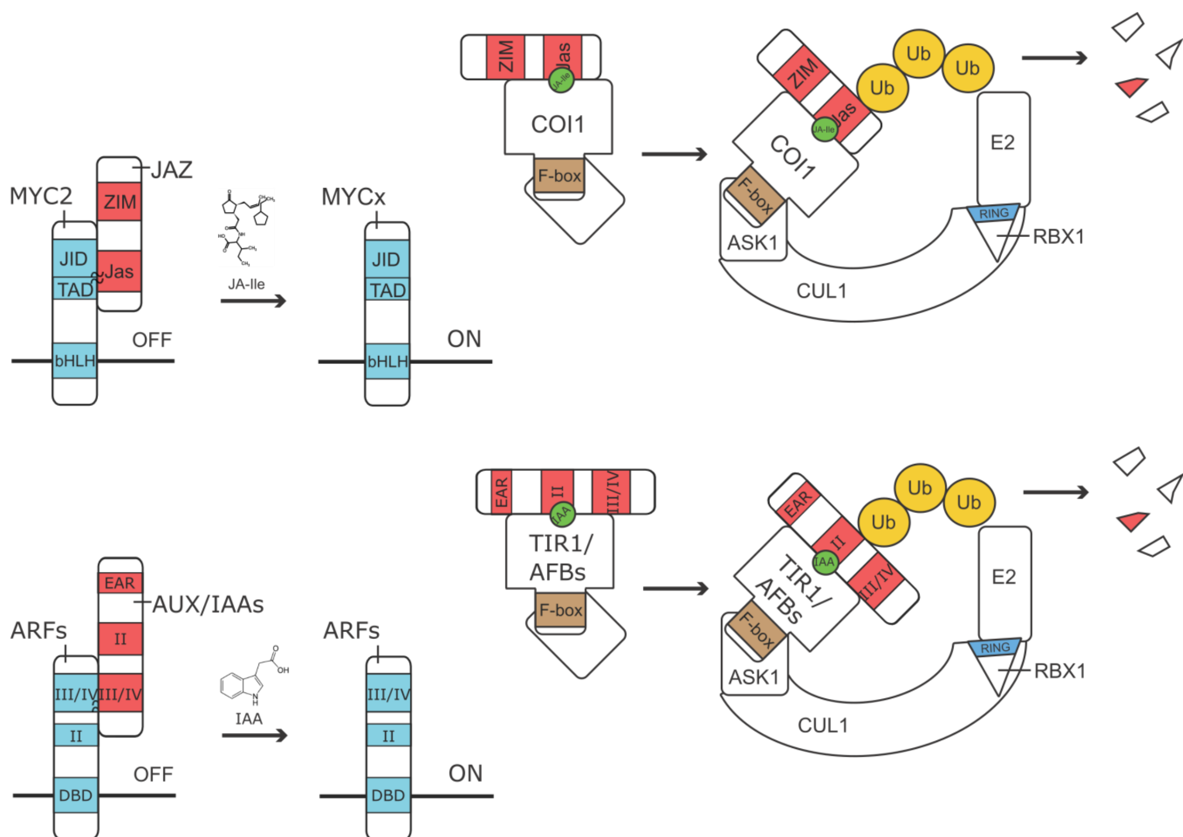

Figure 1: Model of the molecular machinery of jasmonic acid (top) and auxin (bottom) signalling.

Yeast two-hybrid (Y2H) is a user-friendly and fast method for testing direct protein-protein interactions and is often used to validate hormone-mediated interaction between the constituents of the reception complex as seen for JA, abscisic acid (ABA), auxin, strigolactones and gibberellin (Ueguchi-Tanaka *et al.*, 2005; Thines *et al.*, 2007; Fonseca *et al.*, 2009; Park *et al.*, 2009; Prigge *et al.*, 2010; Calderón Villalobos *et al.*, 2012; Hamiaux *et al.*, 2012; Wang *et al.*, 2015). The auxin receptor proteins TIR1/AFBs assemble into an SCF complex to be functional (Gray *et al.*, 1999). The SCF complex contains a stable core

composed of a large subunit CULLIN1 (CUL1) and the REALLY INTERESTING NEW GENE (RING) finger protein RING-BOX1 (RBX1), which is essential for the ubiquitination capacity of SCF complexes. An F-box protein determines substrate specificity of the complex and is connected to CUL1 subunit via the S PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1) adaptor (Hua & Vierstra, 2011). A crystal structure of the human SCF^{SKP2} complex pointed out that the F-box protein, SKP2, is also directly involved in interaction with CUL1 via some conserved residues in the first helix of the F-box domain of SKP2 (Zheng *et al.*, 2002).

Due to the conservation of the Skp1 and Cullin components of the SCF complex among eukaryotes, TIR1 was shown to be functional in yeast, leading to auxin-mediated degradation of AUX/IAA proteins in yeast (Nishimura *et al.*, 2009; Yu *et al.*, 2013). Recently, Y2H analysis showed that mutations in the first helix of the F-box domain of the TIR1 and AFB2 receptor proteins could increase interaction with AUX/IAA proteins in the absence and presence of auxin (Yu *et al.*, 2015). These mutated versions of TIR1 were still able to interact with ARABIDOPSIS SKP1 HOMOLOGUE (ASK1) but not with CUL1. This resulted in increased stability for the mutated TIR1 constructs, probably due to decreased auto-ubiquitination (Yu *et al.*, 2015). As a consequence, the AUX/IAA proteins are also protected from SCF^{TIR1}-mediated ubiquitination. Altogether, the higher stability of both TIR1/AFB2 and the AUX/IAA proteins leads to an increase in detectable interaction in Y2H analysis.

Here, we expanded the Y2H results shown for the mutated versions of TIR1 and AFB2 (Yu *et al.*, 2015) and changed the respective conserved amino acids in the F-box domain of the TIR1 homologues AFB1, AFB3, AFB4 and AFB5. The F-box protein of the JA signalling core machinery, COI1, is closely related to TIR1 (Tan *et al.*, 2007) and therefore we introduced similar mutations in COI1 to inquire their effect on the interaction with JAZ proteins in the presence of COR, a mimic of JA-Ile. Similar to the mutated versions of TIR1 and AFB2, we could detect an increased interaction of mutated AFB1 and AFB5 with AUX/IAA proteins in the absence and/or presence of auxin. Using these mutated constructs, we established a small molecule (sm) Y2H system that has increased sensitivity for auxin-mediated interaction between TIR1/AFB F-box proteins and their substrates, the AUX/IAA proteins. This system can be applied to screen chemical libraries and detect new compounds mediating or blocking interaction between TIR1/AFB and AUX/IAA proteins. Furthermore, this system could allow the identification of new targets of TIR1/AFB proteins in the presence of auxin, using a cDNA library. In the future, this concept could be further extended to the perception mechanisms of other hormone pathways like the COI1-JAZ co-receptor complex of JA signalling.

RESULTS

Perturbations in the F-box domain of TIR1/AFB proteins enhance interaction with IAA7

Perturbations in the first helix of the F-box domain of TIR1 and AFB2 resulted in increased stability of these F-box proteins and their AUX/IAA targets. This goes along with an enhanced interaction during Y2H (Yu *et al.*, 2015). To verify if these results could be observed in our hands, we repeated these Y2H experiments testing the same mutated TIR1 and AFB2 constructs for interaction with the AUX/IAA protein, IAA7. Interaction strength can be evaluated by the level of β-galactosidase reporter activity on X-gal-containing medium leading to a blue colour. We first compared auxin-mediated interaction of TIR1 with IAA7 in the presence of 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 µM 1-naphthaleneacetic acid (NAA). The interactions could be detected most clearly using NAA (Fig. 2), consistent with similar Y2H experiments testing the effect of multiple auxins on the interaction between TIR1 and IAA7 (Yu *et al.*, 2013).

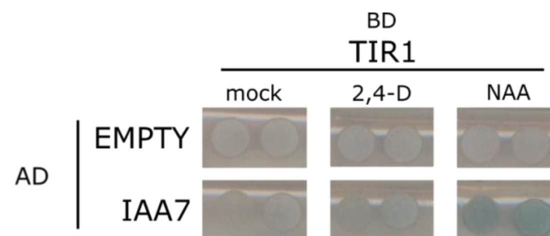

Figure 2: Interaction between TIR1 and IAA7 in the presence of 2,4-D and NAA. TIR1 fused to LexA-BD was tested for interaction with IAA7 fused to the B42-AD. Yeasts transformed with both plasmids were selected on SD-Ura-Trp-His+X-GAL medium supplemented with 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 100 µM 1-naphthaleneacetic acid (NAA) or DMSO (mock) and tested for β-galactosidase activity (blue colour). Empty vector was used as negative control.

Next we verified interaction strength of the three mutated TIR1 and AFB2 constructs, described in Yu *et al.* (2015), with IAA7 in the presence of 100 µM NAA. Mutated residues in the first helix of the conserved F-box domain of TIR1 and AFB2 are indicated in Fig. 3. Native TIR1 and AFB2 interact weakly with IAA7 in the presence of NAA (Fig. 4). All three mutated versions of both TIR1(E12K, E15K and F18L) and AFB2(E7K, E10K and F13L) increased the strength of this NAA-mediated interaction. The mutated versions TIR1(E12K) and AFB2(E7K) showed the strongest increase in interaction. In the case of AFB2, the mutated versions interacted with IAA7 without the need for NAA, still this interaction was clearly increased when NAA was present. These first results were in agreement with the observations of Yu *et al.* (2015) confirming the functionality of our Y2H assay.

					E12K			E15K			F18L		
TIR1	9	F	P	E	E	V	L	E	H	V	F	S	19
AFB1	5	F	P	P	K	V	L	E	H	I	L	S	15
AFB2	4	F	P	D	E	V	I	E	H	V	F	D	14
AFB3	4	F	P	D	E	V	I	E	H	V	F	D	14
AFB4	54	V	L	E	N	V	L	E	N	V	L	Q	64
AFB5	54	V	L	E	N	V	L	E	N	V	L	Q	64
COI1	16	T	V	D	D	V	I	E	Q	V	M	T	26

Figure 3: Alignment of the C-terminal part of the first helix of the F-box domain of the TIR1/AFB proteins and COI1. The amino acid residues mutated in TIR1 are indicated in the black boxes together with the respective residues in the other AFB proteins and COI1.

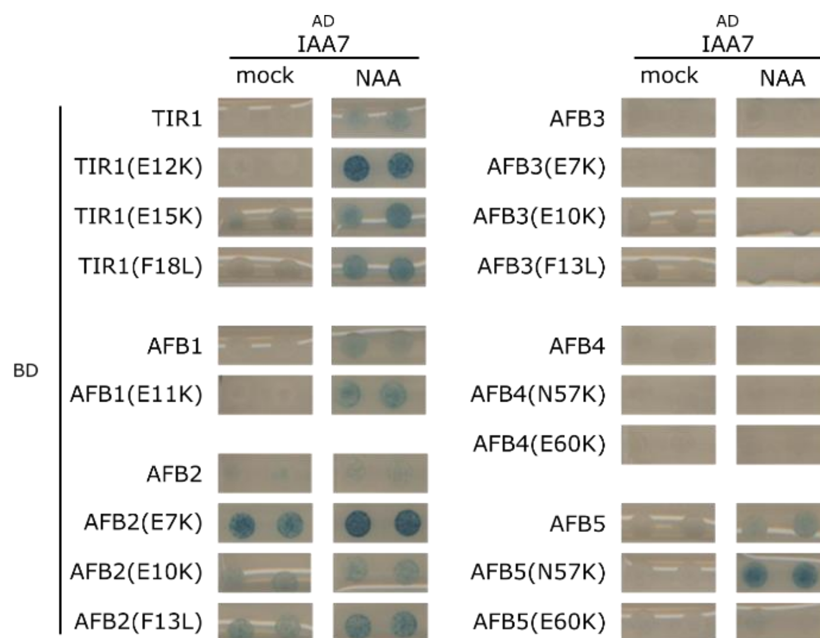

Figure 4: Perturbations in the first helix of the F-box domain of TIR1, AFB1, AFB2 and AFB5 enhance the interaction with IAA7. Wild-type and mutated constructs of TIR1/AFB fused to LexA-BD were tested for interaction with IAA7 fused to the B42-AD. Yeasts transformed with both plasmids were selected on SD-Ura-Trp-His+X-GAL medium supplemented with 100 µM 1-naphthaleneacetic acid (NAA) or DMSO (mock) and tested for β-galactosidase activity (blue colour).

The other members of the TIR1/AFB family are also involved in auxin perception as a co-receptor complex together with the AUX/IAA repressors (Dharmasiri *et al.*, 2005; Calderón Villalobos *et al.*, 2012; Prigge *et al.*, 2016). Comparison of the amino acid sequence of the first helix of the F-box domain of the AFB proteins with the sequence of TIR1, showed some conservation among the different auxin receptors (Fig. 3). The three amino acids that were mutated in the stable constructs of TIR1 and AFB2, were also conserved in the sequence of AFB3. However, out of these three amino acids, only one was conserved in the F-box domain of AFB1, AFB4 and AFB5. Furthermore, AFB1 contains already a Lys and a Leu at the respective TIR1(E12K) and TIR1(F18L) position and AFB4 and AFB5 contain a Leu at the

respective TIR1(F18L) position (Fig. 3). This was in agreement with the finding that TIR1, AFB2 and AFB3 are very unstable proteins compared to AFB1 (Parry *et al.*, 2009; Yu *et al.*, 2015), though data about AFB4 and AFB5 are missing here. We generated mutated constructs of AFB1(E11K), AFB3(E7K, E10K and F13L), AFB4(N57K and E60K) and AFB5(N57K and E60K) according to the amino acid changes that were made in the mutated TIR1 and AFB2 constructs.

Neither the mutated nor the wild-type versions of AFB3 and AFB4 could induce interaction with IAA7, whether or not in the presence of NAA (Fig. 4). Nevertheless, AFB1(E11K) and AFB5(N57K) were able to increase interaction with IAA7 in the presence of NAA, in contrast to AFB5(E60K), which diminished interaction of AFB5 with IAA7 (Fig. 4). To verify whether these results can also be observed for other AUX/IAA repressors, we tested the best performing TIR1/AFB mutants for interaction with IAA1, IAA3 and IAA17 in the presence or absence of NAA, leading to similar observations (Fig. 5). The constructs of AFB3 and AFB4 did not show interaction with any of the AUX/IAA proteins tested. In contrast, the TIR1(E12K), AFB1(E11K), AFB2(E7K) and AFB5(N57K) constructs all increased NAA-mediated interaction with IAA1, IAA3 and IAA17 compared to their wild-type counterparts. In the case of IAA1 and IAA17, these mutated TIR1/AFB constructs were able to increase interaction without the need for NAA, although the effect is more pronounced in the presence of NAA (Fig. 5).

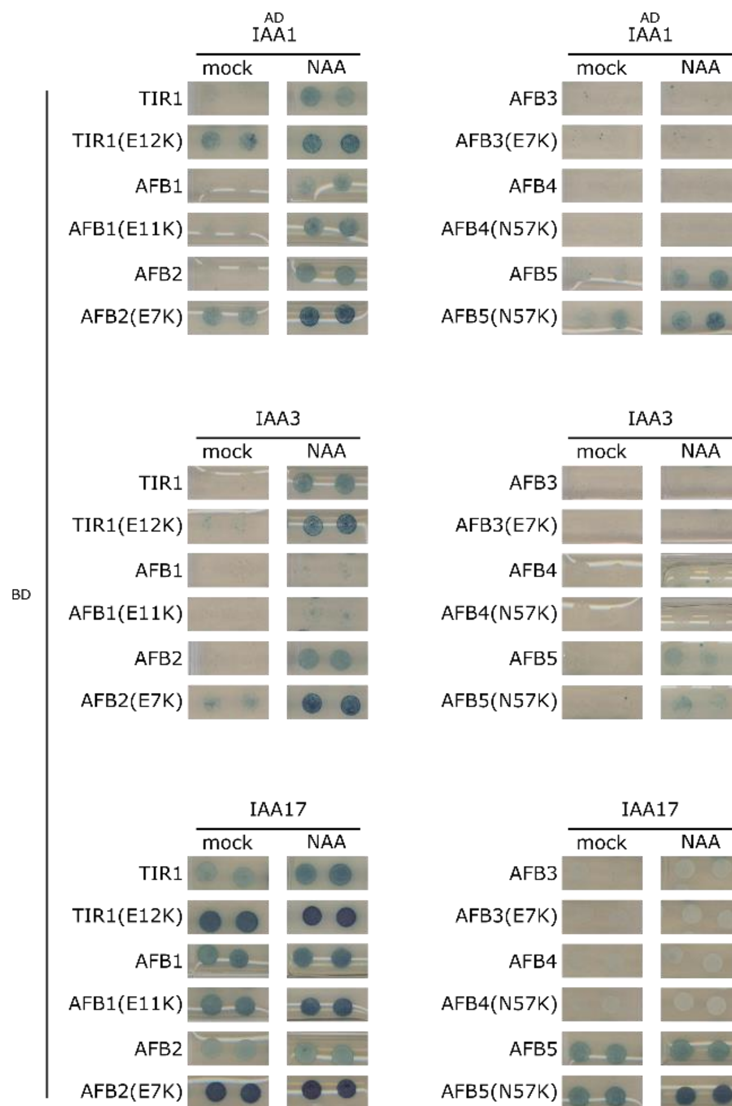

Figure 5: Perturbations in the first helix of the F-box domain of TIR1, AFB1, AFB2 and AFB5 enhance the interaction with IAA1, IAA3 and IAA17. Wild-type and mutated constructs of TIR1/AFB fused to LexA-BD were tested for interaction with IAA1, IAA3 and IAA17 fused to the B42-AD. Yeasts transformed with both plasmids were selected on SD-Ura-Trp-His+X-GAL medium supplemented with 100 µM 1-naphthaleneacetic acid (NAA) or DMSO (mock) and tested for β-galactosidase activity (blue colour).

Extension of the smY2H to JA signalling

The closest homologue of the TIR1/AFB family is COI1, an F-box protein playing a role in the core JA signalling module. Alignment of the amino acid sequences of the first helix of the F-box domain of COI1 and TIR1 shows that the TIR1E12, E15 and F18 positions are occupied by respectively an Asp, Glu and Met in the sequence of COI1 (Fig. 3). COI1 constructs with mutations at these positions (D18K, E22K and M25L) did not show a tremendous increase in interaction with JAZ12 in the presence of COR, though a slight increase could be observed for COI1(E22K) (Fig. 6).

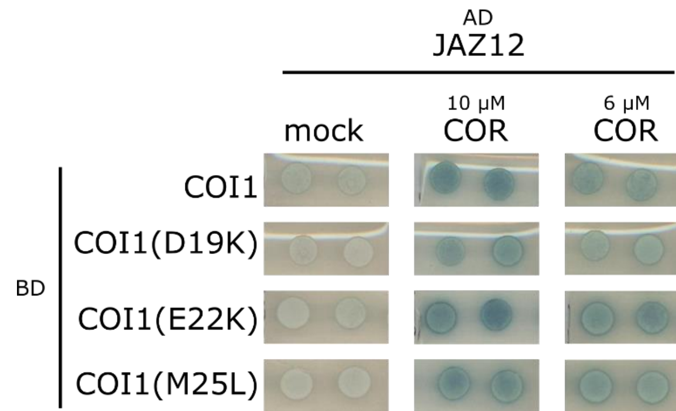

Figure 6: Interaction of COI1 constructs, carrying mutations in the first helix of the F-box domain, with JAZ12. Wild-type and mutated constructs of COI1 fused to LexA-BD were tested for interaction with JAZ12 fused to the B42-AD. Yeasts transformed with both plasmids were selected on SD-Ura-Trp-His+X-GAL medium supplemented with 6 µM or 10 µM coronatine (COR) or DMSO (mock) and tested for β-galactosidase activity (blue colour).

DISCUSSION

Hormone signalling is important for the development and survival of the plant. Screens using big chemical libraries to identify and characterize new bioactive compounds help to improve our understanding of hormonal pathways. The use of agonists or antagonists of hormonal compounds can modify the hormone signalling processes to explore the function and possibilities of hormones. Furthermore, these compounds can be potentially used in the agrochemical or pharmaceutical field to improve the performance of crops or increase the production of specialized metabolites in medicinal plants.

Based on the experiments performed in Yu *et al.* (2015), we developed a highly sensitive smY2H system containing mutated constructs of TIR1, AFB1, AFB2 and AFB5 that show enhanced interaction with AUX/IAA proteins, increasing the sensitivity for small compounds as was shown here for the auxin NAA. However, we were not able to show interaction of AFB3 and AFB4 with the tested AUX/IAA proteins, neither in the absence nor the presence of NAA. This is in agreement with the lack of Y2H assays using AFB3 or AFB4, in contrast to reported Y2H assays showing interaction of TIR1, AFB1, AFB2 and AFB5 with AUX/IAA proteins in the presence of different auxins (Calderón Villalobos *et al.*, 2012). The increased stability by mutations in the first helix of the F-box domain of TIR1/AFB proteins was explained by increased protein stability due to loss of direct interaction of TIR1 with CUL1, and hence diminished autocatalytic degradation (Yu *et al.*, 2015). In contrast, loss of interaction with ASK1 by e.g. a deletion in the F-box domain leads to destabilization of TIR1 (Dezfulian *et al.*, 2016). In this case, a SCF^{TIR1}-independent degradation mechanism is likely responsible for this loss of TIR1 protein stability. This was also observed for COI1, where deletion of the F-box led to decreased stability in a non-autocatalytic manner (Yan *et al.*,

2013). However, loss of interaction between ASK1 and CUL1 also led to degradation of the COI1 protein.

Here, we added COI1 to the smY2H system. One mutated construct, COI1(E22K) showed a slight increase in interaction with JAZ12 in the presence of COR. The respective amino acid change in TIR1 showed to reduce interaction with CUL1 and not with ASK1 (Yu *et al.*, 2015). However, a similar mutant construct, COI1(E22A), was shown to abolish interaction with ASK1 (Xu *et al.*, 2002). Additional mutational research to increase the stability of COI1 could further optimize our smY2H assay. F-box proteins are often very unstable due to an autocatalytic degradation mechanism (Galan & Peter, 1999; Bosu & Kipreos, 2008; Schmidt *et al.*, 2009). It would be interesting to investigate in the future whether the smY2H can be extended to other hormonal pathways, such as gibberellin and strigolactone signalling, where the F-box proteins, respectively SLEEPY1 (SLY1) and MORE AXILLARY BRANCHES2 (MAX2), are important for hormone perception (Wallner *et al.*, 2016).

The smY2H system established here is highly sensitivity towards NAA and COR, mediating interaction of respectively TIR1/AFBs and COI1 with their substrates. We tested our smY2H system using an unknown auxin-like compound that induced a partial auxin response in Arabidopsis plants (Vain *et al.*, unpublished). However, no interaction could be mediated between TIR1/AFBs and AUX/IAA repressors. The transcriptome of plants treated with this compound was analysed and suggested VQ MOTIF-CONTAIING PROTEIN33 (VQ33) as alternative repressor that could be bound by TIR1/AFB proteins. Unfortunately, no interaction could be detected between TIR1/AFBs and VQ33 in the absence or presence of the unknown compound. Finally, a second auxin-like unknown compound, which showed a typical JA-induced early transcriptional response in Arabidopsis plants (Geelen *et al.*, unpublished), was tested in our smY2H system. However, this compound could not mediate interaction between COI1 and the JAZ repressors. Still, the mutated F-box constructs of the smY2H result in a high sensitivity for small compounds. Lower concentrations of the compounds are needed to induce formation of the co-receptor complex. For instance, interaction between TIR1 and IAA7 is almost undetectable at relatively high concentrations of different auxins (Yu *et al.*, 2013; Yu *et al.*, 2015), in contrast to mutated, stable constructs of TIR1, which enhanced the interaction during Y2H.

In conclusion, smY2H is a suitable technique for the validation of identified compounds and F-box protein substrates involved in the perception of hormonal compounds. Furthermore, it enables chemical libraries to be screened for new compounds that can mediate or block interaction between particular F-box proteins and their substrates. Interesting compounds can then be applied in the field in a tissue- and time-controlled manner to target individual proteins or protein families in plants, without affecting other processes. In addition, cDNA

libraries can be used to identify new substrates that are bound by a particular F-box protein in the presence of a specific compound.

MATERIALS ANS METHODS

Gene cloning

All cloning was carried out by Gateway® recombination (Thermo Fisher Scientific, Waltham, MA, USA). The point mutations in TIR1(E12K, E15K and F18L), AFB1(E11K), AFB2(E7K, E10K and F13L), AFB3(E7K, E10K and F13L), AFB4(N57K and E60K), AFB5(N57K and E60K) and COI1(D18K, E22K and M25L) were generated with the GeneTailor™ Site-Directed Mutagenesis system (Thermo Fisher Scientific).

Yeast two-hybrid

Y2H analysis was performed as described (Cuéllar Pérez *et al.*, 2013). Bait and prey were fused to the B42-AD or LexA-BD via cloning into pB42ADgate or pGILDAgate respectively. The *Saccharomyces cerevisiae* EGY48 yeast strain (Estojak *et al.*, 1995) was co-transformed with bait and prey using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) media lacking SD media lacking Ura, Trp and His (Clontech, Saint-Germain-en-Laye, france). Two individual colonies were grown overnight in liquid cultures at 30°C and 10-fold dilutions were dropped on control media (SD-Ura-Trp-His) and selective media containing X-GAL (Duchefa, Haarlem, The Netherlands). To test for interaction between bait and prey, 2,4-D (100 µM), NAA (100 µM), COR (10 µM or 6 µM), compound X (100 µM), compound Y (100 µM) or DMSO were added to the medium.

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Chapter 7

Isolation of protein complexes from the model legume *Medicago truncatula* by tandem affinity purification in hairy root cultures

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Published in:

Jonas Goossens[1], Nathan De Geyter[1], Alan Walton, Dominique Eeckhout, Jan Mertens, Jacob Pollier, Jennifer Fiallos-Jurado, Annick De Keyser, Rebecca De Clerq, Jelle Van Leene, Kris Gevaert, Geert De Jaeger, Sofie Goormachtig and Alain Goossens. ***Isolation of protein complexes from the model legume Medicago truncatula by tandem affinity purification in hairy root cultures.*** *Plant Journal 2016, 88(3):476-489.*

[1]These authors contributed equally to this work

[2]Author Contributions: Molecular cloning, Y2H assays, systematic Y2H screen, data interpretation and writing (Abstract, TAP for protein complexes with MtCKS1 and MtCAF1, Validation of TAP-MS data for MtCKS1 and MtCAF1 by a systematic Y2H screen for binary interactions, TAP for protein complexes with MtJAZ1, Discussion, Tables 1, 2, S7, S9, S10 and S11).

ABSTRACT

Tandem affinity purification coupled to mass spectrometry (TAP-MS) is one of the most powerful techniques to isolate protein complexes and elucidate protein interaction networks. Here, we describe the development of a TAP-MS strategy for the model legume *Medicago truncatula*, which is widely studied for its ability to produce valuable natural products and to engage in endosymbiotic interactions. As biological material, transgenic hairy roots, generated through *Agrobacterium rhizogenes*-mediated transformation of *M. truncatula* seedlings, were used. As proof of concept, proteins involved in the cell cycle, transcript processing and jasmonate signalling were chosen as bait proteins, resulting in a list of putative interactors, many of which confirm the interologue concept of protein interactions, and which can contribute to biological information about the functioning of these bait proteins in planta. Subsequently, binary protein–protein interactions among baits and preys, and among preys were confirmed by a systematic yeast two-hybrid screen. Together, by establishing a *M. truncatula* TAP-MS platform, we extended the molecular toolbox of this model species.

INTRODUCTION

Protein–protein interactions modulate many, if not all, cellular processes and, therefore, it is a key goal in the post-genomic era to define protein complexes and elucidate the overall network of protein–protein interactions, collectively called the 'interactome' (Morsy *et al.*, 2008; Braun *et al.*, 2013). Yeast two-hybrid (Y2H) and, to a lesser extent, (co-) immunoprecipitation or bimolecular fluorescence complementation (BiFC) screens used to be the methods of choice, but with the advent of high-throughput, ultrasensitive mass spectrometry (MS) and protein sequence databases, techniques based on *in situ* affinity purification (AP) have come into favour, also in plants (Fukao, 2012; Dedecker *et al.*, 2015).

The tandem affinity purification (TAP) method is a powerful AP-MS technique for the systemic identification of protein complexes and protein networks (Wodak *et al.*, 2009; Pflieger *et al.*, 2011; Braun *et al.*, 2013). This technique encompasses the generation of a translational fusion of the protein of interest, also known as the 'bait', to a double-affinity tag; the introduction of this transgenic construct into the host system; and the subsequent execution of a double affinity purification procedure to fish out the natively assembled protein complexes. Afterwards, protein interactors, also known as 'preys', are typically identified using ultrasensitive MS (Li, 2011). Since the development of TAP in *Saccharomyces cerevisiae* (yeast) by (Rigaut *et al.*, 1999), the technique has been implemented in other model systems, i.e. bacteria (Gully *et al.*, 2003), insects (Forler *et al.*, 2003) and mammals (Knuesel *et al.*, 2003), leading to the development of a vast array of different affinity tags and elution conditions (Li, 2010; Xu *et al.*, 2010; Li, 2011).

In plants, the use of TAP-MS has seen a steady rise since its initial use to study protein interactions by transient assays in tobacco (Rohila *et al.*, 2004; Pflieger *et al.*, 2011). It has been successfully implemented in *Arabidopsis thaliana* to study the interactome of, for example, cell cycle proteins, signal transducers, transcription machinery elements and enzymes both in cell suspension cultures (Van Leene *et al.*, 2007; Nelissen *et al.*, 2010; Pauwels *et al.*, 2010; Van Leene *et al.*, 2010; Fernández-Calvo *et al.*, 2011; Bassard *et al.*, 2012; Antoni *et al.*, 2013; Di Rubbo *et al.*, 2013; Sauer *et al.*, 2013) and plants (Rubio *et al.*, 2005; Xing & Chen, 2006; De Lucia *et al.*, 2008). Likewise, TAP was used in *Oryza sativa* (rice) in cell cultures (Abe *et al.*, 2008; Nallamilli *et al.*, 2013) and stably transformed plants (Rohila *et al.*, 2006; Dong *et al.*, 2013).

Medicago truncatula is a well-established model legume that is of particular interest for the study of endosymbiotic interactions and specialized metabolite production (Colditz & Braun, 2010; Oldroyd, 2013; Gholami *et al.*, 2014). Legumes (Fabaceae) are distinctive in their ability to enter into an intricate symbiosis with rhizobial bacteria that fix atmospheric nitrogen for the plant, in exchange for a protective niche and a source of fixed carbon (Oldroyd, 2013). Also the more widespread endosymbiotic association with arbuscular mycorrhizal fungi, by which many land plants acquire mineral nutrients among which phosphate, has been extensively studied in *M. truncatula* (Maillet *et al.*, 2011; Gutjahr & Parniske, 2013). Furthermore, *M. truncatula* has been widely studied for the production of two classes of valuable specialized metabolites, i.e. triterpene saponins and flavonoids (Pollier *et al.*, 2013; Gholami *et al.*, 2014), playing a vital role for the plant but also having many potential uses for humans (Augustin *et al.*, 2011; Osbourn *et al.*, 2011). The regulation of these important biological processes involves dynamic protein networks and signalling pathways, for which our knowledge remains relatively limited (De Geyter *et al.*, 2012; Oldroyd, 2013).

Here, we developed a TAP-MS strategy to study the protein interactome of *M. truncatula* in transgenic hairy roots, which combine the benefits of *in vitro* cultured and whole-plant cultivated tissues, i.e. easy access to an unlimited supply of biomass and sufficient differentiation to allow the study of relevant processes such as specialized metabolism and plant–microbe interactions (Boisson-Dernier *et al.*, 2001; Georgiev *et al.*, 2012; Sharma *et al.*, 2013).

RESULTS

Bait selection

As proof of concept, three bait proteins, belonging to known conserved multimeric protein complexes in other plants or eukaryotes, were selected. The first bait was *Medtr4g057040*, encoding a protein that has the highest amino acid sequence similarity to CYCLIN-DEPENDENT KINASE (CDK) SUBUNIT1 (CKS1) from Arabidopsis (Fig. S27), a central component in cell cycle control and known to be a docking factor for regulators of CDK activity (Jacqmard *et al.*, 1999; Boudolf *et al.*, 2001; De Veylder *et al.*, 2001). A list of interactors of the Arabidopsis CKS1 has been identified by performing TAP-MS experiments on Arabidopsis cell suspension cultures (Fig. S28; Van Leene *et al.*, 2007), and several of these proteins have been proven to be direct interactors of CKS1 (Fig. S28; Boruc *et al.*, 2010b; Van Leene *et al.*, 2010).

The second bait was an *M. truncatula* homologue of CARBON CATABOLITE REPRESSOR (CCR4) ASSOCIATED FACTOR 1 (CAF1), encoded by *Medtr4g006800* (Fig. S29). CAF1 is an mRNA deadenylase and one of the nine core subunits of the CCR4-NEGATIVE ON TATA (NOT) complex that is highly conserved across the eukaryotic kingdom (Collart, 2003; Basquin *et al.*, 2012; Xu *et al.*, 2014). Initially, this megadalton complex has been discovered and characterized in yeast. It is known to be involved in many processes, ranging from transcription and mRNA degradation to protein modification and ubiquitination (Collart & Panasenko, 2012; Villanyi & Collart, 2015). For this multifunctional complex, TAP-MS experiments have been performed in yeast and human cell lines, yielding numerous interactors, only some of which were shown to be directly interacting with CAF1 (Gavin *et al.*, 2002; Gavin *et al.*, 2006; Krogan *et al.*, 2006; Lenssen *et al.*, 2007; Azzouz *et al.*, 2009; Lau *et al.*, 2009; Mauxion *et al.*, 2013). In plants, the Arabidopsis, rice and pepper (*Capsicum annuum*) orthologues of *CAF1* have been studied to some extent (Sarowar *et al.*, 2007; Liang *et al.*, 2009; Walley *et al.*, 2010; Chou *et al.*, 2014), but neither the study of the other components of the CAF1 complex nor the identification of CAF1 interactors has been carried out.

The third bait, a *M. truncatula* homologue of JASMONATE ZIM-DOMAIN 1 (JAZ1), encoded by *Medtr2g042900* (Fig. S30), belongs to the family of JAZ proteins that are key components of the jasmonate (JA) signalling cascade that is very conserved throughout the plant kingdom (Browse, 2009; Pauwels & Goossens, 2011). These transcriptional repressor proteins are degraded in the presence of the JA-Ile conjugate, thereby relieving the steady-state repression of JA-mediated responses (Chini *et al.*, 2007; Thines *et al.*, 2007). A variety of protein–protein interaction techniques, such as TAP, Y2H, BiFC and pull-down, have been

used to show that JAZ proteins interact with many different proteins in their role as JA signalling hubs (Pauwels & Goossens, 2011).

The *Medicago truncatula* TAP strategy

An overview of the *M. truncatula* TAP strategy to study protein complexes from *M. truncatula* hairy roots ectopically expressing TAP fusions is given in Fig. 1. For the efficient generation of the TAP translational fusions, Gateway®-compatible vectors were used for both N- and C-terminal tagging (Fig. 1A; Karimi *et al.*, 2002; Van Leene *et al.*, 2008). Moreover, it was opted to bring the fused proteins under control of the strong constitutive *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter, because they need to compete with their endogenous counterparts for incorporation into protein complexes. The use of the *CaMV 35S* promoter has already been tested and found to be successful for TAP in Arabidopsis (Van Leene *et al.*, 2007). The affinity tag used here is the GS-tag, which consists of the double immunoglobulin G (IgG) binding domain of protein G from Streptococcus sp. and the streptavidin-binding peptide (SBP), separated by two tobacco etch virus (TEV) cleavage sites (Bürckstümmer *et al.*, 2006). This tag has been tested in multiple model systems and was found to be superior to the original yeast TAP-tag, consisting of two IgG binding units of *Staphylococcus aureus* protein A and the calmodulin-binding peptide, in terms of both specificity and complex yield (Bürckstümmer *et al.*, 2006; Kyriakakis *et al.*, 2008; Van Leene *et al.*, 2008).

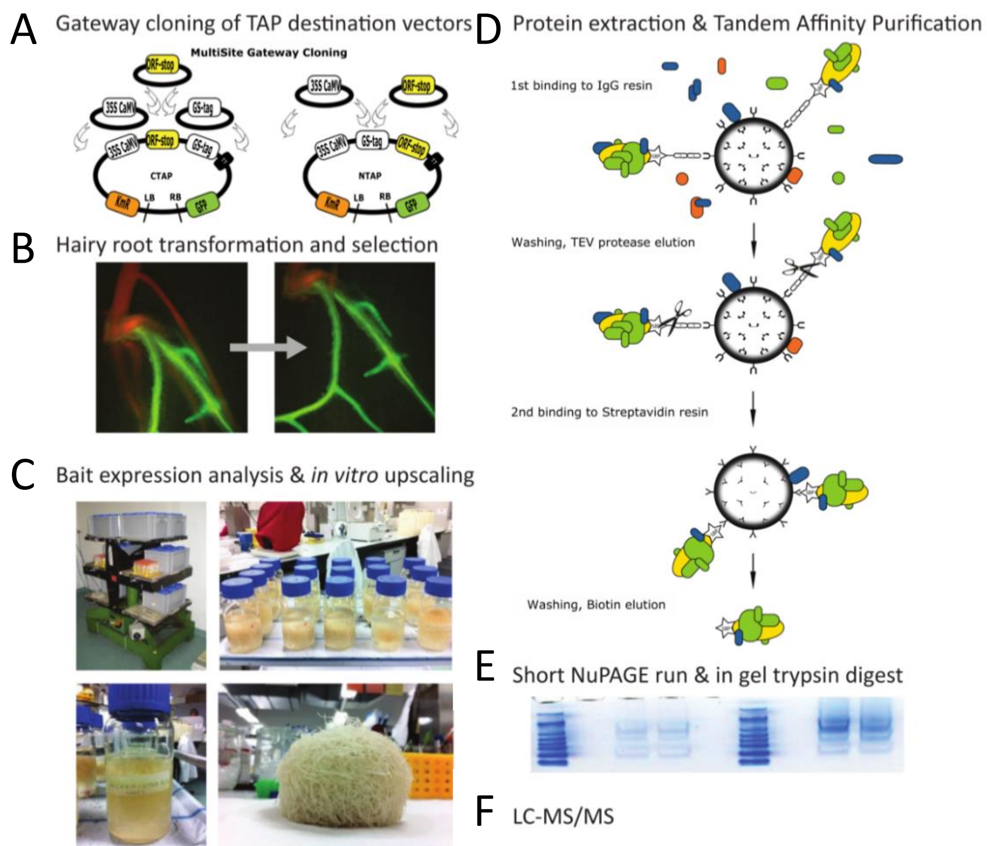

Figure 1: Overview of the general *Medicago truncatula* TAP-MS workflow. A) Schematic representation of the multisite Gateway cloning strategy to fuse the GS-tag to the C- or N-terminus of the bait protein. To obtain the C-terminal fusion, a three-fragment recombination reaction is performed, whereas the N-terminal fusion only requires a two-fragment recombination reaction, because the pKNTAP destination vector already carries the GS-tag. TT, *CaMV 35S* transcription terminator; KmR, kanamycin resistance gene for selection of transformed hairy roots; GFP, *GFP* expression cassette as a visible marker for transformation; LB and RB, left and right border for T-DNA insertion. B) Pictures illustrating the screening of transformed *M. truncatula* hairy roots under a stereomicroscope with a blue-light source and a GFP Plus filter set. During the screening, roots that do not show *GFP* expression are removed to force the production of co-transformed *GFP*-expressing roots. C) After verification of bait expression at the transcript and protein level in individual roots, roots expressing the baits were further propagated in an optimized *in vitro* upscaling system. D) Schematic representation of the *M. truncatula* TAP purification procedure as modified from (Van Leene *et al.*, 2008). Protein extracts are incubated with IgG resin, and native protein complexes assembled around the transgenic bait are captured through binding with the IgG binding domain in the first affinity step. The GS-tagged proteins are washed and eluted by specific cleavage by adding TEV protease (scissors). The IgG eluates are incubated with streptavidin resin that binds the remaining affinity handle, i.e. the streptavidin-binding peptide (SBP). The captured protein complexes are washed to get rid of residual TEV protease and contaminating proteins, and finally eluted through competitive binding with biotin. E) Contaminants are removed on a NuPAGE gel and the fraction for liquid chromatography is determined, based on the intensity of the Coomassie-stained protein bands. Finally, an in-gel tryptic digestion is carried out. F) Peptides derived from the sliced gel plugs are separated on liquid chromatography and identified by LTQ-Orbitrap ultrasensitive mass spectrometry.

The generation of transgenic hairy root cultures and bait expression analysis (Fig 1.B-C) are explained in the following sections. Typically, for each independent transformant, 15 g of crushed hairy root material was split up to prepare two protein extracts, each containing about 25 mg of total protein, thus representing two technical repeats (Van Leene *et al.*, 2015). The actual TAP procedure (Fig. 1D) has been modified from (Van Leene *et al.*, 2008) and is explained in more detail in the Experimental Procedures section. Purified samples were loaded onto precast 4–12% gradient NuPAGE gels (Fig. 1E), followed by a short electrophoresis run and staining with Coomassie Brilliant Blue G-250. Broad zones, containing all eluted proteins per TAP purification, were cut out and in-gel digested with trypsin. Resulting peptides were separated on a nano liquid chromatography (LC) system and analysed on an LTQ Orbitrap™ Velos mass spectrometer (Fig. 1F). The mass spectra were examined using the mascot search engine against the Medicago Mt4plus protein database, containing all entries from the improved Mt4 *M. truncatula* genome release (Tang *et al.*, 2014), i.e. Mt4RC1_ProteinSeq_20130326_1624, concatenated with sequences of all types of possible contaminants in TAP or proteomics experiments in general. These include the sequences of the common repository of adventitious proteins (cRAPs), a list of proteins frequently identified in proteomics studies, present either by accident or by inevitable contamination of protein samples (The Global Proteome Machine, www.thegpm.org/crap/). Additionally, frequently used tag sequences and classical TAP contaminants, such as sequences derived from the resins or the proteases used, were added. The Medicago

Mt4plus database contains 85 446 sequence entries, and is accessible at www.psb.ugent.be/tapdata.

Expression of TAP-tagged proteins in hairy root cultures

Transgenic hairy roots generated through *Agrobacterium rhizogenes*-mediated transformation of *M. truncatula* seedlings were used to produce the tagged proteins, because these can be generated relatively fast, give an unlimited supply of biomass and are ideal tissue to study endosymbiosis and specialized metabolite production (Boisson-Dernier *et al.*, 2001; Floß *et al.*, 2008; Floss *et al.*, 2008; Pollier *et al.*, 2011; Pollier *et al.*, 2013). Two-day-old wild-type *M. truncatula* seedlings were infected with *A. rhizogenes* strains harbouring binary vectors containing the chimeric TAP constructs. For the following 4 weeks, non-transformed roots were removed from the infected plants to force them to produce transgenic roots (Fig. 1B). This selection was based on *green fluorescent protein* (*GFP*) expression, as the TAP destination vectors carry a *prolD:GFP* expression cassette as a visible marker for transformation (Fig. 1A). Several independently transformed hairy root lines were selected per bait and cut from the composite plantlets to be further propagated *in vitro* on medium supplemented with sucrose. The expression levels of the tagged baits were analysed at the transcript level using quantitative real-time polymerase chain reaction (qRT-PCR) and compared with the endogenous transcript levels (Fig. 2A). As expected, some variation could be observed between lines resulting from positional effects of the T-DNA insertion into the genomic region of the infected cells, but each line displayed strong transgene expression. Protein extracts of selected lines were analysed by immunoblotting to verify the protein levels of the fused baits (Fig. 2B). Although *MtJAZ1* was highly expressed in all TAP lines (Fig. 2A), no or only low levels of tagged protein could be detected in either configuration, while all other N- and C-terminal fusion proteins were clearly detectable at the expected molecular weight. Ultimately, two selected lines per bait were further propagated *in vitro* to obtain enough input material for TAP experiments. In order to obtain enough biomass in an efficient way, a streamlined *in vitro* upscaling system was established making use of wide mouth bottles with membrane caps, placed on an orbital shaker (130 rpm) in the dark (Fig. 1c). As inoculum, 3-week-old liquid-grown cultures were used in a start volume of 100 mL fresh Murashige and Skoog (MS) medium supplemented with sucrose. Every week, fresh medium was added to double the culture volume and, after 4 weeks of upscaling, the cultures could be harvested for TAP experiments. This cultivation procedure was found to yield roots from which higher concentrations of proteins could be obtained than from those used previously for metabolite profiling studies (Pollier *et al.*, 2011; Pollier *et al.*, 2013).

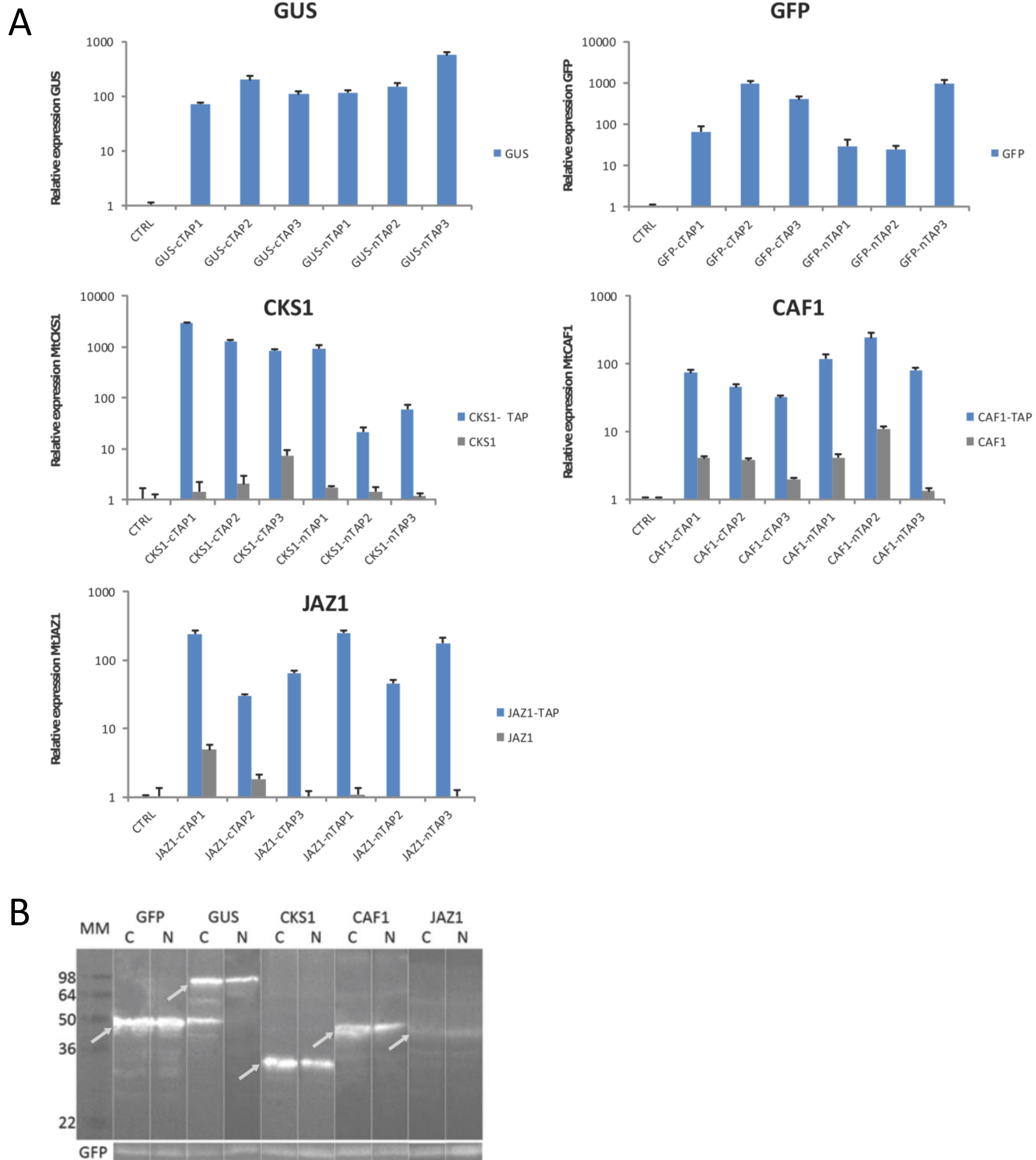

Figure 2: Expression analysis of TAP-tagged constructs. A) Expression analysis of TAP-tagged genes in *Medicago truncatula* hairy roots by means of qRT-PCR. For each construct, three N- and three C-terminal lines were tested and, where possible, transgene transcript levels (in blue) compared with the endogenous transcript levels (shown in grey). The graphs show the values in log scale, normalized to the transcript levels of the control sample (CTRL), which expresses *p35S:GUS* without tag. Error bars, ± SEM (n = 3). B) Analysis of the protein levels of the TAP-tagged constructs expressed in *M. truncatula* hairy roots by means of immunoblotting. The total protein extract of one N- and one C-terminal line of each construct was used, and detection was performed using the PAP antibody against the GS-tag after SDS–PAGE. The molecular weight of the tagged proteins (indicated by grey arrows) is 48.6 kDa for GFP, 89.6 kDa for GUS, 32.2 kDa for MtCKS1, 53.4 kDa for MtCAF1 and 49.1 kDa for MtJAZ1. Molecular marker (MM) values are given in kDa. The same protein samples were also immunoblotted with GFP antibody as loading control. Representative immunoblots are shown for one sample of each construct in each configuration.

Filtering *bona fide* interactors from false-positives and non-specific interacting proteins

Discriminating *bona fide* interactors from background proteins, consisting of false-positives that bind to the resins, and co-purifying non-specific interacting proteins, remains a challenge. To do so, we established a two-step filtering procedure. First, we made a background protein list by performing mock purifications with GFP and β-glucuronidase (GUS). In total, four purifications were carried out per mock bait, corresponding to material from two independent lines each in two technical repeats, which yielded a background list of 170 proteins (Table S5) including abundant proteins, such as ribosomal proteins, cytoskeletal proteins and heat shock proteins. Proteins from this background list were systematically subtracted from the list of proteins that were obtained from purifications of baits of interest. Then, because typical background proteins are highly abundant and conserved among species, and depend on the type of purification used, we took benefit from the available plethora of Arabidopsis GS purifications to further identify the GS background in *M. truncatula* (Van Leene *et al.*, 2015). The orthologous Arabidopsis proteins of all proteins identified in the *M. truncatula* TAP experiments were determined by Plaza 3.0 (Proost *et al.*, 2015), and if the orthologous Arabidopsis protein was part of the GS background in Arabidopsis, also the *M. truncatula* protein was considered as background and removed from the final prey list. Proteins that were excluded in this way are listed in Table S6.

TAP for protein complexes with MtCKS1 and MtCAF1

A total of four purifications were performed for MtCKS1, on material from two independent lines, each in two technical repeats. Proteins that were identified by at least two matched high-confident peptides, kept after the two-step background filtering and co-purified with the bait in at least two independent TAP purifications, are shown in Table 1A. The proteins that were recovered include four CDKs, two D-type cyclins, one A-type cyclin and some other regulators of the cell cycle, such as CDK inhibitor siamese related (SMR)-proteins. Overall, this interactome appeared very similar to that obtained by TAP on Arabidopsis CKS1 (Table 1A and Fig. S28; Van Leene *et al.*, 2007), indicating that the purifications with the GS-tag were successful in purifying relevant cell cycle protein complexes from hairy root cultures of *M. truncatula*. Additionally, hitherto unreported potential interactors were recovered in the MtCKS1 TAP, which would be novel and/or specific for *M. truncatula*.

Table 1: List of identified protein interactors for MtCKS1 (A) and MtJAZ1 (B)

A. MtCKS1				
ID[a]	**Annotation/Description**	**Mtr TAP N-GS (# ID/4)[b]**	**Ath TAP C-GS[c]**	**Y2H[d]**
Medtr4g057040	CKS1	4	Bait	n/a
Medtr5g032550	CYCD2	4	y	n
Medtr2g032060	CDKA;1	4	y	y
Medtr4g094430	CDKA;2	4	y	y
Medtr1g075610	CDKB2	4	y	y
Medtr0002s1060	Narbonin	4	n	n/a
Medtr7g087250	GrpE-like protein	4	n	n
Medtr1g018680	CYCA3	3	n	n
Medtr1g090837	SMR6C	2	y	y
Medtr5g033220	SMR6B	2	y	y
Medtr8g069510	SMR6A	2	y	y
Medtr4g007750	CDKB1	2	y	y
Medtr5g015670	CYCD5	2	y	Y[e]
Medtr1g041685	Transducin/WD40 domain-like protein	2	y	n
Medtr2g035100	pathogenesis-related class 10 protein	2	n	n
Medtr2g035130	pathogenesis-related class 10 protein	2	n	n/a
Medtr2g035320	pathogenesis-related class 10 protein	2	n	n/a
B. MtJAZ1				
ID[a]	**Annotation**	**Mtr TAP N-GS (# ID/2)[b]**	**Ath TAP C-GS[c]**	**Y2H[f]**
Medtr2g042900	JAZ1	2	Bait	n/a
Medtr6g087140	NINJA	2	y	y

Shown are the proteins that were identified with at least two matched high-confident peptides and co-purified with the bait in at least two independent TAP purifications. The result from the systematic Y2H screen and classic pair-wise Y2H is also provided.

[a] Gene ID and annotations/descriptions are derived from the Mt4 release of the *M. truncatula* genome sequence (Tang *et al.*, 2014) that can be found on the website of JCVI, but manually inspected and curated.

[b] # ID/# denotes the number of times a protein was recovered out of the total amount of purifications that were performed on that particular bait in the *M. truncatula* TAP (Mtr TAP). N-GS denotes that the proteins were identified in the purification of an N-terminally tagged version of the bait.

[c] The table also indicates whether the orthologous interaction was already identified experimentally in other species, i.e. in TAP data from Arabidopsis (Ath TAP with C-terminally tagged version of the bait, C-GS; Van Leene *et al.* (2007) for CKS1;Pauwels *et al.* (2010) for JAZ1). y, yes; n, no.

[d] Indicates whether interaction could be confirmed by systematic Y2H (see Table S7 for the OD_{600} values). y, yes; n, no; n/a, not assessed.

[e] Indirect interaction. Interaction is detected with another bait-interacting prey.

[f] Indicates whether interaction could be confirmed by a classical pair-wise Y2H.

For MtCAF1, purifications were carried out on two independent lines of both the N- and C-TAP constructs, each in two technical repeats. Seven putative orthologues of CCR4/NOT subunits known in other species were found (Tables 2 and S8). First, two orthologues of human NOT6 or yeast CCR4 were identified (Fig. S34), proteins known to directly bind CAF1 and together forming the nuclease core of the CCR4-NOT complex (Collart, 2003; Basquin *et al.*, 2012; Mauxion *et al.*, 2013). Second, the orthologue of NOT1 was found (Fig. S35), which is the very large scaffold protein that holds the CCR4-NOT complex together (Basquin *et al.*, 2012). Third, the orthologue of NOT10 was found, which is a core component of the CCR4-

NOT complex in human (Mauxion *et al.*, 2013) and Drosophila (Bawankar *et al.*, 2013). In both species, it was shown that NOT10 by itself cannot bind NOT1, but together with NOT11 (C2ORF29) it forms a module that binds the N-terminal part of NOT1. The NOT11 orthologue was also present in two MtCAF1 TAP experiments, though with only one significant but unique peptide sequence per identification (Table 2). Further, orthologues of NOT9 (RCD1) and NOT3 were found in one MtCAF1 TAP experiment only, the latter only with one significant but unique peptide. Hence, *M. truncatula* TAP allowed linking MtCAF1 to homologous known elements of eukaryotic CCR4-NOT complexes. Moreover, this confirms the genericity of the CCR4-NOT complex among eukaryotes, including plants. Furthermore, 19 additional proteins were identified and retained after subtraction of the background proteins (Table S9). The CCR4-NOT complex has been reported to play a multifunctional role in important general processes conserved in eukaryotes, such as ubiquitination, transcription, translation and protein modification (Collart & Panasenko, 2012). The extensive list of putative MtCAF1-associated proteins suggests that also in *M. truncatula* this protein might play such a multifunctional role, potentially with plant-specific aspects.

Table 2. List of identified protein interactors for MtCAF1

		Mtr		**Sce**	**Hsa**	**Y2H[d]**
ID[a]	**Annotation[1]**	**C-GS**	**N-GS**			
Medtr4g006800	CAF1	4	4	bait	bait	n
Medtr1g054535	CCR4A/NOT6	4	4	y	y	y
Medtr7g107240	CCR4B/NOT6L	4	4	n/a	y	y
Medtr8g005820[e]	NOT1	1	2	y	y	y
Medtr4g115350[e]	NOT10	0	2	n/a	y	n
Medtr3g053170[e]	NOT9/RCD1	0	1	y	y	n
Medtr1g041405[e]	NOT11	0	2[f]	n/a	y	n
Medtr7g085350[e]	NOT3	0	1[f]	n	y	y[g]

Only known CCR4/NOT subunits, as described in yeast or human (Table S8), which were identified with at least two matched high-confident peptides and co-purified with the bait in at least two independent TAP purifications, are shown, with the exception of NOT3, NOT9 and NOT11 that were identified in only one experiment and/or with only one unique peptide. The list of other identified proteins can be found in Table S9. The result from the systematic Y2H screen is also provided.

[a] Gene ID and annotations are derived from the Mt4 release of the *M. truncatula* genome sequence (Tang *et al.*, 2014) that can be found on the website of JCVI. CCR4/NOT subunits are named according to their orthologues in yeast or human.

[b] # ID/# denotes the number of times a protein was recovered out of the total amount of purifications that were performed with MtCAF1 in the *M. truncatula* TAP (Mtr TAP). C-GS and N-GS denote whether the protein was identified in the purification of a C- or N-terminally tagged version of the bait, respectively.

[c] The table also indicates whether the orthologous interaction was already identified experimentally in other species, i.e. in *S. cerevisiae* (Sce TAP; Gavin *et al.*, 2006) and *H. sapiens* (Hsa TAP; Mauxion *et al.*, 2013). y, yes; n, no; n/a, not applicable. For a complete list of known CCR4/NOT subunits purified with CAF1 TAP in yeast or human, and nomenclature in the different species, see Table S8.

[d] Indicates whether interaction could be confirmed by the systematic Y2H screen (see Table S10 for the OD_{600} values). y, yes; n, no.

[e] Orthologues in Arabidopsis are in the Arabidopsis GS background list, but not filtered out here, because they are known subunits of the yeast and/or human CCR4-NOT complex.

[f] Identified with only one significant, but unique peptide.

[g] Indirect interaction. Interaction is detected with another bait-interacting prey.

In summary, expected as well as potentially novel interacting proteins were recovered in both the MtCKS1 and MtCAF1 TAPs, indicating that our platform allows identifying *bona fide* interactors and protein complexes with *M. truncatula* bait proteins.

Validation of TAP-MS data for MtCKS1 and MtCAF1 by a systematic Y2H screen for binary interactions

Commonly, TAP results are validated using complementary methods such as Y2H or BiFC, which allows verifying the binary protein–protein interactions in the isolated complex. Here, we implemented a systematic Y2H screen not only assessing interactions between the bait and the majority of the preys, but also among the preys. This semi-automated mating-based screen was performed as described before (Boruc *et al.*, 2010a). Genes encoding fusions with activating and binding domains were cloned for all tested *M. truncatula* proteins. However, the fusion constructs of CKS1, NOT9 and NOT11 with the GAL4 binding domain resulted in auto-activation and were therefore excluded from the assay. Likewise, results with CCR4B fused to the GAL4 binding domain were considered unreliable because all tested interactions, including the negative control, gave high growth ratios (Table S10). Growth of mated yeast was measured in OD_{600} and interaction was considered positive if the ratio of growth on selective medium to that on control medium exceeded 60%, which was the minimal ratio for our positive controls (Table S11).

For CKS1, we observed direct interaction with seven of the 12 preys tested, i.e. with CDKA;1, CDKA;2, CDKB2, SMR6C, SMR6B, SMR6A and CDKB1 (Tables 1A and S7). Two of these, SMR6B and CDKB1, could also interact with another prey, CYCD5, pointing to indirect interaction between CKS1 and CYCD5. Remarkably, all of the CDKs but none of the cyclins directly interacted with CKS1 in our Y2H. Analogous results were obtained in a Y2H to characterize the Arabidopsis core cell cycle binary protein–protein interaction network, in which Arabidopsis CKS1 and CKS2 interacted with most CDKs but rarely with cyclins (Fig. S28; Boruc *et al.*, 2010a; Van Leene *et al.*, 2011).

For CAF1, we were only able to confirm direct interaction with the known core subunits of the CCR4-NOT complex, CCR4A, CCR4B and NOT1 (Tables 2 and S10). Notably, plant CCR4 proteins do not contain the leucine-rich repeat domain (LRR) that is responsible for interaction with CAF1 in other eukaryotes (Winkler & Balacco, 2013), indicating that direct interaction between *M. truncatula* CCR4 and CAF1 proteins occurs in an LRR-independent manner. Furthermore, a potential indirect interactor of CAF1 is NOT3, which interacted with CCR4B. A possible reason for the low detection of (in)direct interactors of CAF1 is the necessity of the subunits of the CCR4-NOT complex to be assembled, hence interaction would only occur when all or at least some subunits are present. Accordingly, for yeast, CAF1

is required for the interaction between CCR4 and other subunits of the complex (Bai *et al.*, 1999; Chen *et al.*, 2001).

TAP for protein complexes with MtJAZ1

In contrast to MtCKS1 and MtCAF1, tagged MtJAZ1 protein could not be readily detected, in either configuration, by immunoblot analysis in protein extracts from transformed *M. truncatula* hairy roots (Fig. 2B). Therefore, for MtJAZ1, instead of the usual 25 mg of total protein input, 200 mg of total protein input was used to increase the chance of identifying interactors. For one line, this resulted in the successful purification of one interactor in two technical repeats, i.e. the *M. truncatula* homologue of NINJA (Fig. S36 and Table 1B). The low number of identified interactors was expected, as bait proteins could hardly be detected by immunoblotting. In Arabidopsis, NINJA is known to associate directly with JAZ1 and other proteins to recruit the transcriptional repressor TOPLESS to repress JA-mediated gene expression (Pauwels *et al.*, 2010). Accordingly, we confirmed direct interaction between MtJAZ1 and MtNINJA by Y2H (Fig. 3).

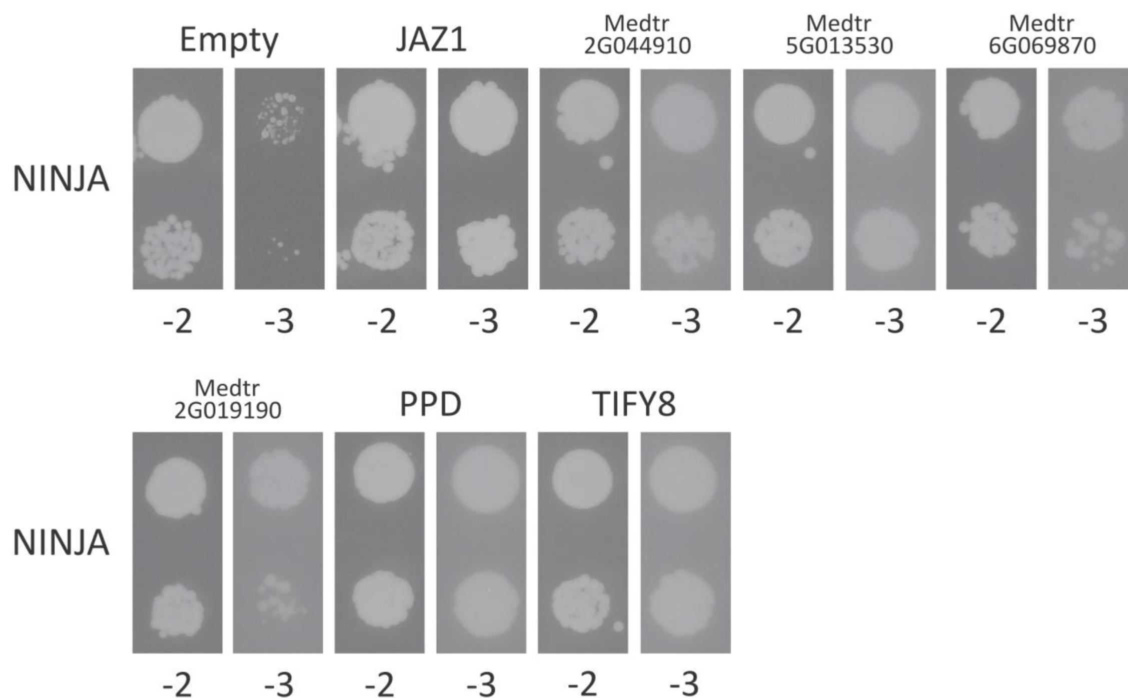

Figure 3: Verification of interaction between MtJAZ1 and MtNINJA by Y2H. Other TIFY proteins were also tested for interaction with MtNINJA. MtNINJA fused to GAL4-BD was tested for interaction with MtJAZ1 and other TIFY proteins fused to GAL4-AD. *M. truncatula* homologues of Arabidopsis TIFY proteins are represented by four JAZ (Medtr2g019190, Medtr2g044910, Medtr5g013530 and Medtr6g069870), one PPD (Medtr1g102900) and one TIFY8 (Medtr3g107950) protein. Transformed yeasts were dropped in 10- and 100-fold dilutions on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium. Empty vectors were used as negative control.

The JAZ proteins belong to the TIFY protein family, which, besides 12 JAZ proteins, also comprises TIFY8 and the two PEAPOD (PPD) proteins in Arabidopsis, all of which can interact with the Arabidopsis NINJA (Pauwels *et al.*, 2010; Pauwels & Goossens, 2011; Cuéllar Pérez *et al.*, 2014). To assess whether MtNINJA displayed similar protein affinities, we first mined the *M. truncatula* genome for homologues of JAZ, PPD and TIFY8. For all TIFY clades existing in Arabidopsis, one or more members could be found in the *M. truncatula* genome, indicating that the family structure is largely conserved between the two species (Fig. S30). A representative set of *M. truncatula* TIFY proteins was cloned, including several MtJAZs, MtPPD and MtTIFY8, and subjected to a 'classic' Y2H analysis. In agreement with the findings in Arabidopsis, all were found to be able to directly interact with MtNINJA (Fig. 3).

DISCUSSION

As a well-established model legume, *M. truncatula* is studied particularly for its ability to engage in endosymbiotic interactions and for its production of valuable, specialized metabolites (Colditz & Braun, 2010; Oldroyd, 2013; Gholami *et al.*, 2014) . These biological processes are often regulated by the dynamic interaction of proteins in complexes and signalling pathways. Here, we describe the establishment of a *M. truncatula* TAP-MS strategy enabling the survey of protein–protein interaction networks from *M. truncatula* hairy roots in an unprecedented way. The procedure is based on a combined approach comprising the relatively fast generation and upscaling of transgenic hairy root cultures, the use of a procedure adapted for high to low expressing baits, protein identification by ultrasensitive tandem MS and data analysis.

To express the TAP-tagged constructs in *M. truncatula* hairy roots, we used an overexpression approach, by far the most commonly used strategy for TAP in plants, and demonstrated in Arabidopsis to lead to superior complex recovery as compared with expression with endogenous promoters in a wild-type background (Van Leene *et al.*, 2007). In our experience from TAP in Arabidopsis seedlings, expression with endogenous promoters only leads to successful complex purification when the TAP construct had been brought into a plant line that contains a knock-out in the bait protein encoding gene (Van Leene *et al.*, 2015). This may be due to the cloned endogenous promotor, which may lose (part of) its activity because of the absence of one or more enhancer sequences, or to a poor competition with the untagged endogenous protein for being taken up in complexes. Nonetheless, constitutively overexpressed proteins may exhibit protein misfolding, mislocalization and/or misregulation on a cellular level, and eventually false-positive interactors that would not be identified after expression under control of endogenous promotor in a mutant background. In this regard, the ease and speed with which transformed *M. truncatula* hairy roots can be generated and upscaled certainly creates

avenues for exploring TAP in mutant backgrounds or with different growth conditions, allowing the survey of the perturbation or re-organization of protein complex assembly and dynamics in different protein environments. Eventually, TAP could also be carried out on transgenic plants rather than hairy roots. However, because generating stably transformed transgenic lines in *M. truncatula* requires a long and laborious process, this would dramatically hamper the throughput of TAP analysis, hence supporting our choice for hairy root cultures as a model. Likewise, in Arabidopsis, TAP is also efficiently being carried out in transformed cell suspension cultures generating relevant novel biological insights that can consistently be confirmed at the whole-plant level (Van Leene *et al.*, 2015).

A challenge associated with TAP, and AP-MS in general, is figuring out a robust way to separate *bona fide* interactions from non-specific background associations (Braun, 2012; Pardo & Choudhary, 2012; Mellacheruvu *et al.*, 2013). Because this study focused on a new species, no large data sets were available for frequency filtering approaches or intensity-based label-free quantitative LC-MS/MS to remove the background. Therefore, a two-step filtering approach was applied, which combines filtering via a *de novo* generated background list of 170 co-purifying proteins from four mock purifications with GUS and GFP, with removal of *M. truncatula* proteins of which the Arabidopsis orthologues are known Arabidopsis GS background proteins. This allowed reducing the size of the lists of potential interaction partners. The remaining candidates were validated by a systematic Y2H screen, leading to the identification of direct interactors matching the described interaction behaviour of their orthologues in other organisms. Nonetheless, because experiments performed in heterologous systems such as yeast lack essential subunits to assemble complexes, our Y2H screen might have overlooked interactions. Hence, preys not validated by Y2H should not all be considered as potential false-positives.

Also reproducibility is a major acceptance criterion in many TAP or AP-MS studies (Pardo & Choudhary, 2012). In general, only interactions that are identified in at least two independent purifications are considered for further study. This selection criterion does, however, not rule out that interactors that were identified only in a single TAP experiment are genuine. For example, in the MtCKS1 purifications, two more D-type cyclins were identified in only one experiment, likely representing genuine, but possibly more transient associations. Hence, biological information about the identified interactors might further contribute to the selection of *bona fide* interactors for follow-up studies. In this regard, it is important to take into account that data obtained from TAP-MS do not necessarily reveal the composition of a single individual protein complex, but rather resemble a snap shot of a mix of discrete protein complexes in which the bait operates. For instance, given what is known about, for example, CAF1 in yeast and humans (Collart & Panasenko, 2012; Mauxion *et al.*, 2013) and the extensive putative interactor list obtained by TAP on MtCAF1

(Tables 2 and S9), this is undoubtedly the case for this protein. Intensity-based label-free quantitative LC-MS/MS analysis might be used in these cases in order to identify genuine interactors of bait proteins in specific conditions (Smaczniak *et al.*, 2012; Keilhauer *et al.*, 2015). This approach could also be applied to further reduce false-positives.

In conclusion, we have established a TAP-MS strategy for purifying protein complexes from hairy root cultures of *M. truncatula*. We identified interactors of all three bait proteins and provide a systematic Y2H screen that can be used to prioritize genuine interactors above potential false-positives.

MATERIALS AND METHODS

Cloning

Open reading frames (ORFs) were amplified (for primer sequences, see Table S12) with the high-fidelity Kapa HiFi PCR kit (Kapa Biosystem, Woburn, MA, USA) from *M. truncatula* (ecotype Jemalong J5) cDNA and recombined in the pDONR221 vector (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Sequence-verified entry clones were used to generate the TAP destination vectors using the standard MultiSite Gateway® cloning technology as described (Van Leene *et al.*, 2007). TAP expression vectors were transferred to *A. rhizogenes* ARqua1 by electroporation.

Hairy root transformation and upscaling

Transformation of *M. truncatula* (ecotype Jemalong J5) hairy roots and maintenance of the hairy roots was carried out as described (Pollier *et al.*, 2011). To stimulate the growth of co-transformed transgenic hairy roots, non-*GFP*-expressing roots were cut from the plants 5 days after the plants were transferred to plates containing 100 mg L^{-1} cefotaxime. For upscaling, 5 mL of liquid MS medium (pH 5.8) supplemented with vitamins (Duchefa) and 1% sucrose (w/v) was inoculated with transgenic hairy roots and placed on an orbital shaker for 3 weeks in the dark at 25°C (130 rpm). After 7 days, 5 mL of fresh MS medium was added and, 14 days after inoculation, another 15 mL of MS medium was added to a final volume of 25 mL. After 21 days, some cultures were harvested in liquid nitrogen and stored at −70°C for bait expression analysis, while the best growing cultures were transferred to 1 L, wide mouth bottles with membrane caps (Duran Group GmbH, Meinz, Germany) containing 100 mL of MS medium. Inoculated bottles were placed on an orbital shaker (130 rpm) in the dark at 25°C. Every week, the volume of the medium was doubled by adding MS medium until a final culturing volume of 800 mL was obtained. One month after inoculation of the bottles, the hairy roots were harvested in liquid nitrogen and stored at −70°C.

qRT-PCR

Total RNA was extracted from hairy roots with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) and cDNA prepared with SuperScript™ II Reverse Transcriptase (Life Technologies). qRT-PCR was carried out with a Lightcycler 480 (Roche Applied Science, Penzberg, Germany) and the Lightcycler

480 SYBR Green I Master kit (Roche). Gene-specific qPCR primers (Table S12) were designed with Beacon Designer version 4.0 (Premier Biosoft International, Palo Alto, CA, USA). As reference genes, *40S ribosomal protein S8* (*40S; Medtr7g053450*) and *translation elongation factor 1*α (*ELF1*α*; Medtr6g021800*) were used. For the relative quantification with multiple reference genes, qBase was used (Hellemans *et al.*, 2007).

Protein extraction

Plant material of 3-week-old (1–2 g) and 7-week-old (15 g), liquid-grown hairy root cultures was ground to homogeneity in liquid nitrogen with mortar and pestle for immunoblot and TAP analysis, respectively. Crude protein extracts were prepared with 15 mL of extraction buffer [25 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM *p*-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% NP-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 µM E64, EDTA-free Ultra Complete tablet Easypack (1/10 mL; Roche Diagnostics, Brussels, Belgium), 5% ethylene glycol] with an Ultra-Turrax T25 mixer (IKA Works, Wilmington, NC, USA) at 4°C. For immunoblot analysis, samples were centrifuged as described in (Dedecker *et al.*, 2016). Protein concentrations were determined by Bradford assay (Bio-rad, Hercules, CA, USA). For TAP, the soluble protein fraction was obtained by a double centrifugation at 36 900 ***g*** for 20 min at 4°C. The extract was passed through a 0.45 µm syringe filter (Alltech, Deerfield, IL, USA) or through four layers of Miracloth (Merck KGaA, Darmstadt, Germany) and kept on ice.

Immunoblot analysis

Total protein extract (30 µg) was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 0.75 mm 12% Mini-PROTEAN® TGX™ precast gels; Bio-Rad), and immunoblot analysis was executed as described in (Dedecker *et al.*, 2016). Bound antibody was detected using detection substrate (Western Lightning Plus-ECL; Perkin Elmer, Waltham, MA, USA) and X-ray films (Amersham hyperfilm™ ECL; GE Healthcare, Wauwatosa, WI, USA).

TAP

Purifications were performed as described by (Van Leene *et al.*, 2008) with modifications. Briefly, 25 mg of total protein extract was incubated for 1 h at 4°C under gentle rotation with 25 µL IgG Sepharose 6 Fast Flow beads (GE Healthcare), pre-equilibrated with 3 × 250 µL extraction buffer. The IgG Sepharose beads were transferred to a classic mobicol column with a 35 µm pore size (MoBiTec GmbH, Göttingen, Germany) and washed once with 100 column volumes or 2.5 mL IgG wash buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% NP-40, 1 µM E64, 1 mM PMSF, 5% ethylene glycol), and washed again with 50 column volumes or 1.25 mL tobacco (*Nicotiana tabacum* L.) etch virus (TEV) buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 µM E64, 1 mM PMSF, 5% ethylene glycol). Bound complexes were eluted in an Eppendorf in 100 µL TEV buffer via AcTEV (Life Technologies) digest (2 × 20 Units, second boost after 30 min) for 1 h at 16°C. Eluate was collected by passing it on a classic mobicol column with a 35 µm pore size (MoBiTec GmbH) through centrifuging for 1 min at 239 *g* at 4°C and beads were washed with 100 µL TEV buffer, and this wash step was collected together with the eluate. This eluate was incubated for 1 h at 4°C under gentle rotation

with 25 µL Streptavidin Sepharose High-Performance beads (GE Healthcare), pre-equilibrated with 3 × 250 µL TEV buffer. Streptavidin beads were transferred to a classic mobicol column with a 35 µm pore size (MoBiTec GmbH) and washed with 100 column volumes or 2.5 mL TEV buffer. Bound complexes were eluted in 40 µL NuPAGE sample buffer containing 20 mM desthiobiotin (Sigma-Aldrich, Saint-Louis, MO, USA) by 5 min incubation on ice, followed by a centrifugation at 239 *g* at 4°C. Purified proteins were migrated for 7 min on 4–12% gradient NuPAGE Bis-Tris gels (Life Technologies), fixed in 50% EtOH/2% H_3PO_4 and visualized with colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Saint-Louis, MO, USA).

Proteolysis and peptide isolation

Proteolysis and peptide isolation were basically executed as described in (Dedecker *et al.*, 2016). NuPAGE gels with the protein samples were de-stained twice in HPLC-grade water (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h and transferred to reducing buffer (6.66 mM DTT, 50 mM NH_4HCO_3 in HPLC-grade water) for 40 min. Next, the thiol groups were alkylated by incubating the gel lanes for 30 min in alkylating buffer (55 mM iodoacetamide, 50 mM NH_4HCO_3 in HPLC-grade water) before they were washed with HPLC-grade water. Broad zones containing all eluted proteins per TAP sample were cut out, sliced into 16 smaller gel plugs, but kept together and processed as one sample. The gel pads were dehydrated in 95% acetonitrile for 10 min, rehydrated with HPLC-grade water and dehydrated again in 95% acetonitrile for 10 min. Dehydrated gel plugs were rehydrated in 90 µL of trypsin digest buffer [12.5 µg mL^{-1} trypsin (MS gold; Promega, Madison, WI, USA) in 50 mMNH_4HCO_3:10% CH_3CN (v/v) in HPLC grade water] for 30 min at 4°C. Next, gel plugs were incubated for 3.5 h at 37°C for in-gel digestion with Trypsin. Peptide samples were sonicated for 5 min in a sonication bath and the upper fraction was kept aside. Remaining gel plugs were dehydrated in 95% acetonitrile for 10 min, and this solution was added to the first fraction of the trypsin digests that were then completely dried in a SpeedVac for 2–3 h at 4°C.

LC-MS/MS

The obtained peptide mixtures were analysed by an LC-MS/MS system, the Ultimate 3000 RSLC nano (Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) as described by (Van Damme *et al.*, 2014). Peptides were solubilized in 15 µL Solvent A (0.1% trifluoroacetic acid, 2% acetonitrile), and 5 µL was loaded on a trapping column [made in-house, 100 µm internal diameter (I.D.) × 20 mm (length), 5 µm C18 Reprosil-HD beads; Dr Maisch GmbH, Ammerbuch-Entringen, Germany]. After back-flushing from the trapping column, the sample was loaded on a reverse-phase column (made in-house, 75 µm I.D. × 150 mm, 5 µm C18 Reprosil-HD beads; Dr Maisch). Peptides were loaded with solvent A (0.1% trifluoroacetic acid, 2% acetonitrile), and separated with a 20 min linear gradient from 2% solvent A′ (0.1% formic acid) to 50% solvent B′ (0.1% formic acid and 80% acetonitrile) at a flow rate of 300 nL min^{-1}, followed by a wash step reaching 100% solvent B′.

The LTQ Orbitrap™ Velos was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition for the 10 most abundant peaks in a given MS spectrum. Full-scan MS spectra

were acquired in the Orbitrap™ at a target value of 1E6 with a resolution of 60 000. The 10 most intense ions were isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 20 sec. Target value for filling the ion trap was set to 1E4 ion counts.

Protein identification

From the MS/MS data in each LC run, Mascot Generic files were created with Mascot Distiller (version 2.4.1, Matrix Science, www.matrixscience.com) and, if possible, a maximum intermediate scan count of five was allowed and grouping of spectra with a maximum intermediate retention time of 30 sec and a 0.005 Da precursor tolerance. No de-isotoping was used and a relative signal-to-noise limit of two. A peak list was generated only when the MS/MS spectrum contained more than 10 peaks. Mascot Daemon interface was used to search the peak lists with the Mascot search engine (version 2.4.1, MatrixScience, www.matrixscience.com) against the Medicago Mt4plus protein database, constructed from the Mt4 release of the *M. truncatulab*genome sequence (Tang *et al.*, 2014) and extended with cRAP protein sequences (The Global Proteome Machine, www.thegpm.org/crap/) and other non-*M. truncatula* protein sequences commonly found in TAP purifications. The next steps were performed as described by (Vercruyssen *et al.*, 2014). Variable modifications were set to methionine oxidation and methylation of aspartic acid and glutamic acid. Fixed modifications were set to carbamidomethylation of cysteines. Mass tolerance on MS was set to 10 ppm (with Mascot's C13 option set to 1) and the MS/MS tolerance at 0.5 Da. The peptide charge was set to 2+ and 3+, and the instrument setting was set to ESI-TRAP. Trypsin was set as the protease used, allowing for one missed cleavage, and also cleavage was allowed when arginine or lysine is followed by proline. Only high-confident peptides, ranked one and with scores above the threshold score, set at 99% confidence, were withheld. Only proteins with at least two matched high-confident peptides were retained. Next, background proteins were subtracted in two subsequent steps. In the first step, all identifications that were present in the *M. truncatula* background list, compiled from four mock TAP experiments with GUS and GFP, were removed. In the second step, all identifications of which the Arabidopsis orthologue was present in the Arabidopsis GS background list were removed.

Y2H

The mating-based systematic Y2H screen was performed as described (Boruc *et al.*, 2010a). Classic Y2H to test interaction between JAZ and NINJA was carried out as described (Cuéllar Pérez *et al.*, 2013).

Phylogenetic analysis

Protein sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and aligned with ClustalW. The Neighbour-Joining method with bootstrapping with 10 000 replicates was executed with the MEGA5 software (Tamura *et al.*, 2011). The evolutionary distances were analysed with the Poisson correction method. The positions containing gaps and missing data were eliminated from the data set (complete deletion option).

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Discussion and perspectives

Characterization of the interactome of JA-related TF complexes

The quest to characterize JA-related TF complexes was initiated by the visualisation of the network of protein-protein interactions that occur in these complexes. For this purpose, throughout the course of this research, two protein interaction techniques were combined, TAP-MS and Y2H. TAP-MS was performed as genome-wide analysis to identify interactors of a particular bait. The advantage here is the capability of TAP-MS to detect indirect protein interactions, which allows characterization of larger protein complexes, e.g. TF complexes. The endogenous environment of the bait proteins was approximated by the use of Arabidopsis cell cultures leading to the presence of endogenous proteins, cofactors and/or post-translational modifications that might be necessary to ensure the formation of bait protein complexes. As data obtained from TAP-MS represent a snap shot of the complete interaction behaviour of the bait, poor interactors might not be detected. However, recent improvement of the TAP tag combined with ultrasensitive MS definitely has increased the sensitivity of the technique (Van Leene *et al.*, 2015).

Problems associated with TAP experiments often are addressed to tag placement. The presence of GS^{rhino} tag can, for instance, interfere with protein structure and localisation and have detrimental effects on protein complex assembly. In chapter II.1, protein expression of N-terminal fusion constructs of the baits could not be observed. Normally, N-terminal fusion constructs are expected to lead to better protein translation because of the efficient translation initiation sites in the tag (Costa *et al.*, 2014). However, gene expression of the baits should be analysed to exclude the possibility that the N-terminal fusion constructs were inefficiently transcribed. A possible reason for decreased stability of tagged proteins can be that particular domains are revealed due to structural changes caused by the tag. For instance, the N-terminal part of MYC2 contains a destruction element in its activation domain that might be more exposed due to structural changes leading to decreased stability of MYC2 (Zhai *et al.*, 2013).

The baits were brought under control of the strong *CaMV* 35S promoter. Overexpression leads to better sensitivity but it can also lead to the purification of uncomplexed bait proteins, resulting in lack of detection of co-purifying interactors or to the detection of aspecific proteins. Better specificity of the results can be achieved by expressing the bait under control of its endogenous promoter, however, this should be done in a mutant background containing a knock-out in the endogenous bait gene. The Arabidopsis cell cultures used for TAP experiments are available in the lab and preparing new, mutated, cell

cultures would have required a lot of time. Nevertheless, protein expression of the baits was very moderate or low, hence, overexpression of the baits is most likely not the problem for the low detection of interactors.

Low solubility of chromatin-bound proteins might interfere with the purification of their interactors. As all baits in chapter II.1 are TFs, improving the solubility of chromatin-associated protein complexes might improve the results of the TAP analyses. This can be done by the preparation of a nuclear extract and re-solubilisation of chromatin-associated proteins, however, this approach does not allow to identify interactors of the soluble portion of the bait proteins in the same TAP experiment and adds multiple steps to the protocol. Therefore, nuclease treatment and/or sonication to fragment the chromatin would increase the solubility of the chromatin-bound proteins without affecting the detection of the interactors of the soluble portion of the bait proteins and is compatible with the TAP protocol (Lambert *et al.*, 2014).

To unravel the composition of the identified protein complexes by TAP-MS, we applied Y2H analysis. As it only detects binary protein-protein interactions, the mutual relationships among the proteins of the complex can be clarified. Furthermore, Y2H also serves as a validation method for the identified interactors of the TAP-MS analysis. Genome-wide TAP-MS analysis and binary Y2H have been proven to be a good combo, e.g. for resolution of the cell cycle interaction network (Boruc *et al.*, 2010; Van Leene *et al.*, 2010) and for characterization of the repression mechanism of JA-related TF complexes (Pauwels *et al.*, 2010; Van Leene *et al.*, 2011). The Y2H method has a good rating for accuracy (related to false positives), however, as a consequence the sensitivity (related to false negatives) is quite low (Braun *et al.*, 2009). Therefore, it should be noted that a negative result during Y2H does not imply a negative interaction in plants. Other binary interaction assays could also have been used as validation technique, such as luminescence-based mammalian interactome mapping (LUMIER), nucleic acid programmable protein array (NAPPA) or split yellow fluorescent protein. Nevertheless, all these techniques have similar accuracy and sensitivity scores to the Y2H system (Braun *et al.*, 2009; Braun *et al.*, 2013).

As Y2H is performed in a heterologous system, the absence of an endogenous environment can lead to omission of true interactors. Furthermore, also in this technique, tag placement can lead to abrogation of interactions in yeast. Therefore, it is advised to check both possible combinations of tags for the tested proteins. However, auto-activation of many TFs fused to the GAL4 DNA binding domain frequently occurs. A split-ubiquitin Y2H system that does not rely on interaction-induced transcription of the reporter gene, can be an appropriate alternative to verify interactions with TFs (Deslandes *et al.*, 2003). Nevertheless, Y2H is a quick and easy-to-handle method, which allows clear evaluation of the occurrence or lack of

binary protein-protein interactions, and furthermore, enables the characterization of interaction domains. Therefore, Y2H suits the scope of our research, i.e. to acquire knowledge about the regulation of TF complexes by changing involved protein-protein interactions via site-directed mutagenesis.

Generation of hyperactive MYC2 constructs

In chapters II.2 and II.3, two hyperactive constructs of MYC2 were generated by change of a conserved amino acid in the JID ($MYC2^{D105N}$; Goossens *et al.* (2015)) and in the TAD ($MYC2^{E165K}$; Gasperini *et al.* (2015)). These constructs both lost interaction with almost all JAZ proteins resulting in increased transcriptional activity and enhanced downstream responses. It was remarkable to see that a change in the TAD could also mediate loss of JAZ interaction. Recently, Zhang F *et al.* (2015) revealed the crystal structure of a MYC3 construct, encompassing the JID and TAD, in complex with the Jas peptide of JAZ9. They showed that the TAD is also important for interaction with the JAZ proteins. Moreover, a Glu in the TAD of MYC3, corresponding to the E165 in MYC2, is involved in one of the main interactions with the Jas peptide. Also the Asp in the JID of MYC3, corresponding to D105 in MYC2, was shown to be important for interaction with JAZ9 (Zhang F *et al.*, 2015).

We have shown that the D105N mutation in the JID of MYC2 is transferable to paralogues. It would be interesting though, to assess the use of the hyperactive MYC2 in crops or medicinal species. *Catharanthus roseus* for instance, produces the anti-tumour MIAs vinblastine and vincristine. In *C. roseus*, MYC2 controls the expression of ORCA3, which at its turn activates expression of several genes in the MIA biosynthetic pathway (van der Fits & Memelink, 2000; van der Fits & Memelink, 2001; Zhang *et al.*, 2011). In our research group, Arabidopsis $MYC2^{D105N}$ and the *C. roseus* orthologue of MYC2, carrying a point mutation at the corresponding conserved Asp in its JID, are being tested in *C. roseus*. Preliminary results show that both hyperactive constructs outperform wild-type *C. roseus* MYC2. Altogether, we have a proof of concept, showing the possibility of changing the activity of TFs in Arabidopsis by the change of their interaction behaviour and eventually using these constructs in other plant species. Other TFs in *C. roseus*, like the bHLH TFs BIS1 and BIS2, would also be interesting targets for our approach. BIS1 and BIS2 both control the iridoid branch of the MIA pathway and overexpression of BIS1 or BIS2 leads to increased accumulation of MIAs in *C. roseus* cell suspension cultures (Van Moerkercke *et al.*, 2015; Van Moerkercke *et al.*, 2016).

Specificity in the JAZ family

The JAZ family is often described as a family of redundant JAZ proteins. This is attributed to a lack of phenotype in many single *jaz* loss-of-function mutants (Thines *et al.*, 2007). However, more and more observations evidence specificity among the JAZ proteins. As such, JAZ5, JAZ6, JAZ8 and JAZ13 can directly bind TPL without the need for the adaptor NINJA (Causier *et al.*, 2012; Shyu *et al.*, 2012; Thireault *et al.*, 2015). In addition, JAZ8 and the newly characterized JAZ13, lack a canonical degron, leading to increased stability in the presence of MeJA (Shyu *et al.*, 2012; Thireault *et al.*, 2015). Moreover, JAZ13 is the only JAZ protein that does not contain a TIFY motif, and hence does not homo- or heterodimerize with other JAZ proteins (Thireault *et al.*, 2015).

JAZ10 had already been described to contain an, N-terminally located, CMID that can mediate interaction with MYC2 but not with COI1 (Moreno *et al.*, 2013). In chapter II.2, we contributed to the concept of JAZ-specificity by the characterization of an N-terminal divergent CMID in JAZ1, mediating interaction with MYC2 (Goossens *et al.*, 2015). Whether this CMID lost interaction with COI1, similar to JAZ10, still has to be investigated. However, amino acid substitutions in the C-terminal Jas domain of JAZ1 can abrogate interaction of full length JAZ1 with COI1 (Melotto *et al.*, 2008), so most likely the CMID of JAZ1 is not able to interact with COI1. The hyperactive $MYC2^{D105N}$ lost interaction with most of the JAZ proteins except for JAZ1 and JAZ10 (Chapter II.2) and only JAZ1 was still able to bind the hyperactive $MYC2^{E165K}$ (Chapter II.3). The CMID of JAZ1 and JAZ10 was responsible for this remaining interaction. Probably, the CMID interacts with the JID of MYC2 in a different manner than the C-terminal Jas domain, increasing the affinity for MYC2. Recently, Zhang *et al.* (2017) could show via the crystal structure of a MYC3 construct in complex with the CMID of JAZ10, that the CMID indeed binds more tightly to MYC3 than the Jas domain of JAZ10. They also indicated the presence of conserved CMID residues in JAZ2, JAZ5 and JAZ6. So far, only the residues essential for the interaction with MYC3 have been determined for the CMID of JAZ10 and not for the other CMID-containing proteins (Zhang *et al.*, 2017).

Chapter II.4 described interaction of KEG specifically with JAZ12, leading to increased stability of JAZ12 (Pauwels *et al.*, 2015). A stretch of five amino acids (X_5-stretch) in the C-terminal part of the Jas domain is responsible for this interaction and is conserved among JAZ12 orthologues, but not among Arabidopsis JAZ proteins. Until now, no interaction has been dedicated yet to this X_5-stretch in the other JAZ proteins. This stretch is followed by a PY sequence that is conserved among the JAZ proteins. The C-terminal part of the Jas domain, including the X_5-stretch and the PY sequence, has been shown to be essential for interaction with COI1. However, mutation of PY to two Ala residues in JAZ2 and JAZ10 does not abolish the interaction with COI1 (Chung *et al.*, 2010). Also the X_5-stretch of JAZ12 does

not interfere with COI1 binding as was shown by Y2H analysis in chapter II.4. Possibly, lack of the C-terminal part of the Jas domain induces a conformational change of the JAZ proteins resulting in loss of interaction with COI1.

The positive effect of KEG on the stability of JAZ12 occurs uniformly in as well the cytoplasm as the nucleus, implying that JAZ12 is constantly translocated between nucleus and cytoplasm and that KEG does not tether JAZ12 outside the nucleus. This suggests that KEG is a negative regulator of a specific JAZ12-involved JA response. However, no JA hypersensitivity was observed in the *KEG* amiRNA lines. To investigate further the function of this specific interaction, phenotypes of Arabidopsis plants overexpressing *JAZ12* can be compared to overexpression of *JAZ12* in a knock-down line of *KEG*. Specific phenotypes would be less pronounced in the *KEG* amiRNA line. A negative role for KEG in both ABA and JA signalling is in agreement with the positive crosstalk observed between ABA and JA signalling. MYC2 was shown before to be a positive player in the ABA pathway (Chapter II.2) and accordingly, the ABA receptor PYRABACTIN RESISTANCE1-LIKE6 (PYL6) could interact with MYC2 in the presence of ABA (Aleman *et al.*, 2016). In addition, ABA induces biosynthesis of JA during plant defence (Adie *et al.*, 2007).

PPD signalling: a copycat of JAZ?

PPD1 and *PPD2* arose in vascular plants and probably diverged from the *JAZ* genes (Bai *et al.*, 2011). Accordingly, the PPD proteins have a protein architecture similar to JAZ. They also contain a TIFY motif-containing ZIM domain and a C-terminal Jas domain. Correspondingly, the ZIM domain of the PPD proteins is responsible for heterodimerization with the JAZ proteins. PPD proteins can also homodimerize and bind NINJA, presumably via the ZIM domain (Cuéllar Pérez *et al.*, 2014; Gonzalez *et al.*, 2015). The PPD proteins are targeted to the proteasome by SAP-mediated ubiquitination (Wang *et al.*, 2016), however, the domain responsible for this interaction still has to be determined. Remarkably, no protein interaction have been appointed yet to the Jas domain of the PPD proteins.

In chapter II.5, we generated a mutated construct of PPD2, which carried a Tyr to Phe change in the Jas domain. Y2H analysis showed that wild-type PPD2 interacts with JAM1, a negative regulator of JA signalling. In addition, the mutated PPD2 mediated direct interaction with MYC2, MYC3 and JAM2. It was hypothesized that the Tyr in the Jas domain of PPD2 is phosphorylated in yeast, interfering with the interaction with the JID of JAM2, MYC2 and MYC3. Still, wild-type PPD2 was able to interact with JAM1 in yeast. Alignment of the amino acid sequence of the JID of JAM1, JAM2, MYC3 and MYC4, shows a conserved Tyr that is occupied by a Cys in JAM1 (Fig. S2). This Tyr might be phosphorylated in yeast and, together with the phosphorylated Tyr of PPD2, block interaction of PPD2 with JAM2, MYC2 and MYC3.

Transient expression assays showed that PPD2, co-expressed with KIX8 and KIX9, could confer repression activity to JAM2 at the promoters of *DFL1* and *JAZ1*, and that this was dependent on interaction between PPD2 and JAM2. Nevertheless, there is no plant phenotypic link observed yet that could biologically explain the interaction between JAM2 and PPD2. Currently, we are looking deeper into this by performing phenotypic assays on knock-down lines of *PPD1* and *PPD2* and knock-out lines of *JAM2*. Still, interaction of JAM2 only happens with the mutated version of PPD2 in yeast. Therefore, it is important to confirm this interaction via alternative techniques, e.g. co-immunoprecipitation, assuring the biological relevance of the interaction observed in yeast.

Co-expression of PPD2/KIX8/KIX9 and JAM2 mediated repression on the promoter of *CYCD3;2* in transient expression assays, although, these repression effects were not dependent on interaction between JAM2 and PPD2. Therefore, we assume that another TF is bound by PPD2 at the promoter of *CYCD3;2*, which is a cell cycle-related gene. *JAZ1*, in contrast, is a gene typically involved in JA signalling. Moreover, the *DFL1* promoter can be induced by MYC2 in our transient expression assays wherefore *DFL1* is also thought to be involved in the JA pathway. Altogether, we propose that PPD2-JAM2 binds JA-related promoters and that PPD2 binds alternative TFs at other promoters, such as *pCYCD3;2*, to mediate repression.

Via a genome-wide Y2H screen followed by next generation sequencing, two candidate TFs were found, WOX5B and NGAL2, that would be able to interact with PPD2, though interaction could not be confirmed via binary Y2H. Further investigation by mapping of the detected reads to the Arabidopsis genome has shown that the quality of the library used in this project was not optimal. Many genes were only partially cloned in the library, e.g. the *NGAL2* clone only contained its first exon. This could lead to structural changes, and hence, changes in interaction behaviour and therefore, these partial genes will be checked in the future via binary Y2H. Furthermore, other TFs identified during the Y2H screen, i.e. TCP20, ANAC042 and bHLH106 are also currently being tested for interaction via Y2H and for activity in transient expression assays. It should be noted though, that the library used for our screen has been proven to work nicely using other baits. Furthermore, data analysis of the Y2H-seq method is being optimized by comparing the data of all these Y2H screens. Alternatively, a Y2H library suited for PPD2 can be generated by using RNA isolated from a pool of leaves and shoots harvested at different stages during stomatal development.

Alternative splicing of *JAZ* pre-mRNAs, via retention of the Jas intron, led to stable JAZ proteins. *PPD* pre-mRNAs are also subjected to alternative splicing. Interestingly, multiple splicing strategies are used by PPD1 and PPD2 that result in truncated constructs lacking partially or fully the Jas domain. Based on the knowledge of JAZ proteins, we hypothesize

that the Jas domain of PPD1 and PPD2 serves to interact with SAP and G-box binding TFs and that alternatively spliced PPD constructs lose interaction with SAP while they continue the repression of target TFs. To further investigate this hypothesis, the alternative splice variants of *PPD* could be overexpressed in Arabidopsis. The resulting phenotypes would be expected to be more severe compared to overexpression of wild-type *PPD*.

Our data emphasizes again the similarities between PPD and JAZ protein architecture and suggests similar modes of action for PPD2 and the JAZ proteins. In our proposed model, PPD2 confers repression to bound TFs, like JAM2, via interaction with KIX8, KIX9 and/or NINJA, which connect the TPL repressors to the TFs. An unknown signal then mediates binding of SAP to the PPD proteins, mediating their degradation and releasing the bound TFs. It was shown before that JAM proteins exhibit their repression capacity by competing with the MYC TFs for binding target promoters (Fonseca *et al.*, 2014; Qi *et al.*, 2015). Both repression mechanisms might exist and can be compatible with each other.

Development of the TAP technology in *Medicago truncatula* hairy roots

In chapter II.7, a TAP-MS strategy was developed in the model plant *Medicago truncatula*. Baits were overexpressed in *M. truncatula* hairy roots and TAP-MS analysis was performed in a similar manner to TAP-MS in Arabidopsis cell cultures (Van Leene *et al.*, 2015). The *M. truncatula* orthologues of Arabidopsis CKS1, CAF1 and JAZ1 were picked as proof of concept because these proteins are very conserved among different plant species or even eukaryotes. Furthermore, protein interaction analyses on these baits had already been performed in other organisms.

The TAP results were validated using a systematic Y2H technique. Alternative binary methods using a plant endogenous background, such as co-immunoprecipitation or bimolecular fluorescence complementation assays, are more laborious and are therefore less appropriate for high-throughput experiments. Therefore, it was opted to use the systematic Y2H technique, which allowed to verify interactions among and led to more information about the constitution of the bait protein complexes.

CKS1 is involved in the cell cycle control as docking factor for CDK/cyclin complexes. The systematic Y2H screen could confirm many of the identified interactors of CKS1, though only a few direct interactions among preys could be detected. For instance, interaction between CDK proteins and cyclins could be expected, as substrate-specificity of the CDKs is determined by interaction with the cyclins (Inzé & De Veylder, 2006; De Veylder *et al.*, 2007). However, in this analysis only direct interaction between CDKB1 and CYCD5 was detected. Accordingly, a high-throughput binary Y2H screen of the Arabidopsis cell cycle core proteins has shown that the orthologues of the CDKs and cyclins, identified in this screen, do not

interact, except for the orthologue of CYCD5, which interacted with the orthologues of CDKA1 and CDKB1 (Boruc *et al.*, 2010).

CAF1 is a component of the big CCR4-NOT1 complex, involved in a diverse array of regulatory processes. Only the key components of the CCR4-NOT1 complex could be confirmed by the systematic Y2H screen. As this system works with single components of a big complex, it might be possible that some basic components of the CCR4-NOT1 complex are essential to mediate interactions among the other proteins of the complex. Apparently, the presence of the yeast orthologues of these components is not sufficient. Therefore, above-discussed interaction techniques, such as co-immunoprecipitation, might be more appropriate to confirm TAP results of the CCR4-NOT1 complex. An alternative is to perform Y2H while basic components of the complex are co-expressed in the yeast cells.

JAZ1 is involved in JA-related TF complexes as transcriptional repressor and TAP-MS analysis in Arabidopsis led to the identification of MYC3, JAZ12, COI1 and NINJA (Pauwels *et al.*, 2010; Van Leene *et al.*, 2011). Here, only NINJA could be identified during TAP-MS of *M. truncatula* hairy roots and was confirmed as direct interactor of JAZ1 via Y2H. The low prey detection was probably caused by low stability of JAZ1 as protein levels in the hairy root cultures were low, in contrast to *JAZ1* transcriptional expression. Treatment of the hairy root cultures by JA could lead to identification of JA-induced interactors of JAZ1, such as COI1. As JAZ proteins are very unstable in the presence of JA, it is recommended to perform the treatment only for 1 min (Geerinck, 2010).

Altogether, these three baits demonstrated the reliability of TAP-MS in *M. truncatula* hairy roots and once again demonstrated the functionality of combining an initial TAP-MS analysis followed by Y2H validation. Recently, a knock-out mutant of the *M. truncatula* orthologue of the Arabidopsis *PPD* genes, *BIG SEEDS1* (*BS1*), had an increase in weight and size of the seeds and leaves due to increased primary cell proliferation (Ge *et al.*, 2016). It would be interesting to investigate its interaction behaviour by performing TAP on BS1 in *M. truncatula* hairy root cultures. *M. truncatula* is a model legume and resulting information can be relevant for increasing the yield of grain crops and forage crops in the future. Finally, TAP of hairy root cultures could be transferred to other plants, such as *C. roseus* and tomato, and lead to valuable information there.

Development of a small molecule yeast two-hybrid system

The molecular mechanism of auxin signalling is strikingly similar to JA signalling (Cuéllar Pérez & Goossens, 2013). In particular the way the hormone is perceived is almost identical. Both auxin and JA effectuate the formation of a co-receptor complex consisting of an F-box protein, respectively TIR1 and COI1, and a transcriptional repressor, respectively an AUX/IAA

protein and a JAZ protein. Yu *et al.* (2015) changed amino acids in the F-box domain of TIR1 and AFB2 to lower autocatalytic degradation, and hence to increase the stability of TIR1 and AFB2. Y2H analysis in the presence of auxin, showed an increase in interaction between these mutated constructs and AUX/IAA proteins.

The Y2H system available in our lab was suited to show NAA- or 2,4-D-dependent interaction between TIR1 and AUX/IAA proteins and COR-mediated interaction between COI1 and JAZ proteins. The strength of the interaction can be semi-quantified by the presence of X-gal in the growth medium, which is converted in a blue colour by the reporter enzyme β-galactosidase. Since interaction between TIR1 and AUX/IAA proteins was not always clearly detectable in the presence of NAA and 2,4-D, relative high amounts of these auxins are needed to mediate interaction via Y2H (Yu *et al.*, 2013). In chapter II.6, we made our Y2H system more sensitive by integration of the stable TIR1 and AFB2 constructs, described by Yu *et al.* (2015), in this Y2H system. Moreover, we extended it with mutated constructs of AFB1 and AFB5, which also increased interaction with the AUX/IAA proteins.

The stable TIR1/AFB constructs contained mutations in the first helix of the F-box domain. These perturbations in the F-box domain did not mediate a loss of interaction with ASK1. Remarkably, direct interaction between the mutated TIR1 constructs and CUL1 was reduced, probably leading to a decreased autocatalytic degradation (Yu *et al.*, 2015). This was also observed in yeast and human, where perturbations or deletion of the F-box domain increases stability of the F-box proteins by blocking autocatalytic degradation (Galan & Peter, 1999; Wirbelauer *et al.*, 2000). As COI1 shows amino acid sequence similarity to TIR1, mutated constructs of COI1 corresponding to the mutations inserted in the F-box domain of the TIR1/AFB proteins, were generated. A slight increase in interaction with JAZ12 could be observed for COI1(E22K). Whether this led to a change in COI1 stability was not examined. To look deeper into the effect of mutations in the first helix of the F-box domain of F-box proteins, it is necessary to investigate the stability of the different TIR1/AFB and COI1 constructs in yeast and plants via immunoblotting and to verify interaction of the constructs with ASK1 and CUL1.

In this project, a directed approach was used to increase the stability of COI1 in the Y2H system. A more general approach to identify COI1 mutants with increased stability in yeast could be to perform an error-prone PCR to create a randomized library of COI1 constructs, containing point mutations. This library could then be screened for increased interaction with a JAZ protein in the presence of COR. To avoid COI1 constructs that interact better specifically with JAZ proteins, without an increase in COI1 stability, mutations in the F-box domain would be preferred. Alternatively, the randomized COI1 library can be screened for reduced interaction with CUL1.

Altogether, we established a sensitive Y2H system to screen for small compounds that can mediate interaction between F-box proteins and their targets. So far, the smY2H has only been shown to work for the F-box proteins TIR1/AFB and COI1 with, respectively, AUX/IAAs and JAZs as substrates. It would be interesting to extend this system to other F-box proteins and to perform effective screens using chemical libraries, to identify bioactive compounds, and cDNA libraries, to identify new substrates. The interaction between SAP and PPD2, for instance, could be dependent on a natural compound. Therefore, a chemical library of hormonal compounds could be used to screen for compounds that might mediate interaction between SAP and PPD2. Also COI1 might bind additional compounds and substrates. As such, COI1 is involved in the resistance against the pathogen *Verticillium longisporum*, however, in a JA-Ile-independent manner (Ralhan *et al.*, 2012) suggesting an alternative compound that can mediate interaction between COI1 and JAZ. Alternative protein substrates for COI1 are suggested by the characterization of JA-ASSOCIATED VQ MOTIF GENE1 (JAV1), which is degraded in the presence of JA in a COI1-dependent way (Hu *et al.*, 2013).

The smY2H system has also the potential to be a simple and quick validation method for compounds thought to mediate or abolish interaction between the components of hormonal co-receptor complexes. For instance, rational design of agonists or antagonists of hormones, such as auxinole and COR-MO (Hayashi *et al.*, 2012; Monte *et al.*, 2014), could be easily assessed by this smY2H system. The value of such a Y2H system to evaluate quantitative differences in binding strength was demonstrated by the generation of a mutated COI1 construct with less affinity for JAZ proteins in the presence of COR but not in the presence of JA-Ile (Zhang L *et al.*, 2015). Still, validation of the small molecule Y2H system using small compounds, other than auxins or COR, is needed in the future.

Final conclusion

In this research, we have shown that interaction methods -to generate a blueprint of the interaction network of protein complexes- combined with site-directed mutagenesis -to change the interaction behaviour of involved proteins- is an appropriate strategy to characterize protein complexes. Furthermore, this strategy allows to modulate the activity of TFs, leading to hyperactive TFs with potential applications in metabolic engineering of crops or medicinal plants. Finally, we developed two techniques, TAP-MS in hairy roots of *M. truncatula* and smY2H, that can contribute in the future to the characterization of protein complexes and hormonal pathways.

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Thank you!

My journey in the crazy jasmonate world arose during my first master thesis 7 years ago. I still remember the title of the project that was assigned for me: "The role of the E3 Ub ligase KEEP ON GOING in the jasmonate pathway" guided by Amparo. At that time, I was freaking out because I had to write and speak English. However, with both you and Laurens as guiding persons, it was a very interesting and fun period. The people of the group even made such an impact on me that the year after, I chose to do my final master thesis also in the metabol lab. Jasmonate signalling intrigued me and the metabollers were a dream team of colleagues and the idea of starting a PhD here was itching. I managed to get a PhD grant, which, at that time, was one of the toughest achievements I could imagine. Well … now I know better ☺.

Alain, thank you for offering me the chance to do my PhD in such a fun lab, where the best team buildings ever are organized! It was nice to have a boss with who I could talk to about travelling, family, sports and many more. I'm grateful for all the opportunities I had here, for the science, the confidence you had in me, the experience, all the beers we shared and the competitive sport clashes we passed through ;-). Also **Laurens**, thanks for all your scientific input and the support during all my research projects. You told me in the beginning that the metabollers are hard workers but also can party hard and I must say, I got introduced in both concepts very well. I appreciate a lot your initiatives to go with the lab to the 'kerstmarkt', 'Gentse feesten' and the organization of the PhD movies. Such activities are very important for the atmosphere in the group and you really motivated me to do the same.

Of course, I would not have been in this position without the guidance of **Astrid** during my master thesis. Thank you for this, for the support when I was nearly 'scooped' and the burger-ping-pong-barbecues at your place. **Michele**, you were one of the founders of the famous OCR and you learned me the bad behaviour of drinking strong coffee. Together with **Andres** and **Amparo**, you made my early years at the lab really fun. **Alex**: Parov Stelar and dancing on the benches … I don't have to tell you more I guess. Thanks for the after work distraction! **Fabi**, I will always remember the first drink with you at PSB! As one of the island members, you worked on my nerves many times ;-), but I really enjoyed our conversations about science, work and life. I would never have thought that I would hang out at the station that much without taking the train. Btw: thanks for the Tomme cheese! To the other island member, **Sabrina**, I would like to say thanks for not annoying me. You made the island very pleasing and sorry for not watering the plants when you were away! 'Mayor' **Robin**, thanks for the many protoplast assays, your knowledge and presence at the VIB quizzes, 'de ronde

van Vlaanderen', all excellent team buildings, the food, the drinks and the fun we shared! **Rebecca**, thank you for all the Y2Hs you performed and for doing experiments for me while I was preparing my thesis. It was nice working and chatting with you!

The second year of my PhD, I guided two students at the same time: **Joan** and **Gwen**. This period was really busy for myself but I had real fun guiding you two. **Joan**, you were just one crazy student! **Gwen**, sorry for all the error-prone PCR experiments, which were indeed too error-prone. I'm glad this didn't stop you of joining the lab! I should also still say thanks to the people who helped me guiding the students during the practical course plant physiology I teached: **Thomas**, **Stefanie**, **Lisa**, **Marie-Laure**, **Michiel**, **Caroline**, **Alexandra**, **Lukas**, **Gwen** and **Matthias**. In particular, thanks to **Eveline** for doing all preparations for the experiments, it was fun to work with you! I realize now I forgot to give a drink to thank you all, so I hope you will join the party after my defence!

PhD periods have their ups and downs, as well on a professional as on a personal level. **Marie-Laure**, my BCBT buddy at PSB: I cannot imagine how many cola's we shared, but it was really nice to have someone to talk to about everything. Many important decisions were made easier because of you. It was cool to have you around! Speaking about buddies … **Phil**, I think I might say that I shared most moments with you: moving, living together, moving, many dinners, parties, squash battles, good moments, bad moments, reflecting life at the Leie, etc. Thanks man, really, for all moments shared, it meant a lot to me! Playing sports was a very important constant during my time here and therefore I want to thank our little squash gang: **Nathan**, **Michiel**, **Fabi**, **Phil** and **Alan**. Not only during squash but also after work, all you guys were important to forget stress at work and to enjoy life in Ghent. Noteworthy, most different disciplines of sports I shared with **Jelle**: petanque, bowling, football, basketball, squash, rafting, hiking and running. The moments we shared in Vancouver and the surrounding national parks were memorable, thank you! **Karel** (and **Minna**), I can't imagine who else (yes, you **Maite**) can stay 'keep on going' as long as you guys. We shared many long and pleasant evenings! **Piti**, the little 'Marvin' of our lab. I think you are the nicest person on earth! Thank you for make me feel at ease, in particular during the last months! **Jamer**, thanks for the boardgame evenings and for the after-work drinks! **Jacob**, thank you for beating you at 'de muur' ;-) and for some memorable mental pictures I have of parties with you! **Maite**, thank you for the coffee breaks at the coffee(!) lounge, the conversations and to learn me the concept of an aubergine burger. **Bianca**, **Tessa**, **Trine**, **Clara**, **Evi**, **Yuechen** and **Linlin**, it was very nice to have shared many enjoyable lunches and evenings together with you!

The outcome of my projects would not have been the same without collaborations. Thank you **Debora** and **Ted** for the MYC2 collaboration. Thank you **Nathalie** and **Debora** to reflect and discuss about PPD2 not being a transcription factor ;-).

During the last five years (and already many years before), my friends from Merchtem have been indispensable in my life. **Joost**, thanks for all the joy and laughter, the holidays we spent together and the many moments where we were heading home but only arrived many hours later due to discussions about life, less significant discussions or throwing a potato on my head. You are very important in my life! **Steven**, whenever I am out with you, something crazy happens. Dreaming about exotic futures, singing 'High and dry' at high frequencies and high volumes, unforgettable festivals and fun, a lot of fun! I can always count on you and that makes you very unique! **Levin**, you are one of a kind, of a special kind! Thanks for many, many late night conversations, for the road trips and for having lived at the Molenaarsstraat with me. We were eventually not the perfect match to share a house with paper walls, but our good friendship survived this and we can be proud of that! My other road trip partner, **Lanos**, thank you for the multiple dinners and parties in Ghent and for joining me and Jelle in the national parks in Canada! Also all people from 'het sappige druivencollectief': **Yelle**, **Sofie**, **Serge**, **Annelore**, **Steven**, **Joost**, **Caro**, **Nore, Janne**, **Wietse**, **Valerie**, **Seth** and **Valerie**. I always enjoy seeing each one of you, the hugs, the weekends at the 'Ardennen', the parties, the dinners. You guys are the best friends one can imagine and a lot of shared happiness is yet to come!

Important during a PhD life is a fixed weekly moment to get away from everything. The '**drankdinsdag groep**' from Ghent, is the most diverse group of friends that I can imagine. Without any obligations, on Tuesdays, I had the possibility to have some people around to talk, laugh and support each other! Also the yearly men's weekend is each time a great experience. Thanks guys!

Sil and **Ellen**, I am very happy to have you as brother and sister. During our youth it was remarkable already how good we came along. This even became more valuable, the older we got; besides siblings, both of you also are true friends! Together with **Delphine** and **Pieter**, we shared many good moments together. Sil and Delphine, I am very proud to be uncle and 'peter' of the cutest kids in the world: **Lene** and **Gus**!

But the last stretch of the PhD, the toughest one, would not have been the same if you would not have been at my side, **Liesbeth**. You make everything way easier, you comfort me and you make me happy at moments that I should be sad. I guess this is a gift from your family as I felt at home already quite soon in your family, thanks for this **Sabine**, **Hugo**, **Jolien**, **Mare**, **Maarten**, **Britt**, **Jerom** and **Oliver**! Liesbeth, I know I was not the most relaxed

guy the last months, but you always stayed positive! I am glad that I finally found you, having lived so close to me all my life, but hidden like a precious diamond! I love you girl!

Moeke en vake, zo'n hecht gezin vormen als wij is echt bewonderenswaardig. Jullie hebben ons alle kansen gegeven en juiste waarden meegegeven. Vanaf ik op kot mocht in Gent, heb ik het altijd leuk gevonden om 'thuis' te komen, gezellig in de zetel te zitten, frietjes eten en babbelen over alle belevenissen van de week. Nu nog altijd, is het steeds thuiskomen met dat zelfde warm gevoel als ik bij jullie ben. Moeke, merci om mij op moeilijke momenten zoveel mogelijk in de watten te hebben gelegd. Vake, merci voor de zware maar leuke fietstrip naar Compostella vorig jaar, dit was een heel belangrijk deel van mijn doctoraat en zal ik nooit vergeten! Moeke en vake, jullie betekenen veel voor me! **Meter** ... ik vind het superspijtig dat je dit niet meer kan meemaken, ik had zo graag taart en quiche van jou kunnen opdienen op mijn doctoraatsfeestje, ik had zo graag nog zoveel willen vertellen van ons, van de verhuis, van de toekomst. Ik heb zo hard genoten van die vele bezoekjes bij jou, van jouw speelse insteken op onze gesprekken! Meter, bedankt!

Curriculum vitae

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PROFESSIONAL EXPERIENCE

2012 - current	Doctoral researcher (personal IWT grant) - Research in plant biotechnology Flemish Institute for Biotechnology - University Ghent
2015	Head lecturer Plant Physiology - Practical course 2nd Bachelor Biology (40 students) University Ghent

EDUCATION

2017	PhD in Biochemistry and Biotechnology - University Ghent
2012	Master Biochemistry and Biotechnology - University Ghent obtained with great distinction
2010	Bachelor Biochemistry and Biotechnology - University Ghent

SCIENTIFIC PUBLICATIONS

- Goossens J, Swinnen G, Vanden Bossche R, Pauwels L, Goossens A. (2015) Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity. New Phytologist 206: 1229-1237.
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- Goossens J, Fernandez-Calvo P, Schweizer F, Goossens A (2016) Jasmonates: signal transduction components and their roles in environmental stress responses. Plant Mol Biol 91: 673-689.

OTHER SCIENTIFIC COMMUNICATIONS

- Goossens J, Pauwels J, Vanden Bossche R, Goossens A. (2014) Generation of a superactive MYC2 transcription factor to increase the jasmonate response in plants. Poster presentation International Conference on Arabidopsis Research (Vancouver, 2014)
- Goossens J, Gasperini D, Swinnen G, Vanden Bossche R, farmer EE, Pauwels L, Goossens A (2015) Generation of superactive MYC2 transcription factors to increase the jasmonate response in plants. Selected talk and poster presentation PhD Summerschool Environmental
Signalling in Plants (Utrecht, 2015)
- Goossens J, Gasperini D, Swinnen G, Vanden Bossche R, farmer EE, Pauwels L, Goossens A (2015) Single amino acid substitutions modify the composition and activity of jasmonate-related transcription factor complexes. Poster presentation International Conference
in Plant Growth and Substances (Toronto, 2016)

TRAININGS AND GUIDANCE

- Scientific writing and oral presentation (2015) Lectured by Dr. Shirley Ellis
- Career guidance, 2016. Lectured by Dr. Lucia Smit
- Supervision of two Master students in the course of their final project/thesis during their 2nd master Biochemistry and Biotechnology
- Organization of Wetenschap in de Kijker

SCIENTIFIC EXPERTISE

Protein interaction

- TAP-MS
- Immunoprecipitation
- Yeast one-hybrid
- Y2H (binary and screen)

Plant genetics

- Transformation (e.g. CRSIPR)
- Genotyping
- Phenotyping
- Crossing

Molecular Biology

- qPCR/RT-PCR
- Immunoblotting
- Molecular cloning
- Transient expression assay

QUALITIES

- Enthusiastic
- Result-oriented
- Fast-learner
- Reliable
- Organizer

INTERESTS

- Sports (squash, basketball, cycling)
- Travelling
- Music
- Food and drinks
- Quiz

OTHER ACHIEVEMENTS

- Cycling trip Compostela (2300 km, 2016)
- Coordinator youth movement JKAJ Merchtem (2005-2007)
- Organizing team buildings (2012-now)
- European driver's license B (2008)

LANGUAGES

Dutch	●●●●●
English	●●●●○
French	●●●○○

SOFTWARE

MS Office	●●●●●
Illustrator	●●●●○
Photoshop	●●●○○
Inkscape	●●●●●
Movie maker	●●●●○

SCIENTFIC SOFTWARE

Endnote	●●●●●
UGene	●●●○○
ImageJ	●●●●○
CLC main workbench	●●●●●

Supplementary data

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Chapter II.7	Figure S27-S33 Table S5-S12[*]

[*]Tables S7 and S10 of Chapter II.7 are available as Datasets S2 and S3 in the online supporting information of Goossens and De Geyter et al. Isolation of protein complexes from the model legume *Medicago truncatula* by tandem affinity purification in hairy root cultures. Plant Journal (2016).

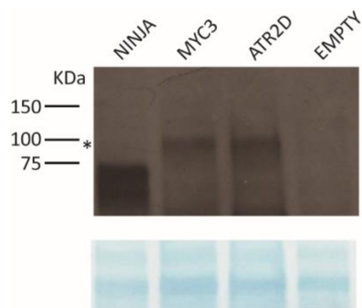

Figure S1: The D94N mutation does not affect MYC3 protein stability in transformed yeasts. The top panel shows the immunoblot analysis with the anti-GAL4-AD antibody on the transformed yeasts from the Y2H analysis presented in Fig. 1A and producing MYC3 and ATR2D fused to GAL4-AD. Protein extracts from wild-type yeasts (control) and transformed yeasts producing NINJA fused to GAL4-AD were included as negative and positive controls, respectively. The asterisk indicates the position of the tagged MYC3 proteins. The bottom panel visualizes the amount of total protein loaded by Coomassie blue staining of the immunoblot.

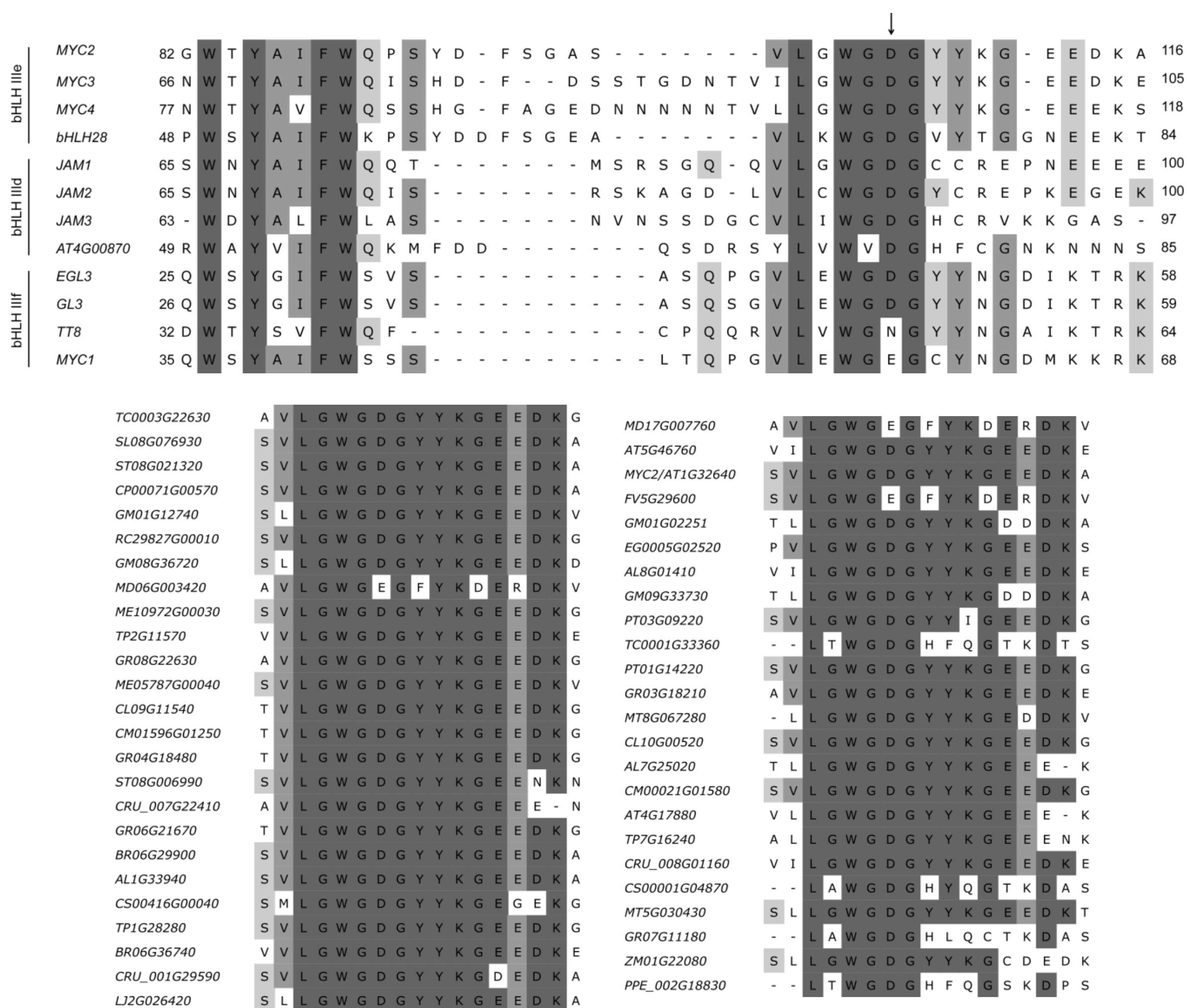

Figure S2: Comparison of the JID sequence of different MYC homologues. Top panel: Alignment of Arabidopsis bHLH IIId and bHLH IIIf proteins. The arrow indicates the mutated Asp in atr2D and MYC2^{D105N}. The sequences of TT8 and MYC1 do not contain an Asn at this position. Lower panel: Alignment of MYC orthologues identified by PLAZA 3.0 (online access). Shading of the residues represents the sequence conservation among the shown bHLH proteins. The sequences were aligned by MUSCLE and edited with UGene.

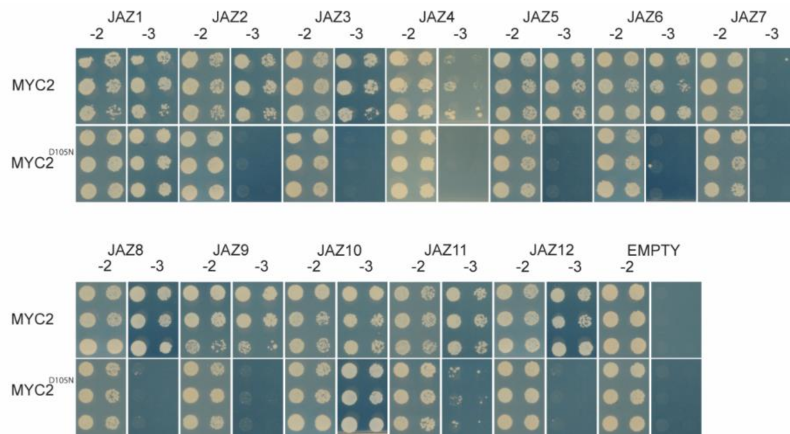

Figure S3: MYC2^{D105N} loses interaction with most of the JAZ proteins. MYC2 and MYC2^{D105N} fused to GAL4-AD were tested by Y2H for interaction with 12 JAZ proteins fused to GAL4-BD. Empty vectors were used as negative control. Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium.

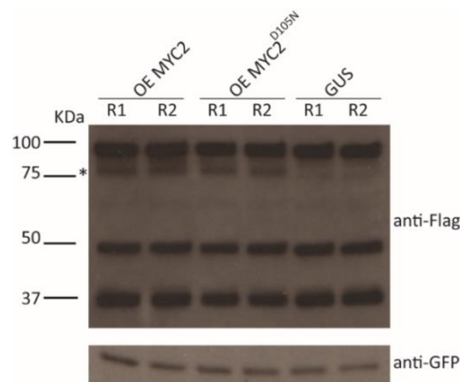

Figure S4: The D105N mutation does not affect MYC2 protein stability in transfected tobacco protoplasts. Immunoblot analysis of total protein extracts from transfected tobacco protoplasts producing MYC2 or MYC2^{D105N} fused to a 3xFlag-6xHis-tag. Extracts from tobacco protoplasts transfected with the GUS effector plasmid were included as a negative control. Effector plasmids expressing GFP were co-transfected to correct for the transfection efficiency. Anti-Flag (top panel) and anti-GFP (bottom panel) were used for detection of tagged MYC2 and GFP proteins, respectively. R1 and R2 represent two repeats that each consist of a pool of transfected protoplasts from 8 biological repeats. The asterisk indicates the position of the tagged MYC2 proteins.

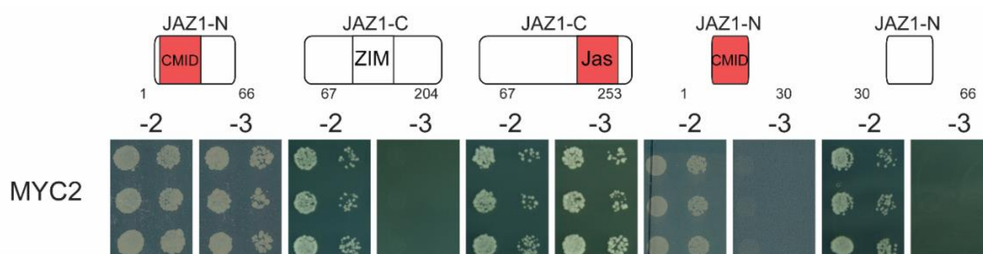

Figure S5: Y2H analysis with JAZ1 fragments. MYC2 fused to GAL4-AD was tested for interaction with truncated versions of JAZ1 fused to GAL4-BD. Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium.

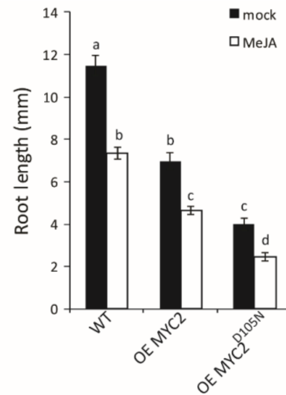

Figure S6: MYC2 or MYC2^{D105N} overexpressing plants are not hypersensitive to exogenous JA. Root length measured in wild-type (WT) and MYC2 or MYC2^{D105N} overexpressing (OE) plants, grown for 7 days in DMSO (mock) or 1 µM MeJA. Values represent the mean (±SE) from 20 to 60 seedlings. Letters represent significant differences according to a Tukey's Honestly Significant Difference (HSD) test (*P<0.05).

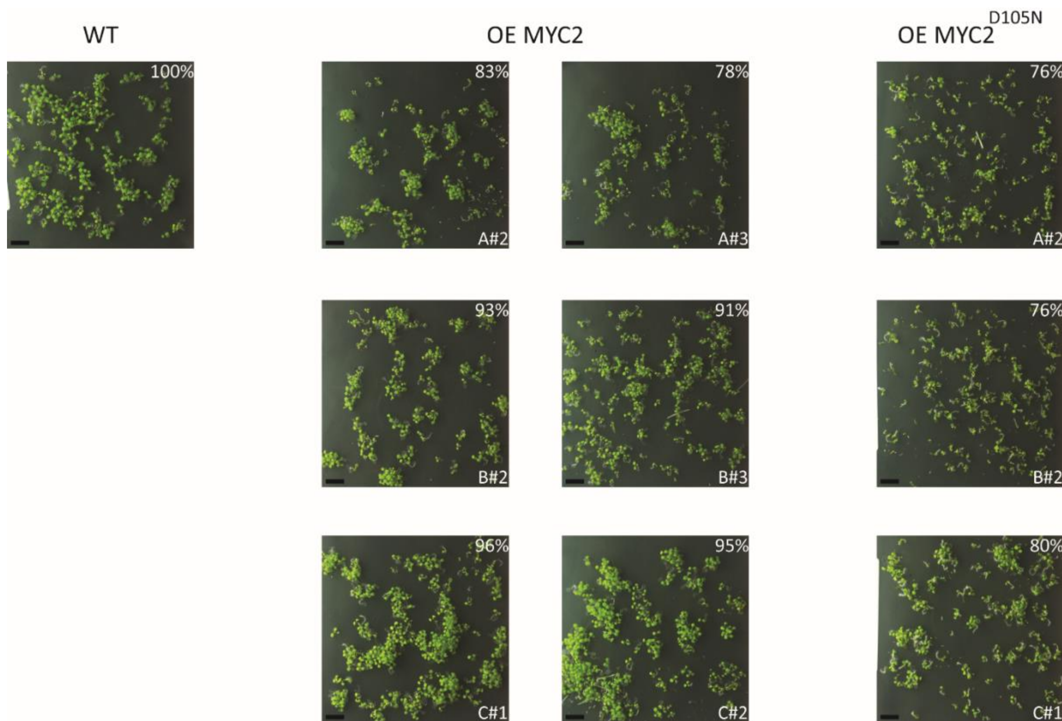

Figure S7: MYC2^{D105N} overexpression decreases germination efficiency. The germination efficiency of plants overexpressing (OE) MYC2^{D105N} was compared to that of plants overexpressing MYC2 and wild-type (WT) plants and represented as a percentage in the top right corner of the plate photograph. Different seed batches (#1, #2 and #3) of different plants of the OE lines shown in Fig. 4C, as well as one additional transgenic line (C) for both genotypes, were tested. All lines shown have similar expression of MYC2/MYC2^{D105N} (ca. 5- fold) except line 'OE MYC2-A' (ca. 40-fold, see Fig. 4d). Bars = 1cm.

Table S1: Primers used for qRT-PCR analysis.

Genes	qRT-PCR Primers
MYC3 (AT5G46760)	**Fw** 5’-AGGTTGGGATGTGATGATACG-3’ **Rv** 5’-AACCTAGCACCGGGATGAT-3’
OPR3 (AT2G06050)	**Fw** 5’-GCTCAAAGCTCGCTTACCTT-3’ **Rv** 5’-TGCCTTCCAGACTCTGTTTG-3’
AOS (At5G42650)	**Fw** 5’-CACCGGCGTTAGTCAAATCT-3’ **Rv** 5’-CGGCGGATTCTAAGAAAAACT-3’
JAZ10 (At5g13220)	**Fw** 5’-CGCTCCTAAGCCTAAGTTCCA-3’ **Rv** 5’-TCGAAATCGCACCTTGAATA-3’
TAT3 (AT2G24850)	**Fw** 5’-GCACCATGAACAGTGGAAAG-3’ **Rv** 5’-AGTCCTGAAGTTGGAATGGG-3’
TSB1 (AT5G54810)	**Fw** 5’-AGCTCCTTCCTCTTCTTCCC-3’ **Rv** 5’-GATGATGACTTGGAGCGAGA-3’
VSP2 (At5G24770)	**Fw** 5’-CCTAAAGAACGACACCGTCA-3’ **Rv** 5’-TCGGTCTTCTCTGTTCCGTA-3’
PDF1.2 (AT5G44420)	**Fw** 5’-TGTTCTCTTTGCTGCTTTCG-3’ **Rv** 5’-GCAAACCCCTGACCATGT-3’
MYC2 (At1G32640)	**Fw** 5’-TCCGAGTCCGGTTCATTCT-3’ **Rv** 5’-TCTCGGGAGAAAGTGTTATTGA-3’A
LOX2 (AT3G45140)	**Fw** 5’-CTCTTCAGAGCAACGCTACG-3’ **Rv** 5’-GAAGATGGAGGGAAGAGCTG-3’

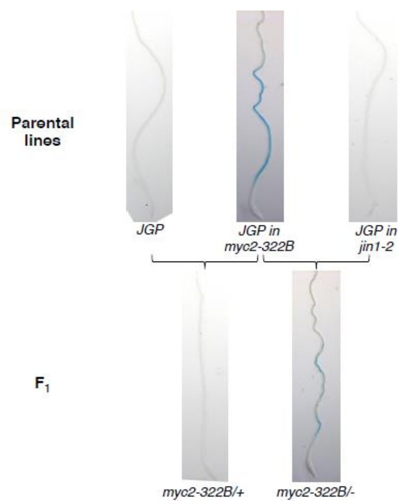

Figure S8: *myc2-322B* inheritance. Root details from GUS stained 5-do seedlings in uniform *JGP* backgrounds. The F1 progeny (*myc2-322B/+*) of a cross between *myc2-322* and the WT *JGP* line does not show ectopic *JGP* activity, whereas the F1 progeny (*myc2-322B/-*) between *myc2-322B* and a *myc2* null mutant (*jin1-2*) displays constitutive *JGP* activity, similar to that of *myc2-322B*.

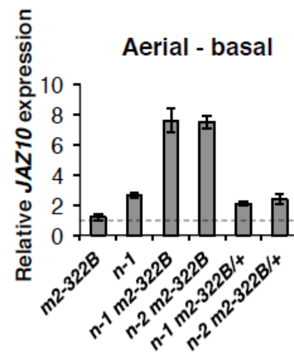

Figure S9: qRT-PCR of *JAZ10* expression in aerial organs of 5-do *myc2-322B* lines. Abbreviations are as follows: *m2-322B* is *myc2-322B*, *n-1* is *ninja-1, n-2* is *ninja-2*. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression WT controls set to 1 (dashed lines). Bars represent the means of three biological replicates (±SD), each containing a pool of organs from ~60 seedlings.

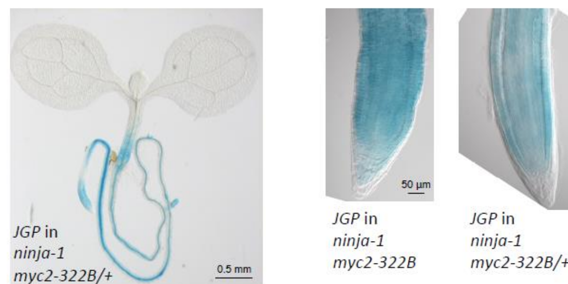

Figure S10: *JGP* expression in 5-do seedlings and primary root meristem of *ninja-1 myc2-322B/+.* Note the weaker GUS staining in the *ninja-1 myc2-322B/+* heterozygous mutant compared to the *ninja-1 myc2-322B* double mutant in Fig 2C.

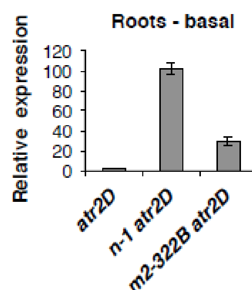

Figure S11: qRT-PCR of *JAZ10* expression in root organs of 5-do seedlings of mutant combinations with *atr2D*. Indicated genotypes: *m2 322B* is *myc2-322B*, *n-1* is *ninja-1*. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression in WT controls set to 1 (dashed lines). Bars represent the means of three biological replicates (±SD), each containing a pool of organs from ~60 seedlings.

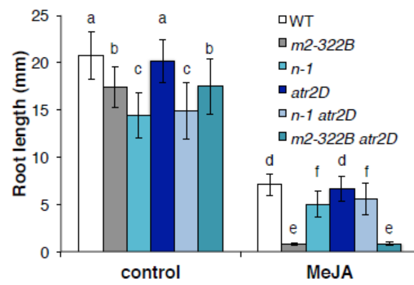

Figure S12: Root length of 7-do seedlings of mutant combinations with *atr2D* grown in control conditions or in the presence of 25 µM MeJA. *m2-322B* refers to *myc2-322B* and *n-1* to *ninja-1*. Data shown are means (± SD) from 27–52 plants. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test (P < 0.001).

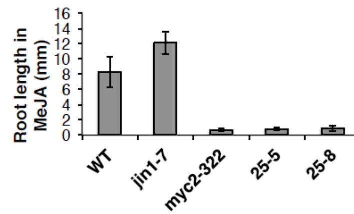

Figure S13: Functionality of the MYC2^{E165K}-CITRINE fusion protein. Root length of 7-do WT, a *myc2* null mutant (*jin1-7*), *myc2-322B* and two independent lines of *jin1-7* transformed with a *MYC2$_{pro}$-MYC2^{E165K}-CITRINE* construct (25–5, 25–8) grown on MS media supplemented with 25µM MeJA. Note the JA hypersensitive phenotype in the *jin1-7* transformed lines (for explanations, refer to Fig 4). Data shown are means (± SD) from 13–27 plants.

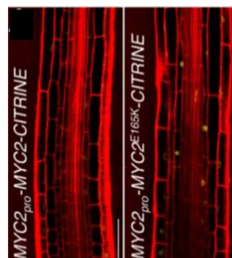

Figure S14: MYC2^{E165K}-CITRINE accumulates at higher levels than MYC2-CITRINE. C2 Confocal microscopy images representing the expression pattern of functional *MYC2$_{pro}$-MYC2-CITRINE* and *MYC2$_{pro}$-MYC2^{E165K}-CITRINE* fluorescent reporters (yellow) in the elongation zone of 5-do *jin1-7 (myc2* null mutant) roots stained with propidium iodide (red). Scale bar = 50 µm.

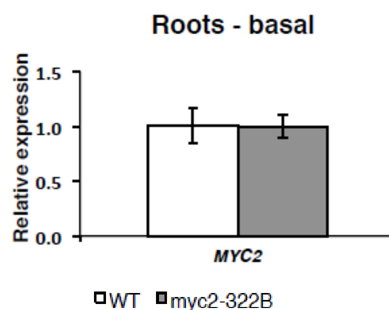

Figure S15: qRT-PCR of *MYC2* expression in 5-do roots of WT and *myc2-322B*. Transcript levels were normalized to those of *UBC21* and displayed relative to the expression in the WT control. Bars represent the means of three biological replicates (±SD), each containing a pool of ~60 roots.

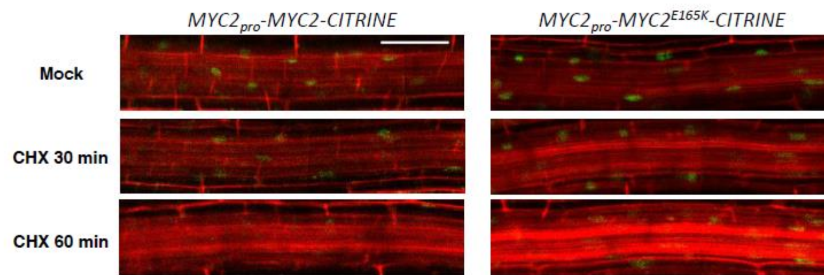

Figure S16: MYC2-CITRINE and $MYC2^{E165K}$-CITRINE are degraded similarly following cycloheximide (CHX) treatment. 5-do *jin1-7* (*myc2* null mutant) seedlings transformed with either *$MYC2_{pro}$-MYC2-CITRINE* or *$MYC2_{pro}$-$MYC2^{E165K}$-CITRINE* were treated with 100 µM CHX for the indicated times, after which primary roots were stained with propidium iodide (red) and examined by confocal microscopy. Details of the vascular tissues in the elongation zone where the florescent (green) signal was more intense are shown. The experiment was repeated three times with two independent lines for each reporter. Scale bar = 50 µm. Note: because the expression of chimeric proteins is under the control of *MYC2* endogenous promoter, their expression level was too low to be detected in Western blots from 5-do seedlings. Moreover, although we analysed 24 independent T2 lines, we failed to recover transgenic lines overexpressing $MYC2^{E165K}$ protein under the *UBIQUITIN 10* promoter (At4g05320), suggesting that this protein version may be harmful to plants if expressed constitutively.

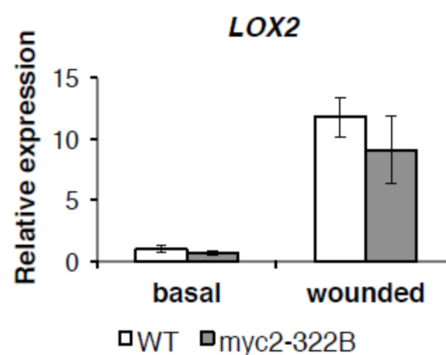

Figure S17: qRT-PCR of basal and 1 h after wounding one cotyledon *LOX2* expression in 5-do WT and *myc2-322B* entire seedlings. Transcript levels were normalized to those of *UBC21* and displayed relative to the expression in the unwounded WT control. Bars represent the means of three biological replicates (±SD), each containing a pool of ~40 seedlings.

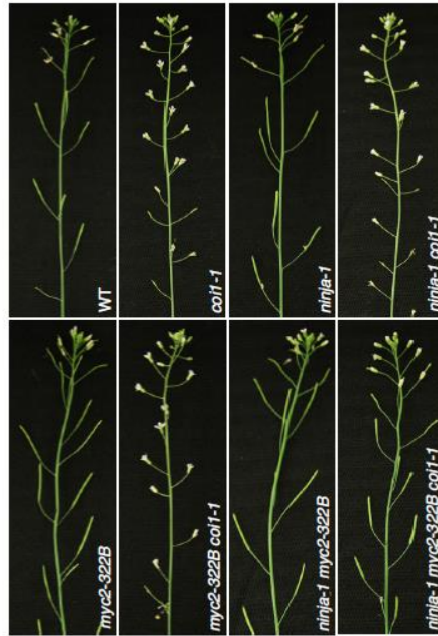

Figure S18: Fertility phenotypes of mutant combinations with *myc2-322B*. Main inflorescences from 5 week-old plants of indicated genotypes. Note the lack of sterility in the *ninja-1 myc2-322B coi1-1* triple mutant.

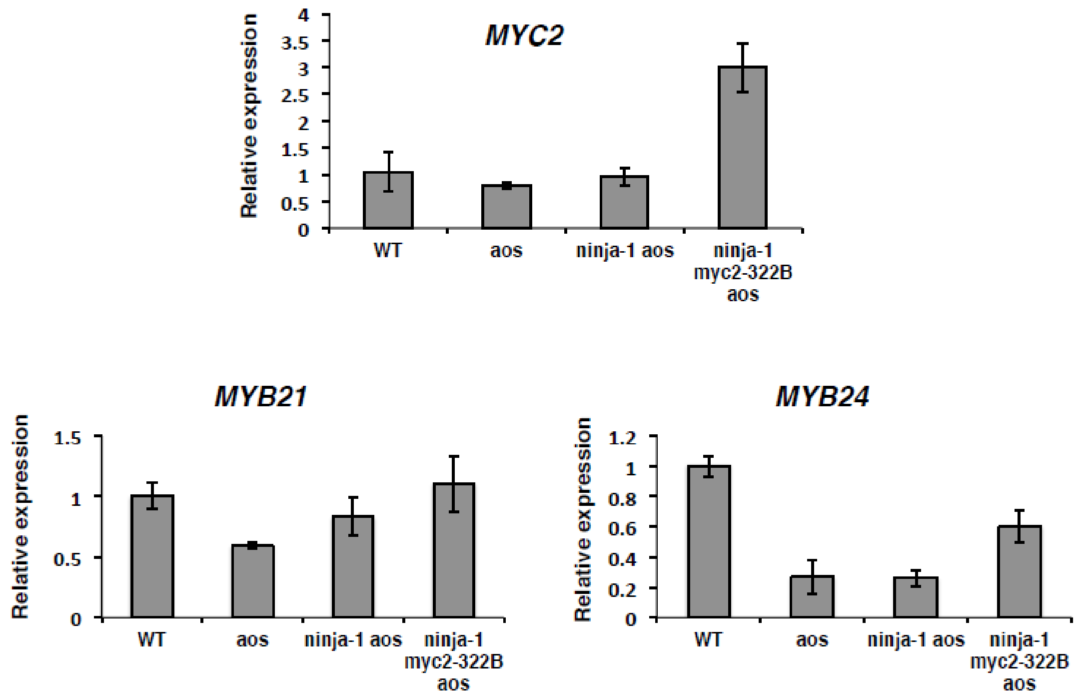

Figure S19: qRT-PCR of *MYC2*, *MYB21* and *MYB24* expression in floral organs of mutant combinations with *myc2-322B*. Transcript levels were normalized to those of *UBC21* and displayed relative to the expression in the WT controls. Bars represent the means of three biological replicates (±SD), each consisting of equivalent stage 12 flower buds from 3–4 inflorescences of the same plant.

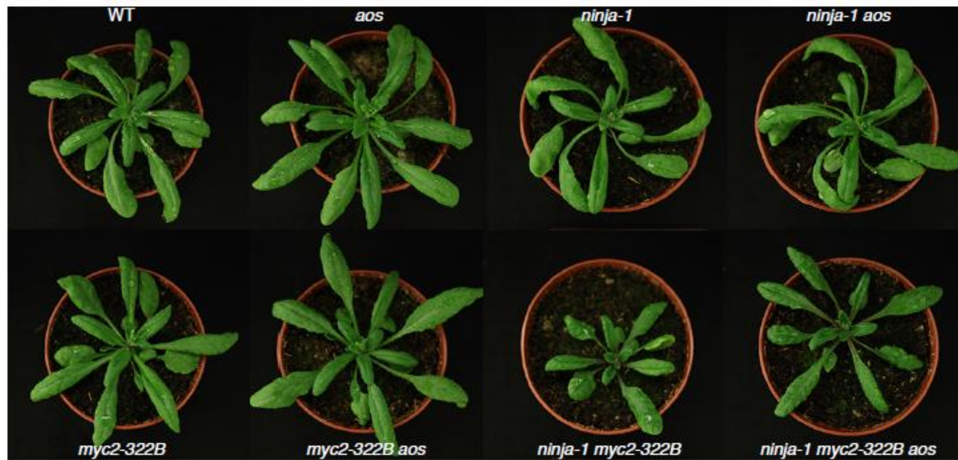

Figure S20: Rosette phenotypes of mutant combinations with *myc2-322B*. Plants were grown in continuous days for 4 weeks. Scale (diameter of each pot) = 7cm.

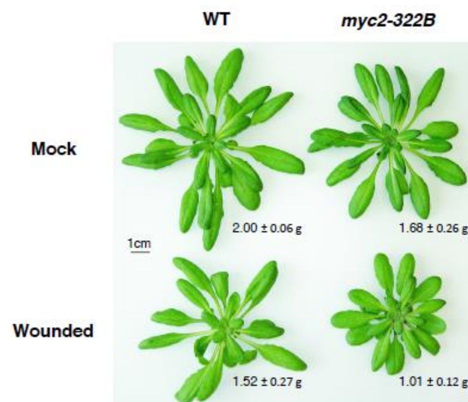

Figure S21: *myc2-322B* is hypersensitive to repetitive wounding in adult rosettes. Rosette phenotypes of WT and *myc2-322B* plants grown in short days for 5 weeks. At the age of two weeks, plants were wounded 5 times on different leaves at 3-d intervals or gently touched on the same leaf (Mock). Leaves were treated in the following order: leaf 2 (L2), L4, L5, L6 and L8. Numbers below plants indicate rosette mean fresh weight ± SD, n = 6.

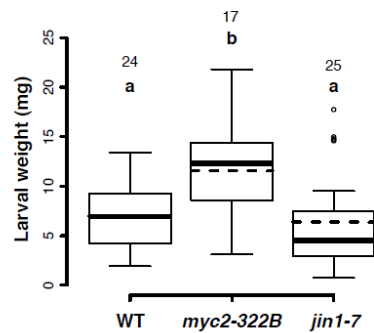

Figure S22: Box plot summary of *S. littoralis* larval weights after feeding for 10 d on adult *myc2-322B* plants. Medians and means are represented inside the boxes by solid and dotted lines respectively. Circles depict outlier data points beyond ±1.5X the interquartile range defined by the whiskers; numbers indicate n. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test.

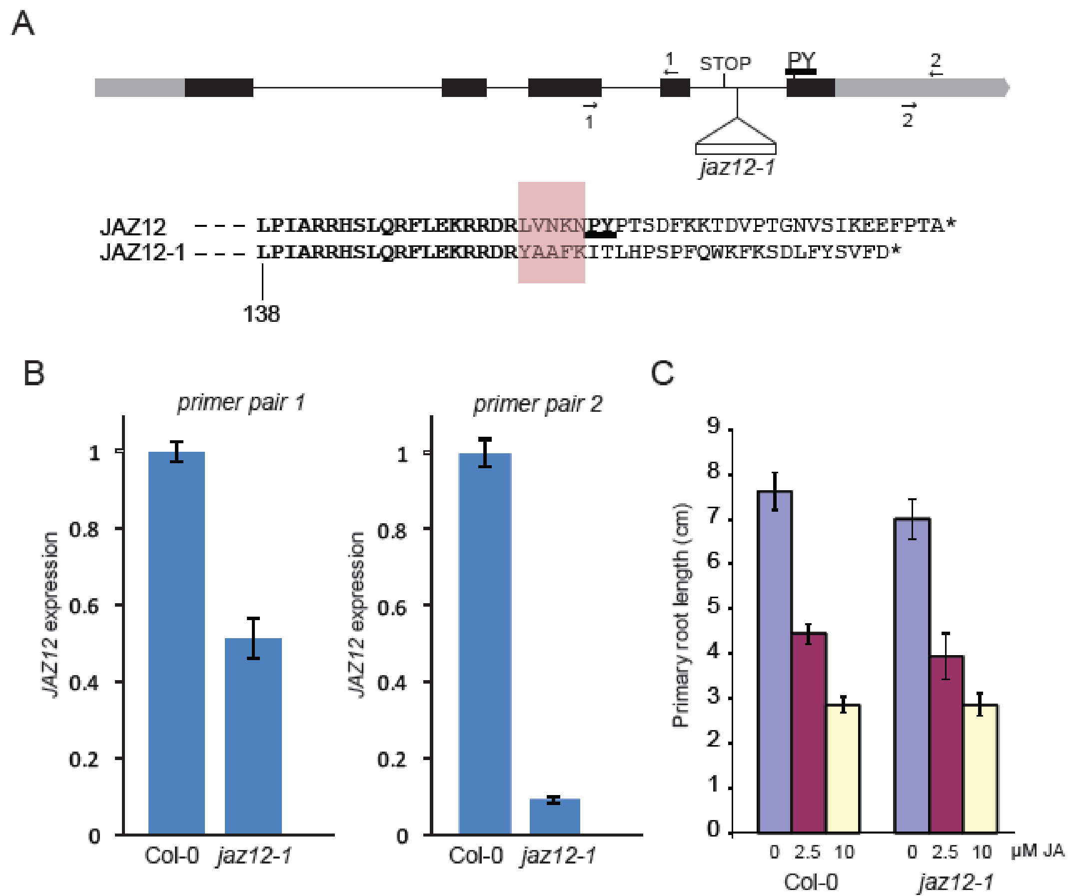

Figure S23. Characterization of the *jaz12-1* T-DNA insertion line. A) Schematic diagram of the *JAZ12* (AT5G20900) locus. Black bars, black lines and grey bars represent exons, introns and the untranslated regions, respectively. The T-DNA in the *jaz12-1* line (SALK_055032) is inserted in the Jas intron. The 5th exon contains the conserved PY motif. Arrows and numbers indicate different primer combinations covering different regions of *JAZ12*. Primer sequences can be found in Table S2. An alignment is shown between the WT JAZ12 and the JAZ12-1 amino acid sequences. While the N-terminal part of the Jas motif (in bold) is unaffected, the sequence essential for KEG interaction (highlighted in pink) and the PY motif (in bold underlined) are lost. B) RT-qPCR analysis of *JAZ12* expression in Col-0 and *jaz12-1* seedlings. Error bars represent +/- SE of four biological replicates. C) Primary root length of Col-0 and *jaz12-1* seedlings grown on MS with 0, 2.5 or 10 µM JA. Error bars represent +/- SE (12≤n<20). For B) and C) seedlings were grown for 10 days in continuous light.

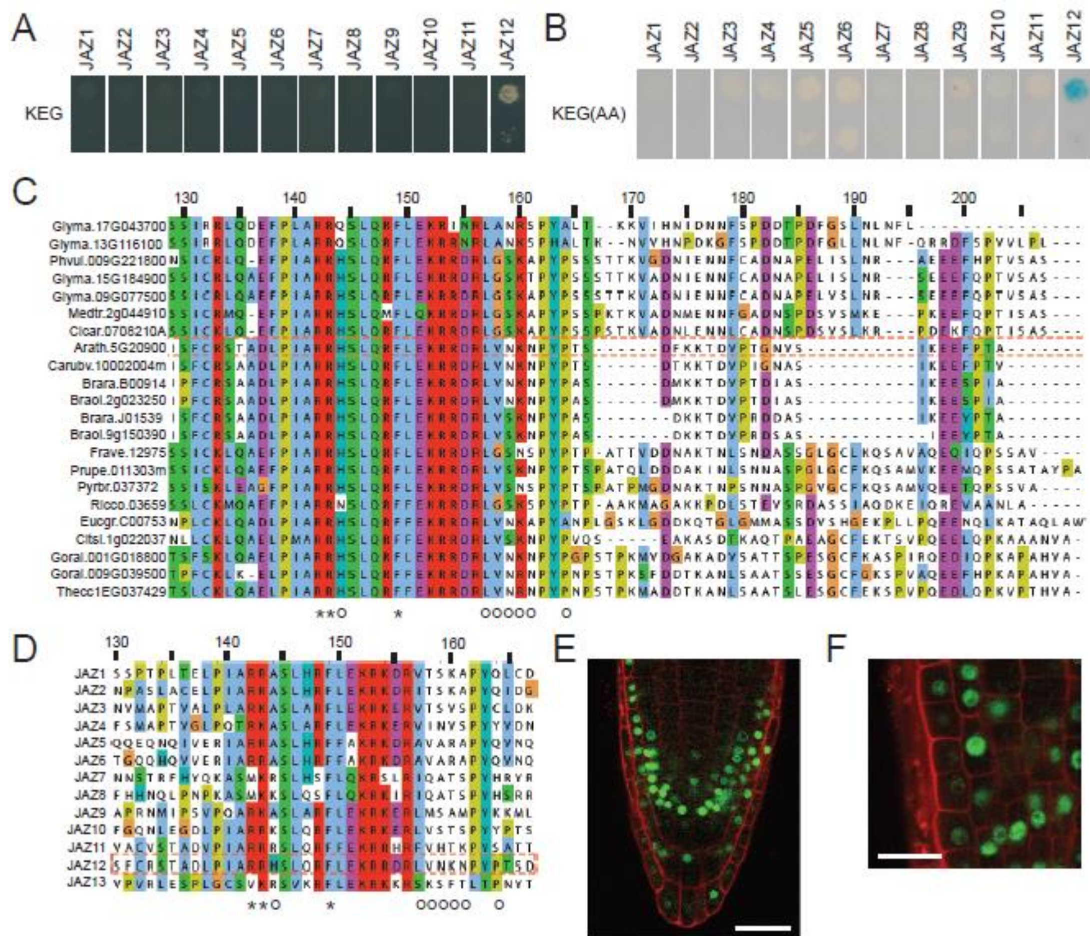

Figure S24. KEG interacts specifically with JAZ12. A) Only JAZ12 interacts directly with KEG in the GAL4-based Y2H system. The PJ69-4A yeast strain was co-transformed with JAZ proteins in pGBKT7 and KEG in pGADT7gate. 10x and 100x dilutions of transformed yeasts were spotted on selective medium lacking His (-3). B) Only JAZ12 interacts directly with the RING domain mutant KEG(AA) in the LexA-based Y2H system. The EGY48 (p8opLacZ) yeast strain was co-transformed with KEG(AA)in pGILDA and JAZ in pB42AD. Transformed yeasts were spotted on inducing Gal/Raf medium supplemented with X-Gal. C) partial alignment of the amino acid sequences of JAZ12 and its orthologues from other plant species. D, alignment of the Jas motif of Arabidopsis JAZ proteins. C-D) Amino acids essential for COI1 interaction are marked with an asterisk, those mutated to test KEG interaction with a circle. Arabidopsis JAZ12 is boxed. E)-F) Confocal root tip imaging of Arabidopsis seedlings stably overexpressing the JAZ12-GFP fusion protein. Propidium iodide staining was used to enhance visualization of the cells. Scale bars are 30 µm (E) and 15µm (F).

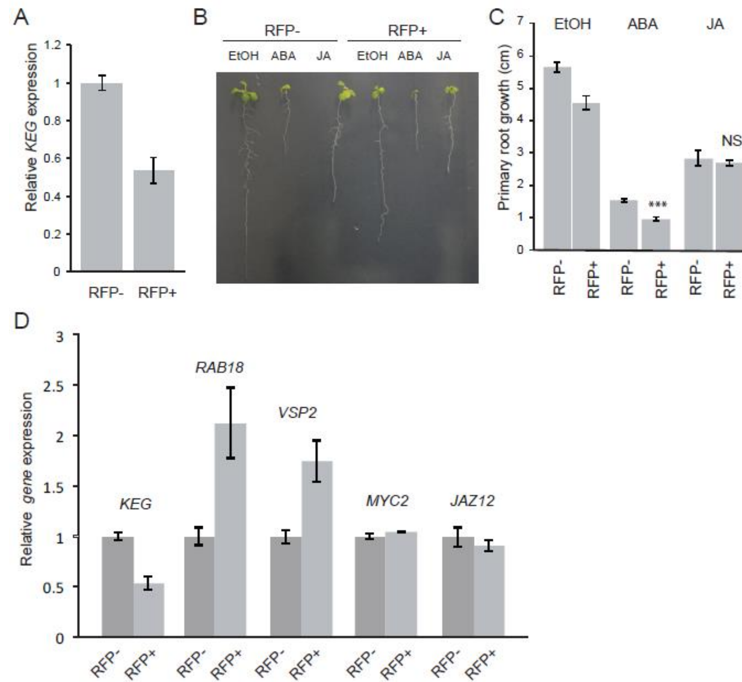

Figure S25. *KEG* knock-down lines are hypersensitive to ABA. Seeds of *KEG* amiRNA Line 2 were separated based on RFP fluorescence using an epifluorescence microscope. Plants were grown for 4 days and transferred to media containing EtOH (control), 5 µM ABA or 2.5 µM JA for 6d. A) relative *KEG* expression. B) Representative 11-day-old WT and *KEG* knock-down lines. C) quantification of increase in primary root length after transfer in (A). Error bars depict standard error (n=3). Treatment × genotype interaction effect (two-way ANOVA) is indicated (***, p-value < 0.001). There were also a significant effects for ABA treatment and of the genotype (P<0.001), and for JA treatment (p<0.05). D) expression of *KEG*, *RAB18*, *VSP2*, MYC2 and J*AZ12* in Line 2. Error bars depict standard error (n=3).

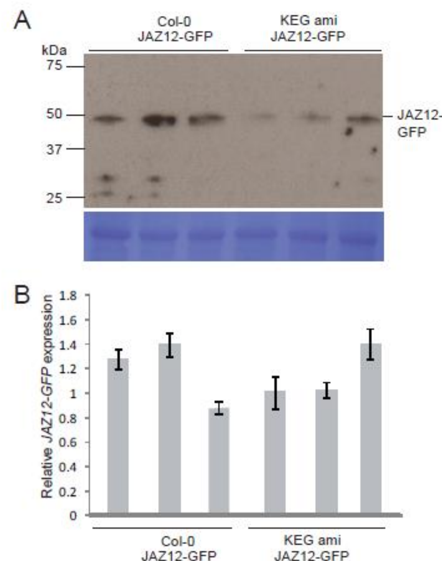

Figure S26. KEG is a positive regulator of JAZ12 stability. A) Immunoblot showing JAZ12-GFP levels in 3 biological repeats of a JAZ12-GFP line and its cross with the *KEG* amiRNA line 14. 6-day-old seedlings were used. Membrane was stained with Coomassie to inspect equal loading. B) *JAZ12-GFP* transcript levels in samples used in (A). Error bars depict standard error of 3 technical replicates.

Table S2: Primers used in this study.

ID		Sequence	Use
JAZ12 pair 1	Fw	CATCTAATGTGGCATCACCAG	qPCR
JAZ12 pair 1	Rv	TGCCTCCTTGCAATAGGTAGA	qPCR
JAZ12 pair 2	Fw	CTATCATGTACGCTGCTGTGTG	qPCR
JAZ12 pair 2	Rv	CCACTCCCAGACATGGAAAC	qPCR
JAZ12(ZIM)	Rv	AGAAAGCTGGGTTTTTAGCAGCAATACGAAGGA	Cloning
JAZ12(Jas)	Fw	AAAAAGCAGGCTACGCAATGGAGACAAAGAATTC	Cloning
JAZ12-1	Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCM**ATCAAAAACTGAATAAAATAG	Cloning
JAZ12(RR142/143AA)	Fw	CGGCTGATCTACCTATTGCA**GCGGCG**CATTCGCTTC	Mutagenesis
JAZ12(RR142/143AA)	Rv	TGCAATAGGTAGATCAGCCGTGGATCTGCA	Mutagenesis
JAZ12(F149A)	Fw	AGGCATTCGCTTCAACGAGCCCTCGAGAAAAGA	Mutagenesis
JAZ12(F149A)	Rv	TCGTTGAAGCGAATGCCTCCTTGCAATAGG	Mutagenesis
JAZ12(H144A)	Fw	CTACCTATTGCAAGGAGGGCTTCGCTTCAACG	Mutagenesis
JAZ12(H144A)	Rv	CCTCCTTGCAATAGGTAGATCAGCCGTGGA	Mutagenesis
JAZ12(P164A)	Fw	GTCAACAAAAACCCTTACGCTACTTCAGACT	Mutagenesis
JAZ12(P164A)	Rv	GTAAGGGTTTTTGTTGACCAATCTGTCCCG	Mutagenesis
JAZ12 (LVNKN157-161AAAAA)	Fw	GAGAAAGACGGGACAGAGCGGCTGCCGCAGCCCCTTACCCTACT	Mutagenesis
JAZ12 (LVNKN157-161VTSKA)	Fw	GAGAAAAGACGGGACAGAGTGACCTCCAAAGCCCCTTACCCTACT	Mutagenesis
JAZ12 (LVNKN157-161)	Rv	TCTGTCCCGTCTTTTCTCGAGGAATCGTTG	Mutagenesis
KEG amiRA	miR-s	GATTCTCTACTAACGTACAGCTATCTCTCTTTTGTATTCC	Cloning
KEG amiRA	miR-a	GATAGCTGTACGTTAGTAGAGAATCAAAGAGAATCAATGA	Cloning
KEG amiRA	miR*s	GATAACTGTACGTTACTAGAGATTCACAGGTCGTGATATG	Cloning
KEG amiRA	miR*a	GAATCTCTAGTAACGTACAGTTATCTACATATATATTCCT	Cloning
jaz12-1	Fw	AGTTATGGCACACTCCCATTG	Genotyping
jaz12-1	Rv	AGCATCAGTCCTGTCTCATCG	Genotyping
SALK LB1.3	-	ATTTTGCCGATTTCGGAAC	Genotyping
KEG	Fw	TTTGATGGACAGGTGCTTTG	qPCR
KEG	Rv	GAGCAACATCAGCCCCATA	qPCR
RAB18	Fw	GGCTTGGGAGGAATGCTT	qPCR
RAB18	Rv	TTGATCTTTTGTGTTATTCCCTTCT	qPCR
VSP2	Fw	ATGCCAAAGGACTTGCCCTA	qPCR
VSP2	Rv	CGGGTCGGTCTTCTCTGTTC	qPCR
MYC2	Fw	TCCGAGTCCGGTTCATTCT	qPCR
MYC2	Rv	TCTCGGGAGAAAGTGTTATTGAA	qPCR
GFP	Fw	GAAGCGCGATCACATGGT	qPCR
tNOS	Rv	ATTGCCAAATGTTTGAACGA	qPCR
UBC	Fw	CTGCGACTCAGGGAATCTTCTAA	qPCR
UBC	Rv	TTGTGCCATTGAATTGAACCC	qPCR
PP2A	Fw	TAACGTGGCCAAAATGATGC	qPCR
PP2A	Rv	GTTCTCCACAACCGCTTGGT	qPCR
JAZ10	Fw	AAAAAGCAGGCTCGATGTCGAAAGCTACCATAGA	qPCR
JAZ10	Rv	AGAAAGCTGGGTTTAGGCCGATGTCGGATAGT	qPCR
AFB2	Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAATTATTTCCCAGATG	Cloning
AFB2	Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCMGAGAATCCACACAAATGGCG	Cloning
COI1	Fw	AAAAAGCAGGCTATCCGATGGAGGATCCTGATA	Cloning
COI1	Rv	AGAAAGCTGGGTATATTGGCTCCTTCAGGACT	Cloning

Table S3: Results from the yeast-two hybrid screen using PPD2 as bait, followed by next generation sequencing.

tracking_id	PPD2 FPKM	gene_alias	annotation	PPD2/NINJA	NINJA FPKM
AT5G15090	31076,3	ATVDAC3	ATVDAC3 VDAC3 voltage dependent anion channel 3	113,9946151	272,612
AT4G11360	20574,4	RHA1B	RHA1B RING-H2 finger A1B	13,3253886	1544
AT3G31161	17900,4			261,0845414	68,5617
AT2G40765	16978,3			28,38682171	598,105
AT3G49580	14542	LSU1	LSU1__response to low sulfur 1	77,91177999	186,647
AT5G58290	12131	RPT3	RPT3 regulatory particle triple-A ATPase 3	218,543106	55,5085
AT5G60390	10589,9		GTP binding Elongation factor Tu family protein	10,1721306	1041,07
AT5G21020	10526,2			6,742507222	1561,17
AT3G11260	7810,76	WOX5WOX5B	WOX5 WOX5B WUSCHEL related homeobox 5	42,2921066	184,686
AT3G49570	7215,01	LSU3	LSU3 response to low sulfur 3	35,50047482	203,237
AT3G46040	6699,22	RPS15AD	RPS15AD__ribosomal protein S15A D	8,424254618	795,23
AT5G66670	4181,08		Protein of unknown function (DUF677)	4222,366298	0,990222
AT1G07930	3742,87		GTP binding Elongation factor Tu family protein	13,33158802	280,752
AT3G55620	3164,78	eIF6Aemb1624	eIF6A_emb1624__Translation initiation factor IF6	10,62712809	297,802
AT5G17340	3073,81		Putative membrane lipoprotein	33,20352839	92,5748
AT5G47030	2643,55		ATPase, F1 complex, delta/epsilon subunit	8,531763962	309,848
AT4G14800	2500,13	PBD2	PBD2__20S proteasome beta subunit D2	6,758185765	369,941
AT2G37130	2144,47		Peroxidase superfamily protein	38,88064951	55,1552
AT3G11930	1893,71		Adenine nucleotide alpha hydrolases-like superfamily protein	51,3718437	36,8628
AT1G07920	1775,07		GTP binding Elongation factor Tu family protein	9,015221156	196,897
AT1G07940	1556,18		GTP binding Elongation factor Tu family protein	6,330644341	245,817
AT2G39280	1176,5		Ypt/Rab-GAP domain of gyp1p superfamily protein	53,82788802	21,8567
AT3G11580	1087,45	NGAL2	AP2/B3-like transcriptional factor family protein	22,03800642	49,3443
AT1G63840	1031,04		RING/U-box superfamily protein	9,507404607	108,446
AT4G39404	1019,84		other RNA	43,47310852	23,4591
AT4G32690	997,716	ATGLB3	ATGLB3_GLB3__hemoglobin 3	7,13627878	139,809
AT4G01850	949,556	AtSAM2MAT2SAM-2	AtSAM2 MAT2 SAM-2 SAM2 S-adenosylmethionine synthetase 2	9,765738933	97,2334
AT4G28910	946,8	NINJA	NINJA novel interactor of JAZ	49,38838319	19,1705
AT3G22680	928,626	RDM1	RDM1 RNA-DIRECTED DNA METHYLATION 1	6,439356221	144,211
AT3G03180	830,298		Got1/Sft2-like vescicle transport protein family	181,5394707	4,57365
AT3G15110	820,456			68,64361969	11,9524
AT3G27830	789,387	RPL12RPL12-A	RPL12_RPL12-A__ribosomal protein L12-A	33,87214706	23,3049
AT1G12520	581,447	ATCCS	ATCCS_CCS__copper chaperone for SOD1	9,512114123	61,127
AT5G11670	571,331	ATNADP-ME2	ATNADP-ME2_NADP-ME2__NADP-malic enzyme 2	13,65096242	41,8528
AT3G55430	506,815		O-Glycosyl hydrolases family 17 protein	8,683111779	58,3679
AT5G26110	495,462		Protein kinase superfamily protein	14,14785139	35,0203
AT4G23570	480,642	SGT1A	SGT1A__phosphatase-related	24,85235187	19,3399
AT5G17620	447,087	AUG7	AUG7	25,42477267	17,5847
AT5G59060	438,345			46,27064971	9,4735
AT2G39290	432,837	PGP1PGPS1PGS1	PGP1_PGPS1_PGS1__phosphatidylglycerolphosphate synthase 1	15,94989185	27,1373
AT4G20480	391,773		Putative endonuclease or glycosyl hydrolase	12,81480706	30,5719
AT4G20360	385,239	ATRAB8DATRABE1BRABE1b	ATRAB8D ATRABE1B RABE1b RAB GTPase homolog E1B	37,60814175	10,2435
AT1G35580	353,32	A/N-InvGCINV1	A/N-InvG_CINV1__cytosolic invertase 1	14,15924948	24,9533
AT3G04730	351,039	IAA16	IAA16 indoleacetic acid-induced protein 16	116,2153751	3,02059
AT1G73380	339,253			33,98358184	9,98285
AT1G65030	330,525		Transducin/WD40 repeat-like superfamily protein	19,34150238	17,0889
AT3G18800	327,587			13,30280929	24,6254
AT2G20230	304,68		Tetraspanin family protein	9,256654322	32,9147
AT3G19508	286,103			7,576017562	37,7643
AT3G21610	278,023		Acid phosphatase/vanadium-dependent haloperoxidase-related protein	8,141563637	34,1486

AT4G17050	267,967	UGLYAH	UGLYAH__ureidoglycine aminohydrolase	6,904550865	38,8102
AT3G17210	263,055	ATHS1	ATHS1 HS1 heat stable protein 1	18,83188007	13,9686
AT5G66680	262,36	DGL1	DGL1__dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kDa subunit family	11,58644568	22,6437
AT2G33590	254,34	AtCRL1	AtCRL1 CRL1 NAD(P)-binding Rossmann-fold superfamily protein	8,902906028	28,5682
AT1G32700	247,911		PLATZ transcription factor family protein	346,7199427	0,715018
AT3G29090	239,782	ATPME31	ATPME31 PME31 pectin methylesterase 31	22,24549815	10,7789
AT3G02200	239,734		Proteasome component (PCI) domain protein	32,03355497	7,48384
AT4G32295	239,707	KIX9		12,50812718	19,1641
AT3G26470	228		Powdery mildew resistance protein, RPW8 domain	23,09244269	9,87336
AT1G04870	226,463	ATPRMT10	ATPRMT10 PRMT10 protein arginine methyltransferase 10	14,74435684	15,3593
AT2G21250	214,453		NAD(P)-linked oxidoreductase superfamily protein	7,165152021	29,93
AT3G54680	204,582		proteophosphoglycan-related	10,5227911	19,4418
AT2G47550	202,802		Plant invertase/pectin methylesterase inhibitor superfamily	62,82473064	3,22806
AT2G40530	199,975			6,084388853	32,8669
AT2G16780	198,057	MSI02MSI2NFC02NFC2	MSI02_MSI2_NFC02_NFC2__Transducin family protein / WD-40 repeat family protein	8,934405153	22,1679
AT1G12200	181,681	FMO	FMO Flavin-binding monooxygenase family protein	46,01639236	3,94818
AT2G01060	179,638		myb-like HTH transcriptional regulator family protein	7,819016736	22,9745
AT1G08160	179,201		Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	65,63129482	2,73042
AT4G24030	173,749			4217034,734	4,1202E-05
AT3G27010	169,201	AT-TCP20ATTCP20PCF1	AT-TCP20_ATTCP20_PCF1_TCP20__TEOSINTE BRANCHED 1, cycloidea, PCF (TCP)-domain	21,11736668	8,01241
AT4G32960	162,969			10,81657441	15,0666
AT3G01345	161,463		Expressed protein	9,08928682	17,7641
AT4G06744	161,249		Leucine-rich repeat (LRR) family protein	8,532596042	18,898
AT2G41130	149,599		basic helix-loop-helix (bHLH) DNA-binding superfamily protein	9,506558044	15,7364
AT5G23380	149,535		Protein of unknown function (DUF789)	83,46869401	1,79151
AT1G31180	148,509	ATIMD3IPMDH1	ATIMD3_IMD3_IPMDH1__isopropylmalate dehydrogenase 3	6,369239083	23,3166
AT5G58560	146,291	FOLK	FOLK Phosphatidate cytidylyltransferase family protein	9,378229374	15,599
AT3G17000	141,746	UBC32	UBC32__ubiquitin-conjugating enzyme 32	20,09968591	7,05215
AT3G51790	130,826	AtCCMEATG1	ATG1_AtCCME_G1__transmembrane protein G1P-related 1	27,51728954	4,75432
AT1G80940	125,573			6,424551566	19,5458
AT5G57490	125,44	ATVDAC4	ATVDAC4 VDAC4 voltage dependent anion channel 4	10,86587436	11,5444
AT5G20280	121,364	ATSPS1FSPSA1	ATSPS1F_SPS1F_SPSA1__sucrose phosphate synthase 1F	52,35020338	2,31831
AT3G08740	113,293		elongation factor P (EF-P) family protein	43,8734137	2,58227
AT4G22470	109,902		protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	9,912779948	11,0869
AT2G43000	107,527	ANAC042JUB1	ANAC042 JUB1 NAC042 NAC domain containing protein 42	18,72617366	5,74207
AT1G29840	104,74		alpha/beta-Hydrolases superfamily protein	7,86832537	13,3116
AT4G25180	102,057		RNA polymerase III RPC4	13,55493456	7,52914
AT5G41580	98,7968		RING/U-box superfamily protein	36,92123713	2,67588
AT2G30170	96,6534	PBCP	PBCP Protein phosphatase 2C family protein	28,25494919	3,42076
AT3G55020	96,3275		Ypt/Rab-GAP domain of gyp1p superfamily protein	7,374523434	13,0622
AT2G20495	91,1067			8,048721664	11,3194
AT2G40780	89,3645		Nucleic acid-binding, OB-fold-like protein	10,3550385	8,63005
AT1G51170	88,0884	AGC2-3UCN	AGC2-3_UCN__Protein kinase superfamily protein	18,54906569	4,74894
AT5G67380	86,9604	ATCKA1	ATCKA1_CKA1__casein kinase alpha 1	6,630555619	13,1151
AT5G13000	84,2391	ATGSL12CALS3	ATGSL12_CALS3_GSL12__glucan synthase-like 12	8,768393891	9,60713
AT5G12080	82,4243	ATMSL10	ATMSL10 MSL10 mechanosensitive channel of small conductance-like 10	14,04291025	5,86946
AT5G41992	82,1024	CPuORF58	CPuORF58__conserved peptide upstream open reading frame 58	7,506711041	10,9372
AT1G15170	75,6147		MATE efflux family protein	18,47424126	4,09298
AT3G19663	74,948			#N/A	#N/A
AT1G53310	74,4374	ATPEPC1ATPPC1	ATPEPC1 ATPPC1 PEPC1 PPC1 phosphoenolpyruvate carboxylase 1	7,897373003	9,42559
AT5G35460	73,35			14,19338609	5,1679
AT2G42840	72,9741	PDF1	PDF1 protodermal factor 1	12,27823608	5,94337
AT4G29810	70,1459	ATMKK2MK1	ATMKK2_MK1_MKK2__MAP kinase kinase 2	21,58700703	3,24945

AT1G05620	69,1093	NSH2URH2	NSH2_URH2__uridine-ribohydrolase 2	9,296093879	7,43423
AT5G09800	65,5628		ARM repeat superfamily protein	6,046276571	10,8435
AT3G54970	64,9741		D-aminoacid aminotransferase-like PLP-dependent enzymes superfamily protein	6,348784944	10,2341
AT3G12150	64,8199			13,75909031	4,71106
AT5G25265	64,3723			11,09052661	5,80426
AT4G29570	62,2964		Cytidine/deoxycytidylate deaminase family protein	42,26923599	1,4738
AT3G12630	60,2768	SAP5	SAP5__A20/AN1-like zinc finger family protein	15,93241843	3,78328
AT5G06730	60,2631		Peroxidase superfamily protein	6,888998632	8,74773
AT1G71480	58,9522		Nuclear transport factor 2 (NTF2) family protein	14,76732622	3,99207
AT5G23405	58,5115		HMG-box (high mobility group) DNA-binding family protein	109,4131001	0,534776
AT3G55760	56,3432			17,36067417	3,24545
AT1G06510	55,2608			13,37641998	4,13121
AT2G35980	55,1375	ATNHL10YLS9	ATNHL10_NHL10_YLS9__Late embryogenesis abundant (LEA) hydroxyproline-rich	57,78376532	0,954204
AT2G29580	53,7311	MAC5B	MAC5B CCCH-type zinc fingerfamily protein with RNA-binding domain	19,26280845	2,78937
AT2G17800	50,4349	ARAC1ATGP2ATRAC1ATROP3	ARAC1_ATGP2_ATRAC1_ATROP3_ROP3 Arabidopsis RAC-like 1	10,98660736	4,59058

The resulting fragments per kilobase of transcript per million fragments mapped (PPD2 FPKM) values were standardized (PPD2/NINJA) to the FPKM results of a Y2H screen where NINJA was used as bait (NINJA FPKM). A cut-off value of six was used to remove potential background proteins. The remaining genes were then ordered by highest PPD2 FPKM value with a minimum value threshold of 50. The candidate genes tested for PPD2 interaction are highlighted by a dotted box. NINJA and KIX9, two positive controls, are highlighted by a lined box.

Table S4: Primer sequences used

Sequence	Type	Gene/plasmid	Comment
TGGATGTCGGAGTTTCACCG	Fw	PPD1	RT-PCR
TGGCCTTTGAGAATTTTCTGTCT	Rv	PPD1.1	RT-PCR; Spanning exon 7 and 8
GCGATTTTGGAATAAGCGTAAGT	Rv	PPD1.2	RT-PCR; Jas intron specific
TTGGCCGGAATCTGACAAGG	Fw	PPD2	RT-PCR
GAAAATCTTCTGTCTTTCCGCTT	Rv	PPD2.1	RT-PCR; Spanning exon 7 and 8
GGAATAAGCAAGCACACAGCA	Rv	PPD2.2	RT-PCR; Jas intron specific
GCGTATAACGCGTTTGGAAT	Fw	pDEST™22	
AGCCGACAACCTTGATTGGAGAC	Rv	pDEST™22	

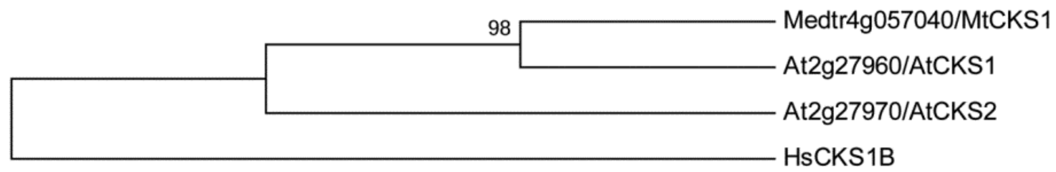

Figure S27. Sequence analysis of MtCKS1 encoded by *Medtr4g057040*. Neighbour-joining phylogenetic tree showing the relation of MtCKS1 with the CKS sequences used to make the multiple sequence alignment. Bootstrap value is indicated in percentage at branch nodes. For the sequence alignment we refer to Fig. S1 in the online supporting information of this article.

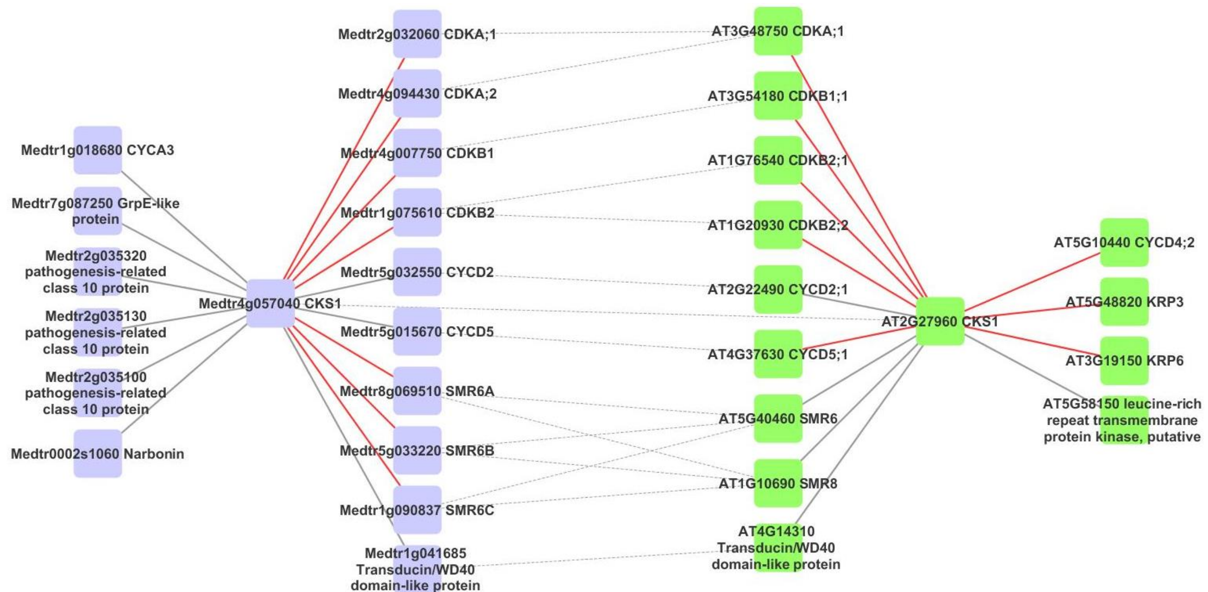

Figure S28. Overview of the protein interactors of CKS1 identified by TAP in Arabidopsis and *M. truncatula*. *M. truncatula* and Arabidopsis proteins are represented in purple and green, respectively. Full lines (black and red) indicate interactions identified by TAP. CKS1 interactors in Arabidopsis and *M. truncatula* have been identified in Van Leene *et al.* (2010) and this study, respectively. Dotted lines represent orthologous gene pairs between Arabidopsis and *M. truncatula* based on Plaza 3.0 (Proost *et al.*, 2015). Red lines represent TAP interactions that have been confirmed in binary Y2H assays for both Arabidopsis (Boruc *et al*., 2010a; Van Leene *et al*., 2011) and *M. truncatula* (this study).

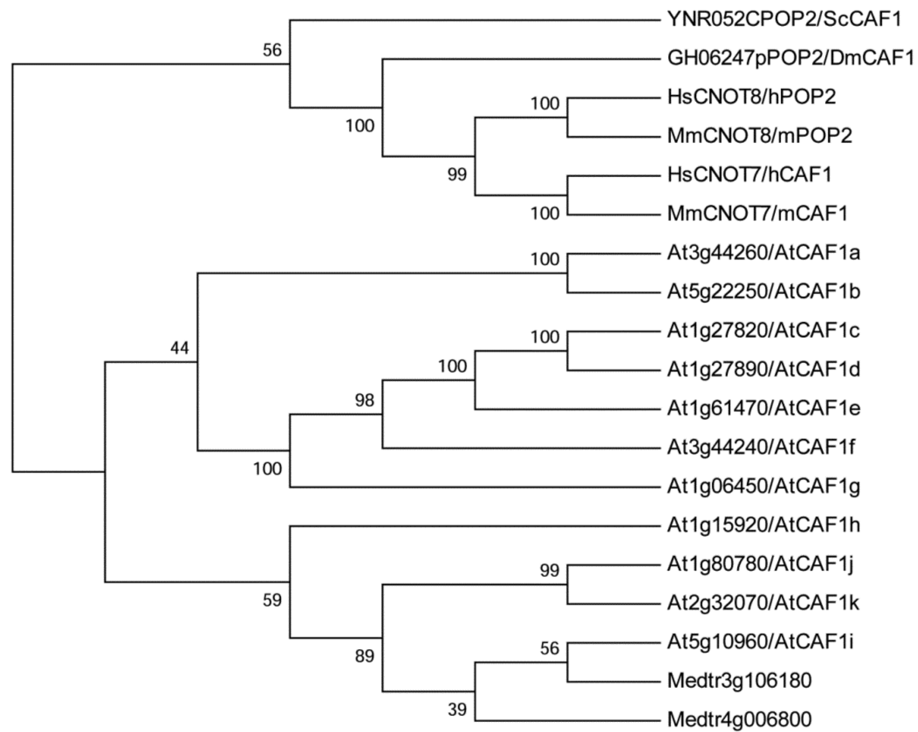

Figure S29. Sequence analysis of MtCAF1 encoded by *Medtr4g006800*. Neighbour-joining phylogenetic tree showing the relation of two MtCAF1 members (Medtr3g106180 and Medtr4g006800) and the 11 CAF1 family members (AtCAF1a-k) from A. thaliana (Walley et al., 2010) and CAF1 from yeast (ScCAF1), human (HsCNOT7 and HsCNOT8), mouse (MmCNOT7 and MmCNOT8) and fly (DmCAF1). Bootstrap values are indicated in percentage at branch nodes. For the sequence alignment we refer to Fig. S3 in the online supporting information of this article.

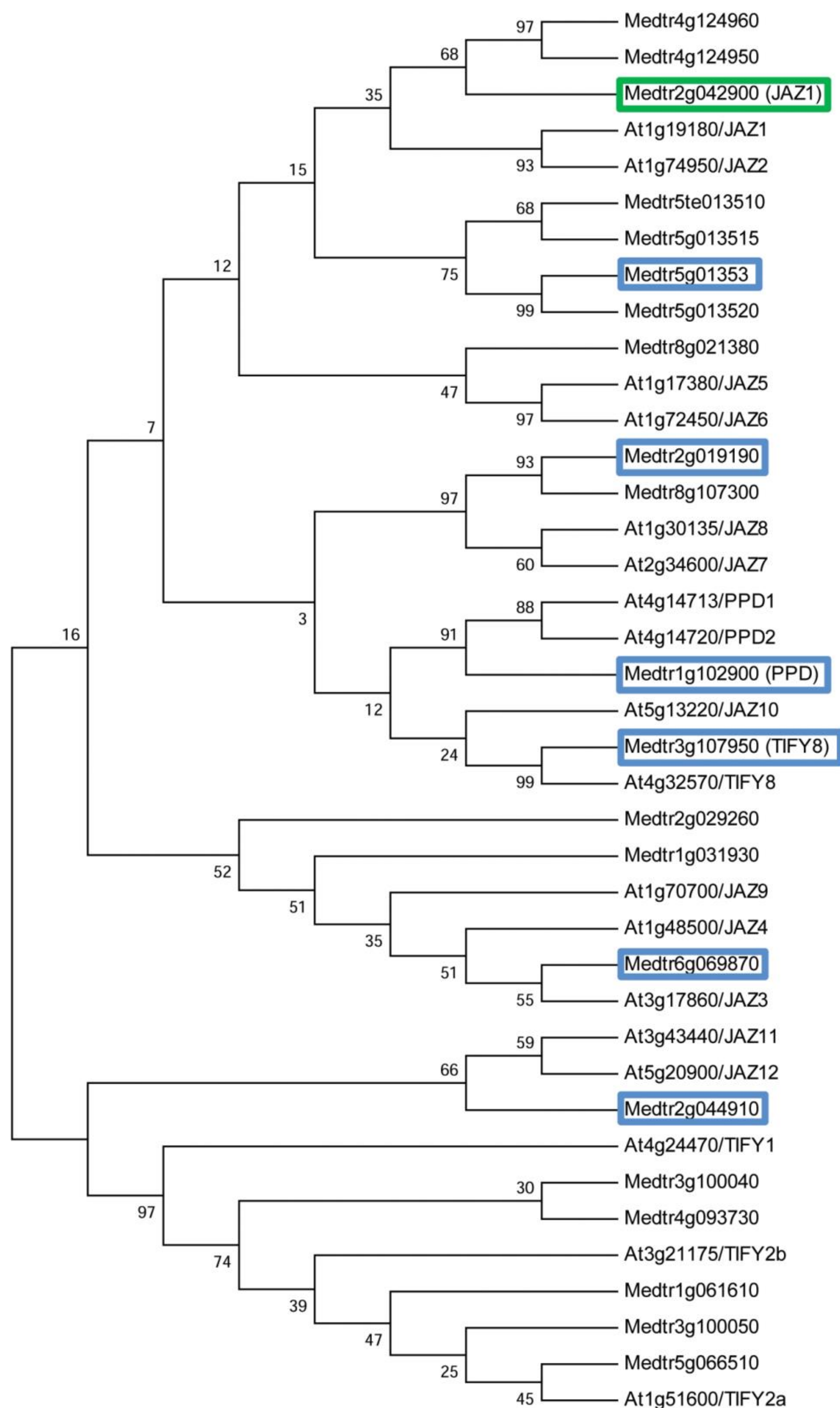

Figure S30. Sequence analysis of MtJAZ1 encoded by *Medtr2g042900*. Unrooted neighbour-joining phylogenetic tree showing the relation of MtJAZ1 (in green) with the Arabidopsis TIFY proteins (Pauwels and Goossens, 2011) and *M. truncatula* homologues thereof encountered in the *M. truncatula* genome by BLAST searches. Bootstrap values are indicated in percentage at branch nodes. TIFY proteins found to interact with MtNINJA are highlighted in blue. For the sequence alignment we refer to Fig. S4 in the online supporting information of this article.

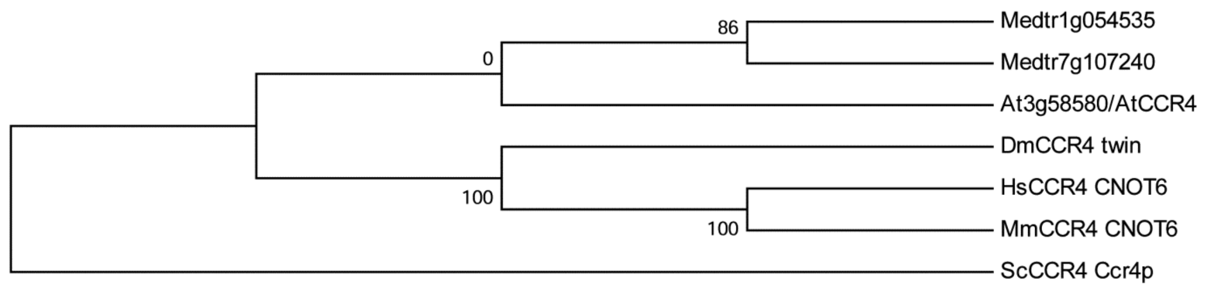

Figure S31. Sequence analysis of MtCCR4 encoded by *Medtr1g054535* and *Medtr7g1072140*. Neighbour-joining phylogenetic tree showing the relation of MtCCR4 (Medtr1g054535 and Medtr7g1072140) and CCR4 from *A. thaliana* (AtCCR4) and CCR4 from yeast (ScCCR4), human (HsCCR4), mouse (MmCCR4) and fly (DmCCR4), Bootstrap values are indicated in percentage at branch nodes. For the sequence alignment we refer to Fig. S5 in the online supporting information of this article.

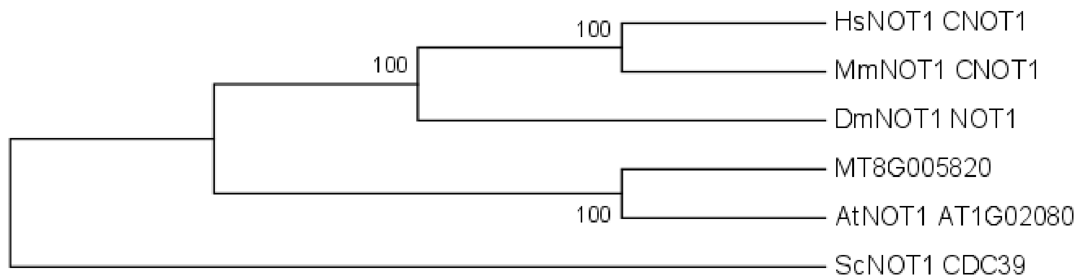

Figure S32. Sequence analysis of MtNOT1 encoded by *Medtr8g005820*. Neighbour-joining phylogenetic tree showing the relation of MtNOT1 with NOT1 from *A. thaliana* (AtNOT1), yeast (ScNOT1), human (HsNOT1), mouse (MmNOT1) and fly (DmNOT1). Bootstrap values are indicated in percentage at branch nodes. For the sequence alignment we refer to Fig. S6 in the online supporting information of this article.

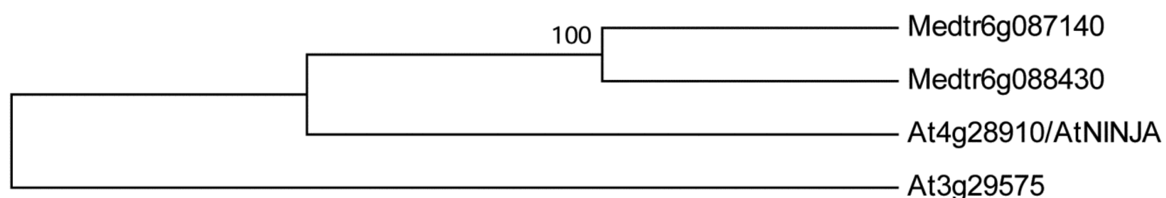

Figure S33. Sequence analysis of MtNINJA encoded by *Medtr6g087140* or *Medtr6g088430*. (a) Alignment of the amino acid sequences of MtNINJA and NINJA from *A. thaliana* (Pauwels *et al.*, 2010), generated with ClustalW. Shown in blue is the conserved region containing the EAR domain (LxLxL), necessary for binding the transcriptional repressor protein TOPLESS (Pauwels *et al.*, 2010). **(b)** Neighbour-joining phylogenetic tree showing the relation of MtNINJA with NINJA from *A. thaliana* (Pauwels *et al.*, 2010). In addition, the amino acid sequence of At3g29575 corresponding to ABI five binding protein 3 (AFP3) was chosen as an outgroup, because it was retrieved as the next best hit, after NINJA, when performing a BLAST search with MtNINJA against the *A. thaliana* genome. Bootstrap values are indicated in percentage at branch nodes and the scale bar corresponds to the number of amino acid substitutions per site.

Table S5: List of co-purifying background proteins obtained with mock purifications.

Gene ID	Annotation/Description
Medtr0014s0290	copia-type LTR gag-polypeptide protein | scaffold0014:147884-148615 | 20130326
Medtr0015s0070	gag-pol polyprotein, putative | scaffold0015:28758-23922 | 20130326
Medtr0015s0130	actin | scaffold0015:66389-69266 | 20130326
Medtr0023s0180	hypothetical protein, putative | scaffold0023:80031-78129 | 20130326
Medtr0040s0170	hypothetical protein, putative | scaffold0040:103080-106580 | 20130326
Medtr0046s0150	hypothetical protein, putative | scaffold0046:58027-57635 | 20130326
Medtr0129s0070	F-box associated protein | scaffold0129:18149-16938 | 20130326
Medtr0254s0010	copia-type LTR gag-polypeptide protein | scaffold0254:6733-2001 | 20130326
Medtr0313s0040	integrase, putative | scaffold0313:6945-8138 | 20130326
Medtr0313s0060	gag-pol polyprotein | scaffold0313:10727-15616 | 20130326
Medtr1g023000	40S ribosomal protein S3 | chr1:7280201-7279209 | 20130326
Medtr1g030560	hypothetical protein, putative | chr1:10595964-10596203 | 20130326
Medtr1g044660	40S ribosomal protein S3 | chr1:16863314-16865972 | 20130326
Medtr1g051390	retroviral aspartyl protease | chr1:20173126-20171727 | 20130326
Medtr1g052450	hypothetical protein | chr1:21305987-21298735 | 20130326
Medtr1g059690	ubiquitin-60S ribosomal protein L40 | chr1:25948399-25950003 | 20130326
Medtr1g066390	polyubiquitin | chr1:28610364-28611065 | 20130326
Medtr1g079490	germin-like protein, putative | chr1:35313009-35313858 | 20130326
Medtr1g085500	germin, putative | chr1:38185415-38184207 | 20130326
Medtr1g101130	tubulin beta chain | chr1:45454817-45452294 | 20130326
Medtr1g107405	transmembrane protein, putative | chr1:48697650-48698027 | 20130326
Medtr1te011370	integrase | chr1:1988353-1986410 | 20130326
Medtr1te012310	gag-pol polyprotein, putative | chr1:2409461-2405961 | 20130326
Medtr1te038880	gag-pol polyprotein, putative | chr1:14476064-14471822 | 20130326
Medtr1te043160	gag-pol polyprotein, putative | chr1:16156059-16160754 | 20130326
Medtr2g005690	heat shock protein 70 | chr2:272429-275305 | 20130326
Medtr2g008050	actin | chr2:1263563-1261671 | 20130326
Medtr2g031310	tubulin beta chain | chr2:11734534-11731744 | 20130326
Medtr2g032550	heat shock protein 70 | chr2:12244311-12246622 | 20130326
Medtr2g034480	glucan endo-1,3-beta-glucosidase, basic protein isoform | chr2:13158862-13161213 | 20130326
Medtr2g039960	eukaryotic initiation factor 4A | chr2:17536004-17533214 | 20130326
Medtr2g040160	disease resistance (TIR-NBS-LRR class) family protein, putative | chr2:17616530-17622587 | 20130326
Medtr2g040260	disease resistance (TIR-NBS-LRR class) family protein, putative | chr2:17660775-17667449 | 20130326
Medtr2g062840	isocitrate dehydrogenase | chr2:26561308-26566797 | 20130326
Medtr2g088060	B-cell receptor-associated 31-like protein | chr2:37075304-37074036 | 20130326
Medtr2g096840	actin | chr2:41387108-41388947 | 20130326
Medtr2g099620	eukaryotic initiation factor 4A | chr2:42716789-42719210 | 20130326
Medtr2g451550	integrase, putative | chr2:22903582-22904367 | 20130326
Medtr2g460560	hypothetical protein, putative | chr2:24956919-24954499 | 20130326
Medtr2te026350	gag-pol polyprotein | chr2:9512929-9508046 | 20130326
Medtr2te042790	gag-pol polyprotein | chr2:18652394-18657277 | 20130326
Medtr2te064150	gag-pol polyprotein | chr2:27169151-27164262 | 20130326
Medtr2te104720	gag-pol polyprotein | chr2:45015652-45020409 | 20130326
Medtr3g022570	NBS-LRR type disease resistance protein | chr3:6720913-6724455 | 20130326
Medtr3g023990	hypothetical protein, putative | chr3:7360210-7360857 | 20130326
Medtr3g037360	gag-pol polyprotein, putative | chr3:13720591-13719441 | 20130326
Medtr3g043980	hypothetical protein, putative | chr3:14688139-14688855 | 20130326
Medtr3g048687	kinase, putative | chr3:18078172-18077860 | 20130326
Medtr3g065110	nicastrin | chr3:29362214-29370700 | 20130326
Medtr3g084800	DNA topoisomerase | chr3:38297625-38308819 | 20130326
Medtr3g092130	ubiquitin-60S ribosomal protein L40 | chr3:42075576-42077473 | 20130326
Medtr3g093600	transmembrane protein, putative | chr3:42769885-42772551 | 20130326
Medtr3g094570	60S ribosomal protein L6 | chr3:43155663-43152936 | 20130326
Medtr3g094860	60S ribosomal protein L6 | chr3:43296220-43298355 | 20130326
Medtr3g095530	actin | chr3:43648121-43646717 | 20130326
Medtr3g102850	hypothetical protein, putative | chr3:47391929-47391437 | 20130326
Medtr3g113970	tubulin beta chain | chr3:53148532-53145891 | 20130326
Medtr3g464580	asparagine synthetase | chr3:25968601-25964057 | 20130326
Medtr3te019250	gag-pol polyprotein, putative | chr3:5382576-5378293 | 20130326
Medtr3te028310	gag-pol polyprotein | chr3:8974022-8978905 | 20130326
Medtr3te030170	gag-pol polyprotein | chr3:9538311-9543196 | 20130326
Medtr4g008250	coatomer subunit beta | chr4:1471099-1475724 | 20130326
Medtr4g015460	beta-glucosidase | chr4:4630246-4623007 | 20130326
Medtr4g017630	tubulin beta chain | chr4:5530428-5532941 | 20130326
Medtr4g019090	tubulin beta chain | chr4:5923374-5921092 | 20130326
Medtr4g019110	tubulin beta chain | chr4:5936783-5934552 | 20130326
Medtr4g059730	glutathione S-transferase tau | chr4:22039553-22040403 | 20130326
Medtr4g063710	heat shock protein 70 | chr4:23586439-23589045 | 20130326

Medtr4g063720	heat shock protein 70 | chr4:23595272-23597897 | 20130326
Medtr4g073580	polyubiquitin | chr4:27908181-27911975 | 20130326
Medtr4g080797	polyubiquitin | chr4:31262869-31259563 | 20130326
Medtr4g080920	polyubiquitin | chr4:31306680-31305985 | 20130326
Medtr4g081010	polyubiquitin | chr4:31288443-31286842 | 20130326
Medtr4g085890	methylcrotonyl-CoA carboxylase beta chain, putative | chr4:33593022-33589655 | 20130326
Medtr4g088485	polyubiquitin | chr4:35102028-35100464 | 20130326
Medtr4g088495	polyubiquitin | chr4:35111369-35108856 | 20130326
Medtr4g091580	polyubiquitin | chr4:36293871-36295472 | 20130326
Medtr4g094535	tubulin beta chain | chr4:38179173-38176891 | 20130326
Medtr4g103920	glyceraldehyde-3-phosphate dehydrogenase | chr4:42993174-42990271 | 20130326
Medtr4g108920	gag-pol polyprotein, putative | chr4:45162609-45165776 | 20130326
Medtr4g113150	RNA-binding protein | chr4:46512445-46508778 | 20130326
Medtr4g124660	sucrose synthase | chr4:51649619-51653478 | 20130326
Medtr4g125970	eukaryotic initiation factor 4A | chr4:52326348-52329664 | 20130326
Medtr4g130540	heat shock protein 70 | chr4:54397225-54395264 | 20130326
Medtr4g131940	binding | chr4:55091594-55102617 | 20130326
Medtr4te006160	integrase, putative | chr4:521078-519081 | 20130326
Medtr4te020810	gag-pol polyprotein | chr4:6693394-6698277 | 20130326
Medtr4te023280	gag-pol polyprotein, putative | chr4:7845623-7843086 | 20130326
Medtr4te036160	reverse transcriptase, putative | chr4:11803453-11805793 | 20130326
Medtr4te038220	copia-type LTR gag-polypeptide protein | chr4:16254414-16255719 | 20130326
Medtr5g033960	ubiquitin/ribosomal protein S27a | chr5:14677042-14677509 | 20130326
Medtr5g035330	RNA recognition motif (A.K.A RRM, RBD, or RNP domain) protein | chr5:15369336-15366199 | 20130326
Medtr5g069050	fructose-bisphosphate aldolase | chr5:29235057-29233162 | 20130326
Medtr5g069055	fructose-bisphosphate aldolase | chr5:29239164-29236912 | 20130326
Medtr5g071360	asparagine synthetase | chr5:30276449-30271337 | 20130326
Medtr5g077070	isocitrate dehydrogenase | chr5:32875463-32879808 | 20130326
Medtr5g083170	ferritin | chr5:35889949-35887143 | 20130326
Medtr5g092700	polyubiquitin | chr5:40493181-40492257 | 20130326
Medtr5g098090	60S ribosomal protein L6 | chr5:42944062-42944766 | 20130326
Medtr5te041830	integrase, putative | chr5:18362453-18364017 | 20130326
Medtr5te047860	gag-pol polyprotein | chr5:20949031-20953920 | 20130326
Medtr5te063160	gag-pol polyprotein | chr5:26201490-26196606 | 20130326
Medtr5te065240	gag-pol polyprotein | chr5:27413324-27418163 | 20130326
Medtr5te079390	gag-pol polyprotein | chr5:33940496-33945379 | 20130326
Medtr5te086070	gag-pol polyprotein, putative | chr5:37212719-37214768 | 20130326
Medtr6g004000	actin | chr6:60515-58842 | 20130326
Medtr6g005310	germin-like protein, putative | chr6:703106-702268 | 20130326
Medtr6g005330	germin-like protein, putative | chr6:711366-710537 | 20130326
Medtr6g005340	germin-like protein, putative | chr6:712332-713170 | 20130326
Medtr6g005360	germin-like protein, putative | chr6:718399-717570 | 20130326
Medtr6g011760	death receptor interacting protein, putative | chr6:3491481-3499624 | 20130326
Medtr6g021800	elongation factor 1-alpha | chr6:7519328-7520766 | 20130326
Medtr6g021805	elongation factor 1-alpha | chr6:7528699-7530303 | 20130326
Medtr6g053070	hypothetical protein, putative | chr6:19029850-19030566 | 20130326
Medtr6g061930	polyubiquitin | chr6:24038016-24040000 | 20130326
Medtr6g086785	gag-pol polyprotein, putative | chr6:32705718-32708018 | 20130326
Medtr6g092235	copia-type LTR gag-polypeptide protein | chr6:34766655-34767725 | 20130326
Medtr6g453170	copia-type LTR gag-polypeptide protein | chr6:18705208-18704507 | 20130326
Medtr6g453190	copia-type LTR gag-polypeptide protein | chr6:18711143-18710442 | 20130326
Medtr6g471170	disease resistance (TIR-NBS-LRR class) family protein | chr6:25492987-25491516 | 20130326
Medtr6te046420	gag-pol polyprotein, putative | chr6:16776884-16772752 | 20130326
Medtr6te046710	gag-pol polyprotein | chr6:16899122-16904005 | 20130326
Medtr6te052080	gag-pol polyprotein, putative | chr6:18161241-18162230 | 20130326
Medtr6te084000	gag-pol polyprotein | chr6:31358737-31362879 | 20130326
Medtr7g024390	heat shock protein 70 | chr7:8016210-8018722 | 20130326
Medtr7g024580	heat shock cognate 70 protein | chr7:8104250-8101523 | 20130326
Medtr7g026230	actin | chr7:8688031-8690767 | 20130326
Medtr7g035035	copia-type LTR gag-polypeptide protein | chr7:13284588-13282096 | 20130326
Medtr7g056163	hypothetical protein, putative | chr7:19592784-19592475 | 20130326
Medtr7g071055	gag-pol polyprotein, putative | chr7:26304732-26310785 | 20130326
Medtr7g078170	quinoprotein amine dehydrogenase beta chain-RIC1-like guanyl-nucleotide exchange factor | chr7:29558486-29569548 | 20130326
Medtr7g082240	peptidyl-tRNA hydrolase family protein | chr7:31516289-31513955 | 20130326
Medtr7g086300	vitamin-b12 independent methionine synthase, 5-methyltetrahydropteroyltriglutamate-homocysteine protein | chr7:33471661-33466402 | 20130326
Medtr7g089120	tubulin beta chain | chr7:34839568-34842010 | 20130326
Medtr7g093260	actin | chr7:37051714-37049953 | 20130326
Medtr7g094240	BSD domain protein | chr7:37505155-37506360 | 20130326
Medtr7g099630	hypothetical protein, putative | chr7:39961872-39963162 | 20130326

Medtr7g099680	heat shock protein 70 | chr7:39987149-39984502 | 20130326
Medtr7g100925	hypothetical protein, putative | chr7:40689749-40689240 | 20130326
Medtr7g104550	transducin/WD40 repeat-like superfamily protein | chr7:42371981-42367441 | 20130326
Medtr7g105080	ubiquitin-60S ribosomal protein L40 | chr7:42609768-42608579 | 20130326
Medtr7g105430	hypothetical protein | chr7:42755239-42758394 | 20130326
Medtr7g110310	S-adenosylmethionine synthase | chr7:45207679-45208863 | 20130326
Medtr7g116360	polyubiquitin | chr7:47988401-47990311 | 20130326
Medtr7te017020	gag-pol polyprotein | chr7:5390336-5386197 | 20130326
Medtr7te020910	gag-pol polyprotein | chr7:6535660-6540536 | 20130326
Medtr7te025550	gag-pol polyprotein | chr7:8502225-8507109 | 20130326
Medtr7te028290	gag-pol polyprotein | chr7:9544405-9539534 | 20130326
Medtr7te045770	gag-pol polyprotein | chr7:16141349-16136493 | 20130326
Medtr7te071170	gag-pol polyprotein, putative | chr7:26362086-26359426 | 20130326
Medtr7te083460	gag-pol polyprotein | chr7:32099556-32104439 | 20130326
Medtr7te109690	gag-pol polyprotein | chr7:44898475-44903416 | 20130326
Medtr8g005185	gag-pol polyprotein | chr8:87331-91593 | 20130326
Medtr8g007635	hypothetical protein, putative | chr8:1730720-1729756 | 20130326
Medtr8g018230	polyubiquitin | chr8:6167713-6169314 | 20130326
Medtr8g018400	eukaryotic initiation factor 4A | chr8:6251688-6249104 | 20130326
Medtr8g059345	Cc-NBS-LRR resistance protein, putative | chr8:20818416-20812862 | 20130326
Medtr8g059745	gag-pol polyprotein | chr8:21212135-21207252 | 20130326
Medtr8g079450	disease resistance (Cc-NBS-LRR class) family protein | chr8:33990047-33993441 | 20130326
Medtr8g081020	polyubiquitin | chr8:35000822-34999677 | 20130326
Medtr8g085910	peptidyl-tRNA hydrolase family protein | chr8:35644817-35638618 | 20130326
Medtr8g085980	tubulin alpha chain, putative | chr8:35670764-35668785 | 20130326
Medtr8g088060	ubiquitin/ribosomal protein S27a | chr8:36459859-36460326 | 20130326
Medtr8g091910	60S ribosomal protein L6 | chr8:38352374-38354087 | 20130326
Medtr8g095500	peptidyl-tRNA hydrolase family protein | chr8:39974433-39971740 | 20130326
Medtr8g098360	tubulin beta chain | chr8:40919952-40916172 | 20130326
Medtr8g106790	guanine nucleotide-binding subunit beta-like protein | chr8:45078023-45079996 | 20130326
Medtr8g464550	copia-type LTR gag-polypeptide protein | chr8:22832182-22832901 | 20130326
Medtr8g479250	polyubiquitin | chr8:33772938-33775487 | 20130326
Medtr8te059820	retrotransposon, Ty3-gypsy subclass protein, putative | chr8:21915351-21922702 | 20130326

Table S6: List of background proteins removed because the orthologue in Arabidopsis is a known GS background protein.

ID	Protein Description	Orthologous genes in Arabisopsis thaliana
Medtr0212s0040	ATP synthase subunit alpha	AT2G07698
Medtr0212s0040	ATP synthase subunit alpha	AT2G07698
Medtr1g013680	elongation factor 1-alpha	AT1G07920 AT1G07930 AT1G07940 AT5G60390
Medtr1g014140	aspartate-tRNA ligase	AT4G31180 AT4G26870
Medtr1g016750	26S proteasome non-ATPase regulatory subunit, putative	AT1G64520
Medtr1g018840	cysteine protease	AT1G47128 AT3G19390 AT3G19400 AT5G43060
Medtr1g018840	cysteine protease	AT1G47128 AT3G19390 AT3G19400 AT5G43060
Medtr1g048000	elongation factor	AT1G56070 AT1G06220 AT3G12915 AT5G25230
Medtr1g054310	40S ribosomal protein S5	AT2G37270 AT3G11940
Medtr1g064070	isocitrate dehydrogenase (NAD(+)) protein	AT5G03290 AT3G09810
Medtr1g069105	26S proteasome regulatory subunit 4-like protein	AT4G29040 AT2G20140
Medtr1g083330	26S protease regulatory subunit 6A-like protein	AT3G05530 AT4G02480 AT1G09100
Medtr1g083810	importin subunit alpha	AT3G06720 AT1G02690 AT1G09270 AT3G05720 AT4G02150 AT4G16143 AT5G49310
Medtr1g086480	ADP-ribosylation factor	AT1G10630 AT3G62290 AT5G14670 AT1G02430 AT2G47170
Medtr1g090130	chaperonin CPN60	AT3G23990 AT2G33210
Medtr1g090827	coatomer alpha subunit	AT1G62020 AT2G21390
Medtr1g095660	26S proteasome regulatory subunit	AT1G53750
Medtr1g106005	tubulin alpha chain, putative	AT1G50010 AT1G04820 AT4G14960
Medtr1g106005	tubulin alpha chain, putative	AT1G50010 AT1G04820 AT4G14960
Medtr1g108765	ATP synthase subunit beta	AT5G08680 AT5G08670 AT5G08690
Medtr1g111970	40S ribosomal protein S27	AT3G61110 AT5G47930
Medtr1g114170	RNase L inhibitor 1	AT4G19210 AT3G13640
Medtr1g116500	2-isopropylmalate synthase	AT1G74040 AT1G18500 AT5G23010 AT5G23020
Medtr2g009080	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	AT4G33510 AT1G22410 AT4G39980
Medtr2g019670	40S ribosomal protein S14	AT3G11510 AT2G36160 AT3G52580 AT4G18270
Medtr2g034900	importin subunit alpha	AT4G16143 AT1G02690 AT1G09270 AT3G05720 AT3G06720 AT4G02150 AT5G49310
Medtr2g040220	disease resistance (TIR-NBS-LRR class) family protein, putative	AT3G09440
Medtr2g044070	sucrose synthase	AT3G43190 AT4G02280 AT5G20830 AT5G49190
Medtr2g065470	glyceraldehyde-3-phosphate dehydrogenase	AT1G79530 AT1G13440 AT1G16300 AT3G04120
Medtr2g067250	26S proteasome regulatory subunit rpn2, putative	AT2G32730 AT1G04810
Medtr2g102277	coatomer beta~ subunit	AT1G52360 AT1G79990 AT3G15980
Medtr3g028590	ADP,ATP carrier protein, putative	AT5G13490 AT3G08580 AT4G28390
Medtr3g062510	26S protease regulatory subunit 6B	AT5G58290
Medtr3g070940	clathrin heavy chain	AT3G11130 AT3G08530
Medtr3g076660	elongation factor Tu	AT4G20360 AT4G02930
Medtr3g083690	DEAD box ATP-dependent RNA helicase, putative	AT2G42520
Medtr3g084310	serine hydroxymethyltransferase	AT4G13930 AT4G13890
Medtr3g085850	glyceraldehyde-3-phosphate dehydrogenase, cytosolic protein	AT1G13440 AT1G16300 AT1G79530 AT3G04120
Medtr3g085850	glyceraldehyde-3-phosphate dehydrogenase, cytosolic protein	AT1G13440 AT1G16300 AT1G79530 AT3G04120
Medtr3g089940	alcohol dehydrogenase	AT1G77120
Medtr3g093110	60S ribosomal protein L9	AT1G33140 AT1G33120 AT4G10450
Medtr3g101470	isocitrate dehydrogenase (NAD(+)) protein	AT5G03290 AT3G09810
Medtr3g102830	ATP-dependent Clp protease	AT5G50920 AT4G14670 AT3G45450 AT3G48870 AT5G51070
Medtr3g112260	26S proteasome regulatory particle triple-A ATPase	AT5G43010 AT5G52882 AT1G64110 AT3G16290 AT5G15250
Medtr3g114480	26S proteasome non-ATPase regulatory subunit 3	AT1G20200 AT1G75990
Medtr3g118070	glutamate-1-semialdehyde 2,1-aminomutase	AT3G48730 AT5G63570
Medtr4g013770	disease resistance-responsive (dirigent-like) family protein	AT1G58170 AT3G13650 AT1G55210 AT3G13660 AT3G13662 AT5G49040
Medtr4g014810	elongation factor 1-alpha	AT1G07920 AT1G07930 AT1G07940 AT5G60390
Medtr4g024630	transketolase	AT2G45290 AT3G60750

Medtr4g045577	transport protein SEC31	AT3G63460 AT1G18830
Medtr4g069920	coatomer subunit gamma	AT4G34450
Medtr4g071130	coatomer alpha subunit	AT1G62020 AT2G21390
Medtr4g074640	alanyl-tRNA synthetase	AT1G50200
Medtr4g076100	gamma aminobutyrate transaminase	AT3G22200
Medtr4g078780	26S proteasome regulatory subunit	AT2G20580 AT4G28470
Medtr4g094270	26S proteasome AAA-ATPase RPT-like subunit	AT5G19990
Medtr5g064500	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	AT1G22410 AT4G33510 AT4G39980
Medtr5g066710	E3 ubiquitin protein ligase UPL-like protein, putative	AT1G70320 AT1G55860
Medtr5g096430	heat shock protein 90	AT5G56010 AT2G04030 AT3G07770 AT4G24190 AT5G52640 AT5G56000 AT5G56030
Medtr5g097320	heat shock protein, putative	AT3G07770 AT2G04030 AT4G24190 AT5G52640 AT5G56000 AT5G56010 AT5G56030
Medtr7g006560	transaldolase	AT5G13420
Medtr7g011990	O-methyltransferase family protein	AT5G54160
Medtr7g063650	delta 1-pyrroline-5-carboxylate synthetase	AT3G55610 AT2G39800
Medtr7g079180	late embryogenesis abundant Lea-like protein	AT1G01470
Medtr7g080110	translation initiation factor IF2/IF5 protein	AT5G36230 AT1G65220
Medtr7g083790	phosphate transporter	AT5G14040 AT3G48850
Medtr7g094460	elongation factor Tu	AT4G02930
Medtr8g027450	importin-alpha re-exporter, putative	AT2G46520
Medtr8g036880	ADP,ATP carrier protein, putative	AT5G13490 AT3G08580 AT4G28390
Medtr8g078370	vacuolar H+-ATPase subunit H, putative	AT3G42050
Medtr8g081510	serine hydroxymethyltransferase	AT4G13930 AT1G22020 AT1G36370 AT4G13890
Medtr8g086070	oxoglutarate/malate carrier protein, putative	AT5G19760
Medtr8g092390	26S proteasome regulatory subunit, putative	AT5G64760 AT5G09900
Medtr8g092450	26S proteasome regulatory subunit, putative	AT5G64760 AT5G09900
Medtr8g098325	GMP synthase	AT1G63660
Medtr8g099185	6-phosphogluconate dehydrogenase, decarboxylating protein	AT1G64190 AT3G02360 AT5G41670
Medtr8g099795	heat shock protein 70	AT5G42020 AT1G09080 AT1G16030 AT1G56410 AT3G09440 AT3G12580 AT5G02490
Medtr8g101330	acetyl-CoA carboxylase, biotin carboxylase subunit	AT5G35360
Medtr8g106120	26S proteasome regulatory subunit rpn2, putative	AT2G32730 AT1G04810
Medtr8g464820	glyceraldehyde 3-phosphate dehydrogenase	AT4G24820

Table S7: OD600 values from the systematic Y2H screen covering interactions between CKS1 and the majority of the preys identified by TAP, and interactions among the preys.

For these data we refer to the Dataset S2 of the online supporting information of Goossens et al. (2016).

Table S8: List of known CCR4/NOT subunits purified with CAF1 TAP in yeast or human and nomenclature in the different species.

yeast	Gavin 2006 POP2-TAP	human	Mauxion 2013 CNOT7-TAP	Medicago	Mt4RC1 accession	Mtr TAP
Caf1p (POP2)	bait	CNOT7 (hCAF1, CAF1a); CNOT8	bait (CNOT7)	CAF1A; CAF1B	Medtr4g006800, Medtr3g106180	bait (CAF1A)
Ccr4p	y	CNOT6 (CCR4A); CNOT6L (CCR4B)	y (CNOT6+CNOT6L)	CCR4A; CCR4B	Medtr1g054535, Medtr7g107240	y (CCR4A+CCR4B)
Not1p (CDC39)	y	CNOT1	y	NOT1	Medtr8g005820	y
Not2p (CDC36)	n	CNOT2	y	NOT2	Medtr4g061960	n
Not4p (MOT2)	n	CNOT4	n	NOT4	Medtr4g061180, Medtr2g093100	n
Not3p; Not5p	n	CNOT3	y	NOT3	Medtr7g085350	y
Caf40p	y	CNOT9 (RQCD1, RCD1)	y	NOT9 (RCD1)	Medtr3g053170	y
Caf130p	y	/	n/a	/	/	n/a
/	n/a	CNOT10	y	NOT10	Medtr4g115350	y
/	n/a	CNOT11 (C2ORF29)	y	NOT11	Medtr1g041405	y

Abbreviations: y, yes; n, no; n/a, not applicable.

Table S9: List of additionally identified proteins for MtCAF1 TAP. Only proteins that were identified with at least two matched high-confident peptides and co-purified with the bait in at least two independent TAP purifications are shown. The result from the systematic Y2H binary interaction assay is also provided.

		Mtr TAP		Y2H[3]
ID[1]	Description[1]	C-GS (# ID/4)[2]	N-GS (# ID/4)[2]	
Medtr4g019800	adenine nucleotide alpha hydrolase-like protein	4	2	n
Medtr5g020760/	isoflavone reductase	4	1	n
Medtr4g068860	non-symbiotic hemoglobin class 1	3	1	n
Medtr6g008500	cytochrome P450 family 82A-like protein	4	0	n
Medtr2g094270	plasma membrane intrinsic PIP2 protein	4	0	n
Medtr4g051622	pyridine nucleotide-disulfide oxidoreductase	4	0	n
Medtr4g059390	plasma membrane intrinsic 2	4	0	n
Medtr4g129790	ATP synthase gamma chain	4	0	n
Medtr5g075450	cinnamate 4-hydroxylase	4	0	n
Medtr5g082070	plasma membrane intrinsic protein	4	0	n
Medtr1g088640	universal stress family protein	3	0	n
Medtr4g019800	adenine nucleotide alpha hydrolase-like protein	3	0	n
Medtr7g084150	ubiquinol-cytochrome C family reductase	3	0	n
Medtr8g098910	monodehydroascorbate reductase	0	2	n
Medtr0002s1060	Narbonin	0	2	n
Medtr4g125690	myosin XI, putative	2	0	n/a
Medtr7g066870	acetyl-CoA carboxylase carboxyltransferase	0	2	n/a
Medtr7g074070	transmembrane protein, putative	2	0	n/a
Medtr8g058330/	transport Sec61 alpha subunit	2	0	n/a

[1]Gene ID and annotation descriptions are derived from the Mt4 release of the Medicago genome sequence (Tang *et al*., 2014) that can be found on the website of JCVI, but manually inspected and curated.

[2]# ID/# denotes the number of times a protein was recovered out of the total amount of purifications that were performed on that particular bait in the Medicago TAP (Mtr TAP). C-GS and N-GS denote whether the protein was identified in the purification of a C- or N-terminally tagged version of the bait, respectively.
[3]Abbreviations: n, no direct interaction; n/a, not assessed.

Table S10: OD_{600} values from the systematic Y2H screen covering interactions between CAF1 and the majority of the preys identified by TAP, and interactions among the preys

For these data we refer to the Dataset S3 of the online supporting information of Goossens et al (2016).

Table S11: OD_{600} values for combinations of preys and baits known to interact. The minimum OD_{600} was set as threshold for positive interactions in the systematic Y2H screen. LTH: Leu, Trp, His-depleted medium.

pDEST32 (GAL4-BD)		pDEST22 (GAL4-AD)				
ID	**Name/description**	**ID**	**Name/description**	**OD^{600} (LT)**	**OD^{600} (LTH)**	**LTH/LT**
AT1G17380	JAZ5	AT1G32640	MYC2	0,647	0,464	0,717
AT1G17380	JAZ5	AT1G32640	MYC2	0,656	0,492	0,750
AT1G17380	JAZ5	AT1G32640	MYC2	0,682	0,517	0,758
AT1G70700	JAZ9	AT1G32640	MYC2	0,714	0,544	0,762
AT1G70700	JAZ9	AT1G32640	MYC2	0,729	0,612	0,840
AT1G70700	JAZ9	AT1G32640	MYC2	0,681	0,48	0,705
AT5G20900	JAZ12	AT1G32640	MYC2	0,67	0,432	0,645
AT5G20900	JAZ12	AT1G32640	MYC2	0,608	0,497	0,817
AT5G20900	JAZ12	AT1G32640	MYC2	0,693	0,542	0,782

Table S12: Primers Used.

Name	Primer Sequence
For cloning	
attB1-MtCKS1-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGGTCAGATCCAGTAC
attB2-MtCKS1-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCATTTGACCAGCATGCCC
attB2-CKS1-nostop-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTTGACCAGCATGCCCTGCTGG
attB1-MtCDKA-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGAACAGTACGAGAAAGTTGAG
attB2-MtCDKA-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCATGGGACAAACTTAATGTCTTTG
attB1-MtCDKB-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGAGAAACCTGGTGAGACA
attB2-MtCDKB-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGAGATAGGTCTTGTCTAGGTCATCA
attB1-MtCAF1-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGCCGCTAATTTTACCTCAAA
attB2-MtCAF1-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAATGAGTACTCTGTCCATTCTCAAC
attB1-MtNOT1-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGCAACCTTTTCATCCAC
attB2-MtNOT1-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATGTCAGACCCCAACCC
attB1-MtJAZ1-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGTCTACCTCATCGGAATATTCA
attB2-MtJAZ1-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAATTTGAGTTGATTTTGCA
attB1-MtNINJA-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGAGGACGATAGCGGG
attB2-MtNINJA-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAACTGTGAGAAGAGGAACCAA
attB1-MtWD40like-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGTCCACTCGTCGCTCC
attB2-MtWD40like-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAACTATTAGATGCCTCCACA
attB1-MtPR10like_130-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGGTGTTTTCAATTTTGAGG
attB2-MtPR10like_130-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTAATCAGGATTTGCCAAAA
attB1-MtDEADRNAhel-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGCGATCTTCATGGGCT
attB2-MtDEADRNAhel-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGTCCCATGCACTAGTCAC
attB1-MtGuaBinP-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGCTGAGGGTCTTGTTCTT
attB2-MtGuaBinP-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTAACGCCCGATTCCC
attB1-MtIsoRed-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGCAACTGAAAACAAAATCC
attB2-MtIsoRed-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGACAAATTGATTCAAATATTCATCA
attB1-Medtr6g069870-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCAGTGGTCATTTTCAAATAAG
attB2-Medtr6g069870-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATCTAATCACTTCCATACATG
attB1-Medtr2g019190-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAGGAGGAATTGCAACTTG
attB2-Medtr2g019190-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGTGATAATATGGAGATGCTTC
attB1-Medtr2g044910-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATGGTGTTACTGTTAAG
attB2-Medtr2g044910-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAGATGCAGATATAGTTGG
attB1-Medtr5g013530-FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAATCATCACAATATAACACC
attB2-Medtr5g013530-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTACTACAAAAATAACACAGAGGTG
attB1-MtPPD-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAACGGCGGAAGCACC
attB2-MtPPD-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGCATTCTTGAACATCTTTATC
attB1-MtTIFY8-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGGTTCTGAGAATG
attB2-MtTIFY8-Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACTGCGCTCTTTTGTCTTC
For qRT-PCR	
qPCR-mTAP-Cterm-Fw	GATGCCACCAAGACCTTC
qPCR-mTAP-Cterm-Rev	GCCGTCTACACCGTTATC
qPCR-mTAP-Nterm-Fw	CCGAGACAGCCGAGAAGG
qPCR-mTAP-Nterm-Rev	TCGTGGCGTCGTCATAGG
qPCRMtCAF1UTR-Fw	TCTCTCTCCCCATTCTCTCTCAAC
qPCRMtCAF1UTR-Rev	CAAGGTTATCGCTCCAAACTTCAC
qPCRMtJAZ1UTR-Fw	TCTCTCTTGAATATCATCATCTTC
qPCRMtJAZ1UTR-Rev	GGTCTGTGTTGTTGTTGTTG
qPCRMtCKS1UTR-Fw	CAGCAGCAGGACAACCAG
qPCRMtCKS1UTR-Rev	GAGAAGTACACCAACAAACATTAG
qPCRMtELFa-Fw	ACTGTGCAGTAGTACTTGGTG
qPCRMtELFa-Rev	AAGCTAGGAGGTATTGACAAG
qPCRMt40S-FW	GCCATTGTCCAAGTTTGATGCTG
qPCRMt40S-RV	TTTTCCTACCAACTTCAAAACACCG