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Faculty of Sciences, Ghent University

Department of Biomedical Molecular Biology
Department of Plant Biotechnology and Bioinformatics

Unraveling the functional roles of endogenous phytohormones in mammals

Seo Woo Kim

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Promotors:
Prof. Dr. Rudi Beyaert
Prof. Dr. Alain Goossens
Prof. Dr. ir. Jens Staal

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Unraveling the functional roles of endogenous phytohormones in mammals

By Seo Woo Kim [1, 2, 3, 4]

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Examination committee

Promotors	Prof. Dr. Rudi Beyaert [1, 2]
	Prof. Dr. Alain Goossens [3, 4]
	Prof. Dr. Ir. Jens Staal [1, 2, 5]
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	Prof. Dr. Els Prinsen [10]
	Prof. Dr. Elena Zocchi [11]

1 Unit of Molecular Signal Transduction in Inflammation, VIB-UGent Center for Inflammation Research, Ghent, Belgium

2 Department of Biomedical Molecular Biology, Faculty of Sciences, Ghent University, Ghent, Belgium

3 Group of Specialized Metabolism, VIB-UGent Center for Plant Systems Biology

4 Department of Plant Systems Biology, Faculty of Sciences, Ghent University, Ghent, Belgium

5 Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium

6 Mouse Genetics in Inflammation Unit, VIB-UGent Center for Inflammation

Research, VIB-UGent Center for Inflammation Research, Ghent University, Ghent, Belgium

7 Laboratory of Immunoregulation and Mucosal Immunology, VIB-UGent Center for Inflammation Research, Ghent, Belgium

8 Department of Internal Medicine and Pediatrics, Faculty of Medicine and Health Sciences, Ghent University Hospital, Ghent, Belgium

9 Group of Bio-Energy and Bio-Aromatics, VIB-UGent Center for Plant Systems Biology

10 Laboratory for Integrated Molecular Plant Physiology Research, Department of Biology, University of Antwerp, Antwerp, Belgium

11 Department of Experimental Medicine, Section of Biochemistry, School of Medical and Pharmaceutical Sciences, University of Genova, Genova, Italy

Summary

Plants produce a variety of phytohormones that control important physiological processes such as growth and stress tolerance. Many traditional plant hormones and specialized metabolites were shown to have biological activities in humans or animal models. For example, the plant hormones salicylic acid (SA) and abscisic acid (ABA) have shown promising anti-inflammatory effects, suggesting these phytohormones can be used as potential multifunctional nutraceuticals against inflammation-associated diseases, metabolic syndrome (MetS) in particular. Interestingly, animal cells have long been known to produce SA and ABA. However, the physiological relevance of these endogenous molecules is poorly understood. Although their anti-inflammatory activities point to involvement in immune homeostasis, the functional characterization of these endogenous molecules has been hampered because of a lack of knowledge about their metabolism and signaling pathways.

Therefore, this PhD research aimed to gain insight into the role of endogenous SA and ABA by exploiting these hormones' known metabolic and signaling pathways in other organisms like plants and bacteria.

Chapter 1 introduces the current state-of-the-art of phytohormones as anti-inflammatory and pro-metabolic compounds against diseases relevant to MetS. Also, we present how these bioactive phytohormones can be generated endogenously and through the gut microbiome.

Chapter 2 introduces thymic T cell development and function in the context of immunometabolism. As in the results section (**Chapter 3**) we mainly focus on thymic T cell development and diverse T cell subsets are closely tied to metabolic homeostasis, this introductory chapter mainly focuses on these aspects of the immune system.

In **Chapter 3**, we aimed to unravel the role of endogenous SA. To get a glimpse of endogenous SA functions, we assessed the level of SA in different mice tissues (**Chapter 3.1**). SA was shown to accumulate in the thymus under normal feeding conditions. We then investigated the effect of SA treatment on the physiological function of the SA-accumulating thymus. The administration of SA in mice altered the differentiation of $CD4^+CD8^+$ double-positive thymocytes into $CD4^+$ and $CD8^+$ single-positive thymocytes, suggesting a role of SA for thymic T cell development.

In **Chapter 2.2**, to further delineate the function of endogenous SA in the thymus and immune homeostasis, we set out to generate transgenic

SA-deficient mice. Because of the unknown SA biosynthesis pathway, we used the SA metabolic pathway in bacteria, in which salicylate hydroxylase NahG degrades SA into catechol. However, the SA and catechol levels were maintained in blood and tissues of interest in transgenic NahG mice under normal feeding conditions.

In **Chapter 3**, we investigated the role of endogenous ABA. A cell-based ABA biosensor was engineered as a tool that enables monitoring ABA levels in large-scale studies to screen mechanisms that influence ABA metabolism (**Chapter 4.1**). ABA inactivation by *Arabidopsis* ABA 8'-hydroxylase (AtCYP707A3), anti-ABA scFv in the endoplasmic reticulum, or *Arabidopsis* UDP-glucosyltransferase71C5 (AtUGT71C5) confirmed the selectivity of the biosensor system for ABA.

Chapter 4.2 exemplifies a technical concern that we identified when using our engineered ABA biosensor. We suggest additional approaches and controls to improve the credibility and reliability of future experiments using our biosensor system.

In **Chapter 4.3**, we tried to determine whether ABA depletion could alter immune responses or initiate or exacerbate the development of diseases relevant to metabolic syndrome. We generated and immunologically characterized transgenic mice overexpressing genes encoding AtCYP707A3 and the phaseic acid (PA) reductase AtABH2 that converts the AtCYP707A3 ABA degradation product PA to dihydrophaseic acid (DHPA). Unfortunately, we could not verify ABA depletion in CYP-ABH2 mice due to the technical challenges in profiling ABA in mice tissues. We were unable to show an effect of CYP-ABH2 expression on the health of mice and immune homeostasis under basal conditions.

In line with previous studies showing that dietary ABA protects against dextran sulfate sodium (DSS)-induced colitis in mice, we planned to investigate whether the preventive role of ABA in DSS-induced colitis is abrogated in transgenic CYP-ABH2 mice. Given the variability of the DSS-induced colitis model easily introduced by environmental factors, we validated the published results showing a prophylactic effect of ABA in the DSS-induced colitis model by monitoring disease development in ABA-administered WT mice with DSS-induced colitis (**Chapter 5**). However, we could not show the prophylactic effect of ABA against DSS-induced colitis in mice. We describe the potential reasons driving the discrepancy between the previous report and our observation.

Finally, we discuss the obtained scientific results and reflect on the technical challenges we faced. This PhD research forms a basis and

provides proof of concept for future phytohormone research in animal systems.

Samenvatting

Planten produceren een verscheidenheid aan plantenhormonen die belangrijke fysiologische processen reguleren, zoals groei en stresstolerantie. Veel plantenhormonen en gespecialiseerde metabolieten bleken biologische activiteiten te bezitten in mensen of diermodellen. De plantenhormonen salicylzuur (SA) en abscisinezuur (ABA) hebben bijvoorbeeld veelbelovende ontstekingsremmende effecten, wat suggereert dat deze fytohormonen kunnen worden gebruikt als potentiële multifunctionele nutraceuticals tegen ontstekingsgerelateerde ziekten, in het bijzonder metabool syndroom. Interessant is dat het al lang bekend is dat dierlijke cellen SA en ABA produceren. Over de fysiologische relevantie van deze endogene moleculen is echter weinig gekend. Hoewel hun ontstekingsremmende activiteit wijst op betrokkenheid bij immuunhomeostase, wordt de functionele karakterisering van deze endogene moleculen bemoeilijkt vanwege een gebrek aan kennis over hun metabolisme en signaalroutes.

Daarom wilden we in dit doctoraatsonderzoek inzicht verkrijgen in de rol van endogene SA en ABA door gebruik te maken van de gekende metabole routes en signaalroutes van deze hormonen in andere organismen zoals planten en bacteriën.

Hoofdstuk 1 introduceert de huidige stand van zaken van fytohormonen als ontstekingsremmende en pro-metabolische verbindingen tegen ziekten die relevant zijn voor metabool syndroom. Ook presenteren we hoe deze bioactieve fytohormonen endogeen en via het darmmicrobioom kunnen worden gegenereerd.

Hoofdstuk 2 introduceert de ontwikkeling en functie van T-cellen in de thymus in de context van immunometabolisme. Omdat we ons in de resultatensectie (hoofdstuk 3) voornamelijk concentreren op de ontwikkeling van T-cellen in de thymus en diverse subsets van T-cellen die nauw verbonden zijn met metabole homeostase, richt dit inleidende hoofdstuk zich voornamelijk op deze aspecten van het immuunsysteem.

In Hoofdstuk 3 wilden we de rol van endogeen SA ontrafelen. Als eerste aanwijzing voor mogelijke endogene SA-functies, hebben we het niveau van SA in verschillende muizenweefsels bepaald (Hoofdstuk 3.1). Basaal SA bleek te accumuleren in de thymus onder normale voedingsomstandigheden. Vervolgens onderzochten we het effect van SA-behandeling op de fysiologische functie van de SA-accumulerende thymus. De toediening van SA aan muizen veranderde licht de differentiatie van CD4+CD8+ dubbel-positieve thymocyten in CD4+ en CD8+ enkel-positieve thymocyten, wat suggereert dat SA een rol speelt

in de ontwikkeling van thymus-T-cellen.

Om de functie van endogeen SA in de thymus- en immuunhomeostase verder af te bakenen, hebben we in Hoofdstuk 3.2 getracht transgene SA-deficiënte muizen te genereren. Vanwege de ongekende SA-biosyntheseroute hebben we gebruik gemaakt van de SA-metabolische route in bacteriën, waarbij salicylaathydroxylase NahG dat SA afbreekt tot catechol als transgeen in muizen tot expressie werd gebracht. De SA- en catechol-spiegels werden echter gehandhaafd in bloed en weefsels van transgene NahG-muizen onder normale voedingsomstandigheden.

In Hoofdstuk 3 hebben we de rol van endogeen ABA onderzocht. Een celgebaseerde ABA-biosensor werd ontwikkeld als hulpmiddel om ABA-niveaus in grootschalige studies te kunnen opvolgen en zo mechanismen te screenen die het ABA-metabolisme beïnvloeden (Hoofdstuk 5.1). ABA-inactivatie door de expressie van een *Arabidopsis* ABA 8'-hydroxylase (AtCYP707A3), of anti-ABA scFv in het endoplasmatisch reticulum, of *Arabidopsis* UDP-glucosyltransferase71C5 (AtUGT71C5), bevestigde de selectiviteit van het biosensorsysteem voor ABA.

Hoofdstuk 4.2 geeft een voorbeeld van een technisch probleem dat we hebben vastgesteld bij het gebruik van onze geconstrueerde ABA-biosensor. We stellen aanvullende benaderingen en controles voor om de geloofwaardigheid en betrouwbaarheid van toekomstige experimenten met ons biosensorsysteem te verbeteren.

In Hoofdstuk 4.3 probeerden we vast te stellen of ABA-depletie de immuunrespons kan veranderen of de ontwikkeling van ziekten die relevant zijn voor het metabool syndroom kan initiëren of verergeren. We hebben transgene muizen gegenereerd en immunologisch gekarakteriseerd die AtCYP707A3 tot overexpressie brengen, samen met het faseïnezuur (PA)-reductase ABH2 dat het AtCYP707A3 ABA-degradatieproduct PA omzet in dihyrdofasezuur (DHPA). Helaas konden we ABA-uitputting in CYP-ABH2-muizen niet verifiëren vanwege technische uitdagingen bij het profileren van ABA in muizenweefsels. We konden geen effect aantonen van CYP-ABH2-expressie op de gezondheid van muizen en immuunhomeostase onder basale omstandigheden.

In overeenstemming met eerder onderzoek dat aantoonde dat ABA in de voeding beschermt tegen door dextraansulfaat-natrium (DSS) geïnduceerde colitis bij muizen, trachtten we te onderzoeken of de preventieve rol van ABA bij DSS geïnduceerde colitis wordt opgeheven in transgene CYP-ABH2-muizen. Gezien de gekende variabiliteit van

het DSS-geïnduceerde colitismodel omwille van specifieke omgevingsfactoren eigen aan verschillende muisfaciliteiten, hebben we eerst de eerder gepubliceerde resultaten die een profylactisch effect van ABA in het DSS-geïnduceerde colitismodel aantonen proberen te valideren door de DSS-geïnduceerde colitis te volgen in ABA behandelde wild-type muizen (Hoofdstuk 5). We konden het eerder gerapporteerdebeschermend effect van ABA tegen DSS-geïnduceerde colitis bij muizen echter niet aantonen, en beschrijven mogelijke redenen voor de geobserveerde discrepantie.

Ten slotte bespreken we de verkregen wetenschappelijke resultaten en weerspiegelen we de technische uitdagingen waarmee we werden geconfronteerd. Dit promotieonderzoek vormt een eerste aanzet tot en proof-of-concept voor toekomstig plantenhormoononderzoek in zoogdieren.

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List of abbreviations

A

ABA	Abscisic acid
ABI1	Abscisic acid insensitive 1
AD	Atopic dermatitis
AE	Anion exchanger proteins
AHK4	*Arabidopsis* histidine kinases 4
AHR	Aryl hydrocarbon receptor
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
AR	Adenosine receptors

B

BA	Benzoic acid
BA2H	Benzoicacid-2-hydroxylase
BAT	Brown adipose tissues
BR	Brassinosteroids
BW	Body weight

C

Cas9	CRISPR-associated protein 9
CD	Crohn's disease
cDC	Conventional dendritic cell
CFU	Colony forming unit
CHASE	Cyclases/histidine kinases-associated sensing extracellular
CK	Cytokinins
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRABP2	Cellular retinoic acid-binding protein
CRE	Cyclic AMP response element
CRISPR	Clustered regularly interspaced short palindromic repeats
CSs	Complementary surfaces
CXCR5	C-X-C chemokine receptor type 5
CYP-ABH2	AtCYP707A3 and AtABH2
cZR	*cis*-zeatin-9-riboside
cZRP	*cis*-zeatin-9-riboside-5′-phosphate

D

DAI	Disease activity index

	DC	Dendritic cell
	DHPA	Dihydrophaseic acid
	DIDS	4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid
	DMAPP	Dimethylallyl pyrophosphate
	DMEM	Dulbecco's modified Eagle's medium
	DN	Double-negative
	DP	Double-positive
	DSS	Dextran sodium sulfate
E		
	EIC	Extracted ion chromatogram
	ELISA	Enzyme-linked immunosorbent assay
	ENO1	Glycolytic enzymes alpha-enolase
	ER	Endoplasmic reticulum
	EV	Empty vector
F		
	FAD	Flavin adenine dinucleotide
	f-MLP	formyl-methionyl-leucyl-phenylalanine
	FOXP3	Forkhead box P3
	FPP	Farnesyl pyrophosphate
	FRET	Fuorescence resonance energy transfer
	FW	Fresh weight
G		
	GA	Gibberellins
	GAL4BD	GAL4 DNA-binding domain
	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
	GC-MS	Gas chromatography-mass spectrometry
	GFP	Green Fluorescent Protein
	GH3	IAA-amino acid conjugate synthase
	GLUT-4	Glucose transporter type 4
H		
	HAB1	Hypersensitive to ABA 1
	HFD	High-fat diet
	His-Asp phosphorelay	Histidyl-to-aspartyl system
	HMGB1	Human high mobility group box 1
	5-HT	5-hydroxytryptamine
I		
	IAA	Indole-3-acetic acid

IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
IAOx	Indole-3-acetaldoxime
IBD	Inflammatory bowel disease
IC	Isochorismate
IFNγ	Interferon γ
IL	Interleukin
iP	N^6–isopentenyladenine
IP	Intraperitoneal
IPA	Indole-3-pyruvic acid
iPA	N^6–isopentenyladenosine
iPRP	N^6-isopentenyladenine -9-riboside-5′-phosphate
ITC	Isothermal calorimetry

L

LANCL2	Lanthionine synthetase componen C-like protein 2
LC-FTMS	Liquid chromatography -Fourier transform mass spectrometry
LC-MS	Liquid chromatography -mass spectrometry analysis
LiP-MS	Limited proteolysis coupled with MS
LOD	Limit of detection
LPS	Lipopolysaccharide
LSL	LoxP-stop-LoxP (LSL)

M

MADCAM1	Mucosal vascular addressin cell adhesion molecule 1
MAPKs	Mitogen-activated protein kinases
MdCHK2	*Malus domestica* CHASE Histidine Kinase receptor 2
Mekk1	MAP/ERK kinase kinase 1
MEP	2-C-methyl-D-erythritol 4-phosphate
MetS	Metabolic syndrome
2MeSiP	2-methyl-thio-N6–isopentenyladenine
2MeSiPR	2-methyl-thio-N6–isopentenyladenosine
2MeSZ	2-methyl-thio-zeatin
2MeSZR	2-methyl-thio-zeatin-riboside

MHC	Major histocompatibility complex
minCMV	Minimal CMV promoter
MLN	Mesenteric lymph nodes
MMP9	Matrix metalloproteinase 9
moDC	Monocyte-derived dendritic cells (moDCs)
MS	Mass spectrometry
mTORC2	Mechanistic target of rapamycin complex 2
MVA	Mevalonate
Myd88	Myeloid differentiation primary response protein 88

N

NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NaSA	Sodium salicylate
NASH	Non alcoholic steatohepatitis
NK	Natural killer
NPR1	Non-expressor of pathogenesis-related genes 1

P

PA	Phaseic acid
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PKA	cAMP-dependent protein kinase (PKA)
PKM2	Pyruvate kinase isozyme M2
PMA	Phorbol myristate acetate
PP2C	Protein phosphatases 2C
PPARγ	Peroxisome proliferator-activated receptor γ
PPRE	Peroxisome proliferator response element
PYL	Pyrabactin resistance 1-like
PYR1	Pyrabactin resistance 1
RCAR	Regulatory components of ABA receptor

R

RAR	Retinoic acid receptor
RPMI	Roswell Park Memorial Institute Medium
Runx	Runt-related transcription factor
RXRγ	Retinoid X receptor γ

S

SA	Salicylic acid
SCF	Skp-Cullin-F-box containing protein complex
scFv	Single-chain Fv
SD	Standard deviation
Short FAs	Short chain fatty acids
siRNA	Small interfering RNA
SL	Strigolactone
SnRK2	Snf1-related protein kinase 2
Sox2	SRY-box containing gene 2
SP	Single-positive
SPE	Solid-phase extraction
SPF	Specific pathogen free
SPR	Surface plasmon resonance
START	StAR-related lipid-transfer

T

T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAM	Tryptamine
TCA	Trichloroacetic acid
TCR	T cell receptor
TDO	Tryptophan 2,3-dioxygenase
Tfh	T follicular helper cell
ThPOK	T-helper-inducing POZ/Krueppel-like factor
TIC	Total ion chromatogram
TIR1	Transport inhibitor response 1
TMS	Trimethylsilyl
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF6	TNFR-associated factor 6
Tregs	Regulatory T cells
TRIT1	tRNA isopentenyl transferase 1
Trp	Tryptophan

U

UAS	Upstream activating sequence
UC	Ulcerative colitis
UGT	UDP-glucosyltransferase

V

	VCAM1	Vasccular cell adhesion protein 1
	VP16AD	VP16 transactivation domain
	VPR	VP64-p65-Rta
W		
	WAT	White adipose tissues
	WT	Wild-type

PART I: GENERAL INTRODUCTION

Chapter 1. Phytohormones: Multifunctional nutraceuticals against metabolic syndrome and comorbid diseases

Chapter 1

Phytohormones: Multifunctional nutraceuticals against metabolic syndrome and comorbid diseases

Seo Woo Kim [1, 2, 3, 4] , Alain Goossens [3, 4], Claude Libert [1, 2], Filip Van Immerseel [6] , Jens Staal [1, 2, 5] , Rudi Beyaert [1, 2]

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[5]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium
[6]Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

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Commentary

Phytohormones: Multifunctional nutraceuticals against metabolic syndrome and comorbid diseases

Seo Woo Kim[a,b,c,d], Alain Goossens[c,d], Claude Libert[a,b], Filip Van Immerseel[e], Jens Staal[a,b,1,*], Rudi Beyaert[a,b,1,*]

[a] VIB-UGent Center for Inflammation Research, VIB, Ghent, Belgium
[b] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[c] VIB-UGent Center for Plant Systems Biology, VIB, Ghent, Belgium
[d] Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[e] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

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ABSTRACT

Metabolic syndrome is characterized by the co-occurrence of diverse symptoms initiating the development of type 2 diabetes, cardiovascular diseases, and a variety of comorbid diseases. The complex constellation of numerous comorbidities makes it difficult to develop common therapeutic approaches that ameliorate these pathological features simultaneously. The plant hormones abscisic acid, salicylic acid, auxin, and cytokinins, have shown promising anti-inflammatory and pro-metabolic effects that could mitigate several disorders relevant to metabolic syndrome. Intriguingly, besides plants, human cells and gut microbes also endogenously produce these molecules, indicating a role in the complex interplay between inflammatory responses associated with metabolic syndrome, the gut microbiome, and nutrition. Here, we introduce how bioactive phytohormones can be generated endogenously and through the gut microbiome. These molecules subsequently influence immune responses and metabolism. We also elaborate on how phytohormones can beneficially modulate metabolic syndrome comorbidities, and propose them as nutraceuticals.

1. Introduction

1.1. Phytohormones in other organisms – would a rose by any other name smell as sweet?

Phytohormones are essential plant regulators in a myriad of physiological processes, which coordinate growth, reproduction and stress resistance. Given that plants are immotile, unlike animals, finding the right and appropriate strategy in phytohormone actions in order to grow sufficiently and defend themselves against stressful circumstances is highly important. Therefore, their functions and complex signaling mechanisms have been extensively studied in plants. In this regard, small molecules such as abscisic acid (ABA), salicylic acid (SA), indole-3-acetic acid (IAA; the best known auxin), and cytokinins (CK) were grouped and generally termed as 'Phytohormones' [1]. However, these molecules also show biological activities on human cells and in animal

Abbreviations: ABA, Abscisic acid; SA, salicylic acid; IAA, indole-3-acetic acid; CK, cytokinins; T2D, Type 2 diabetes; HFD, High-fat diet; AHR, Aryl hydrocarbon receptor; GA, Gibberellins; MetS, Metabolic syndrome; MEP, 2-C-methyl-D-erythritol 4-phosphate; FPP, Farnesyl pyrophosphate; MVA, Mevalonate; CRABP2, Cellular retinoic acid-binding protein; COX, Cyclooxygenase; AMPK, AMP-activated protein kinase; HMGB1, Human high mobility group box 1; MAPKs, mitogen-activated protein kinases; BA, Benzoic acid; BA2H, Benzoic acid-2-hydroxylase; Trp, Tryptophan; TAM, Tryptamine; IPA, Indole-3-pyruvic acid; IAM, Indole-3-acetamide; IAOx, Indole-3-acetaldoxime; IAN, Indole-3-acetonitrile; iPA, N^6-isopentenyladenosine; iP, N^6-isopentenyladenine; iPRP, N^6-isopentenyladenine-9-riboside-5′-phosphate; cZR, *cis*-zeatin-9-riboside; cZRP, *cis*-zeatin-9-riboside-5′-phosphate; 2MeSZ, 2-methyl-thio-zeatin; 2MeSZR, 2-methyl-thio-zeatin-riboside; 2MeSiP, 2-methyl-thio-N^6-isopentenyladenine; 2MeSiPR, 2-methylthio-N^6-isopentenyladenosine; TRIT1, tRNA isopentenyl transferase 1; DMAPP, Dimethylallyl pyrophosphate; His-Asp phosphorelay, Histidyl-to-aspartyl system; CHASE, cyclases/histidine kinases-associated sensing extracellular; AR, Adenosine receptors; Short FAs, Short chain fatty acids; T1D, Type 1 diabetes; GLUT-4, Glucose transporter type 4; LANCL2, Lanthionine synthetase component C-like protein 2; mTORC2, Mechanistic target of rapamycin complex 2; BR, Brassinosteroids; SL, Strigolactone; IBD, Inflammatory bowel disease; NAFLD, Non-alcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; AD, Atopic dermatitis; 5-HT, 5-hydroxytryptamine; TDO, Tryptophan 2,3-dioxygenase

* Corresponding authors at: VIB-UGent Center for Inflammation Research, Unit of Molecular Signal Transduction in Inflammation, VIB, Ghent, Belgium.
E-mail addresses: Jens.Staal@irc.vib-UGent.be (J. Staal), Rudi.Beyaert@irc.vib-UGent.be (R. Beyaert).
[1] Shared senior authorship

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models. Importantly, these "phytohormones" are not exclusive to plants and have even been shown to be endogenously produced in humans or human cell cultures [2–6]. Since we are constantly exposed to these molecules, they are not foreign to human physiology. This means that they are likely to be exploited during diverse physiological processes. Additionally, several phytohormones can also be produced by microbes and such compounds produced in our gut likely have physiological influences [2,7].

Some phytohormones are anti-inflammatory compounds that inhibit several inflammation-driven disorders. For instance, ABA treatment in humans and animal models show beneficial effects against a wide range of inflammation-related diseases such as type 2 diabetes (T2D), colitis, atherosclerosis, glioma, and depression [8]. Salicylates display pharmacological properties in cardiovascular disease, colon cancer, and diabetes [9]. Administered CK or its derivatives lead to the attenuated oxidative stress in mammalian cells and anti-cytotoxicity in neoplastic cells [10–12]. High production of IAA was shown to dampen a high-fat diet (HFD)- induced liver damage, relying on the aryl hydrocarbon receptor (AHR) [13–15]. Also, phytohormones that are not endogenously produced can have physiological effects and act anti-inflammatory. For example, gibberellins (GA) induce the anti-inflammatory protein A20 in lung epithelial cells, which could protect against asthma [16]. Considering these effects, we propose phytohormones as potential multifunctional nutraceuticals against inflammation-associated diseases, in particular, metabolic syndrome (MetS) and its diverse comorbid symptoms.

1.2. Metabolic syndrome – a complex interaction between nutrition, metabolism, and inflammation

MetS has become a major threat to global health in modern society. MetS is defined as a cluster of conditions, such as obesity, insulin resistance, high blood pressure, and elevated triglycerides, which eventually could increase the chances of developing inflammation-associated comorbidities [17]. Some comorbidities such as psoriasis might be causative and elevate the risk of MetS, whereas others, such as T2D, cardiovascular diseases, asthma, atopic dermatitis, and cancer, are consequences of MetS [18,19]. Metabolism and inflammation are intricately linked, where the activation and differentiation of B-cells, T-cells, dendritic cells, macrophages rely on the primary metabolic pathways [19,20]. Metabolic rewiring like glycolysis, oxidative phosphorylation, fatty acid oxidation, and glutaminolysis largely influence the functional activities of innate and adaptive cells [19,20]. The metabolic shift in macrophages towards a pro-inflammatory state will, in turn, contribute to insulin resistance which raises the blood sugar levels in a vicious circle [21]. As such, homeostasis imbalance in metabolism triggers the dysregulation of immune cells, which reciprocally aggravates metabolic processes [22].

Although currently available therapeutics, like metformin and statins, control symptoms and comorbidity of MetS, a multifunctional drug that hits more than one comorbid disease at multiple targets does not exist to our knowledge. Phytohormones not only trigger anti-inflammatory signals, but also play a role as the nexus among key factors that contribute to disease development and progression of MetS, which are inflammation, gut microbiome, and nutrition. Modulating inflammation either directly or indirectly via phytohormones through the gut microbiome and nutrition could thus be a good way to target some of the most pressing effects from MetS. One possible treatment strategy is the use of medicinal plants [23-25], but also food supplements or conventional drugs based on proven active compounds that are beneficial against MetS should be considered.

Here we will summarize the anti-inflammatory properties of phytohormones and elaborate their potential roles in treating issues related to MetS. Additional sources of phytohormones besides nutrition, such as the endogenous biosynthesis and the gut microbiome, will also be discussed.

2. Endogenous biosynthesis of biologically active phytohormones in humans

2.1. Abscisic acid – regulating sugar responses in three kingdoms of life

Beneficial effects of ABA have been reported in several pathological processes and preclinical disease models such as colitis [26,27], T2D [28], glioma [29], depression [30,31], atherosclerosis [32], neuroinflammation [33,34], hepatitis C virus replication [35], angiogenesis [36], malaria [37], tuberculosis [38], influenza A virus infection [39], and fungal infection [40]. The effect of ABA on glucose homeostasis [41], aspects associated with ischemic retinopathies [42], and interspecies communication in disease transmission [43] were properly reviewed during the recent years. Of note, the reported health benefit on glucose and insulin homeostasis occurs at low doses, 0.5–1 μg/kg body weight, corresponding to normal amounts obtained from fruits and vegetables [44]. The uptake of ABA from fig (*Ficus carica*) fruit extracts reduces plasma glucose and insulin levels in humans, which has been positively evaluated as therapeutic for the treatment of T2D [45].

In our last review, we discussed the physiological effects and relevant signaling pathways of ABA in animal and human cells, and ABA biosynthesis pathways in different organisms [8]. ABA is endogenously produced at low levels (nM or sub-nM concentrations) in various mammalian cells including mesenchymal stem cells, β-cells, macrophages, microglia, monocytes, granulocytes, and keratinocytes (Fig. 1A) [5,46–51]. How endogenous ABA is synthesized in animal or human cells remains to be identified. Plants produce ABA through an 'indirect' plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway from a carotenoid precursor [8]. The phytopathogenic fungus *Botrytis cinerea* produces ABA via a 'direct' pathway from the 15-carbon farnesyl pyrophosphate (FPP) precursor through the mevalonate (MVA) pathway (Fig. 1B) [8]. Four genes involved in ABA biosynthesis, *bcABA1-bcABA4*, have been identified in *B. cinerea* [52], and were recently verified in a heterologous yeast system [53]. In our last review, we claimed that animals are likely to produce ABA via the direct pathway since animals do not have chloroplasts and are closer to fungi in the same Opisthokont group. Also, the precursor FPP is widely used in several MVA pathways to synthesize metabolites in animals, such as sterol. Therefore, human homologs of *Botrytis* genes encoding P450 monooxygenase BcABA1 and BcABA2, and short-chain dehydrogenase/reductase BcABA4 were suggested as candidate genes involved in ABA biosynthesis in humans [8]. However, BcABA genes were absent in other *Botrytis* species, such as *B. fabae* that belongs to the same Clade of *B. cinerea* and other *Botrytis* species in Clade II [54]. Likewise, other ABA-producing fungi, such as *Leptosphaeria maculans*, do not express homologs of all BcABA proteins and seem to generate ABA via a partially different direct pathway [55], demonstrating evolutionary plasticity in the ABA biosynthesis pathway. It is therefore unlikely that BcABA-like genes are involved in human ABA synthesis, and animals may use an alternative pathway, which remains to be elucidated.

Yet, the physiological functions of endogenous ABA have not been revealed much. As the beneficial roles of ABA in inflammation-driven diseases were shown at a relatively higher dose compared to the levels of endogenous ABA (approximately mM or μM versus nM or sub-nM concentrations), the impact and mechanism of action of endogenous ABA might be distinct from the influences of ABA administration in animals. Interestingly, the pathogenesis of T2D might be dependent on the regulation of endogenous ABA as it was demonstrated that the upregulation of ABA in response to glucose challenge stimulates insulin release in pancreatic β-cells [46]. Also, endogenous ABA might be implicated in the pathology of glioma, possibly via its impact on retinoic acid receptor signaling [29]. The concentration of endogenous ABA was shown to be two-fold higher in human low-grade glioma compared to high-grade glioma, where the expression of cellular retinoic acid-binding protein (CRABP2) correlated with the concentration of ABA [29]. Definitely, future elucidation of the endogenous ABA biosynthesis

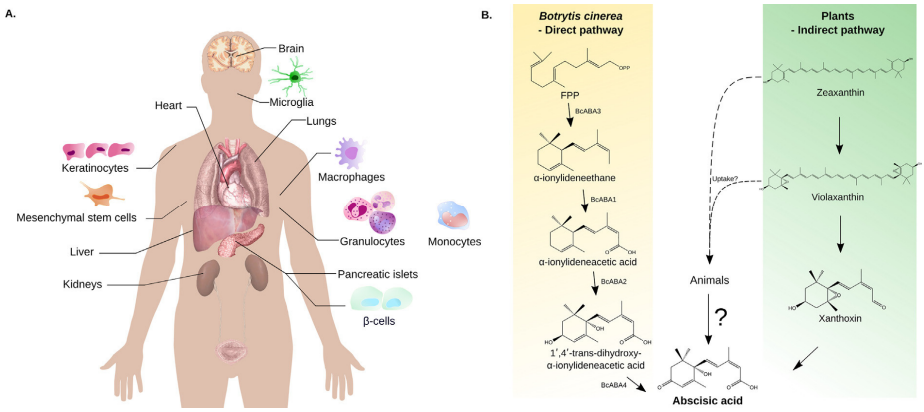

Fig. 1. Suggested model for abscisic acid (ABA) biosynthesis in animals. (A) ABA production in animals. ABA has been shown to be produced or secreted from diverse cell lines (β-cells, macrophages, microglia, keratinocytes), human primary cells (mesenchymal stem cells, monocytes, granulocytes, pancreatic islets), and animal organs (brain, heart, lungs, liver, kidneys). As a summary, the source of ABA production in cell lines, human primary cells, and animal organs is indicated in the human body diagram. (B) Biosynthesis of ABA in phytopathogenic fungus *Botrytis cinerea*, plants and animals. How animals produce ABA remains to be elucidated. *Botrytis cinerea* produces ABA via a 'direct' pathway from the 15-carbon farnesyl pyrophosphate (FPP) precursor and plants produce ABA through an 'indirect' pathway from a carotenoid precursor. Human body diagrams are under Public Domain licensing and were obtained from Wikimedia Commons, the free media repository.

pathway will allow the development of strategies to investigate the physiological relevance of endogenous ABA.

2.2. Salicylic acid – a prehistoric anti-inflammatory drug with multiple targets

The value of SA as an anti-inflammatory drug has been appreciated for a long time, since archaeological evidence indicated the use of salicylate-rich plants for medicinal purposes up to 30.000 years ago [56,57]. The Ebers papyrus also shows that ancient Egyptians used willow to treat fever and pain [58]. Moreover, SA is an active and hydrolyzed metabolite of acetyl-salicylic acid (commonly named Aspirin), generated in 1853 and one of the most sold anti-inflammatory drugs ever [59,60]. The risk of diabetes and cardiovascular disease is reported to be reduced via a low dose regime of aspirin [61]. Cyclooxygenase (COX) inhibition attributes to the anti-inflammatory function of Aspirin, however since free SA is COX-independent, other modes of action should contribute to the anti-inflammatory roles of salicylates [62]. For example, inhibition of IκB kinase-β activity by SA has been shown to block pro-inflammatory NF-κB signaling [63,64].

Aspirin recently received a renewed interest in the context of its protective roles against cancers in the colon, breast, and prostate [65]. Likewise, cancer chemo-preventive activities of SA were discovered along with inhibiting *in vitro* angiogenesis [66], downregulating cdk2/cyclin A2 in multiple cancer cell lines [67], and inhibiting CBP/p300 activities in leukemia cells *in vitro* and *in vivo* [68]. Other anti-cancer associated targets of SA are AMP-activated protein kinase (AMPK), human platelet cathepsin A, human high mobility group box 1 (HMGB1) and p38 mitogen-activated protein kinases (MAPKs) [69–73].

It has been suggested that SA is produced in humans by 2-hydroxylation of benzoic acid (BA), supposedly by a benzoic acid-2-hydroxylase (BA2H) enzyme (Fig. 2) [3]. Plants produce SA mainly via the alternative isochorismate-derived pathway [74]. Yet, the BA2H enzyme activity has also been found in plants, for instance in tobacco, rice, and soybean, but the corresponding gene(s) still remains to be identified [3,75–77]. Whether humans synthesize the precursor BA has not been investigated yet, but BA may originate from the uptake of milk-based diets or food preservatives [78]. The level of endogenous SA in humans is relatively low (~70 nM), in contrast to plants (i.e. 0.49 μM–21 μM in *Arabidopsis*) [3,9]. If and how endogenous SA influences human physiology has not yet been reported. SA producing tissues or cell types, as well as specific stimuli that can elevate SA levels, still remain to be elucidated.

2.3. Auxin – a multi-functional endogenous aryl hydrocarbon receptor (AHR) ligand?

Indole-3-acetic acid (IAA), the best known auxin, is a crucial hormone in controlling plant growth and development. Interestingly, IAA was first identified in human urine, and only later extensively evaluated as a plant growth regulator [79,80]. IAA was also shown to be produced in animal liver, kidney, hippocampus, midbrain, and human cerebrospinal fluid (Fig. 3A) [6,81,82]. Endogenous urinary IAA is massively produced in human patients with neuromuscular diseases, phenylketonuria, hereditary syndrome with symptoms of intermittent cerebellar ataxia and mental deterioration, diabetes mellitus, liver injury and cancer [6,13,83]. A potential receptor for IAA in animals is the AHR, as it has been demonstrated in a yeast model and mouse hepatoma cells that IAA can act as a direct ligand for the AHR [84–87], which is an important regulator of cellular development, immunity, and liver toxicity responses [15,88]. These activities correspond well with known effects of IAA in animals: i.e. IAA has been shown to influence the formation of induced pluripotent stem cells [89] and treatment of animals with IAA can reduce liver damage [90].

IAA in animals was shown to be synthesized from tryptophan (Trp) via two pathways: 1) the tryptamine (TAM) pathway (decarboxylation mechanism) or 2) the indole-3-pyruvic acid (IPA) pathway (transamination metabolism) (Fig. 3B) [6,91]. Animals do not produce Trp endogenously but obtain Trp through the degradation of dietary peptides in protein-rich foods like meat and cheese [14]. Both enantiomers of Trp from dietary sources, L-Trp and D-Trp, can be utilized to produce IAA, but only the transamination of L-Trp is vitamin B_6-dependent [92,93]. The active form of vitamin B_6, pyridoxal phosphate, catalyzes transaminase activity [6,94]. The central nervous system was suggested to exploit the TAM pathway that decarboxylates Trp to TAM, which is subsequently converted to IAA via putative monoamine oxidase activity

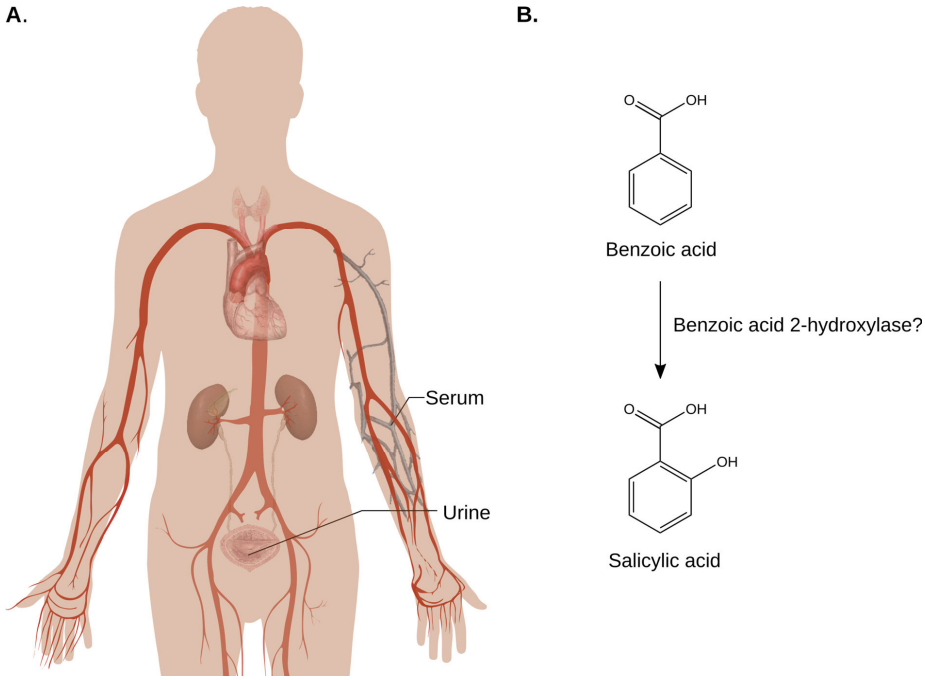

Fig. 2. Suggested model for salicylic acid (SA) biosynthesis in animals. (A) SA production in humans. SA was shown to be secreted in human serum and urine. (B) SA biosynthesis pathway in humans. Based on feeding studies with labeled benzoic acid (BA) precursor in humans, it is postulated that humans produce SA from the BA precursor [3]. The BA2H gene in humans, as well as in plants, still remains to be identified. Human body diagrams are under Public Domain licensing and were obtained from Wikimedia Commons, the free media repository.

[82]. As TAM can diffuse across the blood–brain barrier, the animal brain might produce IAA from TAMs produced in other peripheral tissues [82]. In a rat model of D-galactosamine-induced liver injury, serum IAA levels increase before the increase of serum transaminase activity, thereby indicating that the TAM pathway is more dominant than the IPA pathway with regard to the elevated IAA levels in liver-injured conditions [13]. Yet, genes encoding enzymes involved in both IAA biosynthesis pathways remain to be identified. Plants, several endophytic or phytopathogenic fungi, and bacteria can synthesize IAA via Trp-dependent pathways [95–102]. In plants, indole-3-acetamide (IAM), IPA, TAM, and indole-3-acetaldoxime (IAOx) pathways were suggested in IAA biosynthesis [103]. In a similar way, albeit with some different intermediates, bacteria were shown to produce IAA through IAM, IPA, TAM, and indole-3-acetonitrile (IAN) pathways [95]. Most of the metabolic intermediates, enzymes, and genes encoding enzymes in Trp-dependent pathways have been discovered in plants and bacteria, yet genes and enzymes involved in some steps are missing [103]. IAA biosynthesis pathways in fungi are less well known, but IAM and IPA pathways were shown to be the core IAA biosynthesis pathways [97,102]. In the IPA pathway, genes that encode transaminase and indole-3-acetaldehyde reductase in *Ustilago maydis*, and indole-3-pyruvic acid dehydrogenase and indole-3-acetaldehyde reductase in *Neurospora crassa* have been identified [101,104]. Interestingly, a previous phylogenetic analysis of plant *tryptophan transaminase* and *flavin monooxygenase*, which function in the IPA pathway, postulated that IAA biosynthesis evolved convergently in plants, bacteria, and fungi [105]. The plant IPA pathway might have evolved from an ancient horizontal gene transfer event from bacteria to the most recent common ancestor of land plants, and been shaped as a distinct IAA biosynthesis pathway in order to control the impact of bacteria on plants [105].

IAA plays a prominent role in plant–microbe symbiosis and mycorrhiza [95]. Similarly, human gut microbiota diversity and stability, which can influence the generation of Trp metabolites including IAA, are essential for human gut immunity and health [106]. For example, microbial production of AHR ligands including IAA and microbiota-driven AHR activity were shown to decrease in mice on HFD, and HFD-driven metabolic dysregulation and the intestine barrier dysfunction could be rescued via the administration of AHR ligands or AHR-agonist producing bacteria, indicating the possible involvement of IAA in the interaction between host immunity and gut microbiota in the context of MetS [107].

2.4. Cytokinins – a class of nucleotide-derived signaling molecules

Animals and plants produce CK in a free-base form, a nucleoside form (where a purine ring contains a ribose sugar at N^6), and a nucleotide form (where a phosphate group is attached to the ribose) [108]. CK was discovered as kinetin (N^6-Furfuryladenine) from herring sperm DNA in 1956, but not much is known about its physiological effects in animals [4]. Kinetin in animal DNA is formed from the reaction of an adenine residue with furfural, an oxidative damage product of 2-deoxyribose sugar in DNA, [109]. Besides kinetin, 9 different CKs were determined in canine tissues or HeLa cells (N^6-isopentenyladenosine (iPA), N^6-isopentenyladenine (iP), N^6-isopentenyladenine-9-riboside-5′-phosphate (iPRP), *cis*-zeatin-9-riboside (cZR), *cis*-zeatin-9-riboside-5′-phosphate (cZRP), 2-methyl-thio-zeatin

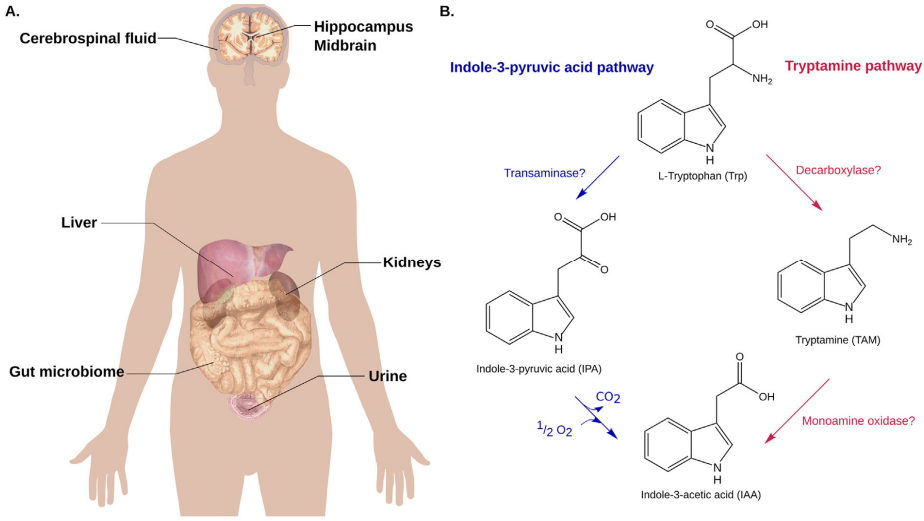

Fig. 3. Suggested model for indole-3-acetic acid (IAA) biosynthesis in animals. (A) IAA production in animals. Organs that produce IAA in animals (hippocampus midbrain, liver, kidneys) are indicated. IAA was shown to be produced in human cerebrospinal fluid, and secreted in human urine. As a summary, the source of IAA production in animals and humans are indicated in the human body diagram. (B) IAA biosynthesis pathways in animals. In animals IAA is synthesized from tryptophan (Trp) through either the indole-3-pyruvic acid (IPA) pathway or the tryptamine (TAM) pathway. In the IPA pathway, transaminase enzyme activity that catalyzes an aminotransfer reaction between the amino group and the keto group was shown, but the corresponding gene has not been identified yet. The conversion of IPA to IAA has not been described in animals so far, and an additional intermediate may still exist. In the TAM pathway, IAA is produced via a putative monoamine oxidase activity from TAM, which is generated upon the decarboxylation of Trp. Human body diagrams are under Public Domain licensing and were obtained from Wikimedia Commons, the free media repository.

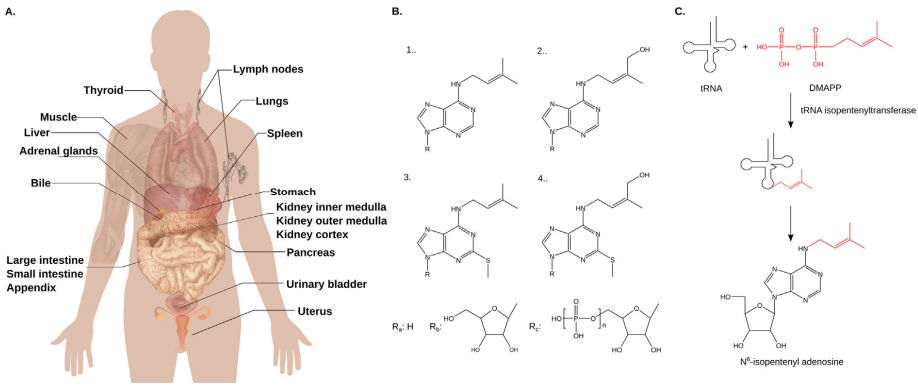

Fig. 4. Source of CK production in animals, molecular structure of mammalian endogenous CKs, and suggested model for tRNA-N^6-isopentenyl adenosine biosynthesis in humans. (A) Production of CKs in animals. Organs that produce CKs in animals are indicated in the human body diagram. **(B)** Adenine compounds with different adducts form free-base CKs **(R_a),** CK nucleosides **(R_b),** and nucleotides **(R_c).** **1Ra.** N^6-isopentenyladenine (iP), **1Rb.** N^6-isopentenyl adenosine (iPA), **1Rc.** N^6-isopentenyladenine-9-riboside-5′-phosphate (iPRP), **2Rb.** *cis*-zeatin-9-riboside (cZR), **2Rc.** *cis*-zeatin-9-riboside-5′-phosphate (cZRP), **3Ra.** 2-methyl-thio-N^6-isopentenyladenine (2MeSiP), **3Rb.** 2-methylthio-N^6-isopentenyladenosine (2MeSiPR), **4Ra.** 2-methyl-thio-zeatin (2MeSZ), **4Rb.** 2-methyl-thio-zeatin-riboside (2MeSZR). **C)** Human tRNA isopentenyl transferase 1 (TRIT1) transfers an isopentenyl residue of dimethylallyl pyrophosphate (DMAPP) to the tRNA adenine-37. N^6-isopentenyl adenosine 5′-diphosphate can be released from prenylated-tRNA through tRNA-degradation, and might be converted to iPA consequently. Human body diagrams are under Public Domain licensing and were obtained from Wikimedia Commons, the free media repository.

(2MeSZ), 2-methyl-thio-zeatin-riboside (2MeSZR), 2-methyl-thio-N^6-isopentenyladenine (2MeSiP), 2-methylthio-N^6-isopentenyladenosine (2MeSiPR) (Fig. 4B) [110,111]. In the first step of putative tRNA-CK iPA biosynthesis, a human tRNA isopentenyl transferase 1 (TRIT1) that transfers an isopentenyl residue from dimethylallyl pyrophosphate (DMAPP) to adenine was identified (Fig. 4C) [108,112]. TRIT1 plays a role in the translational activity of mitochondrial tRNA [113]. Interestingly, mutations in *TRIT1* are associated with mitochondrial diseases, along with developmental delay and epilepsy [113,114]. Also, a suppressive effect of TRIT1 has been suggested in lung tumorigenesis,

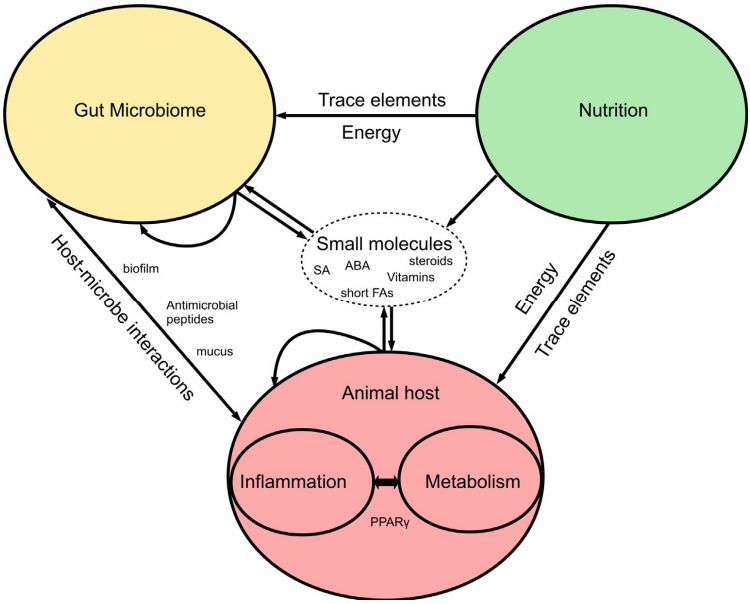

Fig. 5. Complex interplay between nutrition, gut microbiome, metabolism and inflammation. Nutrition does not only provide necessary building blocks (amino acids), energy and trace elements, it is also a source of a complex mixture of small molecules (SA, ABA, …) that can have an impact on the host organism. In addition, nutrition will influence the composition of the gut microbiome, which in turn produce various small molecules that can have an effect on the host organism. The interaction is reciprocal, since also the host organism can produce many small compounds that might influence the composition of the gut microbiome directly or indirectly, as well as have autocrine effects.

as enhancing TRIT gene expression inhibits growth of lung tumor cells and tumor incidence in mice [115]. How CK is generated, degraded and conjugated/deconjugated in mammals is poorly understood. In Hela cells, purine nucleotide phosphorylase is suggested to convert exogenous N^6-benzyladenosine into N^6-benzyladenine [110]. In cultured human leukemia cells, where N6-substituted adenosine has an apoptotic effect, adenosine is converted to mononucleotides by adenosine kinase [116].

Whereas active CKs are most prevalent as free-base (zeatine, dihydrozeatin, iP) in plants, both CK ribosides and free-based CKs are bioactive in animals [10,117,118]. A well-known CK signal transduction pathway in plants is the histidyl-to-aspartyl system (His-Asp phosphorelay), a two component signaling system that is conserved between plants, bacteria and fungi, but not in animals [108,119]. The His-Asp phosphorelay signal transduction in bacteria and fungi broadly leads to adaptive responses to environmental stimuli and the regulation of cell growth [120,121]. In the His-Asp phosphorelay, the CHASE (cyclases/histidine kinases-associated sensing extracellular) domain of histidine-kinase receptors recognizes CK, and an intermediate histidine phosphotransfer protein (known as authentic histidine phsopho-transferase) transfers a phosphate to response regulators to activate transcription or induce a negative feedback response [122]. Interestingly, phytopathogenic bacteria *Xanthomonas campestris* was shown to sense plant CK via a CHASE domain in the adaptation of oxidative stress [123]. In mammals, two subtypes of adenosine receptors (AR), A_3R and A_{2A}R, are involved in CK signaling [124-126]. Racemic ZR and iPA bind to A_3R with a high nanomolar affinity, and the anti-proliferative effect of iPA in the prostate cancer cell line LNCaP depends on A_3R [124]. Also, ZR suppresses serum deprivation-induced cell death and mutant huntingtin-induced protein aggregation in the pheochromocytoma PC12 cell line via activating an A_{2A}R and subsequent protein kinase A signaling [126]. In addition, ZR treatment of mice negatively regulates the production of Th1 and Th2 cytokines in $CD4^+$ and $CD8^+$ T cells, inhibits CD25, CD69, and CD40L expression, and increases cyclic adenosine monophosphate via a A_{2A}R-dependent mechanism [125]. ZR was also shown to protect against thioglycollate-induced peritoneal leukocytosis [125]. A synthetic CK that regulates plant growth and development, 6-benzylaminopurine, activates a P2-purinereceptor and induces a beneficial inotropic effect in cardiac tissue [127].

Intriguingly, iPA shows anti-cancer properties in several studies, where the viability of several neoplastic cell lines and the tumorigenicity in induced pluripotent stem cells could be inhibited by iPA [10,89]. Also, iPA shows anti-glioma effects in primary glioma cells and cell lines, through the AMPK-dependent regulation of epidermal growth factor receptor signaling [128]. Another CK, kinetin riboside, inhibits the *trans*-activation of cyclin D1 and cyclin D2 in primary myeloma cells, which play a role in cell cycle and cancer progression [118]. Anti-proliferative effects of kinetin riboside were also shown in cancer cell lines of human melanoma, colon, and pancreas, along with cellular ATP depletion and DNA damage [129]. Oxidative stress can be attenuated by CK, which can also link to other beneficial roles of CK against cancer [130,131]. The activities of anti-oxidant enzymes, such as total-superoxide dismutase and glutathione peroxidase, are elevated in astrocyte cells and mouse brains upon treatment with kinetin [132]. Finally, the generation of cellular reactive oxygen species in human breast adenocarcinoma cells could be inhibited by iPA, possibly through the activation of NRF2 transcription factor signaling [133].

CK together with auxin is widely used in plant tissue cultures in order to proliferate or regenerate shoots and root apical meristems [134]. Likewise, treatment with iPA and IAA boosts the early reprogramming efficiency of induced pluripotent stem cells by controlling cell cycle and up-regulating known pluripotent marker genes, such as *Oct3/4*, *Utf1*, and *Rex1* [89]. Another common feature of CK treatment of plants and animals is its anti-aging effect. Kinetin is known to delay senescence of leaves and fruits in plants, and aging in hairless dogs, fruit flies, human fibroblasts, and spleen and thymus in rats [135–137]. Gene expression of *Bcl-2* and *Bax*, and production of IL-6 are possible targets of kinetin in protecting against aging processes in rat spleen lymphocytes or thymus [136,138].

3. Complex interplay between nutrition, gut microbiome, inflammation and metabolism

Small molecules present in a plant-based diet can have direct and indirect effects on host immune regulation and metabolism. As shown in Fig. 5, nutrition also influences gut microbiome ecology, which in turn can influence host cell metabolism and inflammation. This could in part be due to the generation of biologically active small molecules like phytohormones or kynurenic acid [139–141]. For example, a fiber-rich diet benefits gut microbes that produce short chain fatty acids (short FAs), which in turn benefit the development of anti-inflammatory regulatory T cells [142]. The phytohormones and other small molecules produced by the gut microbiome can either have a direct effect on the microbes themselves [143], or an indirect effect through modulation of host responses. Of note, food is a highly complex mixture of small molecules, which not only represent plant hormones like SA and ABA that can inhibit inflammation, but also other potent anti-inflammatory plant compounds like berberine [144] and curcumin [145].

The imbalance of immune and metabolic homeostasis could impact on gut microbial composition and the activity of gut microbes that produce small molecules. For example, reduced gut microbiome diversity in children with type 1 diabetes (T1D) was shown to be associated with a higher population of *Bacteroides* microbes [146]. Corroborating that patients with diabetes mellitus secrete higher urinary IAA and the fact that several species of intestinal *Bacteroides (B. thetaiotaomicron, B. eggerthii, B. ovatus, B. fragilis)* produce IAA, intestinal *Bacteroides* could contribute to increased IAA production in T1D [14,83,147,148].

Citrobacter species also produce IAA [148]. Of interest, several *Citrobacter* species (*C. fruendii, C. koseri, C. sedlakii*) are part of the healthy human gut microbiome, and some species (*C. amalonaticus, C. braakii*) are shown to be pathogenic [149]. Among them, IAA-producing species have yet to be discovered. In a mouse model of alcoholic liver disease that induces intestinal dysbiosis, reduced intestinal IAA levels and lower production of IL-22, which is regulated by the IAA-responsive AHR, were observed [150]. Another relevant study showed that AHR-deficient mice are more susceptible to *C. rodentium* infection compared to wild-type mice, along with decreased IL-22 production [151], which may suggest a protective role of *C. rodentium*–derived IAA that triggers AHR-mediated IL-22 expression.

ABA may also influence the gut microbiome via its action on gut bitter taste receptors [140,152–154], which in turn can influence metabolism [155–157]. The influence of ABA on immune cells, and possibly on the gut microbiome, also reflects the beneficial effects that have been found for ABA in experimental models of colitis [26,27]. Whether there are gut microbes that produce ABA at physiologically relevant levels remains to be elucidated. Given that ABA produced from gut microbiome might elicit the same protective roles as dietary ABA, an ABA-producing gut microbe could be highly interesting as a probiotic. For instance, it would be interesting to investigate if *Lactobacillus*, which is highly represented in the gut microbiome, produces ABA, as L. plantarum shows anti-obesity effects. Also, the potential production of ABA by Lactobacillus may reflect the protective effect against drought in wheat plants that has been reported for this microbe [158–161].

4. Protective roles of phytohormones against comorbid diseases of MetS

4.1. Type 2 diabetes

T2D is a major comorbidity of MetS. The disease is characterized by obesity and insulin resistance, which are most likely triggered by inflammatory signals [162]. Several studies indicated that low amounts of ABA can benefit the glycemic response [41,46,163], and PPARγ in immune cells was shown to be essential for the protective effect of ABA [163]. PPARγ is important for the metabolic switch from sugars to lipids, which in turn influences the differentiation and polarization of various immune cells [164,165]. ABA was shown not to bind to the ligand-binding domain of PPARγ and how ABA indirectly activates PPARγ in controlling glucose levels thus remains to be determined [166]. Evidence for an important role of endogenous ABA in glycemic control comes from the observation that T2D is associated with failure to up-regulate ABA upon glucose challenge [28]. The central role of ABA in sugar responses in humans is an interesting parallel to one of the physiological roles of ABA in plants, where ABA controls germination, vegetative development, and stress responses through the glucose signal transduction pathway [167]. ABA treatment of *in-vitro* cultured pre-adipocytes induces the translocation of glucose transporter type 4 (GLUT-4) to the plasma membrane, which increases glucose uptake [49]. Silencing of lanthionine synthetase component C-like protein 2 (LANCL2), which has been identified as an ABA receptor, indicates a role for LANCL2 in this ABA-induced glucose uptake in adipocytes [49,168]. LANCL2 is normally localized at the plasma membrane where it is coupled to the α subunit of a Gi protein, but is imported in the nucleus upon ABA stimulation [169]. In human hepatocyte cell lines, LANCL2 activation induces the phosphorylation of the serine/threonine protein kinase Akt via mechanistic target of rapamycin complex 2 (mTORC2) [170], which has also been shown to induce GLUT4-dependent glucose uptake and to increase glycolysis and several key gluconeogenesis enzymes in an Akt-dependent manner [171]. Together, these findings suggest a model in which ABA binding to LANCL2 triggers mTORC2-dependent Akt phosphorylation and the subsequent translocation of GLUT4 to the plasma membrane leads to glycolysis and glucose uptake. Finally, the protective effects of ABA in T2D may not only reflect its anti-inflammatory activities and role in glycemic control, but also the fact that ABA-induced Akt signaling can turn white fat cells into brown fat cells, and stimulate glucose uptake by brown fat cells [168].

SA induces glycolysis via activating AMPK, which strongly influences diabetes [73]. It was recently reported that SA also might provide health benefits similar to caloric restriction diets through increased mitophagy and cellular rejuvenation [172]. Low dose Aspirin (acetyl-SA) has been shown to be beneficial for glucose tolerance in a rat model of T2D [173], However, T2D can also lead to Aspirin resistance [174], which suggests that some pathological changes in T2D may also reflect resistance to endogenous SA.

Muscle protein anabolism is one of the symptoms caused by insulin resistance in T2D patients [175]. In this context it is worth mentioning that an analog of plant growth regulator brassinosteroids (BR), 28-homobrassinolide, induces protein anabolism and suppresses protein breakdown in rat skeletal muscle cells via Akt signaling [176]. Moreover, the synthetic plant hormone strigolactone (SL) analogue GR24 activates NAD^+ dependent deacetylase, which leads to GLUT4 translocation and mitochondrial biogenesis, glucose uptake, and insulin signaling in rat skeletal muscle cells [177]. Together, these data demonstrate an important regulatory role of several phytohormones in the pathology of T2D.

4.2. Colorectal cancer and inflammatory bowel disease (IBD)

Colorectal cancer incidence and mortality are both increased in patients with MetS [178]. Many anti-inflammatory foodstuffs, for example spices (including curcumin and salicylates) in the Indian diet [179–181], seem to be associated to a lower incidence of colorectal cancer, indicating a causative role of gut inflammation. A protective effect of ABA against colorectal cancer has not been shown yet. However, research of the last years has shown that ABA is a potential pharmaceutical compound against colitis, which may indirectly affect the development of colon cancer. For instance, dietary ABA reduces IBD in mice through the up-regulation of PPARγ in immune cells [26,27]. As obesity is associated with more severe cases of IBD, the described anti-

obesity effects of ABA could also be beneficial in the context of IBD and ultimately colon cancer [182].

Aspirin (acetyl-SA), on the other hand, has been shown to be very effective against the development of colorectal cancer [183], and might act synergistically with a fiber-rich diet [184]. SA directly inhibits the proliferation of human colon HT-29 cancer cells by targeting CDK2 [67]. In addition, several tumor-promoting inflammatory signals, such as NF-κB signaling, are SA sensitive [185].

Other major plant hormones can be also an option to develop as anti-cancer therapeutics. As also mentioned above, the cytokinin kinetin riboside inhibits proliferation of HCT-15 colon cancer cells [186]. Also derivatives of the plant defense-related hormone jasmonic acid (JA), which have a similar structure as prostaglandins in mammals [187], induce cell death and inhibit cell proliferation of several human cancer cell lines including colon cancer cells [188]. The application of SL analogues can also induce apoptosis and G2/M cell cycle arrest in colon cancer cell lines [189]. IAA-administration in mice was shown to induce the degradation of a known-marker for colorectal cancer, β-catenin [190]. A G1-phase arrest can result from the treatment of IAA or BR in human breast cancer cell lines, and SL applications can result in cell death of primary prostate cancer cells [191,192].

4.3. Non-alcoholic fatty liver disease (NAFLD)

The hepatic counterpart of MetS is NAFLD [193]. NAFLD frequently occurs as a consequence of MetS and evokes life-threatening nonalcoholic steatohepatitis (NASH) [193]. The onset of NASH highly correlates to MetS since 88% of biopsy-proven NASH patients are shown to have MetS [193]. Interestingly, patients with liver cirrhosis, an advanced symptom of NASH, produce a higher amount of IAA in lumbar cerebrospinal fluid [82]. Also, D-galactosamine-induced liver injury induces an increase of IAA in rat serum and isolated hepatocytes [13]. The observed increase of IAA in these liver diseases might be important in the control of disease development and delay further pathogenesis in comorbid diseases. Indeed, IAA ameliorates liver injury and improves insulin resistance in HFD-induced NAFLD mice, along with dampened hepatic oxidative and inflammatory stress [90]. Another study also showed an increase in antioxidant enzyme activities in the liver of IAA-supplemented rats [194]. Interestingly, IAA was shown to act as a direct agonist for human AHR that is heterologously expressed in yeast as well as in mouse hepatoma cell lines [84,85]. Since AHR negatively regulates HFD-induced hepatic steatosis, increased IAA levels in response to HFD-induced liver inflammation may be involved in dampening damage via an AHR-dependent pathway [15].

4.4. Atherosclerosis and cardiovascular disease

Cardiovascular disease is a major cause of death in modern society. The risk for cardiovascular disease development is significantly increased by MetS and is further exacerbated when T2D has developed. Also, psoriasis, another comorbidity of MetS (see below), is correlated with cardiovascular disease [195]. Atherosclerosis is an inflammatory disease that relies on foam cell formation by macrophages and pro-inflammatory signals from T cells [196], and ABA shows protective effects against atherosclerosis via activities on both cell types [32]. Low dose Aspirin (acetyl-SA) can have a dual favorable function against cardiovascular disease, since it has both anti-inflammatory and anti-thrombotic activities [197]. Recent results also indicate that Aspirin (or free SA) can have cardioprotective effects via the induction of mitophagy [172].

4.5. Arthritis

Arthritis induced by MetS could partially be due to increased weight-bearing and mechanical stress on joints from obesity [198], but MetS is also more common in patients with psoriatic arthritis [199]. Also, rheumatoid arthritis correlates with the risk of cardiovascular disease [200]. As we mentioned above, SA activates AMPK, which could additionally induce glycolysis to alleviate rheumatoid arthritis [73,201]. Aspirin has long been used as an anti-inflammatory agent against rheumatoid arthritis, but in high ("normal") doses [202]. Also, methyl jasmonate shows anti-oxidative, anti-inflammatory and anti-osteoarthritic effects against LPS-induced arthritis [203]. For now, there is no evidence for benefits from low dose Aspirin or JA in arthritis.

4.6. Psoriasis and atopic dermatitis (AD)

Although immunologically two different diseases, with atopic dermatitis (AD) typically being a Th2-driven disease and psoriasis a Th17-driven disease, both are comorbidities of MetS [204]. *In-vitro* cultured keratinocytes produce ABA upon UV stress [50], but it is currently unknown if ABA plays an important role in the suppression of inflammatory signals leading to psoriasis or AD. A recent study does, however, indicate that stimulation of PPARγ can be beneficial against AD [205]. Since ABA acts upstream of PPARγ in other cell types [163], it is therefore possible that ABA also plays a beneficial role against AD. In contrast to many other comorbidities of MetS, Aspirin treatment might increase the risk or exacerbate AD [206], but not psoriasis [207].

4.7. Depression, memory, and other psychological problems

MetS is not only affecting "physical" health, but also several "mental" comorbidities, like depression [208] and T2D-induced cognitive decline [209]. There is increasing evidence that depression and other mental ailments are linked to inflammatory signals via gut dysbiosis and a leaky gut [210–212]. Apart from its beneficial effects on gut inflammation and glycemic control as outlined above, ABA also seems to have direct beneficial effects on memory and depression [31,213]. Just like ABA can have indirect (via gut inflammation) and direct beneficial effects on depression, it is possible that ABA has a similar dual positive effect against cognitive decline via glycemic control, since memory loss is exacerbated by high blood sugar levels [214]. ABA-administration was shown to protect against the cognitive decline in mice models of Alzheimer's disease and HFD-induced neuroinflammation [215,216]. A rat model of Alzheimer's disease has shown that learning, memory and cognitive functions can be improved by ABA via a PPAR β/δ and PKA–dependent mechanism [34]. Whether ABA treatment can also reduce the risk of cognitive decline associated with other neurodegenerative diseases, such as Parkinson's disease [217], is unknown. Noteworthy, the brain is one of the organs that show the highest abundance of endogenous ABA [218].

With the participation of 5-hydroxytryptamine (5-HT), better known as serotonin, Trp metabolism is strongly implicated in anxiety-related behaviors [81]. An anxiolytic effect was shown as a result of increased levels of Trp metabolites in plasma, midbrain, and hippocampus of tryptophan 2,3-dioxygenase (TDO) deficient mice, which can no longer convert Trp to kynurenine [81]. As plasma IAA levels also significantly increased in $Tdo^{-/-}$ mice, it would be of interest to determine whether IAA plays directly or indirectly a role in the improvement of anxiety-related behaviors [81]. Also, methyl jasmonate reduces depression and aggression in mice via a mechanism in which serotonin has been suggested to be implicated [219,220]. Whether Aspirin treatment could be used to prevent the development of depression is currently under investigation [221].

5. Conclusions and perspectives

To manage and prevent MetS, the most valid strategy is altered behavior like diet and lifestyle, however, this is challenging for patients [222]. As a complement, food supplement pills that can provide some of the benefits of a plant-rich diet could be very useful. For example, self-medication with a daily low dose of Aspirin together with additional

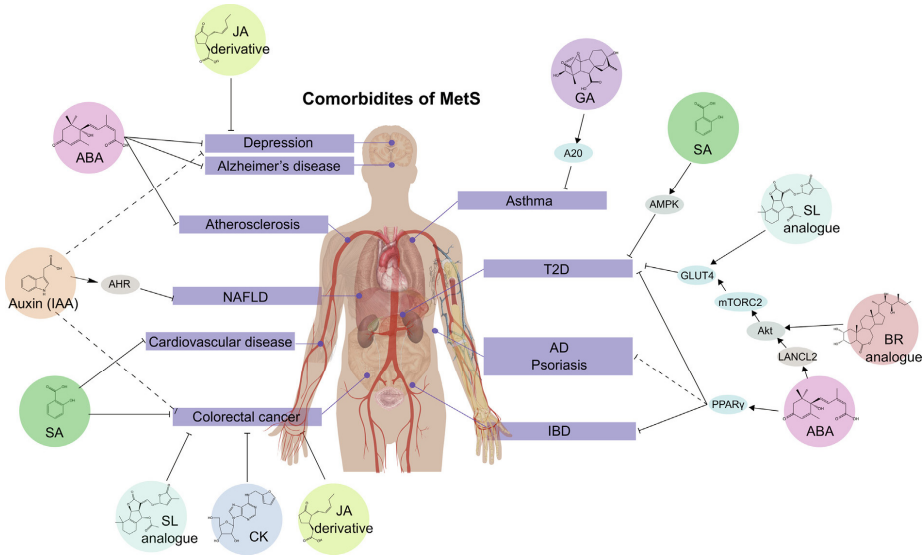

Fig. 6. Overview of the protective effects of phytohormones in comorbid diseases of MetS. MetS is a cluster of metabolic risk factors that increase the chance of developing comorbidities. Major phytohormones, ABA, SA, IAA, CK, GA, BR, SL, and JA were shown to alleviate diverse comorbid diseases and target signaling pathways that are involved in the regulation of immune responses and metabolism, which allows us to propose phytohormones as future multifunctional nutraceuticals against MetS. Human body diagrams are under Public Domain licensing and were obtained from Wikimedia Commons, the free media repository.

plant fibers does seem to have synergistic beneficial effects, for example against colon cancer [184]. As such, we propose that the protective roles of phytohormones could be used to develop future multifunctional plant-based drugs to mitigate comorbid diseases of MetS (Fig. 6). Yet, the optimal formulation and dosage for any phytohormone supplements are left to be determined. The value of dietary ABA has already been appreciated in pharmaceutical and nutraceutical markets. ABA extract of fig fruit was recently released for sugar control against T2D [45]. On top of inflammatory conditions for which ABA is medicated, the identification of other targets of ABA may indicate other disease conditions in which ABA could have a therapeutic value. Apart from the protective roles of endogenous phytohormones against diseases associated with MetS, given that they are endogenous, the development and pathogenesis of other inflammation-driven diseases might be controlled and prevented. A bottleneck to discover additional physiological roles of endogenous human phytohormones is their poorly defined biosynthesis pathways and receptors. The known metabolic pathways of these hormones in other organisms, plants, bacteria, and fungi, could be utilized to deplete endogenous hormones in animals, which will enable us to gain insights on the physiological roles of endogenous human phytohormones [8]. The functional studies of endogenous human phytohormones, in turn, will also offer us a deeper understanding of signaling pathways associated with relevant inflammatory disorders.

CRediT authorship contribution statement

Seo Woo Kim: Conceptualization, Writing - original draft, Visualization. **Alain Goossens:** Supervision, Writing - review & editing. **Claude Libert:** Writing - review & editing, Funding acquisition. **Filip Van Immerseel:** Writing - review & editing, Funding acquisition. **Jens Staal:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Rudi Beyaert:** Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 1. Addendum

Phytohormones: Multifunctional nutraceuticals against metabolic syndrome and comorbid diseases

Brassinosteroids - a class of steroid-derived signaling molecules that trace the evolutionary history

Chapter 1 introduced a variety of phytohormones that show beneficial effects on comorbid diseases of metabolic syndrome (MetS). One such hormone is brassinosteroids (BRs). BRs are derived from plant sterols (phytosterols) and comprise one of the six major phytohormone classes, after auxins, abscisic acid, cytokinins, ethylene, and gibberellin. Approximately 70 natural BRs present as free molecules or conjugates have been isolated in plants [1]. Many studies have reported their functions in controlling resistance against abiotic stresses and developmental processes like growth, fertility, and flowering [1]. Steroids are not solely present in plants but universal as an important hormones and components of cellular membranes in eukaryotes such as fungi and animals (**Figure 1**) [2]. The representative sterols in each eukaryote are cholesterol in animals, phytosterols in plants, and ergosterol in fungi [3]. Their biosynthesis pathways are conserved involving the common precursors dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP) (**Figure 1**) [2]. The first metabolite committed to sterol biosynthesis in plants, fungi, and animals is C_{30} hydrocarbon squalene [2]. First phytosterols, namely sitosterol and campesterol, are synthesized via cycloartenol from squalene [2]. Plants use these phytosterols as starting points to further produce other plant steroids like BRs [2]. In fungi, epoxidation of squalene followed by the cyclization of squalene epoxide leads to the production of lanosterol, which ultimately converts into ergosterol [4]. In animals, lanosterol also serves as a precursor of steroids such as cholesterol, testosterone, androgens, and estrones [5,6]. Animal sex hormones like androgen and estrogen are also present in plants; however, their functions are not revealed yet [2]. What makes steroids interesting is their conservation and diversity among eukaryotic taxa, which allows tracing the evolutionary history of eukaryotes in a geological manner [7,8].

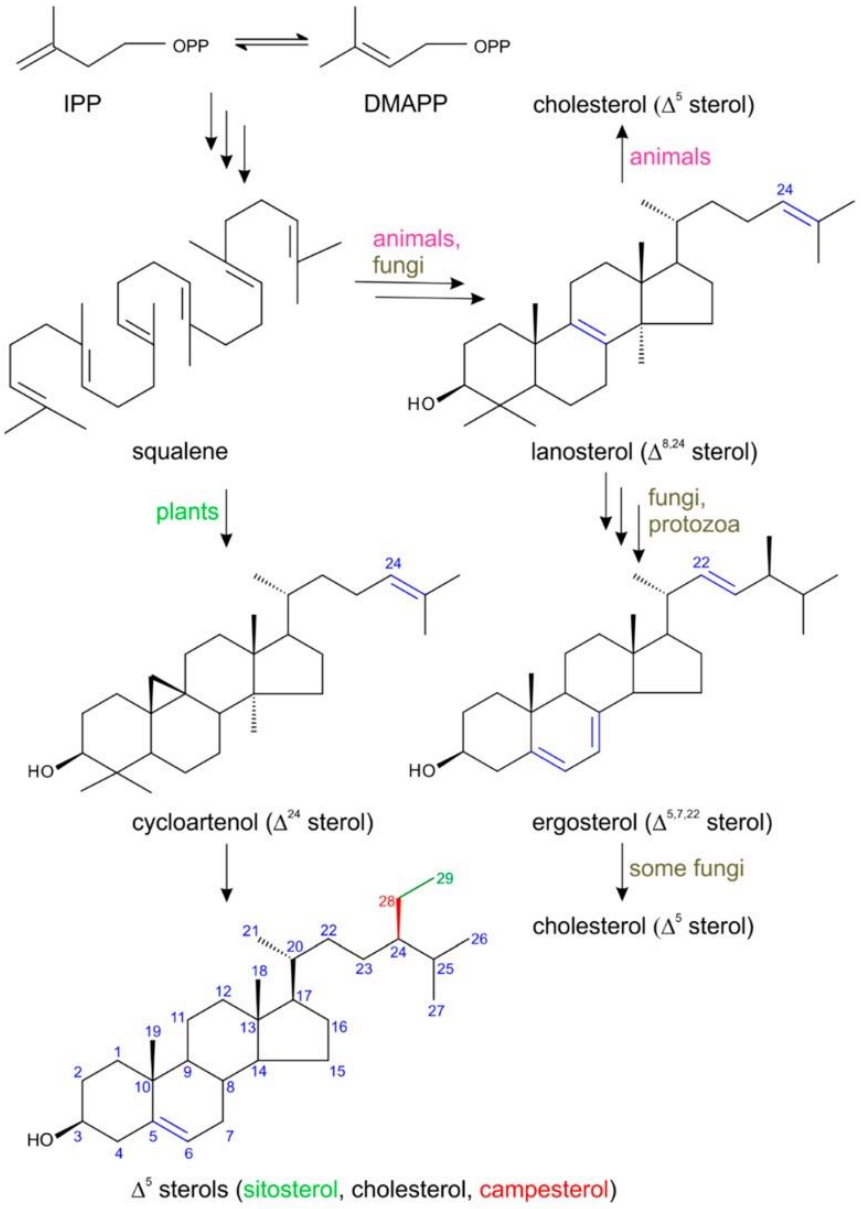

Figure 1. A simplified biosynthetic pathway of natural sterols in animals, plants, and fungi.
This figure is modified from Tarkowská, 2019 [2].

Derivatives of cholesterol, such as glucocorticoids and sex hormones (e.g., androgen and estrogen), exert immunomodulatory functions [9,10]. Under circadian control, the hypothalamus-pituitary-adrenal gland (HPA)

axis regulates glucocorticoid secretion, which controls the inflammatory response via the activation of its intracellular receptor (glucocorticoid receptor or GR) [9,10]. GR controls various gene networks by directly interacting with transcription factors (e.g., nuclear factor-κB or NF-κB and activator protein-1 or AP-1) or by indirectly regulating pro-inflammatory gene expressions (e.g., IL-1 or TNF) [9,11]. GR also regulates the production of pro-inflammatory cytokines or adhesion molecules in immune cells, reflecting glucocorticoids' immunomodulatory function [9,11]. Their immunomodulatory functions thus made synthetic glucocorticoids a universal drug in treating inflammation-driven diseases such as colitis, psoriasis, and rheumatoid arthritis [10,12]. In multiple tissues such as the liver, pancreas, skeletal muscle, and white adipose tissues, glucocorticoids play a role in glucose or lipid metabolism; therefore, glucocorticoid dysregulation or the abnormality in GR signaling leads to the development of MetS [13–15]. Also, the metabolism regulation by glucocorticoids was shown to be influenced by sex-steroids, such as androgen and estrogen, and vice versa, implying the importance of balance between these steroids [16,17].

Interestingly, BRs and other phytosterols have shown promising pro-metabolic and anti-inflammatory effects, thus suggesting their potential in treating issues relevant to MetS. For instance, it has been more than seven decades since pre-clinical and clinical studies reported that dietary phytosterols such as β-sitosterol, campesterol, and stigmasterol lower the levels of low-density lipoprotein (LDL) and total cholesterol in the blood, indicating their antiatherosclerotic effects [18,19]. The primary mechanism behind this is the competition between cholesterol and phytosterols, thereby inhibiting the intestinal absorption of cholesterol [20]. However, studies using a cholesterol-free diet and mice exhibiting very little biliary cholesterol suggest the involvement of an additional mechanism, namely via their anti-inflammatory effects [21,22]. Dietary phytosterol supplementation containing β-sitosterol, campesterol, dihydrobrassicasterol, and stigmasterol was shown to inhibit the pro-inflammatory cytokine production (e.g., interleukin (IL-6) and tumor necrosis factor (TNF)-α) and increase the production of anti-inflammatory IL-10 from cultured splenocytes of atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) mice [21].

Moreover, dietary phytosterol esters, mainly composed of ester forms of β-sitosterol, campesterol, and stigmasterol, lowered levels of hepatic total cholesterol, triglycerides, and fatty acids in a high fat diet-induced non-alcoholic fatty liver disease (NAFLD) mouse model, along with ameliorated hepatic oxidative and inflammatory stress [23]. Furthermore, several studies showed the anti-diabetic effects of phytosterols [24]. β-

sitosterol was shown to induce glucose uptake and decrease intracellular levels of triglyceride and cholesterol in L6 myotube cells via AMP-activated protein kinase (AMPK) activation [24]. In addition, the effect of campesterol derivative (5-campestenone) on glycemic control in diabetic fatty rats and db/db mice was examined [25,26]. Also, phytosterols showed their potential in lowering the risk of colorectal cancer, possibly driven by metabolic dysregulation. The application of a bioactive by-product of BR biosynthesis, epibrassinolide was shown to induce cell death of human colon cancer cell lines in an phosphoinositide-3-kinase/serine-threonine protein kinase (Akt) dependent manner [27]. In conclusion, on top of other phytohormones such as salicylic acid, abscisic acid, cytokinins, and indole-3-acetic acid, the described effect of phytosterols including BRs on immune regulation and metabolic homeostasis shows their potential for therapeutic practices in managing and preventing MetS.

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Chapter 2. Thymic T cell development and function in the context of immunometabolism

Chapter 1 introduced the anti-inflammatory function of phytohormones against comorbid diseases of metabolic syndrome (MetS). We highlighted the importance of discovering additional physiological roles of endogenous mammalian phytohormones, which will eventually offer us a deeper understanding of the pathogenesis of relevant diseases. To achieve that, it is important to comprehend some key features of the immune system. As in the results section I mainly focus on thymic T cell development and because diverse T cell subsets are closely tied to metabolic homeostasis, whose imbalance brings the onset of MetS, I will limit this introductory chapter mainly to these aspects of the immune system.

1.1 The innate and adaptive immune systems

The immune system comprises two major parts: the innate immune system and the adaptive immune system. The development and the organization of lymphoid tissues, such as bone marrow, thymus, spleen, and lymph nodes, contribute to the defense offered by these immune systems [1,2]. Intricate orchestration of innate and adaptive immune responses confer a tight regulation of inflammatory responses to ensure efficient immune protection.

Innate immunity is a non-specific and rapid defense system induced by pattern recognition receptors, conserved in many microorganisms [3]. Innate immune cells include natural killer cells (NK cells) and phagocytes (e.g., dendritic cells or DCs). NK cells kill virally- or tumor-infected cells and secrete pro-inflammatory molecules like tumor necrosis factor (TNF) and interferon-gamma (IFNγ), which regulate the function of other innate and adaptive immune cells [4]. DCs are APCs that link the innate immune system and the adaptive immune system. They capture antigen and present it to the surface of the T cells. DCs are divided into several subsets, such as conventional dendritic cell type 1 (cDC1), conventional dendritic cell type 2 (cDC2), and monocyte-derived dendritic cells (moDCs). cDC1 is effective in priming cytotoxic T cells that can kill tumors and infected cells [5], and cDC2 is the major DC population in the spleen, which is specialized for priming helper T cells. Another distinct subset, moDCs, are differentiated from monocytes upon inflammation and serve as a backup reservoir of APCs.

The adaptive immune response is specific for particular pathogens, involving immunological memory that remembers the pathogens and enhances the immune reaction upon the second encounter of the pathogen [3]. Adaptive immunity is mediated by T cells [6]. There are two main types of T cells: cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells.

Cytotoxic T cells eliminate tumors and infected cells [7]. Helper T cells activate other immune cells like B cells to produce antibodies, macrophages to phagocytose microbes, and cytotoxic T cells to destroy virus- or tumor- infected cells [8]. Cytotoxic and helper T cells become activated when antigens are presented to them by antigen presenting cells (APCs), and these activated T cells also produce immunomodulatory molecules called cytokines [9]. Helper T cells also differentiate into regulatory T cells (Tregs) and memory T cells. Tregs are immunosuppressive and have roles in preventing autoimmune diseases [10]. Memory T cells are antigen-specific T cells playing essential roles in immunological memory. Memory T cells stay after an infection terminates and can rapidly change into effector T cells such as Tregs, cytotoxic T cells, and helper T cells [9]. Another major component of the adaptive immune system is B cells [11,12]. B cells are responsible for antibody production in adaptive immune responses. B cells are produced in the bone marrow and then migrate into the peripheral lymphoid organ spleen and develop into follicular and marginal zone B cells [13]. A small population of B cells also develops into regulatory B cells, which displays immunesuppressive functions. A specialized subset of T cells also collaborates with B cells. For instance, T follicular helper cells (Tfh) are located in the B cell zone of the spleen and help B cell differentiation [14].

1.2 T cell development in thymus

The thymus is a primary lymphoid organ of the immune system [15]. The thymus plays an essential role in developing T cells, crucial for the adaptive immune system [6]. The thymus microenvironment induces the differentiation of the bone marrow-derived hematopoietic stem cells into T cells via distinct maturational stages in tight interaction with thymic epithelial cells and other hematopoietic cells [16]. Distinct maturational stages are characterized based on the presence of cell surface markers (*e.g.*, CD4, CD8, CD25, and CD44) [17]. First, after the progenitors arrive in the thymus, they develop into the $CD4^-CD8^-$ double negative (DN) cells in the cortex [18,19]. The DN stages are further sub-divided into four stages by the presence of CD44 (an adhesion molecule) and CD25 (interleukin-2 receptor α chain) [18]. Next, DN cells progress into $CD4^+CD8^+$ double-positive (DP) cells [18]. DP cells then translocate to the medulla and mature into $CD4^+$ or $CD8^+$ single-positive (SP) cells [18]. A distinct subset of CD4+ SP T cells can differentiate into $CD25^+FoxP3^+$ regulatory T cells (Tregs), suppressing excessive immune response and autoimmunity [20]. Lastly, T cells and Tregs egress from the thymus and enter lymphatics or blood vessels in the medulla, thereby circulating in the bloodstream and the lymphoid tissue to be capable of participating

in the adaptive immune response [18].

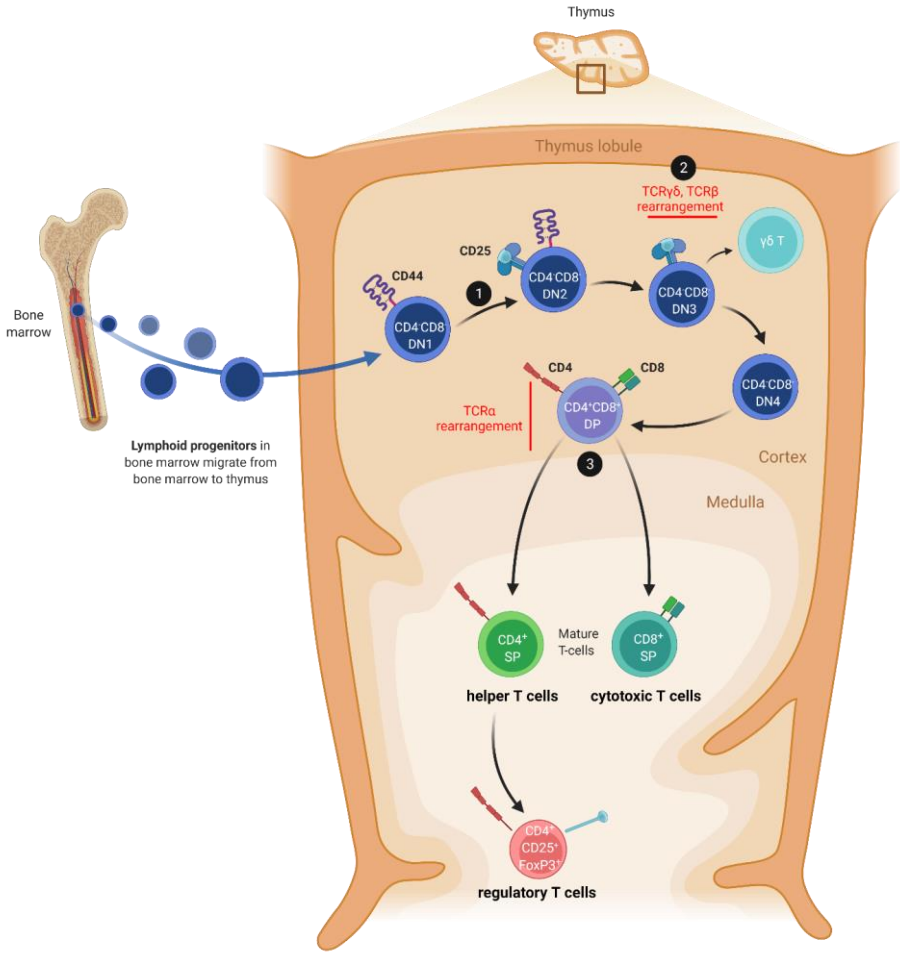

Figure 1. T cell development in thymus.
The figure was adapted from "T cell development in thymus" by BioRender.com (2022) and retrieved from https://app.biorender.com/biorender-templates.

In the journey of T cell precursors in the thymus, three critical checkpoints decide the cell fate of T cells by rescuing them from programmed cell death and inducing their commitment to distinct lineage [18,21]. These checkpoints are indicated with black circled numbers.

❶ Notch signaling: Besides the commitment of DN1 ($CD44^+CD25^-$) cells into the T cell lineage, DN1 cells can also turn into non-T cells such as dendritic cells (DCs), natural killer (NK) cells, and macrophages [22].

Here, to block this alternative lineage, the Notch signaling promotes an initial T-lineage commitment [23,24].

❷ β-selection: One of the primary T cell features is the presence of a T cell receptor (TCR) on their cell surface [25,26]. The TCR is responsible for antigen recognition by recognizing peptides presented by major histocompatibility complex (MHC) molecules [27]. Variations of TCR allow T cells to recognize many peptides presented by MHC [25,28]. The TCR is comprised of two different protein chains. The majority of T cells express the TCR composed of an α and a β chain, while the minority of T cells possess a δ and a γ chain. Each TCR loci consists of sets of gene segments, enabling the production of variable and constant regions [28,29]. Somatic recombination of variable (V), diverse (D), joining (J), and constant (C) gene segments results in the formation of diverse TCR [28,29].

At this step, DN thymocytes undergo receptor gene arrangement, employing a series of DNA-interacting enzymes, and exhibit the commitment to either a pre-αβ or a γδ lineage [30,31]. The β-selection here rescues pre-αβ cells from apoptosis, which successfully rearranged their TCRβ chain locus [21]. The TCRβ chain then pairs with an invariant α-chain (pre-α) [30,31]. Next, the survived pre-αβ DN thymocytes proliferate and differentiate into $CD4^+CD8^+$ DP cells by upregulation of CD4 and CD8 expression [32]. CD4 and CD8 act as coreceptors for MHC I (CD8) or MHC II (CD4)-restricted TCR recognition, respectively [33].

❸ Positive and negative selection: DP cells then undergo the extensive proliferation, followed by the rearrangement of TCRα chain loci, enabling the production of a large number of αβT cells. Next, αβT cells undergo positive selection. In the cortex, DP cells bearing TCR that engage antigen-MHC with an appropriate affinity survive, while cells with weaker affinity interactions undergo apoptosis [34]. These selected thymocytes then migrate into the medulla, where the negative selection occurs. During the negative selection, thymocytes bearing TCR that engage too strong affinity interactions with antigen-MHC die, as they can be self-reactive and cause autoimmunity [34,35].

At the late stage of positive selection, the DP thymocytes differentiate into $CD4^+$ SP or $CD8^+$ DP. The underlying mechanism of the CD4-CD8 cell-fate decisions has been the subject of debate for a long time [33]. Several models for the CD4-CD8 lineage decision have been proposed. First, the classic 'stochastic-selective' model suggests that the downregulation of CD4 or CD8 gene expression occurs randomly, in a TCR-independent manner, and the following selection removes cells bearing the mismatched TCR [33,36]. Second, the 'instructive' model

speculates that the downregulation of CD4 or CD8 gene expression occurs in a TCR-dependent manner [33,36]. The TCR and the matched coreceptor molecule (CD4-MHCII or CD8-MHCI) elicit the signal, which accordingly downregulates the expression of the mismatched coreceptor gene [33]. Third, a relatively recent 'kinetic signaling model' postulates that the lineage choice of both $CD4^+$ SP and $CD8^+$ SP initiates from the transcriptional repression of the CD8 gene, resulting in $CD4^+CD8^{lo}$ intermediate thymocytes expressing lower CD8 levels [37–39]. In MHC-I restricted thymocytes, the TCR signaling is disrupted due to the mismatch between CD4 and MHC-I restricted TCR. The disrupted TCR signaling induces MHC-I $CD4^+CD8^{lo}$ thymocytes to switch coreceptor expression from $CD4^+CD8^-$ to $CD4^-CD8^+$, thereby leading to the further development into $CD8^+$ SP thymocytes [37]. In the MHC-II restricted thymocytes, TCR signaling is not disrupted, and $CD4^+CD8^{lo}$ cells further progress into $CD4^+$ SP cells. [36,37,40].

1.3 Thymic T cells in immunometabolism

Immune cell function is intricately tied to metabolism. Several studies have shown that metabolic alterations play an important role in controlling the differentiation and activation of mature peripheral T cells [41,42]. Metabolic rewiring, such as glycolysis, glutaminolysis, and oxidative phosphorylation (OXPHOS), regulates T cell activation [41,42]. For instance, mitochondrial metabolism and aerobic glycolysis contribute to memory T cells' function and differentiation [42–44]. Compared to naïve T cells, $CD8^+$ memory T cells contain a higher amount of mitochondrial mass, which leads to their rapid and efficient proliferation and the production of cytokines upon activation [43]. In maintaining this increase of mitochondrial mass upon activation, the involvement of increased OXPHOS and aerobic glycolysis was shown [43].

Whereas the roles of metabolic reprogramming in T cell fate and function in the peripheral lymphoid organ were well characterized, the effect of metabolic rewiring on thymic T cell development is relatively poorly understood [45]. At the β-selection checkpoint, the commitment of T cells to either a pre-αβ or a γδ lineage was shown to rely on rapamycin (mTOR) complex 1 (mTORC1)-dependent glycolysis and OXPHOS [46]. Loss of mTORC1 component regulatory-associated protein of mTOR (RAPTOR) DN3 cells yielded increased γδ T cell development and the production of reactive oxygen species (ROS) [46]. Recently, extracellular flux and transcriptomics analyses of human and murine thymocytes and *in vitro* artificial thymic organoid cells from DN to SP stages revealed their metabolic patterns in the context of glycolysis and OXPHOS between stages [45]. It was shown that cells at

the DN stage exhibit the highest glycolytic and OXPHOS activity, which correlates with the expression levels of genes encoding enzymes involved in glycolytic and tricarboxylic acid cycle pathways [45]. Afterward, the activity of glycolysis and OXPHOS decreases at the DP stage, which again eventually increases at the SP stage [45]. Besides primary metabolites like glucose, nutrients such as vitamins and zinc are also reported to affect thymic T cells [47–49]. The effect of malnutrition on the thymic microenvironment causes thymic atrophy, which leads to immune dysfunction [50,51]. Also, the metabolites relevant to phytohormones, which we introduced in **Chapter 1**, were shown to influence the thymic microenvironment [52]. For instance, a tryptophan metabolite derived from the kynurenine metabolic pathway, 3-hydroxyanthranilic and quinolinic acid, was shown to induce thymocyte cell death *in vitro* and *in vivo* in a caspase-8 dependent manner, indicating their potential roles in shaping thymic T cell homeostasis [52]. Several types of steroids are synthesized in the thymus (e.g., glucocorticoids and pregnenolone) [53–55]. It was previously shown that glucocorticoids produced by thymic epithelial cells play a role in thymic homeostasis [56]. Glucocorticoids specifically target DP cells (post positive selection), thereby downregulating the expression of genes involved in negative selection, resulting in decreased negative selection [56]. In addition, aspirin (acetyl-SA) supplementation was reported to induce a decrease in the percentage of $CD4^+CD8^+$ DP cells and an increase in the percentage of $CD4^+CD25^+$ Tregs in the thymus [57], suggesting the potential effect of salicylic acid (SA), a hydrolyzed metabolite of aspirin, on the thymic T cell development. Interestingly, cytokinin-like kinetin was isolated from calf thymus [58], and the kinetin treatment reduces oxidative damage in the thymus of aging rats [59], indicating the potential role of kinetin in delaying thymic involution [60]. However, it is currently unknown whether phytohormones (e.g., abscisic acid, SA, indole-3-acetic acid, cytokinins, and phytosterols including brassinosteroids) directly impact on thymic T cell development.

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PART II: AIM AND OBJECTIVES

Aims and objectives

Salicylic acid (SA), abscisic acid (ABA), cytokinins (CK), and indole-3-acetic acid (IAA) are grouped and generally termed as 'phytohormones', which have high potential as nutraceuticals because of their anti-inflammatory properties. What is less well known is that they are not exclusive to plants and are also endogenously produced and bioactive in animal cells. The functional role of these endogenously produced molecules in animal cells is however largely unknown.

In the 'Molecular Signal transduction in Inflammation' research group of prof. Rudi Beyaert, we are focusing on unraveling the role of molecular signal transduction in response to inflammation. Over the past years, the group gained significant knowledge and expertise in cellular models and mouse models of inflammatory diseases, such as inflammatory bowel disease (IBD) and psoriasis. Because of the already reported anti-inflammatory effects of exogenous ABA in colitis and SA in type 2 diabetes, we decided to focus first on these two molecules. Other important endogenous phytohormones such as CK and IAA are rather known for their cytotoxic activity and effect on nonalcoholic fatty liver disease, respectively.

We defined the following specific aims:

1. Unraveling the functional roles of endogenous SA in immune regulation

    1.1 Investigating tissue distribution of SA and the effect of SA treatment on the physiological function of SA-accumulating tissues (**Chapter 3.1**)

    1.2 Investigating possible immunological alterations induced by depletion of SA in geneticially engineered mice (**Chapter 3.2**)

2. Unraveling the functional roles of endogenous ABA in immune regulation

    2.1 Discovering inflammatory signaling pathway activators that affect ABA metabolism. In detail, we aim to develop a rapid, economical, and reliable method for ABA quantification (**Chapter 4.1**), which can also be used to investigate the effect of specific inflammation mediators on ABA metabolism (**Chapter 4.2**).

    2.2 Investigating immunological alterations induced by depletion of ABA in genetically engineered mice (**Chapter 4.3**)

    2.3 Investigating the physiological relevance of endogenous

ABA in the context of IBD (**Chapter 5**)

Obstacles in developing tools to address the abovementioned aims are the poorly defined biosynthesis and signaling pathways of SA and ABA. This PhD research therefore employed the known metabolic and signaling pathways of SA and ABA hormones in other organisms like plants and bacteria. We have generated transgenic mice overexpressing *Arabidopsis thaliana CYP707A3* and *ABH2* or bacterial *NahG* to degrade ABA or SA, respectively. In addition, to implement a broad cellular screening for diverse stimuli that possibly influence ABA signaling and metabolism, a rapid and economical method for ABA quantification is a milestone. We engineered a novel mammalian ABA biosensor system variant based on the PYR/PYL-ABA-PP2C ternary complex of *Arabidopsis* ABA signaling. The obtained insights in the role of endogenous SA and ABA will contribute to understanding the complex interplay between host immune regulation and metabolism. Also, this will in turn offer us a deeper understanding of the development and pathogenesis of relevant inflammatory disorders.

PART III: RESULTS

Chapter 3. Unraveling the roles of SA in immune regulation

Chapter 3.1. Tissue distribution of salicylic acid in mice and its effect on T-cell development in thymus

Seo Woo Kim[1, 2, 3, 4], Yasmine Driege[1, 2], Sandrien Desmet[5], Jacob Pollier[5], Mira Haegman[1, 2], Ioannis Skordos[1, 2], Alain Goossens[3, 4], Rudi Beyaert[1, 2], Jens Staal[1, 2, 6]

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[5]VIB Metabolomics Core Ghent, VIB, Ghent, Belgium
[6]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

Unpublished results

Author contributions

SWK, AG, RB, and JS designed the research and interpreted data. SWK, YD, SD, JP, MH, and IS performed experiments. SWK, SD, and JP analyzed the data. SWK wrote the paper with the help of AG, RB, and JS. AG, RB, and JS supervised the project and RB and AG provided resources.

1 Abstract

Background
Salicylic acid (SA) was proposed as a potential therapeutic/prophylactic against metabolic syndrome and its comorbidities like type 2 diabetes (T2D), colorectal cancer, and cardiovascular disease. Interestingly, SA is not a foreign molecule to animals. Humans are shown to produce SA, possibly mediated by benzoic acid 2-hydroxylase activity. This raises the interesting question whether the endogenous SA is involved in immune and metabolic homeostasis. So far, the physiological function of endogenous SA remains elusive.

Objective
To understand a possible function of endogenous SA, we wanted to investigate the concentration of SA in different mouse tissues. This information was then used to study the effect of SA treatment on the physiological function of SA-accumulating tissues.

Methods
Various tissues of C57BJ/6J mice were profiled for SA levels using gas chromatography-mass spectrometry (GC-MS). In addition, we studied the effect of SA by *ad libitum* feeding of sodium salicylate (250 mg/L) in drinking water to 4-weeks-old mice for 28 days.

Key Results & Conclusions
GC-MS analysis revealed that SA accumulates in the thymus and brown adipose tissues of mice under normal feeding conditions. Flow cytometry analysis of thymic T cells showed that SA administration slightly altered thymic T cell development by interfering with the transition from the double-positive (DP) stage to the $CD4^+$ single-positive (SP) or $CD8^+$SP stage. These data suggest that SA accumulation in the thymus may be implicated in T cell ontogeny.

2 Introduction

Salicylic acid (SA) is a small phenolic molecule composed of an aromatic ring with an attached carboxylic and a hydroxyl group [1,2]. Salicylates were first discovered as an analgesic and antipyretic [3,4], and ever since then, they have been used as a multifaceted therapeutic given their anti-platelet [5,6], anti-inflammatory [7–11], and cytotoxic activities [12–18]. Among others, a synthetic SA derivative, acetylsalicylic acid (aspirin), has been widely used as one of the top seller anti-inflammatory drugs [19–21]. In **Chapter 1**, we also proposed that SA might be a potential therapeutic/prophylactic against metabolic syndrome and its comorbidities like type 2 diabetes (T2D), colorectal cancer, and cardiovascular disease [17,22]. Interestingly, SA is not a foreign molecule to animals [23]. SA was detectable in plasma of patients who fasted and underwent total proctocolectomy, indicating that humans endogenously produce SA regardless of diet and gut microbiome [23]. It has been suggested that SA is synthesized from benzoic acid (BA), catalyzed by a benzoic acid-2-hydroxylase (BA2H) enzyme [23].

Phenolic compounds are known to occur widespread and ubiquitous in nature [24,25]. Likewise, SA is naturally produced and bioactive in eukaryotes (*i.e.,* plants and animals) and prokaryotes (*i.e.,* bacteria) [1,8,13,26–31]. In plants, SA plays a crucial role in defense mechanisms against biotic stress [2,8,26,29,31,32]. SA induces systemic acquired resistance (SAR), providing broad-spectrum and long-lasting pathogenic resistance to uninfected distal tissues [33–35]. Since host plants accumulate SA to induce resistance and SAR in response to pathogen attack, some plant-pathogenic bacteria (*i.e., Ralstonia solanacearum* and *Pseudomonas putida*) and fungi (*i.e., Ustilago maydis* and *Epichloë festucae*) are capable of SA degradation to enhance their virulence [36–41]. In addition, several bacterial species residing in the humans or rhizosphere incorporate SA to produce SA-derived siderophores, which are crucial in iron acquisition, promoting their growth and full virulence [26,42–45].

SA was proposed to be synthesized via an isochorismate (IC) pathway or phenylalanine ammonia-lyase (PAL) pathway across kingdoms [26,46]. Both pathways utilize chorismate as a precursor, which diverges into isochorismate by isochorismate synthase or cinnamate by chorismate mutase and PAL (**Figure 1**) [46]. Noteworthy, the BA2H, a potential enzyme that might catalyze human SA biosynthesis, is at the last step of the PAL pathway [23]. Whether humans endogenously produce BA has not been known, but humans may mainly derive BA from the uptake of milk-based diets and food preservatives [47]. In

plants, the ICS pathway was a major pathway in *Arabidopsis*, whereas in other plant species such as rice and soybeans, the PAL pathway also contributes to pathogen-induced SA accumulation [48–50]. In bacteria, the SA biosynthesis pathway involves ICS converting the chorismate precursor to IC [26]. Given the anti-inflammatory activity of SA, endogenous SA might be exploited in immune and metabolic homeostasis. To date, the physiological role of endogenous SA remains unexplored.

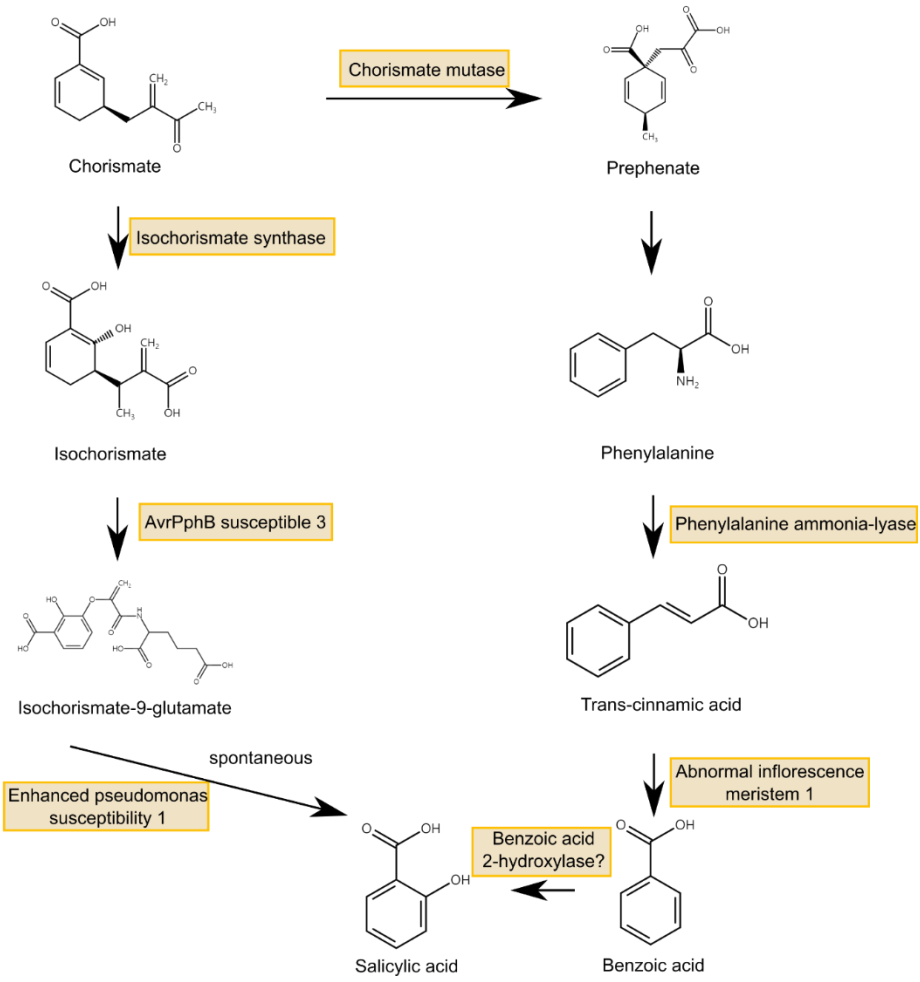

Figure 1. Salicylic acid (SA) biosynthesis pathways in plants (see following page for figure legend)**.**

Figure 1. Salicylic acid (SA) biosynthesis pathways in plants (see previous page for figure)**.**
The figure is modified from Lefevere et al., 2020 **[48].** SA was proposed to be synthesized via an isochorismate (IC) pathway or phenylalanine ammonia-lyase (PAL) pathway, utilizing chorismate as a precursor. In the PAL pathway, chorismate mutase catalyzes the production of prephenate, which converts into phenylalanine via several metabolites such as phenylpyruvate and arogenate **[51]**. Phenylalanine then further converts into trans-cinnamic acid by phenylalanine ammonia-lyase. *Arabidopsis* Abnormal inflorescence meristem 1 was shown to contribute to the conversion of trans-cinnamic acid to benzoic acid. The benzoic acid 2-hydroxylase was proposed to be an enzyme that might catalyze the conversion of BA to SA; however, the gene encoding this enzyme has not yet been identified. In the IC pathway, chorismate first converts into isochorismate by isochorismate synthase. The conversion of isochorismate to isochorismate-9-glutamate and the subsequent salicylic acid was catalyzed by *Arabidopsis* AvrPphB SUSCEPTIBLE3 enzyme and Enhanced pseudomonas susceptibility 1 enzyme (acetyltransferase), respectively. The conversion of isochorismate-9-glutamate to salicylic acid was also shown to occur spontaneously.

In order to reveal the involvement of endogenous SA in immune regulation in animals, we first questioned which tissues could produce or accumulate SA. Considering that animals intake SA from plant-based diets [46,48,49] and possibly from gut microbiome, tissues of mice under normal feeding conditions might also contain exogenous SA. Nevertheless, investigation on tissue distribution of bioactive compounds could help reveal their site of action and functions [52–55]. Determining SA-containing tissues may thus help discover whether SA could be secreted from certain tissues and can elicit endocrine or autocrine or paracrine signals, as well as the functional role of endogenous SA. Thus, in the current chapter, we set out to assess SA levels in different mouse tissues using mass spectrometry. In addition, we further investigated whether the exogenous application of SA could affect the physiological function of the identified SA-accumulated tissue.

3 Results

3.1 Tissue distribution of SA in mice

To validate a method for SA extraction in mouse tissues, we performed an exploratory study, in which we isolated different tissues of non-perfused or perfused mice and semi-quantified SA levels using gas chromatography-mass spectrometry (GC-MS) (**Figure 2**). The extraction was based on previously described methods for metabolomics in mice tissues and profiling SA in tobacco leaves [56,57]. Tissues containing rich blood vessels such as liver and kidney were isolated from mice perfused with phosphate-buffered saline (PBS), assuming that dietary SA in the bloodstream might exceed the level of accumulated SA in blood-rich tissues. This exploratory study not only confirmed the reliable extraction and profiling method but also revealed the tissue distribution of SA in mice. We observed that, except for tissues that might contain SA directly from plant-based diets (*e.g.*, small intestine and stomach), brown adipose tissues (BAT), thymus, and brain of non-perfused mice showed relatively higher levels of SA.

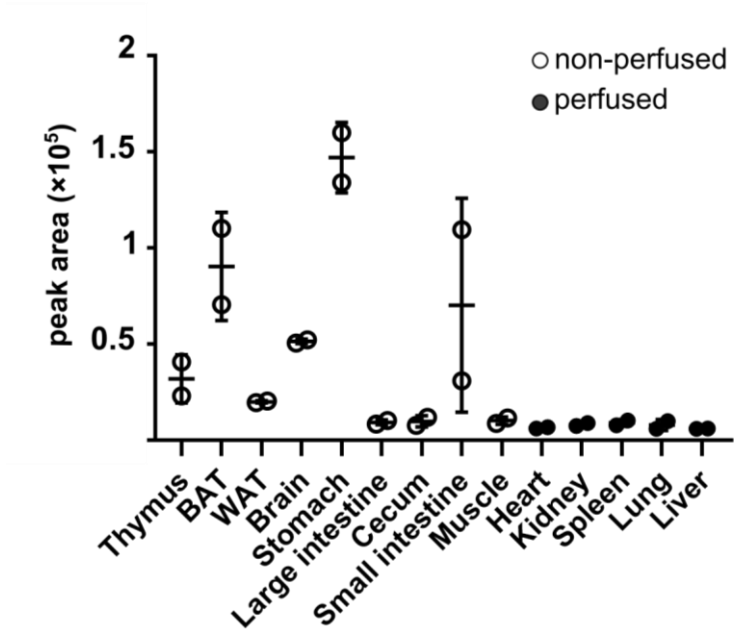

Figure 2. Determination of the salicylic acid (SA) levels in different tissues of non-perfused or perfused mice (see following page for figure legend)**.**

Figure 2. Determination of the salicylic acid (SA) levels in different tissues of non-perfused or perfused mice (see previous page for figure)**.**
GC-MS analysis revealed a peak area of SA (2trimethylsilyl or 2TMS derivative, *m/z* = 267.0867) in the thymus, brown adipose tissues (BAT), white adipose tissues (WAT), stomach, large intestine, cecum, small intestine, and muscle of non-perfused mice, as well as heart, kidney, spleen, lung, and liver of perfused mice. The empty dots represent each biological replicate for corresponding tissues of non-perfused mice, and the black dots represent each biological replicate for corresponding tissues of perfused mice. Error bars represent SD. Data shown are from one experiment (n=2). Peak area represents the area under a peak proportional to the amount of the corresponding metabolite.

Based on this preliminary observation, we assessed SA levels in SA-abundant tissues (*i.e.*, BAT, thymus, and brain) and SA-sparse tissues (*i.e.*, white adipose tissues (WAT) and liver) of both non-perfused mice and perfused mice (**Figure 3**). In line with the preliminary observation, SA levels in the thymus and BAT showed to be relatively higher compared to other tissues such as liver and WAT in both non-perfused and perfused mice (**Figure 3**). These data suggest the potential relevance of SA in the function of the thymus and BAT.

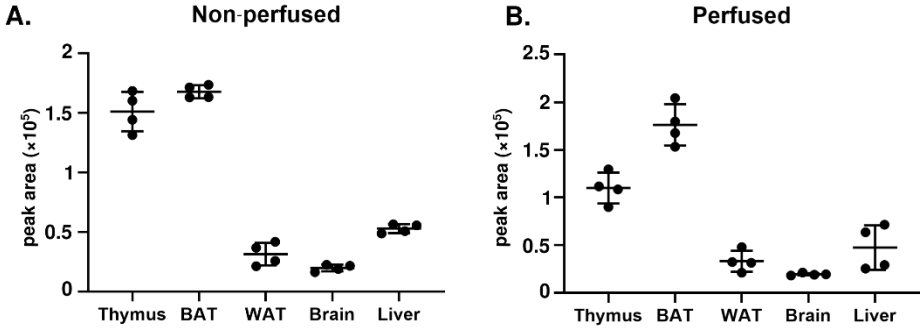

Figure 3. Determination of the level of salicylic acid (SA) in the thymus, brown adipose tissues (BAT), white adipose tissue (WAT), brain, and liver of non perfused (A) and perfused (B) mice.
GC-MS analysis revealed a peak area of SA (2TMS derivative, *m/z* = 267.0867) in the thymus, BAT, WAT, brain, and liver of non-perfused (A) and perfused (B) mice. Error bars represent SD. Data shown are from one experiment (n=4). Each dot represents one biological replicate. Peak area represents the area under a peak proportional to the amount of the corresponding metabolite.

3.2 SA administration has a slight effect on the frequencies of specific T cell subsets in the thymus

Accumulation of SA in the thymus raises the question of whether these tissues' physiological functions rely on SA. The thymus is a primary

lymphoid organ responsible for the maturation of thymus-derived cells (T cells), which mediate adaptive immune responses (see **Box 1**) [58]. We, therefore, first asked whether SA administration for several weeks affects T-cell development in the thymus of mice. 250 mg/L of sodium salicylate (NaSA) or sodium chloride (NaCl), as a negative control, was administered *ad libitum* to 4-weeks-old mice in the drinking water for four weeks. The dose of sodium salicylate in drinking water (250 mg/L) was opted for based on the previous study, which showed the anti-inflammatory effect of salicylic acid in mice fed a high-fat diet [59]. Sodium salicylate treatment reduced the expression of extracellular matrix genes (e.g., biglycan) and anti-inflammatory genes (e.g., IL-6 and TNF-α) in the subcutaneous adipose depot [59]. We opted for four weeks for its treatment, given the time between the entry of a T-cell progenitor into the thymus and the mature progeny release (3 weeks) [60]. The frequencies of T cell subsets in primary thymocytes were assessed by staining cells with T cell-specific surface markers, followed by flow cytometry analysis (**Figure 4**). SA administration did not change the frequencies of $CD4^-CD8^-$ double negative (DN) T cells and their subsets DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) (**Figure 4A**). Minor changes in the frequencies of $TCR\gamma\delta^+$ DN cells and $TCR\beta^+$ DN cells upon SA administration can be observed in the experiment shown (**Figure 4B**), but were however not reproducible in an independent experiment. SA administration slightly but reproducibly increased the frequencies of $CD4^+CD8^+$ double-positive (DP) T cells, while the frequencies of $CD4^+$ single-positive (SP) T cells and $CD8^+$ SP T cells were slightly decreased (**Figure 4A**). These conclusions were further supported by an independent experiment in which 4-weeks-old mice were intraperitoneally (IP) injected with NaSA for four weeks, which showed slight but statistically significant differences in the corresponding thymocyte populations (data not shown). Regulatory T cells (Tregs), characterized by expression of the transcription factor forkhead box P3 (FOXP3) and the IL-2 receptor (CD25), did not change upon SA administration (**Figure 4C**). Collectively, these results demonstrate that SA administration slightly reduces the transition from the DP stage to the $CD4^+$SP or $CD8^+$SP stage.

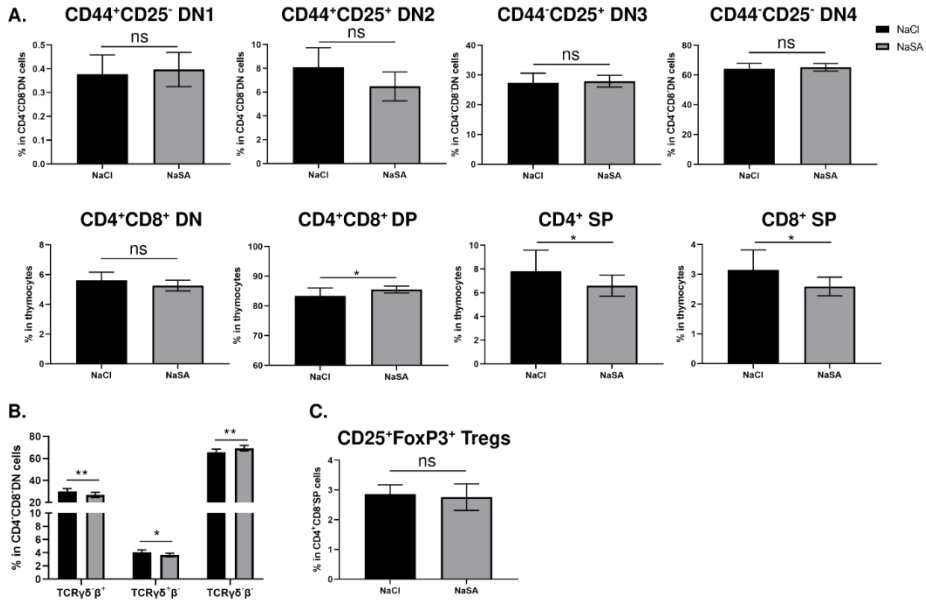

Figure 4. SA administration affects T cell development in the thymus. Thymocytes were isolated from C57BL/6J mice with NaSA or NaCl treatment for four weeks. The percentages of thymocytes in different stages of T cell development (A), the percentages of TCRγδ$^+$ T cells and TCRβ$^+$ T cells (B), and the percentage of Tregs (CD4$^+$CD25$^+$FoxP3$^+$) in the thymus (C) in the thymus of NaSA-treated or NaCl-treated control mice were analyzed using flow cytometry. Results are shown as mean ± SD (n=10). Statistical differences were determined using the Student's T-test (equal variance assumed) or Welch's t-test (equal variance not assumed), (not significant (n.s.) p-value >0.05, *p-value <0.05, **p-value < 0.01) (n=10). The data shown are from one experiment.

3.3 SA administration does not alter the frequencies of splenic T cell subsets

After the maturation of T cells in the thymus, mature thymic T cells egress from the thymus and migrate into the periphery [61]. The alteration in T cell development in the thymus thus can affect the T cell repertoire in the periphery, such as the spleen, the largest secondary lymphatic organ where lymphocytes responding to foreign antigens can initiate the adaptive immune response [62]. Therefore, we next compared the frequencies of splenic T cell subsets of SA-treated mice compared to control mice. However, SA administration did not alter the percentage of CD4$^+$ SP and CD8$^+$ SP T cells (**Figure 5A**). Also, the frequencies of TCRγδ$^+$ or TCRβ$^+$ T cells did not change (**Figure 5B**). Furthermore, similar to the percentage of thymic Tregs, the percentage of splenic Tregs also did not change upon SA administration (**Figure 5C**). These results indicate that SA has a slight effect on the maturation

of T cells in the thymus, which, however, does not alter the T cell repertoire in spleens. This might be due to the short lifespan of naïve T cells in the periphery [63], reflecting a thymocyte-specific effect of SA on T cell differentiation.

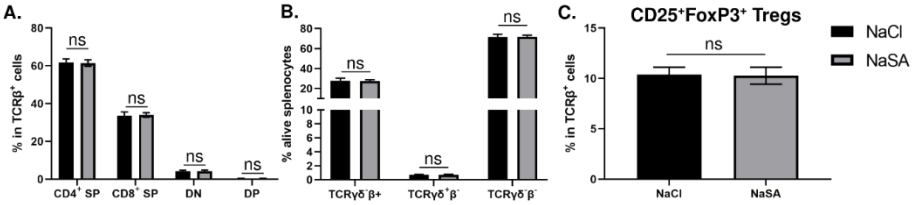

Figure 5. SA did not alter the frequencies of splenic T cell subsets in the spleens of mice

Splenocytes were isolated from C57BL/6J mice with NaSA or NaCl treatment for four weeks. The percentages of splenocytes in the percentage of splenic CD4+ T cells and $CD8^+$ T cells (A), the percentages of $TCR\gamma\delta^+$ T cells and $TCR\beta^+$ T cells (B), and the percentage of splenic Tregs ($CD4^+CD25^+FoxP3^+$) of NaSA-treated or NaCl-treated control mice were analyzed using flow cytometry. Statistical differences were determined using the Student's T-test (equal variance assumed) or Welch's t-test (equal variance not assumed), (not significant (n.s.) p-value >0.05, *p-value <0.05, **p-value < 0.01) (n=10). Data shown are from one experiment.

4 Discussion

In order to unravel the hitherto unknown physiological role of endogenous SA, we investigated its tissue distribution in mice. Here we show that SA is highly abundant in the thymus, implicating a potential contribution of SA to the thymic function. We observed that administration of high-dose SA alters T cell development in the thymus by weakly interfering with the differentiation of $CD4^+CD8^+$ DP T cells into $CD4^+$ SP or $CD8^+$ SP T cells. The unchanged frequencies of splenic T cells subsets reflect the thymus-specific impact of SA on T cell differentiation. Nonetheless, as mature thymic SP T cells egress from the thymus and migrate into the peripheral pool, it can be expected that the altered T cell populations can also affect the T cell repertoire in the peripheral pool, such as peripheral blood, which remains to be investigated [61].

The observed perturbation in thymic T cell differentiation might be explained by a change in the thymic microenvironment upon SA administration, directing the CD4-CD8 lineage [64,65]. In the kinetic signaling model of CD4-CD8 cell-fate decision, DP cells differentiate into the intermediate $CD4^+CD8^{lo}$ cells, subsequently committing to either the CD4 or CD8 lineage [66]. Here, the transcriptional regulation of the *Cd4* and *Cd8* gene expression plays an important role [64,67]. A key mechanism for the CD4 versus CD8 lineage choice is the antagonistic interplay between the transcription factors T-helper-inducing POZ/Krueppel-like factor (ThPOK) and Runt-related transcription factor (Runx) [64,68]. ThPOK was shown to be an essential transcriptional regulator for the CD4 T cell differentiation, while Runx3 was shown to be an essential transcriptional regulator for CD8 differentiation [68–71]. ThPOK limits *Runx3* expression to induce the differentiation toward $CD4^+$ SP, whereas Runx3 represses the *ThPOK* expression toward $CD8^+$ SP [68–71]. Interestingly, the mRNA expression of *ThPOK* is stage-specific [72]. DN and DP thymocytes do not express *ThPOK* mRNA, while the mRNA expression of Thpok was detectable in $CD4^+CD8^{lo}$ [72,73]. In this context, SA administration might interfere with the induction of *ThPOK* expression, thereby hindering the differentiation of DP to $CD4^+CD8^{lo}$. The Runx-dependent *ThPOK* silencer activity might have been maintained upon SA administration [68,74,75]. To corroborate this, a future experiment could check the expression level of *ThPOK* and *Runx3* in $CD4^+CD8^-$ thymocytes of SA-treated and control mice. As various cis-acting elements and trans-acting factors modulate *CD4* and *CD8* expression [68], single-cell RNA sequencing might reveal the relationship between the gene expression profile and the altered T cell development [76,77].

Our findings with SA-treated mice raise the interesting question whether endogenous SA would also affect T cell ontogeny in the thymus. As the effect of SA treatment on T cell development was already very minor, we speculate that endogenous SA concentrations, which are much lower, will not have any impact on T cell development. Future studies using genetically engineered SA-deficient mice may answer this question.

In contrast to our own observations in the present study, aspirin (acetyl-SA) treatment was previously shown to lead to a decrease in the percentage of CD4CD8 DP thymocytes and comparable frequencies of CD4 SP thymocytes [78]. Also, the percentage and cell number of thymic $CD4^+CD25^+$ Treg cells was shown to be increased [78]. In the peripheral blood and spleen, the frequencies of CD4 SP T cells decreased and $CD4^+CD25^+$ Treg cells increased upon aspirin treatment, and athymic mice verified that this altered T cell repertoire is mainly due to the direct effect of aspirin on T cells in the periphery [78]. The distinct major mechanism of action of aspirin (acetyl-SA) versus SA (NaSA) in our experiments might explain these discrepancies. A major effect of aspirin is the inhibition of cyclooxygenase (COX) through acetylation of a serine residue [79], which does not occur in response to NaSA due to the lack of an acetyl group. Whether the effect of aspirin as a potential immune suppressor is COX2-dependent or COX2-independent remains to be determined. Nonetheless, our contradictory observation points out that the effect of aspirin on the thymic and splenic T cell differentiation might be COX2-dependent, and therefore be distinct from our observed NaSA effect on the thymus.

Besides the thymus, we also show that SA highly accumulates in BAT. BAT is a type of fat tissue in charge of energy expenditure and thermogenesis [80]. Unlike WAT, BAT contains a high quantity of mitochondria and small lipid vacuoles, and its induction has been shown to be a promising therapeutic target for obesity and T2D [81–83]. Interestingly, it was previously shown that SA directly activates AMP-activated protein kinase in multiple tissues (e.g., liver, muscle, and WAT), which induces BAT induction and differentiation [84,85]. Also, the lowering effect of SA in plasma free fatty acids was abrogated in mice lacking the β1 subunit of AMPK subunit [85]. Therefore, SA might activate AMPK in BAT, thereby stimulating browning of WAT, which could lower the level of free fatty acid in plasma [84–86]. It would be thus interesting to investigate whether SA also could contribute to the activity of BAT and exert metabolic effects in mice. Noteworthy, SA was shown to significantly activate AMPK at > 1 mM in HEK293T cells, indicating that the low level of SA (~ 70 nM) in mice under normal feeding conditions might not be sufficient to activate AMPK [85]. Therefore, future investigation on the role of SA in the activity of BAT should also

take other pathways into account. For instance, the cAMP-dependent protein kinase (PKA) pathway was shown to be an important alternative pathway for the metabolic effect of salicylates [87,88]. Salsalate, a dimer of SA lacking an acetyl group, showed an anti-diabetic effect by improving glucose tolerance in T2D patients and increasing energy expenditure in patients who underwent a hyperinsulinemic-euglycemic clamp experiment [87,88], presumably by the activation of BAT via the PKA pathway, but independent of AMPKβ1 [89,90].

Interestingly, Rainsford *et al*. studied the distribution of radioactively labeled salicylates in rat tissues in stomach, liver, kidney, brain, bone marrow, inflamed paws, and non-inflamed paws [91]. 3 hours after oral administration of radioactively labeled salicylates (100 mg/kg or 10 mg/kg), SA levels were found to be the highest in inflamed paws of rats treated with 100 mg/kg SA, which was 186 times higher than that in non-inflamed paws [91]. This previous observation raises the question whether SA levels might also increase at sites of inflammation. Therefore, it would be of interest to assess SA levels in inflamed tissues of preclinical mouse models of inflammatory diseases such as T2D, as well as in mouse colon cancer models, where preventive effects of SA are known [17,22].

In conclusion, we show that SA accumulates in the thymus of mice under normal feeding conditions, and that SA administration slightly alters T cell development in the thymus. Future research remains necessary to investigate the role of endogenous SA in T cell ontogeny and to determine physiological functions of SA accumulation in other tissues such as BAT.

5 Materials and methods

5.1 Mice

For profiling SA in multiple tissues of mice (n=4), six to twelve-week-old male or female wild-type (WT) C57BL/6J mice were bred and maintained under specific pathogen-free conditions and housed in individually ventilated cages following the national and institutional guidelines for the care and use of laboratory animals. Mice were divided into two groups: perfused group and non-perfused group. For perfused group mice, mice were transcardially perfused with phosphate-buffer saline using a 20 mL syringe and a 26G needle.

For assessing the effect of SA on T-cell development, four-week-old male (n=10) or female (n=10) mice were purchased from Janvier (Le Genest-St-Isle, France). Mice were bred in the specific pathogen free (SPF) facilities of Pasteur Institute, Lille. Mice were housed under SPF conditions in individually ventilated cages in accordance with the national guidelines and regulations for the care and use of laboratory animals. Animal protocols were approved by the ethical committee of Ghent University (EC2019-025).

5.2 SA treatment

C57BL/6J mice were divided into two groups: Control group and SA-treated group. Sodium salicylate (S3007, Sigma-Aldrich, Life Technologies) was freshly dissolved (250 mg/L) in drinking water every three or four days and continuously given to mice for 28 days. Control mice were treated with sodium chloride (S9888, Sigma-Aldrich, Life Technologies) dissolved in drinking water (250 mg/L). On day 29, mice were euthanized by CO_2.

5.3 Metabolite extraction

After sacrifice, brain, thymus, liver, epididylmal (perigonadal) white adipose tissues, and interscapular brown tissues were harvested and immediately snap-frozen in liquid nitrogen and stored at -70 °C. Tissues were homogenized using Precellys 24 with 2.8 mm Zirconium oxide beads (Bertin technologies, Rockville, US) in 1 mL of 80 % methanol, followed by the overnight incubation at -70 °C in an ultrafreezer. The mixture was further centrifuged at 10,600 x g for 15 min at 4 °C, and the supernatant was evaporated using a SpeedVac. 1 mL of a 50:50 water: cyclohexane solution was added to the dried residue. The phases were separated by centrifugation at 10,600 x g for 5 min, and the aqueous phase was collected. Next, 500 µL of 5% trichloroacetic acid was added to the collected aqueous phase. The mixtures were vortexed and

partitioned three times against 500 µL of ethyl acetate, and the organic layer was collected and evaporated using a SpeedVac.

5.4 GC-MS analysis

The residue obtained from metabolite extraction was trimethylsilylated using 10 µL of pyridine and 50 µL of N-methyl-N-(trimethylsilyl)trifluoroacetamine. GC-MS analyses were performed on a 7890B GC system coupled to 7250 GC/QTOF mass spectrometer (Agilent). Analyses were performed on a VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm; Varian CP9013; Agilent) at a constant helium flow of 1.2 mL/min, using a 1 µL aliquot, which was injected in splitless mode. After injection, the oven was held at 80 °C for 1 min, ramped to 200 °C at a rate of 5 °C/min, subsequently ramped to 320 °C at a rate of 20 °C/min and then held at 320 °C for 5 min. The MS transfer line, the MS ion source, and the quadrupole were set to 280 °C, 230 °C and 150 °C, respectively. A full EI-MS spectrum was generated for each sample by scanning the m/z range of 50 to 500 with a solvent delay of 10 min. Electron ionization energy was 70 eV. For relative quantification, the peak areas were calculated using the default settings of the Masshunter Quantitative Analysis software (version 10.0; NIST). For the SA analysis, 2TMS derivative at *m/z* 267.0868 ($[C_{12}H_{19}O_3Si]+Si)^+$ was used as an quantifier and their derivatives at *m/z* 209.0445 ($[C_9H_{12}O_2Si_2]+H)^+$ and *m/z* 193.0678 ($[C_{10}H_7O_3]+NH_4)^+$ were used as qualifiers. Targeted analysis of salicylic acid was performed by comparing retention times and mass transitions with that of corresponding standard metabolite (Sigma-Aldrich).

5.5 Flow cytometry

5.5.1 Thymus

Thymuses were collected in cold complete RPMI medium supplemented with 2 mM L-glutamine, 0.4 mM sodium pyruvate solution, 10 % heat-inactivated Fetal Bovine Serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Life Technologies), and 50 µM β-mercaptoethanol (Sigma-Aldrich, Life Technologies). Thymuses were smashed with the plunger of the 3 mL syringe in 1 mL of medium. Smashed thymuses were passed over a 70 µm cell strainer, which was further rinsed with 3 mL medium. Cells were spun for 4 min at 400 × g at 4 °C, and the supernatant was removed. The cell pellet was resuspended in 3 mL complete RPMI medium.

To analyze the population of T cells in the thymus, single cells were surface stained with the following antibodies in brilliant stain buffer (BD): fixable viability dye eFluor506 (eBioscience), anti-CD16/CD32 (553142,

BD), anti-TCRβ-FITC (11-5961-81, eBioscience), anti-CD8-BV605 (563152, BD), anti-TCRγδ-APC (17-5711, eBioscience), anti-CD44-Alexa Fluor 700 (560567, BD), anti-CD4-PE-Cy7 (563933, BD), and anti-CD25-BUV395 (564022, BD). Next, to facilitate intracellular staining, cells were fixed and permeabilized using the fixation/permeabilization buffer (00-5123-43, eBioscience). Cells were then stained with anti-FoxP3-PE (12-5773-82, eBioscience). Multi-color samples were measured on an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree star).

5.5.2 Spleen

Spleens were collected in cold complete RPMI medium supplemented with 2 mM L-glutamine, 0.4 mM sodium pyruvate solution, 10 % heat-inactivated Fetal Bovine Serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Life Technologies), and 50 µM β-mercaptoethanol (Sigma-Aldrich, Life Technologies). Single-cell suspensions from these spleens were prepared as described previously [92]. Splenocytes were flushed out by injecting 3 mL of complete medium with a 26 G needle. Spleens were smashed with the plunger of the 3 mL syringe. Smashed spleen as well as flushed out cells were passed over a 70 µm cell strainer, which was further rinsed with 3 mL complete medium. Cells were spun for 4 min at 400 × g at 4 °C, and the supernatant was removed. Red blood cells were lysed in 3 mL ACK lysis buffer (Sigma-Aldrich, Lonza) per spleen at room temperature for 3 min. 8 mL complete RPMI buffer was added, and cells were spun for 4 min at 400 × g at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 3 mL complete RPIM medium.

To analyze the population of T cells in the spleen, single cells were surface stained with the following antibodies in brilliant stain buffer (BD): fixable viability dye eFluor506 (eBioscience), anti-CD16/CD32 (553142, BD), anti-TCRβ-FITC (11-5961-81, eBioscience), anti-CD8-BV605 (563152, BD), anti-TCRγδ-APC (17-5711, eBioscience), anti-CD4-PE-Cy7 (563933, BD), and anti-CD25-BUV395 (564022, BD). Next, to facilitate intracellular staining, cells were fixed and permeabilized using the fixation/permeabilization buffer (00-5123-43, eBioscience). Cells were then stained with anti-FoxP3-PE (12-5773-82, eBioscience). Multi-color samples were measured on a LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree star).

5.6 Statistical analysis

Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the

appropriate statistical test (Student's t-test; equal variances t-test) or (-Welch's t-test; unequal variances t-test) has been performed to determine a significant difference.

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Chapter 3.2. Overexpression of a bacterial salicylate hydroxylase gene does not alter salicylic acid and catechol levels in mice

Seo Woo Kim[1, 2, 3, 4], Yasmine Driege[1, 2], Jacob Pollier[5], Sandrien Desmet[5], Annelies Demeyer[1, 2], Alain Goossens[3, 4], Rudi Beyaert[1, 2], Jens Staal[1, 2, 6]

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[5]VIB Metabolomics Core Ghent, VIB, Ghent, Belgium
[6]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

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Author contributions

SWK, AG, RB, and JS designed the research and interpreted data. SWK, YD, SD, JP, and AD performed experiments. SWK, SD, and JP analyzed the data. SWK wrote the paper with the help of AG, RB, and JS. AG, RB, and JS supervised the project and RB and AG provided resources.

1 Abstract

Background
Despite the appreciated roles of salicylates as an anti-inflammatory drug in animals, the inflammatory roles of endogenous SA have been poorly addressed. In **Chapter 3.1**, we revealed that the thymus is abundant in basal SA. In addition, we showed that SA administration altered the thymic T cell development, interfering with the cell fate decision of $CD4^+CD8^+$ double-positive (DP) cells into $CD4^+$ and $CD8^+$ single-positive (SP) cells. This observation raises the possibility of SA involvement in thymic T cell development. However, the physiological role of basal SA in animals and whether basal SA accumulated in the thymus plays a role in T cell development remain unclear.

Objective
In this chapter, we aim to unravel the role of endogenous SA in thymic T cell development. Also, we ask whether endogenous SA has a role in shaping the immune system by resulting innate and adaptive immune responses.

Methods
Given the unknown SA biosynthesis and catabolic pathways in animals, we made use of *Pseudomonas putida* salicylate hydroxylase (NahG), converting SA to catechol. We generated HEK293T cells stably expressing *NahG* gene and analyzed its SA-degrading activity in mammalian cells by profiling SA and catechol levels by gas chromatography-mass spectrometry (GC-MS). Next, we produced transgenic mice expressing *NahG* gene and profiled SA and catechol levels in different tissues of mice. Finally, we assessed the percentage of T cells in the thymus and T cells, B cells, natural killer cells, and dendritic cells in spleen of transgenic *NahG* mice using flow cytometry analysis.

Key Results & Conclusions
HEK293T cells expressing *NahG* gene convert high-level SA to catechol. However, transgenic *NahG* mice do not deplete basal SA in blood and different tissues of mice. Furthermore, *NahG* mice show a normal population of thymic and splenic immune cell subsets. The maintained basal SA levels in mice are possibly due to the following reasons 1) the low rate of NahG-catalyzed reaction at basal SA level or 2) cell type- or tissue type-specific SA transport or 3) SA and catechol homeostasis. Future studies remain necessary to evaluate whether transgenic *NahG* mice are a suitable tool in investigating the role of basal SA.

2 Introduction

Given the anti-inflammatory activity of salicylic acid (SA), endogenous SA might be exploited in immune homeostasis. However, to date, the physiological role of endogenous SA remains unexplored. In **Chapter 3.1**, we documented the accumulation of SA in mouse thymus and showed that SA administration to mice slightly reduces the differentiation of thymic $CD4^+CD8^+$ double-positive (DP) cells into $CD4^+$ and $CD8^+$ single-positive (SP) cells. These observations prompted us to investigate whether also endogenous SA accumulation in the thymus is involved in thymic T-cell development. To this end, we generated genetically engineered SA-deficient mice. Because the gene encoding benzoic acid 2-hydroxylase (BA2H), which is suggested to be responsible for human SA production, has yet to be identified in animals and plants [1–4], and considering unknown SA receptor and catabolic pathways, we resorted to an alternative strategy which consists of, overexpression of *NahG* encoding *Pseudomonas putida* salicylate hydroxylase [5–7].

The *NahG* gene encodes a flavin-dependent monooxygenase that catalyzes the decarboxylation and hydroxylation of SA into catechol [5–7]. NahG was shown to degrade SA in the naphthalene catabolic pathway in several *Pseudomonas* species, which use naphthalene as a carbon source [8–10]. Several studies have used plants overexpressing *NahG* and successfully revealed the roles of SA in plant growth and development, adaptation to abiotic stresses, and local and systemic acquired resistance [5,11–16]. Likewise, to study the physiological roles of basal SA in animals, we opted to generate transgenic SA-depleted mice by overexpression of *NahG*.

To begin with, we examined the activity of NahG in mammalian cells (HEK293T), which may support the validity of expressing *NahG* to degrade SA in mice. Next, we generated transgenic *NahG* mice and profiled the abundance of SA and catechol in serum and tissues. Simultaneously, we investigated whether the expression of *NahG* alters immunophenotypes of thymocytes and splenocytes. Similar to the flow cytometry analysis performed in **Chapter 3.1**, we assessed the frequencies of immature T cells in different developmental stages in the thymus of wild-type (WT) and transgenic *NahG* mice. Also, we analyzed the percentage of splenic immune cell populations that compose adaptive and innate immune systems (**Figure 1**).

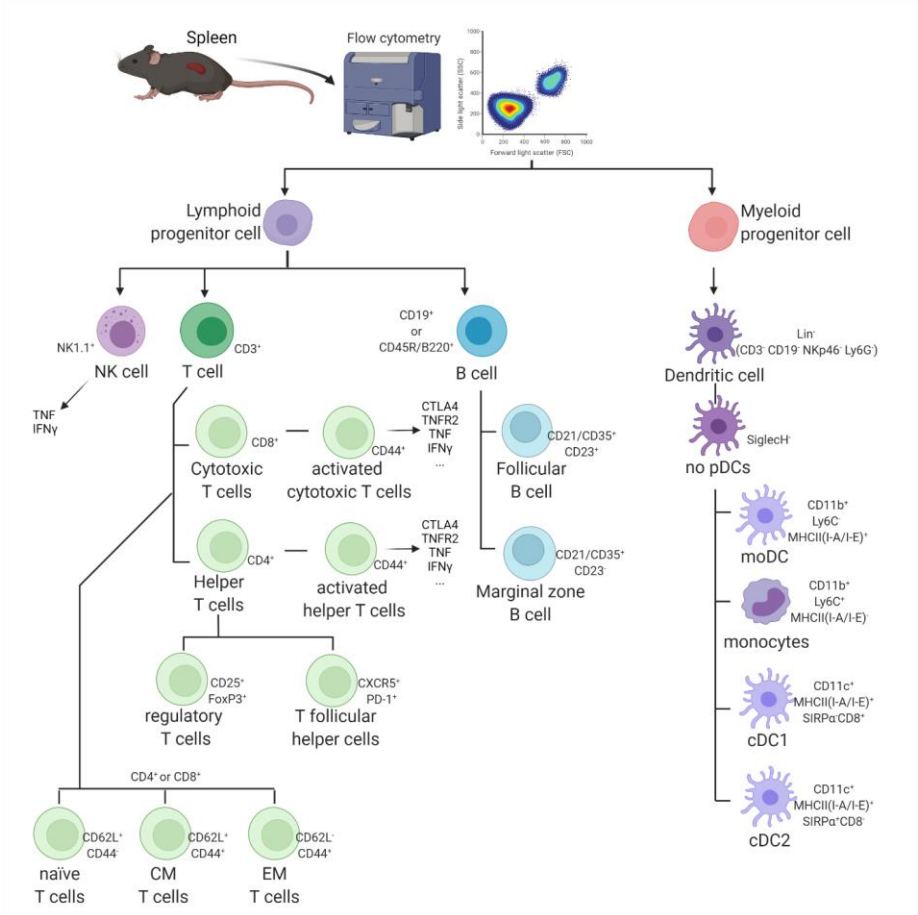

Figure 1. Schematic representation of different cell lineages and their maturation/activation status measured by flow cytometry-mediated immunophenotyping of mouse splenocytes.
The lineage, maturation, and activation status of various immune cells were examined. Cell surface or intracellular markers that we stained to define the different immune cell populations are indicated at the side of each depicted cell type. This figure was created with BioRender.com

3 Results and discussion

3.1 Overexpression of *NahG* in HEK293T cells results in the conversion of SA into catechol

Several studies have used plants expressing *NahG* encoding salicylate hydroxylase to evaluate the role of SA (**Figure 2A**) [5,11–16]. To investigate the physiological role of SA in animals, we therefore aimed to generate conditional transgenic SA-depleted mice by overexpression of *NahG*. To begin with, we first examined the activity of NahG protein in mammalian cells (HEK293T), which may support the validity of utilizing NahG to degrade SA in mice. We opted for overexpression in HEK293T cells because of their fast-growing and cancerous nature eliciting high transfection efficiency.

Wild-type (WT)-HEK293T cells and transgenic *NahG*-HEK293T cells, stably expressing *NahG*, were treated with 500 µm SA (**Figure 2B and 2C**). Subsequently, the conversion of SA into catechol was assessed by gas chromatography-mass spectrometry (GC-MS) (**Figure 2D and 2E**). We observed almost complete SA-conversion to catechol in SA-treated *NahG*-HEK293T cells, which was not observed in WT-HEK293T cells (**Figure 2D-2F**). Of note, the concentration of SA we applied to cells (500 µM) is approximately 10,000 times higher than the basal levels of SA (~70 nM) [1], implying a high chance of complete SA depletion in *NahG* transgenic mice.

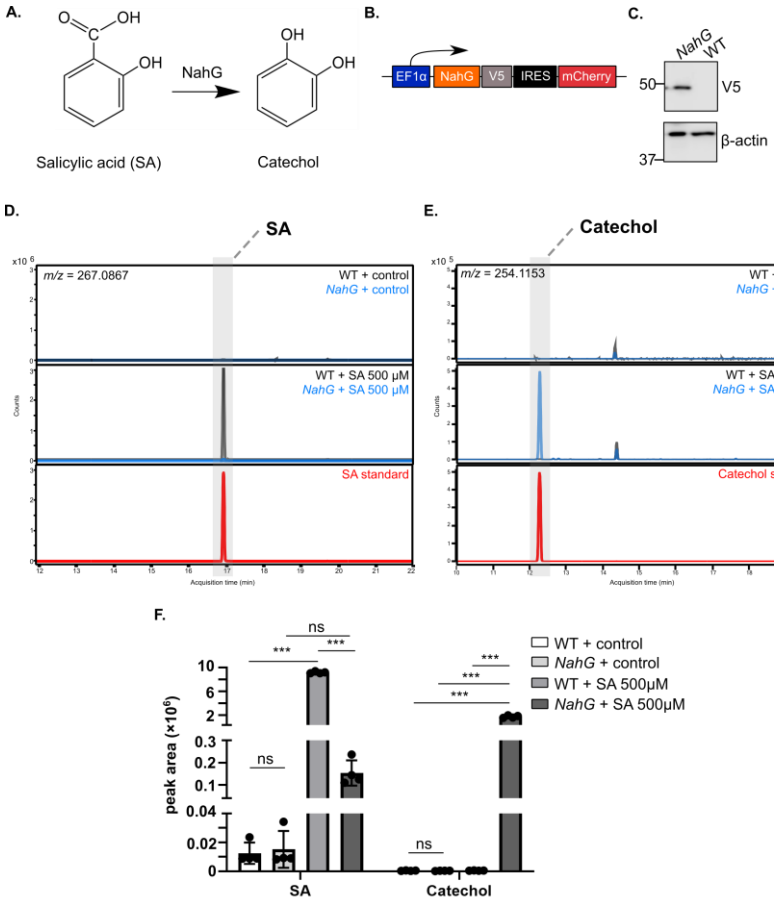

Figure 2. Enzymatic conversion of SA into catechol in HEK293T cells expressing *NahG*.

(A) Outline of the catabolism of SA by overexpressing *NahG*. **(B)** Schematic representatation of the *NahG* transgene construct. **(C)** Representative western blot showing NahG protein levels in HEK293T cells stably expressing *NahG*. β-actin is used as a loading control. **(D)** Representative EIC (2trimethylsilyl or 2TMS derivative, *m/z* = 267.0867) GC-MS chromatograms corresponding to culture media extracts of wild-type (WT) HEK293T cells (gray) or HEK293T cells expressing *NahG* (blue) and of a SA standard (red). **(E)** Representative EIC (2TMS derivative, *m/z* = 254.1153) GC-MS chromatograms corresponding to culture media extracts of WT HEK293T cells (gray) or HEK293T cells expressing *NahG* (blue) and of a SA standard (red). **(F)** Peak area of SA and catechol in culture media extracts of WT HEK293T cells and HEK293T cells expressing *NahG*. Culture media extracts of WT and *NahG* HEK293T cells are subjected to GC-MS analyses for semi-quantification of SA and catechol levels. Results are the means of four biological replicates and are representative of two independent experiments. Statistical significance was calculated using a Home-Sidak's multiple comparisons test (pairwise): *P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

3.2 Overexpression of *NahG* does not alter SA and catechol levels in mice

Given the proven enzymatic activity of NahG in *NahG*-HEK293T cells, we next generated transgenic mice that overexpress *NahG* by introducing in embryonic stem cells a construct expressing *NahG* via the PiggyBac transposon system (*LoxP-stop-LoxP (LSL)-NahG*$^{+/-}$). Here, the expression of NahG was first inactivated by a polyA stop cassette between the promoter and the coding domain sequence to maintain the mouse T0 generation in case the overexpression of NahG would lead to a lethal phenotype (**Figure 3A**). To excise the polyA stop cassette afterwards, we further crossed *LSL-NahG-V5*$^{+/-}$ mice with Sox2Cre deleter mice, which express Cre recombinase under the control of the mouse Sox2 (SRY-box containing gene 2) promoter. Sox2Cre mice provide epiblast-specific Cre-mediated LoxP recombination, thereby activating the transgene expression in the embryo. This resulted in *Sox2Cre*$^{+/-}$; *NahG*$^{+/-}$ mice that express *NahG* under the CAG promoter (**Figure 3A**). *Sox2Cre*$^{+/-}$; *NahG*$^{+/-}$ mice are viable and do not show a lethal phenotype from birth up to 15 weeks old, which indicates that the expression of NahG does not have a considerable impact on mouse health under basal conditions. In addition, as we were not initially targeting particular tissues or conditions to degrade SA, we employed a constitutive transgene expression system. Subsequently, we crossed *Sox2Cre*$^{+/-}$; *NahG*$^{+/-}$ mice with WT mice, allowing Cre-independent *NahG* expression in mice. This generated heterozygous transgenic mice that express *NahG* in the absence of Cre (*Sox2-Cre*$^{-/-}$; *NahG-V5*$^{+/-}$, hereafter called ***NahG* mice**) (**Figure 3A**). Western blot analysis of different tissues (e.g., spleen, thymus, brain, liver, white adipose tissue (WAT), and brown adipose tissue (BAT)) confirmed the accumulation of NahG in *NahG* mice (**Figure 3B and Figure S1**). We detected a V5-immunoreactive band with a size around 50 kDa corresponding to the predicted size (47.9 kDa) of NahG, which is absent in WT.

Next, we assessed whether NahG altered the levels of SA and catechol in the transgenic mice. Relative SA and catechol levels of serum and different tissues of WT and *NahG* mice (thymus, BAT, WAT, brain, and liver) were analyzed by GC-MS (**Figure 3C**). However, unlike our observations in HEK293T cells, SA was not converted into catechol *in vivo* (**Figure 3C**). This may be due to the low rate of NahG-catalyzed reaction at the low concentration of SA in mice (78 nM) [1,6], which was also reflected by the maintained SA levels in untreated *NahG*-HEK293T cells (**Figure 2F**). In *Arabidopsis NahG* plants, a half depletion of the basal SA was described, however, the level of endogenous SA in plants is relatively high compared to animals (2.86 µg g^{-1} fresh weight; 20 µM in *Arabidopsis*) [18]. Future studies testing NahG enzyme activity in cells

at various concentrations of SA will thus be informative.

To partially corroborate this, we assessed whether primary cells derived from transgenic *NahG* mice could convert a high dose of SA. Primary fibroblasts were isolated from WT and *NahG* mice and were subsequently treated with 500 µM SA, reaching the maximum rate reaction (V_{max}) [6]. We applied the same experimental design used before for HEK293T cells (**Figure 2**), allowing identical pH and temperature conditions and concentrations of cofactors, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) [6]. However, in contrast to our observations in HEK293T cells, primary transgenic *NahG* fibroblasts did not convert SA into catechol (**Figure 4A**). This indicates that the enzymatic reaction rate at low SA concentrations may not explain why transgenic *NahG* mice do not deplete SA. Possibly, NahG inactivating mutations or a much lower NahG accumulation in *NahG* transgenic mice and primary fibroblasts derived of these mice, compared to high accumulation in HEK293T cells, can still explain the absence of SA conversion in *NahG* transgenic mice. A missense mutation in a gene can result in altered amino acid, and this might render the enzyme nonfunctional by changing for example the structure of its active site responsible for substrate- and cofactor-binding. A nonsense mutation might result in a nonfunctional truncated enzyme. To rule out this possibility in the case of *NahG*, we sanger sequenced the region of interest in both transgenic mouse and HEK293T cell genomes. However, an identical, full-length, and unmutated *NahG* gene was integrated into both genomes. To exclude the other possibility, i.e. significantly low NahG protein accumulation in transgenic mice, we performed western blotting to compare the intensity of a V5-immunoreactive band of NahG in transgenic primary mouse fibroblasts and HEK293T cells (**Figure 4B**). We observed that NahG accumulation levels in primary fibroblasts and HEK293T cells are comparable (**Figure 4B**). Also, the MW of NahG protein in primary mouse fibroblasts seems identical to that in HEK293T cells (around 50 kDa, corresponding to the size of NahG (47.9 kDa)), again ruling out the possibility that NahG in mice is truncated (**Figure 4B**). Together, these results suggest that the maintained SA levels in transgenic *NahG* mice are not due to too low NahG accumulation or a gene mutation impairing its catalytic activity.

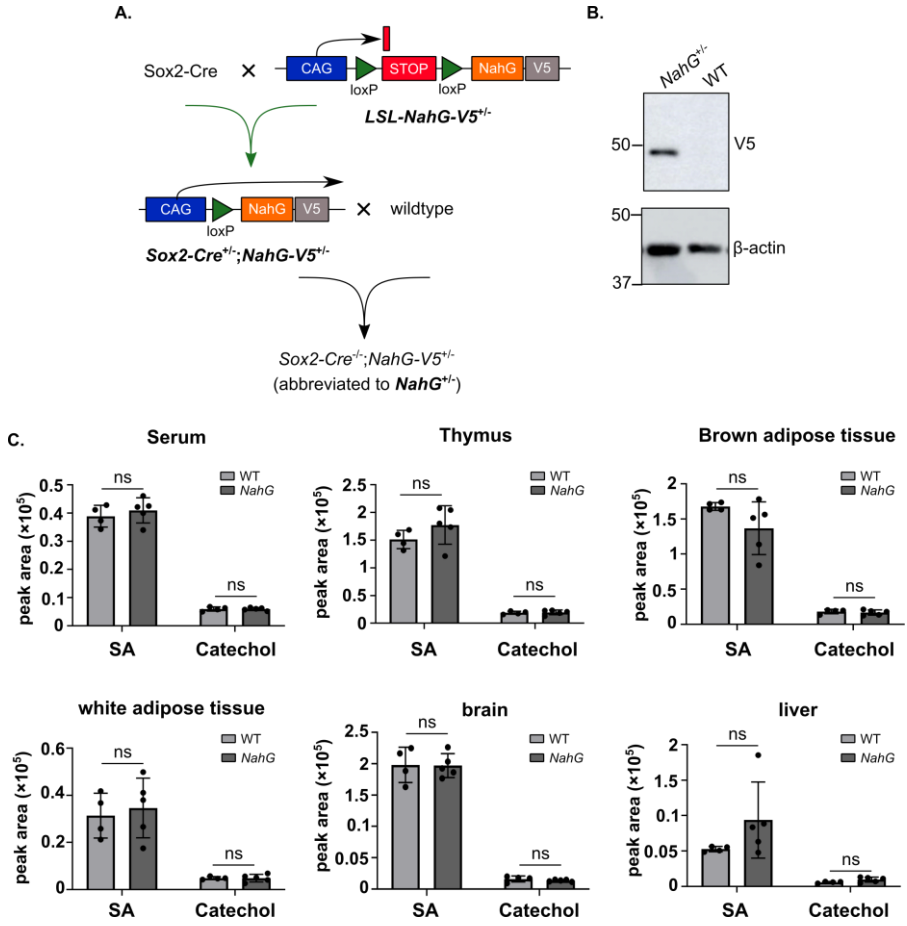

Figure 3. SA and catechol levels in serum and tissues of WT and transgenic *NahG* mice.

(A) Mating scheme to generate transgenic mice expressing *NahG*. Transgenic mice expressing *lox-stop-lox (LSL)-NahG* (abbreviated to *LSL-NahG-V5*$^{+/-}$) were bred with female Sox2-Cre mice. The resulting progeny, *Sox2Cre*$^{+/-}$; *NahG*$^{+/-}$, was bred with wild-type mice. This resulted in *Sox2Cre*$^{-/-}$; *NahG*$^{+/-}$ (abbreviated to *NahG*$^{+/-}$). **(B)** Representative western blot showing NahG-V5 protein levels in spleen lysates of wild-type *NahG*$^{-/-}$ and transgenic *NahG*$^{+/-}$ mice. β-actin is used as a loading control. **(C)** Peak area of SA and catechol in serum, thymus, brown adipose tissue, white adipose tissue, brain and liver of WT and transgenic *NahG* mice. Tissue extracts of serum and tissues are subjected to GC-MS analysis for semi-quantification of SA (2TMS derivative, *m/z* 267.0868) and catechol (2TMS derivative, *m/z* 254.1153) levels. Results are the means of four biological replicates and are representative of two independent experiments (serum and liver) or one experiment (thymus, brown adipose tissue, white adipose tissue, and brain). Statistical significance was calculated using a Student's T-test: *P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

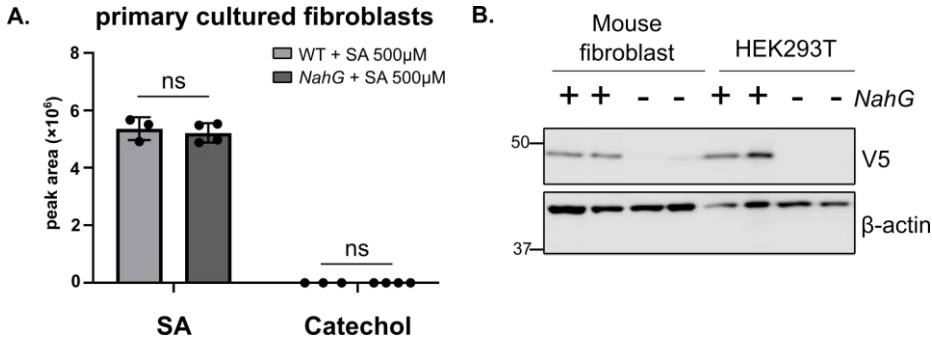

Figure 4. SA and catechol levels in primary fibroblasts derived from WT and transgenic *NahG* mice.
(A) Peak area of SA and catechol in culture media extracts of fibroblasts isolated from WT and transgenic *NahG* mice. Primary fibroblasts, isolated from WT and *NahG* mice, are cultured with 500 μM SA, and culture media extracts are subsequently subjected to GC-MS analysis for semi-quantification of SA (2TMS derivative, *m/z* 267.0868) and catechol (2TMS derivative, *m/z* 254.1153).1160 levels. Results are the means of three (WT) or four (*NahG*) biological replicates and are representative of two independent experiments. Statistical significance was calculated using a Student's T-test: *P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001. (B) Representative western blot showing NahG-V5 protein levels in primary *NahG*-fibroblasts and *NahG*-HEK293T cells. The result shows two biological replicates and is representative of two independent experiments. β-actin is used as a loading control.

The observed discrepancies between human HEK293T cells and primary mouse cells such as fibroblasts might also be explained by a cell type- or species-specific difference in SA transport [19,20]. Several studies have shown that the influx of SA into animal cells might be through both passive and active transport and could vary depending on the cell type [21–25]. Therefore, we question whether the maintenance of SA levels in fibroblasts (**Figure 4**) is merely due to the absence of SA influx. Future *in vitro* enzymatic assays could resolve this by determining the enzymatic activity in an extract from mouse fibroblasts. If this is the case, we could make use of inhibitors of transporters under pH gradient and iso-pH conditions, suggested to be involved in SA transport, such as an inhibitor of organic anion transport (probenecid) [21], monocarboxylic acid transport (α-cyano-4-hydroxycinnamic acid) [22], and organic anion transporting polypeptide (pravastatin) [23–25]. If a transporter responsible for SA in HEK293T cells is also present in mouse fibroblasts, this might imply species-specific difference in the kinetics of SA uptake between orthologous human and mouse transporter protein [26–29].

Another possible explanation for the discrepancy between HEK293T cells and mouse primary cells expressing *NahG* might be the presence

of other still unknown metabolic pathways involved in SA and catechol homeostasis in mice. Interestingly, transgenic *Populus* expressing *NahG* also does not deplete SA [30], in contrast to other plant species [5,11–16,31]. Untargeted metabolite profiling revealed a decrease of salicylic acid-glucoside and an increase of catechol-glucoside in transgenic *NahG* poplar [30]. This indicates that transgenic *NahG* poplar likely maintains SA and catechol levels by deconjugating salicylic acid-glucoside and detoxificates catechol by conjugation, respectively [30]. Also, the level of quinic acid conjugates decreased, reflecting metabolite flux from the shikimic pathway or the phenylpropanoid pathway [30,32]. In this context, we cannot exclude the possibility of other metabolic pathways indirectly regulated by NahG in mice. Furthermore, the presence of a SA-like competitive inhibitor or an allosteric inhibitor of NahG in mice might be another explanation [33], as a metabolite constellation in a living organism is more complex than *in vitro* cell culture environments. NahG does not decarboxylate benzoic acid, which is a benzene ring bearing a carboxyl group, indicating that the catalytic activity of NahG needs the presence of a hydroxyl group *ortho* to the carboxyl group in SA [6]. As SA is a rather simple phenolic compound, it is possible that other natural compounds bearing an aromatic ring with those residues (e.g., amorfrutin [34]) act as the SA-like competitive inhibitor. Future studies should thus determine additional metabolite levels in *NahG* mice via untargeted metabolite profiling.

Collectively, the maintenance of low-level SA in tissues of NahG mice and SA-untreated *NahG*-HEK293T cells might be due to the low rate of NahG-catalytic reaction. However, the maintenance of high-level SA in *NahG*-mouse fibroblasts does not support this, raising the possibility of cell type- or tissue type- or species-specific differences in SA transport. Future experiments determining SA levels in different tissues of NahG mice treated with a high dose of SA might allow to support or exclude a role for the above mentioned low-rate enzymatic reaction and cell type- or tissue type-specific differences in SA transport.

3.3 Transgenic *NahG* mice have a normal population of thymic and splenic immune cell subsets

In parallel with the SA depletion measurements in transgenic *NahG* mice, we examined whether the expression of *NahG* could change the immunophenotype of mice. Given that SA administration in mice slightly interferes with the differentiation of $CD4^+CD8^+$ double-positive (DP) thymocytes into $CD4^+$ single-positive (SP) and $CD8^+$ SP thymocytes, as shown in **Chapter 3.1**, we first analyzed whether *NahG* expression also affected thymic T-cell development. However, we could not detect any difference in the frequencies of $CD4^-CD8^-$ double negative (DN) T cells

and their subsets DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) between WT and *NahG* mice (**Figure 5A**). The frequencies of $CD4^+CD8^+$ double-positive (DP) T cells, of $CD4^+$ single-positive (SP) T cells, and $CD8^+$ SP T cells were also comparable (**Figure 5B**). We also did not observe a difference in the percentage of thymic TCRγδ$^+$ DN cells, TCRβ$^+$ DN cells, and regulatory T cells (Tregs) between WT and *NahG* mice (**Figure 5C and 5D**).

In addition, we analyzed whether expression of *NahG* affects the percentage of T cells, B cells, dendritic cells (DCs), and natural killer (NK) cells in the spleen, which can be indicative of the innate and adaptive immune functions in *NahG* mice under normal basal condition. However, expression of *NahG* did not cause any abnormalities in the immunophenotype of splenic T cells, DCs, and NK cells (**Figure S2 and S3**). In the experiment shown, the frequencies of T follicular helper cells (Tfh), characterized by C-X-C chemokine receptor type 5 (CXCR5) and programmed cell death protein 1 (PD-1), slightly increased in *NahG* mice. However, this result was not reproducible in two other independent experiments. Collectively, the normal population of thymic and splenic immune cells in *NahG* mice might be due to the lack of SA-degrading NahG activity we observed. Also, it may be necessary to apply SA-relevant inflammatory disease models on *NahG* mice to understand the physiological roles of SA. For instance, based on the effect of SA in suppressing glucose production and inducing fatty oxidation [34–36], a type 2 diabetes (T2D)-like high-fat and high-sugar diet model would be an interesting model. Investigating the physiological relevance of SA as an immune regulator in the context of T2D will give knowledge on how basal SA might impact the interplay between immune modulation and T2D. Future application of disease models, however, only makes sense after the confirmation of NahG activity in mice.

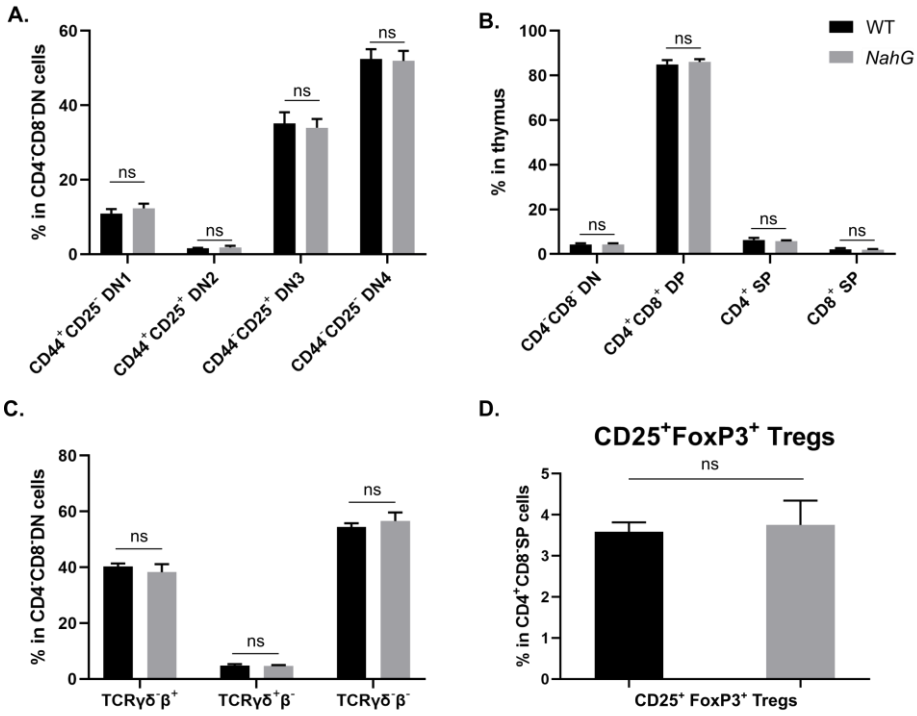

Figure 5. *NahG* expression does not alter the frequencies of thymic T cell subsets in mice.
Thymocytes were isolated from 7-11 week old WT and *NahG* mice. The percentages of thymocytes in different stages of T cell development **(A)-(B)**, the percentages of TCRγδ$^+$ T cells and TCRβ$^+$ T cells **(C)**, and the percentage of Tregs (CD4$^+$CD25$^+$FoxP3$^+$) **(D)** were analyzed using flow cytometry. Results are shown as mean ± SD (n=6). Statistical differences were determined using the Student's T-test, (not significant (n.s.) p-value >0.05, *p-value <0.05, **p-value < 0.01) (n=10). The data shown are from one experiment.

3.3 Limitations of the *NahG* transgenic strategy

The breakdown product of NahG, catechol (1,2-hydroxybenzne) is a foreign molecule to animals, though it can be ingested through plant-based diets [37,38]. Nevertheless, animals secrete catechol derivatives, such as neurotransmitters (e.g., dopamine, adrenaline, noradrenaline, and L-3,4-dihydroxyphenylalanine) [39] and catechol estrogens [40]. Noteworthy, catechol was shown to exert anti-inflammatory effects [41–43]. For instance, it was previously reported that catechol inhibits the activity of cyclooxygenase (COX) and phospholipase A2, which are responsible for producing a pro-inflammatory lipid mediator, prostaglandin [41–43]. Moreover, COX inhibition, in fact, contributes to

the anti-inflammatory function of acetyl-SA (aspirin) [44]. It is thus crucial to delineate whether the effect of NahG on immune or metabolic homeostasis is solely derived from SA depletion and not from the production of catechol. Indeed in *Arabidopsis NahG* plants, their loss of non-host resistance against *Pseudomonas syringae* pv. *phaseolicola*, which is causative of blight, resulted from catechol production, not from SA depletion [17]. To circumvent the possible artifact, it has to be noted that future studies should employ an additional metabolic strategy or investigate the effect of catechol administration in relation to the NahG effect. This additional approach will reveal whether transgenic *NahG* mice are a suitable model for SA research.

In conclusion, we have shown that *NahG* expression in HEK293T cells is able to deplete high-level SA, which suggested the validity of generating transgenic *NahG* mice to reveal the physiological function of basal SA. However, transgenic *NahG* mice did not deplete basal SA in their serum and different tissues. The reason behind unaltered SA levels in mice is unclear yet. We hypothesize this could be due to 1) the low rate of NahG-catalyzed reaction at basal SA level or 2) cell type-, species-, or tissue type-specific SA transport or 3) the existance of other unknown metabolic pathways involved in SA and catechol homeostasis. Resolving these questions will reveal whether transgenic *NahG* mice are a suitable model for SA research. Until then, the role of basal SA still remains elusive.

4 Materials and methods

4.1 Generation of HEK293T cells stably expressing *NahG*

HEK293T cells expressing *NahG* were generated using retroviral transduction. Full-length *NahG* was subcloned into a pLVX-EF1α-IRES-mcherry lentiviral vector (LMBP 07888), resulting in pLVX-EF1α-*NahG*-IRES-mcherry (LMBP 11232). A plasmid of the cloned gene was deposited in the BCCM/GeneCorner plasmid collection along with detailed descriptions of cloning strategy and plasmid sequence (http://bccm.belspo.be/about-us/bccm-genecorner). Viral particles were produced in HEK293T cells (3×10^6 cells in a 75-cm^2 flask) transfected with 5 µg *NahG*-lentivector along with 3.5 µg VSV-G and 1.5 µg packaging plasmids using a calcium phosphate method. Cells were washed 6 h later, and 15 mL of fresh media was added to the flask. The supernatant containing virus particles was collected 24 h, 48 h, and 72 h post-transfection and filtered through a 0.45 µm filter, and 15 mL of which was applied to HEK293T cells seeded at 3×10^6 cells in a 75-cm^2 flask. Mcherry positive cells were sorted using a BD FACS ARIA III (BD Bioscience).

4.2 Generation of pPB-CAG-LoxP-STOP-LoxP-NahG-V5 plasmid

Mouse codon-optimized *NahG* was cloned into mammalian LSL conditional gene expression piggyback vector pPB-LoxP-Stop-LoxP (LSL) by Vectorbuilder (Cyagen Biosciences, Santa Ana, CA, USA). The vector ID of pPB-LSL-NahG-V5 is VB170727-1002ezw, which can be used to retrieve detailed information about the vector on www.vectorbuilder.com. A plasmid of the cloned gene was deposited in the BCCM/GeneCorner plasmid collection (LMBP 10706) along with detailed descriptions of cloning strategy and plasmid sequence (http://bccm.belspo.be/about-us/bccm-genecorner).

4.3 Mice

Mice were bred and maintained under specific pathogen-free conditions and housed in individually ventilated cages following the national and institutional guidelines for the care and use of laboratory animals. Transgenic mice that express *LSL-NahG-V5* mice were first generated by using the PiggyBac transposon technology (Cyagen Bioscience, Santa Ana, CA, USA). In brief, Piggybac transgenic plasmid pPB-CAG-*LSL-NahG-V5* was injected into the pronucleus of fertilized eggs. These eggs were implanted into surrogate mothers, and offspring pups were genotyped by PCR to identify those carrying the transgene. The mice

produced by these surrogate mothers are defined as founders, and pups from two founders (#28 and #31) were genotyped by PCR.

Next, to induce cre excision of the LSL cassette, transgenic mice expressing *LSL-NahG-V5* were bred with Sox2-Cre transgenic mouse strains (C57BL6/J background). This generated transgenic mice that express *NahG-V5* transgene (*Sox2-Cre*$^{+/-}$;*NahG-V5*$^{+/-}$). Then, crossing these transgenic mice with wild-type mice on the C57BL6/J background yielded *Sox2-Cre*$^{-/-}$;*NahG*$^{+/-}$ transgenic mice (here referred as *NahG* mice). As controls, littermates *Sox2-Cre*$^{-/-}$;*NahG*$^{-/-}$ or *Sox2-Cre*$^{+/-}$;*NahG*$^{-/-}$ were used (here referred to as wild-type (WT)).

4.4 Genotyping

To extract genomic DNA of *NahG* mice or littermate control mice, toes or tails were clipped and lysed in 100 µL of 50 mM NaOH at 95 °C for 1 hour while shaking (850-1100 rpm). 10 µL of 1.5 M Tris-HCl (pH 8.8) was added to the lysate, and 1-2 µL of genomic DNA was used per PCR reaction. Genotyping was performed by PCR with Taq master mix (Highqu, Kraichtal, Germany) using the following conditions: one cycle at 95 °C 5 min, 35 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 1 min and a final extension step at 72 °C for 5 min. The primers used to detect the *NahG* transgene were Forward 5-TTCACCGACGGCACAGAGT-3, and Reverse 5- ATCAGCAGGCCATGTAGGCTT-3, which amplified a 332 bp PCR product.

4.5 Western blotting

For confirmation of NahG protein accumulation in mice tissues (spleen, thymus, BAT, WAT, liver, and brain), tissues were harvested and immediately frozen and stored at -70 °C. To make protein lysates, tissues were homogenized and lysed using Precellys 24 with 2.8mm Zirconium oxide beads (Bertin technologies, Rockville, US) in RIPA buffer (50 mM Tris-HCl, pH 7,6, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors. To confirm NahG protein accumulation in HEK293T cells and primary mouse fibroblasts, cells were trypsinized and lysed in RIPA buffer. Debris was removed by a centrifuge at 14000 rpm for 10 min at 4 °C. Pierce™ BCA Protein Assay Kit (23225, ThermoFisher Scientific) was used to determine protein concentration. 5 × Laemmli buffer (250 mM Tris-HCl, pH 8, 10% SDS, 50% glycerol, 0.005% bromophenol blue, 25% β-mercaptoethanol) was added to the samples lysed in RIPA buffer, followed by denaturation for 10 min at 95 °C. Equal amounts of proteins were loaded and separated by 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes with 0.45 µm pores (Protran, Perkin Elmer), and probed with the following specific antibodies: anti-

V5-HRP (R96125, Invitrogen) and anti-β-actin-HRP (sc-47778, Santa Cruz). Western Lightning ECL detection system (Perkin Elmer) was used for protein detection according to the manufacturer's instructions.

4.5 Primary mouse fibroblast cell culture

Primary mouse fibroblasts were isolated and cultured as described previously [45]. Briefly, the left and right pinna from the mouse were collected. Each pinna was soaked in 70 % EtOH for five minutes and transferred to phosphate saline buffer (PBS). Each pinna was subsequently placed in a 24-well plate containing 1 ml of growth media, consisting of Dulbecco's modified Eagle's medium (DMEM) from Gibco supplemented with 10 % heat-inactivated Fetal Bovine Serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Life Technologies), and 2 mM L-glutamine. 4 mg/mL collagenase (17100017, Invitrogen) and 4 mg/ml dispase (D4693-1G, Sigma-Aldrich) was further added. Next, the pinna was minced using scissors. The minced tissue was incubated overnight in a 37 °C incubator. On the next day, cells were passed over a 70 µm cell strainer two times, further centrifuged at 1,020 × g for five minutes and resuspended in 5 mL of growth media. Finally, the cell suspension of both pinnae from a single mouse was combined and seeded in a 150mm plate.

4.6 Metabolite extraction and GC-MS analysis

Metabolite extraction for profiling mouse tissues was performed as described in **Chapter 3.1**, section 5.3.

For profiling HEK293T cells or primary mouse fibroblasts, methanol extraction was performed. Medium was removed from cell culture, and 1800 µL of cold 80 % methanol was added to the 200 µL of medium. Cells were washed with ice-cold 0.9 % NaCl solution. 1 mL of cold 80 % methanol was added to cells, which were subsequently scraped out using a cell scraper. Another 1mL of 80 % methanol was added, and the cell scraping was repeated. Medium and cell mixtures were overnight incubated at -70 °C in an ultra freezer. The mixture was further centrifuged at 10,600 x g for 15 minutes at 4 °C, and the supernatant was evaporated using a SpeedVac.

GC-MS analysis was performed as described in **Chapter 3.1**, section 5.4. In addition, For the SA analysis, *m/z* 267.0868 $([C_{12}H_{19}O_3Si]+Si)^+$ was used as an quantifier and their derivatives at *m/z* 209.0445 $([C_9H_{12}O_2Si_2]+H)^+$ and at *m/z* 193.0678 $([C_{10}H_7O_3]+NH_4)^+$ were used as qualifiers. For the catechol analysis, *m/z* 254.1153 $([C_{12}H_{22}O_2Si]+Si)^+$ was used as an quantifier and their derivatives at *m/z* 239.0918 and *m/z* 151.0211 $([C_7H_6O_2Si]+H)^+$ were used as qualifiers. Target analysis of SA

and catechol was performed by comparing retention times and mass transitions with corresponding standard metabolites (Sigma-Aldrich).

4.7 Flow cytometry

4.7.1 Thymocytes

Analysis of the thymus T cell populations were performed as described in **Chapter 3.1**, section 5.5.1.

4.7.2 Splenotypes

Spleens were collected in cold complete RPMI medium supplemented with 2 mM L-glutamine, 0.4 mM sodium pyruvate solution, 10 % heat-inactivated Fetal Bovine Serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Life Technologies), and 50 µM β-mercaptoethanol (Sigma-Aldrich, Life Technologies). Single-cell suspensions from these spleens were prepared as described previously [46]. Splenocytes were flushed out by injecting 3 mL of complete medium with a 26 G needle. Spleens were smashed with the plunger of the 3 mL syringe. Smashed spleen as well as flushed out cells were passed over a 70 µm cell strainer, which was further rinsed with 3 mL complete medium. Cells were spun for 4 min at 400 × g at 4 °C, and the supernatant was removed. Red blood cells were lysed in 3 mL ACK lysis buffer (Sigma-Aldrich, Lonza) per spleen at room temperature for 3 min. 8 mL complete RPMI buffer was added, and cells were spun for 4 min at 400 × g at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 3 mL complete RPMI medium.

To determine the population of B cells in the spleen, single cells were surface stained with the following antibodies : fixable viability dye eFluor506 (eBioscience), anti-CD16/CD32 (553142, BD), anti-CD21/CD35-eFluor450 (48-0212 (4E3), eBioscience), anti-CD3-PE-Cy5 (55-0031, Tonbo Biosciences), anti-CD23-PEP-Cy7 (25-0232-81, eBioscience), anti-CD4-FITC (11-0041-82, eBioscience), anti-PD-1-APC (17-9985-80, eBioscience), anti-CD18-AF700 (56-0193, eBioscience), and anti-CXCR5-PE (12-7185-80, eBioscience).

To analyze the population of regulatory T cells in the spleen, single cells were surface stained with the following antibodies in brilliant stain buffer (BD): fixable viability dye eFluor506 (eBioscience), anti-CD16/CD32 (553142, BD), anti-CD120b (TNFR2)-BV421 (564088, BD), anti-CD44-AF700 (560567, BD), anti-CD4-APC-eFluor700 (47-0042, eBioscience), anti-CD62L-PE (12-0621-82, Invitrogen), anti-CD3-PE-Cy5 (55-0031-U100, Tonbo Biosciences), anti-CD8-PE-Cy7 (25-0081-82, eBioscience), and anti-CD25-BUV395 (564022, BD). Next, to facilitate

intracellular staining, cells were fixed and permeabilized using the fixation/permeabilization buffer (00-5123-43, eBioscience). Cells were then stained with anti-FoxP3-APC (17-5773, eBioscience) and anti-CD152(CTLA4)-PE-eFluor610 (61-1522, eBioscience).

To analyze the intracellular cytokine production of splenic T cells, single-cell suspensions were stimulated with PMA/ionomycin in the presence of Brefeldin A to inhibit cytokine secretion. 200 µL of single-cell suspensions in complete RPMI were incubated with 50 ng/mL PMA (Sigma), 500 ng/mL ionomycin (Sigma), and 10 µg/mL Brefeldin A (Sigma) for 4 h in a 37 °C, 5% CO_2 cell culture incubator. Cells were surface stained with following antibodies in brilliant stain buffer (BD): fixable viability dye eFluor506 (eBioscience), anti-CD16/CD32 (553142, BD), anti-CD8a-PerCP-Cy5.5 (45-0081, eBioscience), anti-NK1.1-BV650 (564143, BD), anti-CD44-PE (12-0441-81, eBioscience), anti-CD3e-PE-Cy5 (55-0031-U100, Tonbo Biosciences), anti-CD4-BUV395 (563790, BD), and anti-CD45R/B220-BUV496 (612950, BD). Next, to facilitate intracellular staining, cells were fixed and permeabilized using the Cytofix/Cytoperm™ kit (554714, BD). Cells were then stained with anti-GranzymeB-eFlour450 (48-8898-80, eBioscience), anti-IL-17a-BV605 (564169, BD), anti-IL-10-BV711 (564081, BD), anti-IL-4-APC (17-7041-81, eBioscience), anti-TNF-AF700 (558000, BD), anti-IL-2-APC-Cy7 (560547, BD), and anti-IFNγ-PE-Cy7 (25-73311-82, BD).

For phenotyping of dendritic cells in cell suspensions of spleens, the spleen was manually minced into small pieces using scissors and was further digested with complete RPMI medium supplemented with 0.1 mg/ml Liberase (Roche) and 0.1 mg/ml DNase I (Roche) and incubated for 30 min at 37 °C. Spleens were passed over a 70 µm cell strainer, and the remaining lumps were smashed with the plunger of the 3 mL syringe. 6 mL Cold ACK lysis buffer (Sigma-Aldrich, Lonza) was added to the cell suspensions and incubated at room temperature for 3 min. Red blood cell lysis was stopped by adding complete medium up to 40 mL. The suspension was spun for 7 min at 400 × g at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 3 mL complete RPIM medium. Cells were surface stained with following antibodies in brilliant stain buffer (BD): fixable viability dye eFluor506 (eBioscience), anti-CD16/CD32 (553142, BD), anti-CD172a(SIRPα)-PerCP-eFluor710 (46-1721-82, eBioscience), anti-Siglec-H-BV431 (566581, BD), anti-MHCII(I-A/I-E)-V500 (562366, BD), anti-CD8-BV605 (563152, BD), anti-CD3-APC (100236, Biolegend), anti-CD19-APC (152409, Biolegend), anti-CD335(NKp46)-APC (137608, Biolegend), anti-Ly-6G-APC (127613, Biolegend), anti-Ly-6C-AF700 (561237, BD), anti-CD11c-PE-Cy7 (117317, Biolegend), anti-CD4-BUV395 (563790, BD), and anti-CD11b-BUV737 (612800, BD).

Multi-color samples were measured on an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree star).

4.8 Statistical analysis

Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the appropriate statistical test (Student's t-test; equal variances t-test) or (-Welch's t-test; unequal variances t-test) has been performed to determine a significant difference.

Supplementary materials

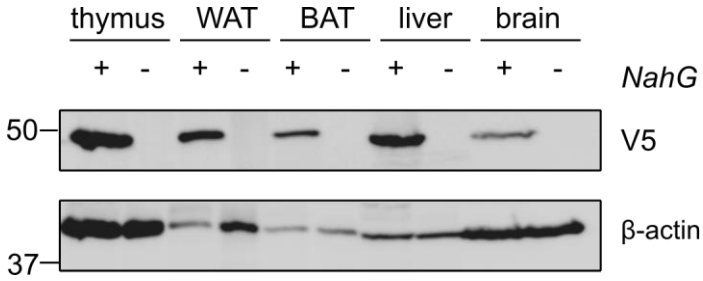

Figure S1. Representative Western blot showing NahG-V5 protein levels in different tissues of mice (thymus, white adipose tissues (WAT), brown adipose tissues (BAT), liver, and brain).
β-actin is used as a loading control. Results are representative of three independent experiments.

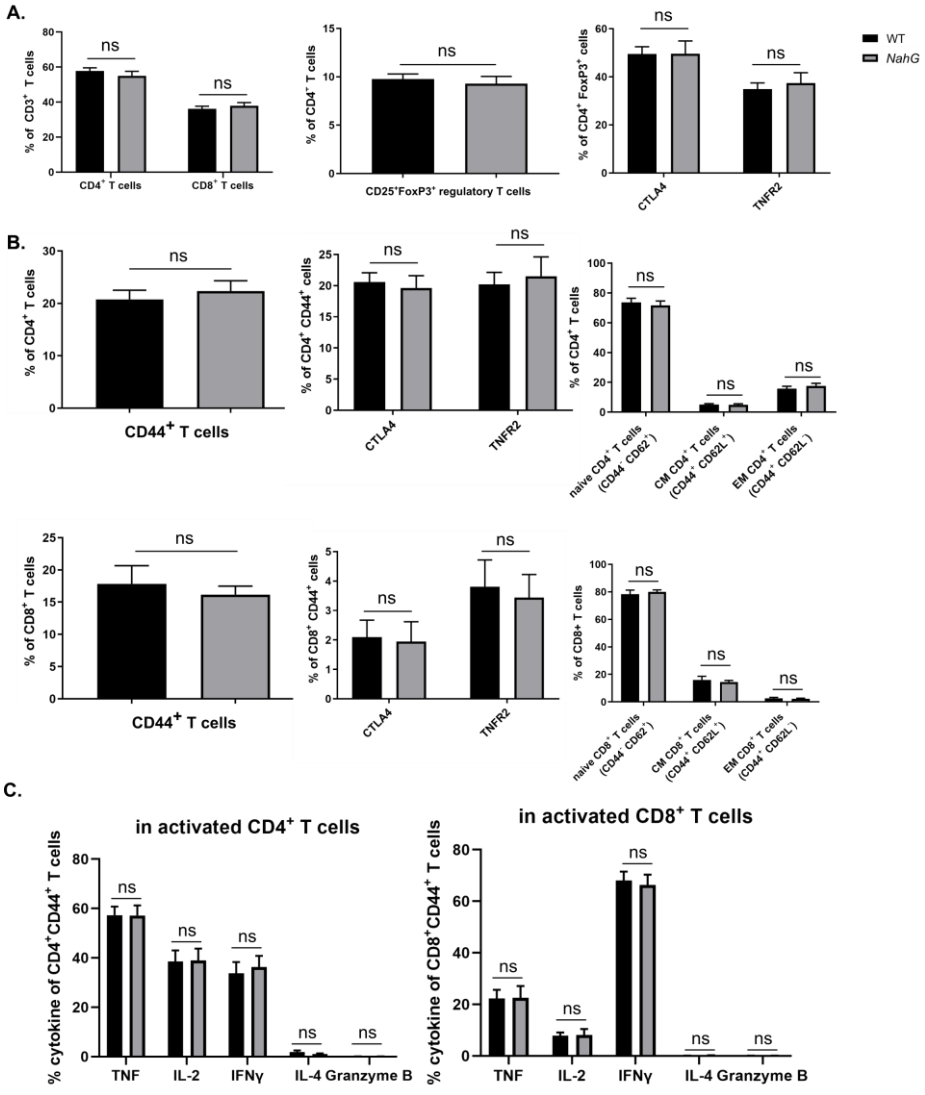

Figure S2. Transgenic *NahG* mice have normal splenic T cell development and activation.

(A) Percentage of splenic helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), Tregs ($CD4^+CD25^+FoxP3^+$), and CTLA4- or Tumor necrosis factor receptor 2 (TNFR2)- expressing Tregs ($CTLA4^+$ or $TNFR2^+$ Tregs) in WT and NahG mice. **(B)** Percentages of activated T cells ($CD44^+CD4^+$ or $CD44^+CD8^+$), CTLA4- or TNFR2- expressing activated T cells ($CTLA4^+$ or $TNFR2^+$ activated T cells), and activation stages (naïve($CD44^-CD62L^+$), central memory (CM;$CD44^+CD62L^+$) and effector memory (EM; $CD44^+CD62L^-$) T cells) of $CD4^+$ and $CD8^+$ T cells in WT and NahG mice. **(C)** Intracellular TNF, Interleukin (IL)-2, Interferon-gamma (IFNγ), IL-4, and Granzyme B cytokine production in activated $CD4^+$ and $CD8^+$ T cells. Error bars represent SD. Statistical differences were determined using

the Student's t-test, (not significant (ns)). Data shown are from one experiment (n=10).

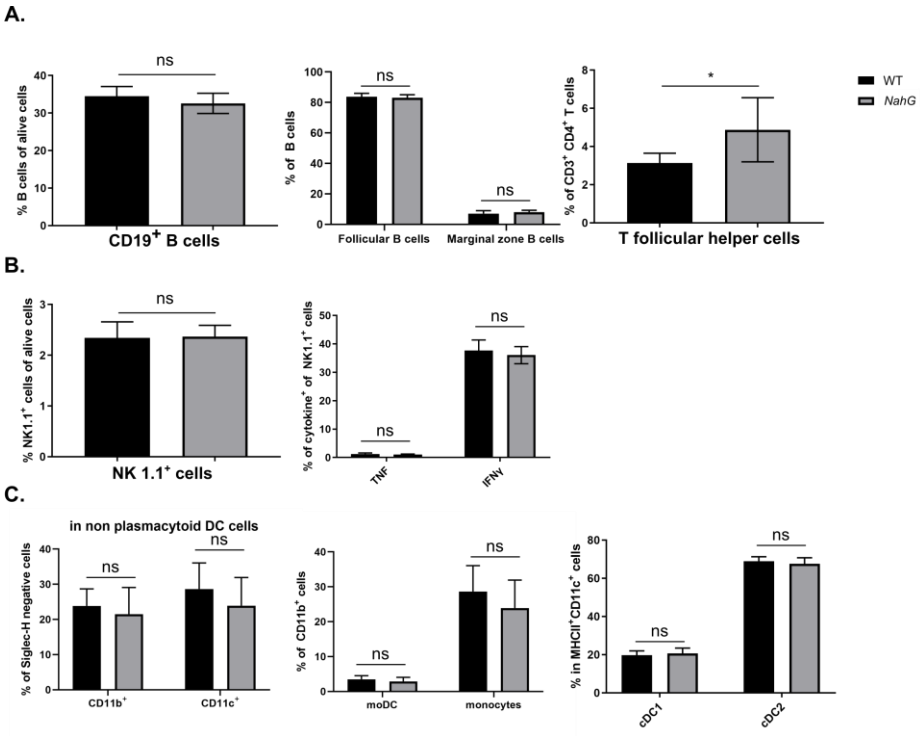

Figure S3. Transgenic *NahG* mice have a normal splenic population of B cell, NK cell, and non-plasmacytoid dendritic cell subsets.
(A) Percentages of B cells (CD45R/B220$^+$ or CD19$^+$) in spleen and the frequencies of follicular (CD19$^+$ CD21/CD35$^+$ CD23$^+$), marginal zone (CD19$^+$ CD21/CD35$^+$ CD23$^-$) B cells, and T follicular helper cells (CD4$^+$CXCR5$^+$PD-1$^+$) of WT and NahG mice. **(B)** Percentages of NK cells (NK1.1+) and TNF- or IFNγ-expressing NK cells (TNF$^+$ NK1.1$^+$ or IFNγ$^+$ NK1.1$^+$) of WT and NahG mice **(C)** Percentages of non-plasmacytoid DC subsets (conventional DC1 and DC2 abbreviated as cDC1 and cDC2), monocyte, and monocyte-derived dendritic cells (moDCs) of WT and NahG mice. cDC1 cells were distinguished by assessing CD11c$^+$MHCII$^+$SIRPα$^-$CD8$^+$ cells, cDC2s by CD11c$^+$MHCII$^+$SIRPα$^+$CD8$^-$, monocytes by CD11b$^+$Ly6c$^+$MHCII$^-$, and moDCs by CD11b$^+$Ly6c$^-$MHCII$^+$. Error bars represent SD. Statistical differences were determined using the Student's t-test (not significant (ns)). Data shown are representative of three independent experiments (A) or from one experiment (B and C) (n=10).

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Chapter 3. Addendum

BA is not the major precursor of SA in HEK293T cells, HeLa cells, and murine primary splenocytes

In **Chapters 1 and 3**, we point out that benzoic acid (BA) was suggested for synthesizing salicylic acid (SA) in humans [1]. In the previous study, a BA-$^{13}C_6$ precursor feeding increased the excretion of urinary SA-$^{13}C_6$ and its urinary metabolite salicyluric acid-$^{13}C_6$ in humans, possibly by the benzoic acid 2-hydroxylase (BA2H) [1]. This suggests that the identification of BA-interacting proteins via a proteome-wide approach, such as limited proteolysis (LiP) with mass spectrometry (MS) [2,3], may allow us to identify a BA2H-encoding gene and reveal the SA biosynthesis pathway. However, this speculation relies on a single study. Verifying whether animals possess BA2H activity is thus necessary. Therefore, we performed the BA2H enzyme assay using BA-α-^{13}C in HeLa cells, HEK293T cells, and primary mouse splenocytes to determine the conversion of BA-α-^{13}C to SA-α-^{13}C (**Figure 1A**). Culturing HEK293T cells, HeLa cells, and primary mouse splenocytes with BA-α-^{13}C (*m/z* 195), however, did not result in an increased abundance of SA-α-^{13}C (*m/z* 268), indicating the lack of BA2H activity in these cells (**Figure 1B**). It is thus questionable whether BA is the major precursor for SA biosynthesis, implicating the synthesis of SA through an alternative pathway in HEK293T cells, HeLa cells, and mouse splenocytes.

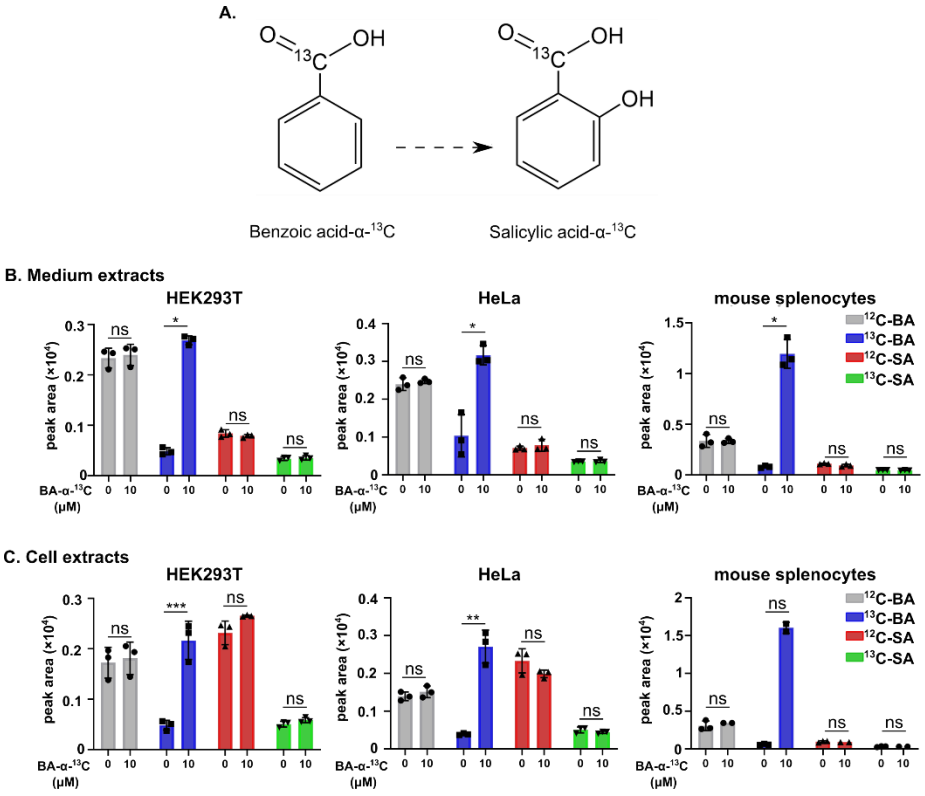

Figure 1. BA is not the major precursor of SA in HEK293T cells, HeLa cells, and murine primary splenocytes.
(A) Putative enzymatic conversion of BA-α-^{13}C into SA-α-^{13}C by benzoic acid 2-hydroxylase. **(B)-(C)** HEK293T cells, HeLa cells, and primary splenocytes from wild-type mice were incubated with a BA-α-^{13}C (10 μM) for 24 hours, and the level of SA (*m/z* = 267.0868, 2trimethylsilyl or 2TMS derivative), SA-α-^{13}C (*m/z* = 268.0868, 2TMS derivative) BA (*m/z* = 194.3025, TMS derivative), and BA-α-^{13}C (*m/z* = 195.3025, TMS derivative) in cell supernatant **(B)** and cell extracts **(C)** were determined by gas chromatography-mass spectrometry (GC-MS). Results are the means of three biological replicates, and the experiment was performed once. Statistical significance was calculated using a Student's T-test: *P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

Material and methods

A single cell suspension from mouse spleen was prepared as described in **Chapter 3.1**, section 5.5.2. Primary splenocytes were seeded at a density of 1.5×10^5 cells in 6-well plates in Roswell Park Memorial Institute Medium (RPMI) from Gibco supplemented with 10% FCS and 1 ng/mL IL-2 (Peprotech). HEK293T cells and HeLa cells were seeded at a density of 1.5×10^5 cells in 6-well plates in Dulbecco's Modified Eagle Medium (DMEM) from Gibco, supplemented with 10 % FCS. Cells were subsequently treated with 10 µM BA-α-^{13}C (Santa Cruz). On the next day, metabolite extraction and GC-MS analysis for profiling SA, SA (*m/z* = 267.0868, 2TMS derivative), SA-α-13C (*m/z* = 268.0868, 2TMS derivative), BA (*m/z* = 194.3025, TMS derivative), and BA-α-13C (*m/z* = 195.3025, TMS derivative) in cell supernatant was performed as described in **Chapter 3.2**, section 4.6 and **Chapter 3.1**, section 5.4. Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the appropriate statistical test (Student's t-test) has been performed to determine a significant difference.

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Chapter 4. Unraveling the roles of ABA in immune regulation

Chapter 4.1. Engineering a highly sensitive biosensor for abscisic acid in mammalian cells

Seo Woo Kim[1, 2, 3, 4], Kübra Alci[2,5], Femke Van Gaever[1, 2], Yasmine Driege[1, 2], Keylla Bicalho[6], Geert Goeminne[6], Claude Libert[1, 2], Alain Goossens[3, 4, *], Rudi Beyaert[1, 2, *], Jens Staal[1, 2, 7*, #]

* shared last authorship.

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[5]BCCM/GeneCorner, Ghent University, Ghent, Belgium
[6]VIB Metabolomics Core Ghent, VIB, Ghent, Belgium
[7]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

[#]Author for correspondence

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SWK: Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization. FVG: Methodology, Analysis. Writing – Review & Editing. KA: Methodology. YD: Validation. KB: Methodology, Validation, Analysis. GG: Methodology, Validation, Analysis. AG: Writing - Review & Editing, Supervision, Project administration. RB: Writing - Review & Editing, Supervision, Project administration, Funding acquisition. JS: Conceptualization, Methodology, Investigation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

1 Abstract

Abscisic acid (ABA) is a signaling molecule conserved in multiple kingdoms such as plants, bacteria, fungi and animals. Recently, ABA has gained attention for its pharmacological activities and its potential as a biomarker for the severity of chronic obstructive pulmonary disease (COPD) and glioma. This prompts the development of a reliable, sensitive, rapid, and cost-effective method to quantify ABA levels in mammalian cells and tissues in a high-throughput format to enable further functional characterization of ABA. The previously described ABA biosensor system based on the ABA-dependent interaction between the plant ABA receptor PYL1 and co-receptor ABI1 is not sensitive enough for the low ABA levels seen in mammals. Therefore, we optimized this system by replacing PYL1 with other high-affinity plant PYL proteins. The optimized biosensor system engineered with the PYL8 receptor enabled the quantification of ABA at low concentrations in HEK293T cells. ABA inactivation by a major *Arabidopsis* ABA 8'-hydroxylase (AtCYP707A3), anti-ABA single-chain Fv antibodies (scFv) in the endoplasmic reticulum, or *Arabidopsis* UDP-glucosyltransferase71C5 (AtUGT71C5) showed the selectivity of the biosensor system for ABA. We envision that this improved sensitive biosensor will be a powerful tool for monitoring ABA levels in large-scale studies deciphering functional roles of ABA in animals and diagnosing the severity of ABA-related diseases.

2 Introduction

The development of biosensors enabled the characterization of various metabolites in many biological processes. Abscisic acid (ABA), a major phytohormone playing an essential role in plant abiotic stress responses, was one such metabolite whose dynamics were revealed in plants using biosensors based on the ABA signaling pathway in plants (**Figure 1A**). For instance, to monitor ABA levels in different tissues of *Arabidopsis thaliana* L. in response to salinity or osmotic stress, a fluorescence resonance energy transfer (FRET)-based biosensor was used [1,2]. This biosensor translates the interaction between the ABA-induced conformationally rearranged the Pyrabactin resistance 1 (PYR1)/PYR1-Like (PYL)/Regulatory components of aba receptors (RCAR) ABA receptors and the co-receptor protein phosphatases 2C (PP2C) (*e.g.,* abscisic acid insensitive; ABI1) into fluorescence intensity [1,2]. In addition, other ABA-responsive reporter systems utilizing downstream ABA signaling components, such as the promoter region of ABA-responsive genes and the ABA-dependent kinase activity of snf1-related protein kinase 2 (SnRK2) family members, allowed deciphering ABA signal transduction as well as ABA homeostasis in plants [3–5].
Recent findings of the beneficial role of ABA administration in humans against type II diabetes (T2D) [6–10] has attracted attention to the use of ABA as a therapeutic or prophylactic agent to prevent T2D. Additionally, the administration of ABA in animal models of several diseases suggested its anti-inflammatory effects in alleviating a wide range of pathological processes such as inflammatory bowel disease [11,12], depression [13,14], and neuroinflammation [15,16]. Moreover, previous studies reported a difference in serum ABA levels between patients diagnosed with moderate and severe chronic obstructive pulmonary disease (COPD) [17], or between human high-grade and low-grade glioma tissues [18], suggesting ABA as a potential biomarker for the severity of these disorders.

Whereas ABA biosynthesis, catabolism, and signaling pathways are well known in plants, they are poorly understood in animals. A limited number of studies have proposed different mammalian ABA receptors [12,18–22]. First, lanthionine synthetase component C-like protein 2 (LANCL2), a peripheral membrane receptor, originally residing in the plasma membrane by N-terminal glycine myristoylation, translocates to the nucleus upon ABA binding [19]. Genetic depletion of LANCL2 specifically in muscle cells eliminates the protective effect of ABA in T2D, demonstrating its importance in an alternative insulin-independent blood sugar regulation mechanism [9]. A second demonstrated target of ABA is the nuclear receptor peroxisome proliferator-activated receptor γ

(PPARγ), which is known to bind free fatty acids, the anti-diabetic drug rosiglitazone, and eicosanoids [20,23]. Several loss-of-function studies showed that PPARγ is necessary for the protective role of ABA against T2D, colitis, and influenza A virus infection [12,21,22]. Although ABA activates PPARγ, it does not bind to its known ligand-binding domain [20]. Previous studies showed that ABA activates PPARγ in a LANCL2-dependent manner [20,24]. Loss of PPARγ in immune cells is sufficient to block the protective effects from ABA in a model of colitis and diabetes [12,21], demonstrating that PPARγ regulates critical responses downstream of ABA. Finally, the retinoic acid signaling pathway was also shown to be involved in a potential inhibitory role of ABA in the pathogenesis of glioma [18]. mRNA expression levels of *retinoic acid receptor β* (*RARβ)* and *retinoic acid receptor γ* (*RARγ)* were negatively correlated with the level of ABA in glioma tissue. A loss of function and the overexpression of retinoic acid receptor α gene (*RARα*) in U87-MG and A172 cells suggested that RARα positively impacts ABA-induced cancer cell apoptosis and differentiation [18]. Of note, retinoic acid and ABA share a similar structure since retinoic acid can be produced via cleavage of β-carotene in animals, which serves as a precursor in the plant ABA biosynthesis pathway [25].

Previous studies of ABA functions in mammals quantified ABA levels by enzyme-linked immunosorbent assay (ELISA) and chromatography-mass spectrometry [26–29]. However, these analytical approaches have drawbacks. Both methods need sample extraction from mammalian tissues or cells, which limits the spatiotemporal resolution. In addition, ELISA is expensive, and its performance is highly influenced by antibody stability, thereby requiring appropriate transport and storage [30]. Tandem mass spectrometry, the most favorable chromatography-mass spectrometry for ABA in the context of sensitivity, is also expensive and requires complex protocols [31]. As an alternative approach, ABA-inducible reporter gene expression in cultured mammalian cells was developed [32]. This rapid and cost-effective system was engineered based on the ABA-dependent interaction of the plant ABA receptor Pyrabactin resistance1-like 1 (PYL1) and the ABA co-receptor PP2C family member, ABI1[32]. The complementary surfaces (CSs) of the PYL1 (PYLcs, amino acid 33 to 209) and the ABI1 (ABI1cs, amino acids 126-423, lacking phosphatase activity) were fused to the VP16 transactivation domain (VP16AD) and GAL4 DNA-binding domain (GAL4BD), respectively (**Figure 1B**). GAL4BD recognizes and binds to an upstream activating sequence (UAS), and VP16AD activates the general transcription factor machinery bound to the minimal promoter region of a downstream gene. In the presence of ABA, the PYL1cs/ABI1cs interaction therefore recruits VP16AD to the GAL4-

dependent promoter and thus leads to the expression of a reporter gene (downstream of UAS) [32]. This mammalian ABA-inducible system has been used in various synthetic biology approaches for the design of on/off switches [33]. However, the detection limit of this system was shown to be relatively high (approximately 1 µM [32]), considering the low basal ABA levels in mammals (sub-nM concentrations) [1,34,35]. Moreover, a reporter gene assay containing a peroxisome proliferator response element (PPRE) and PPARγ was previously used to evaluate the PPARγ activity upon ABA treatment [6,20]. However this system only allowed the detection of ABA at minimum 1.25 µM. Therefore, this limitation establishes the need to engineer a novel sensitive ABA biosensor.

In this study, we optimized the above-described ABA biosensor system by replacing PYL1 with other plant PYL receptors displaying higher affinity for ABA binding, which allowed the improvement of the sensitivity of the biosensor with a stronger signal-to-noise ratio. The ABA-dependent interaction between the PYL8 receptor and the co-receptor ABI1 in the optimized biosensor system allows the determination of ABA levels at low nM concentrations in HEK293T cells. Furthermore, the selectivity of the biosensor system for ABA was verified using the following ABA inactivation strategies: 1) a major *Arabidopsis* ABA 8'-hydroxylase AtCYP707A3, 2) anti-ABA single-chain Fv antibodies (scFv) in the endoplasmic reticulum (ER), and 3) *Arabidopsis* UDP-glucosyltransferase71C5 (AtUGT71C5).

3 Results & Discussion

3.1 Generation of a highly sensitive ABA biosensor based on the ABA-dependent interaction between the *Arabidopsis* PYL ABA-receptor and the co-receptor ABI1

In order to increase the sensitivity of the previously described ABA biosensor that is based on the GAL4DB-ABI1cs/VP16AD-PYL1cs-induced activation of a GAL4-dependent reporter gene [32] (**Figure 1B**), we first added 20 extra repeats of the GAL4-dependent UAS to the original luciferase reporter construct, which only consisted of 2 tandem repeats of UAS in the old sensor system (**Figure 1C**). The resulting 22 x UAS caused a significant increase in the ABA-dependent luciferase reporter gene expression (**Figure 1D**). Next, we replaced PYL1 with other *Arabidopsis* PYL receptors displaying a higher apparent ABA-binding affinity. Compared to dimeric ABA receptors like PYL1, monomeric ABA receptors were reported to show higher ABA binding affinities [34]. For instance, altering dimeric PYR1 to monomeric PYR1 by a point mutation of a histidine 60 to proline (PYR1H60P) increased its apparent ABA affinity [34]. Likewise, a corresponding point mutation in PYL1 (H87P) in the above-mentioned FRET-based biosensor increased its affinity for ABA from Kd ~ 80 µM to Kd ~2 µM [1]. Therefore, we opted for the PYL1cs point mutation of histidine-87 to proline and other monomeric PYLs, PYL6 and PYL8, which will display a higher apparent affinity for ABA compared to PYL1 in the formation of the PYL–ABA–PP2C complex (**Figure 1E**) [1,34,36,37]. As Liang *et al.*, used the truncated variant of PYL1 (PYLcs) that contains star-related-lipid transfer (START) domain responsible for ABA binding [32,38,39], we also made use of the START domain of PYL6 to generate minimal PYL6cs (amino acids 44-183). START domain of PYL8 is not annotated to our knowledge, we made use of its full length. Consistent with the previous report showing the higher affinity of PYL1(H87P) compared to PYL1 [1], the luciferase expression activated by ABA-induced interaction between PYL1(H87P)cs and ABI1 was approximately forty-fold higher at 40 nM ABA in HEK293T cells than that driven by the interaction between PYL1cs and ABI1 in the presence of equimolar ABA (**Figure 1F**). In contrast, a biosensor with the PYL6cs receptor did not respond to ABA (40 nM) in HEK293T cells (**Figure 1F**). It is possible that the truncated PYL6cs does not build a complete binding pocket for ABA. Therefore future studies should examine whether the full-length PYL6 would be functional.

The concentration-response curve for ABA-induced luciferase activation showed that the detection limit of the PYL8-based ABA biosensor in

HEK293T cells is 2.93 nM and its limit of quantification is 8.88 nM (**Figure 1G**). It is worth noticing that ABA-untreated cells already exhibit a clear background signal (**Figure 1F**). Previous studies using a yeast two-hybrid assay and size-exclusion chromatography (SEC) coupled to multiple angle laser light scattering (MALLS) [34,40] showed the partial interactions of monomeric PYLs (e.g., PYL5, PYL6, and PYL8) with PP2C (HAB1) in the absence of exogenous ABA, suggesting the spontaneous formation of a PYL8-ABI1 complex. Thus this background signal possibly reflects ABA-independent formation of the PYL8-ABI1 complex. However, we are not aware whether these proteins spontaneously interact in mammalian cells which grow at a bit higher temperature (37 °C) compared to in plants or yeast. Another possible source for this background could be low amounts of endogenous ABA produced in HEK293T cells or ABA that is present in the serum of the cell culture medium (see also below). Together these data indicate that both PYL1(H87P)- and PYL8-based biosensor systems have enhanced sensitivity to ABA compared to the original PYL1-based biosensor.

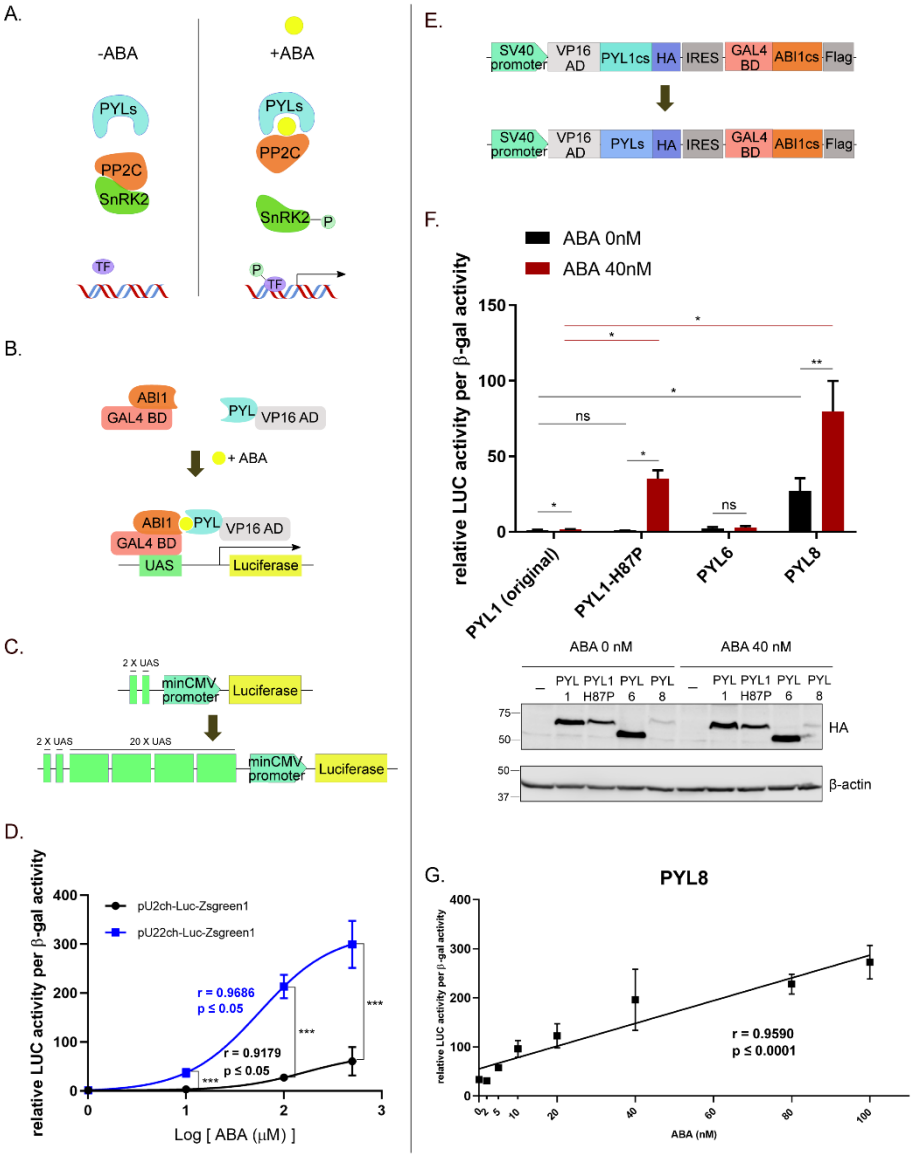


Figure 1. Substitution of PYL1cs to PYL1 mutant and other PYL family receptors in the Abscisic acid (ABA) biosensor construct alters ABA responsiveness (see following page for figure legend).

Figure 1. Substitution of PYL1cs to PYL1 mutant and other PYL family receptors in the Abscisic acid (ABA) biosensor construct alters ABA responsiveness (see previous page for figure)**.**
(A) Overview of the ABA signaling pathway in plants. A signaling complex composed of PYR/PYL/RCAR (PYL), protein phosphatases 2C (PP2C), and snf1-related protein kinase 2 (SnRK2) has been shown as a major component of the core ABA signaling cascade in plants. In the absence of ABA, PP2C negatively regulates SnRK2 activity. Once ABA levels increase in response to environmental stresses, ABA binding to the PYL receptor leads to the formation of an ABA–PYL–PP2C complex. SnRK2 is subsequently released from PP2C inhibition and thus activates downstream components such as ABA-responsive transcription factors by phosphorylation. **(B)** Underlying principle of the ABA-inducible luciferase assay system based on the plant ABA core signaling pathway (figure adapted from [32]). ABA-dependent interaction between the plant ABA receptor PYL and the ABA co-receptor ABI1 brings the VP16AD to the GAL4-dependent promoter, leading to luciferase gene expression. **(C)** Generation of the custom UAS reporter construct with 22 x UAS elements in the promoter region. **(D)** Comparison of the ABA-induced luciferase gene expression in HEK293T cells driven by two or 22 UAS repeats. HEK293T cells were transfected with the PYL1cs-based biosensor construct along with pACTβgal (normalization) and pU2CH-Luc-Zsgreen1 or pU22CH-Luc-Zsgreen1 (reporter) and incubated with ABA at the indicated concentrations. **(E)** Overview of the modifications made in the ABA biosensor construct. The complementary surfaces (CSs) of plant ABA receptor PYL1 in the original construct pSv-ABAactDA [32] were replaced into the hyperactive mutant PYL1 or different members of the PYL receptor family. Protein accumulation of HA-tagged PYL1cs, PYL(H87P)cs, PYL6cs, and PYL8 was analyzed by western blot analysis. β-actin was used as a loading control. **(F)** Comparison of the ABA-induced luciferase gene expression in HEK293T cells expressing different PYL receptor-based biosensors. HEK293T cells were transfected with the indicated different PYL constructs along with pU22ch-Luc-Zsgreen1 and pACTβgal and incubated in the absence or presence of 40 nM ABA. **(G)** Dose-response curve for ABA in the ABA-inducible luciferase assay system utilizing the PYL8 receptor-based biosensor in HEK293T cells. HEK293T cells were transfected with the PYL8-based biosensor construct along with pU22CH-Luc-Zsgreen1 and pACTβgal and incubated with ABA at the indicated concentrations. Fold induction of ABA-induced luciferase gene expression in cells expressing the PYL receptor-based biosensor constructs is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. Error bars represent standard deviation (S.D). Results are the means of four biological replicates and representative of two **(F)** or three **(E, G)** independent experiments. Statistical significance was calculated using a Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001). R-value and P-value in **(G)** were calculated using a simple linear regression analysis.

3.2 ABA inactivation strategies validate the selectivity of the biosensor for ABA

In order to verify the selectivity for ABA of the improved PYL8-based biosensor, we applied the following ABA inactivation strategies: 1) a major *Arabidopsis* ABA 8'-hydroxylase AtCYP707A3, 2) capturing ABA by expressing anti-ABA single-chain Fv antibodies (scFv) in the endoplasmic reticulum (ER), and 3) *Arabidopsis* UDP-glucosyltransferase71C5 (AtUGT71C5).

In *Arabidopsis* ABA catabolism, the C-8' position of ABA is predominantly hydroxylated by the cytochrome P450s (CYP), AtCYP707A1 to AtCYP707A4 [41]. AtCYP707A3 has been shown to catabolize ABA to phaseic acid in insect cells [42]. In agreement with this, the ABA-dependent luciferase activity significantly decreased in ABA-treated HEK293T cells expressing the *AtCYP707A3* gene (**Figure 2A, 2B**). Moreover, this depletion could be reversed by treatment of cells with the CYP707A inhibitor, abscinazole-E3M. The majority of CYP enzymes require the assistance of NADPH-cytochrome P450 reductase for the electron transfer from NADPH [43]. However, the overexpressed AtCYP707A3 depleted ABA in HEK293T cells without expressing additional CYP reductase, indicating the involvement of endogenous CYP reductase. The second strategy, anti-ABA scFv, was kindled by a previous study, showing that accumulation of ER-targeted anti-ABA scFv in tobacco results in an ABA-deficient phenotype [44]. Likewise, ABA-induced luciferase reporter gene expression in HEK293T cells was significantly less when anti-ABA scFv was present (**Figure 2C, 2D**). Expression of an irrelevant transgene *NahG* (encoding an enzyme catalyzing the degradation of salicylic acid) under the same promoter and in an identical backbone vector (pCS2), did not decrease luciferase activity in response to ABA. Finally, a third ABA depletion strategy, *Arabidopsis* UDP-glucosyltransferase71C5 (*AtUGT71C5*), which has a role in ABA homeostasis in *Arabidopsis* through catalyzing ABA glucosylation [45], also significantly decreased ABA-induced luciferase gene expression in ABA-treated HEK293T cells (**Figure 2E, 2F**). Transfection with the empty backbone vector plasmid (pCS2) did not lower ABA-induced luciferase activation. Taken together, these transgenic approaches validate the ABA selectivity of our newly developed PYL8-based ABA biosensor system.

The above described experiments also show that all three ABA-inactivation methods, AtCYP707A3, anti-ABA scFv, and AtUGT71C5, significantly lower the background luciferase signal that is already observed in ABA-untreated HEK293T cells. This suggests that the background signal might derive from ABA that is present in cell culture

medium, or might be endogenous ABA produced in HEK293T cells. Since HEK293T cells show an adrenal and neuronal-like gene expression profile [46], and the brain is one of the most ABA-rich tissues in animals [28], endogenous ABA production by HEK293T cells is not so unlikely. However, as already mentioned above, this background signal may also result from the spontaneous formation of a PYL8-ABI1 complex. Incomplete deletion of background signal upon the expression of ABA-inactivation genes reflect the presence of spontaneous formation of the PYL8-ABI1 complex. Our observation thus indicates that this background signal might be combination of these two factors.

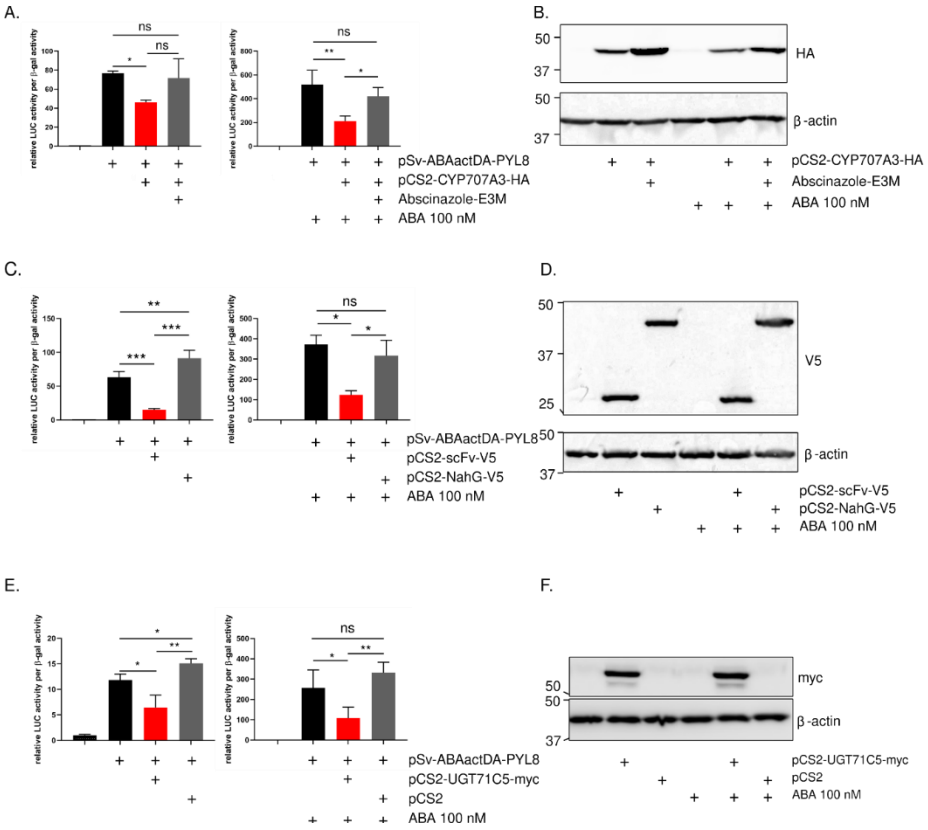


Figure 2. Depletion of ABA in HEK293T cells upon overexpression of plant ABA catabolic genes or ABA-conjugating genes (see following page for figure legend)**.**

Figure 2. Depletion of ABA in HEK293T cells upon overexpression of plant ABA catabolic genes or ABA-conjugating genes (see previous page for figure)**.**
(A) Effect of overexpression of *AtCYP707A3* on ABA-induced luciferase activity in HEK293T cells in the absence or presence of 10 µM of the CYP707A inhibitor abscinazole-E3M. Cells were transfected with the indicated construct along with the PYL8-based biosensor construct, pU22CH-Luc-Zsgreen1 and pACTβgal, and incubated in the presence or absence of 100 nM ABA or 10 µM abscinazole-E3M. **(B)** Western blot analysis of protein accumulation of HA-tagged AtCYP707A3. **(C)** Effect of overexpression of *anti-ABA scFv* on ABA-induced luciferase activity in HEK293T cells. Cells transfected with the irrelevant transgene *NahG* carried in the same backbone vector as anti-ABA scFv were used as a negative control. HEK293T cells were transfected with the indicated construct along with the PYL8-based biosensor construct, pU22CH-Luc-Zsgreen1 and pACTβgal, and incubated in the presence or absence of 100 nM ABA. **(D)** Western blot analysis of protein accumulation of V5-tagged scFv and NahG. **(E)** Effect of overexpression of *AtUGT71C5* on ABA-induced luciferase activity in HEK293T cells. Cells transfected with the control backbone empty vector (EV) plasmid (pCS2) were used as a negative control. **(F)** Western blot analysis of protein accumulation of Myc-tagged UGT71C5. Fold induction of ABA-induced luciferase gene expression in cells expressing the PYL8 receptor-based biosensor construct is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. β-actin was used as a loading control. Error bars represent S.D. Results are the means of three biological replicates and are representative of at least two independent experiments. Statistical significance was calculated using a Home-Sidak's multiple comparisons test (pairwise): *P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

3.3 Evaluation of the use of human LANCL2, PPARγ, and RARs for the generation of novel ABA biosensors

LANCL2 was previously proposed as a mammalian ABA receptor [19,47]] and RAR (α, β, γ) pathways have been linked to the anti-inflammatory activity of ABA and are also proposed as ABA receptors [12,18,20–22]. We therefore hypothesized the potential use of LANCL2, PPARγ and RARs (α, β, γ) for the generation of a novel GAL4-VP16/UAS driven ABA biosensor [12,18–22,47].

LANCL2 is anchored in the cell membrane via myristoylation. Translocation of LANCL2 from the membrane to the nucleus upon binding to ABA has been suggested to result from the loss of myristoylation at the N-terminus [19]. We therefore fused the GAL4BD-VP16AD to the C-terminus of LANCL2 (**Figure 3A, 3B**), and hypothesized that the membrane anchored LANCL2-GAL4BD-VP16AD would translocate to the nucleus upon ABA treatment in HEK293T cells, subsequently transactivating luciferase reporter gene expression.

However, luciferase gene expression was already activated in untreated cells expressing such as LANCL2-GAL4BD-VP16AD fusion protein and did not further increase upon ABA treatment. We speculate that the nuclear localization sequence that is present in GAL4BD overcomes the effect of myristoylation-mediated membrane anchoring, resulting in the ABA-independent nuclear translocation, and making such an approach incompetent (**Figure 3C**).To corroborate this, one could determine a subcellular localization of LANCL2-GAL4BD-VP16AD protein by immunostaining or subcellular fractionation followed by western blotting.

PPARγ and RARs (α, β, γ) are nuclear hormone receptors that elicit transcription-activating functions [48,49]. Hypothesizing their activation by ABA, we therefore fused the GAL4BD to each of these nuclear receptors and measured expression of a GAL4-dependent luciferase reporter gene in HEK293T cells transfected with the corresponding GAL4BD-PPARγ, GAL4BD-RARα, Gal4BD-RARβ, and GAL4BD-RARγ expression plasmids (**Figure 3A, 3B**). Treatment with the PPARγ-ligand rosiglitazone and the RAR-ligand retinoic acid were used as positive controls. However, ABA treatment did not induce GAL4BD-PPARγ or GAL4DB-RARs (α, β, γ)-dependent luciferase activity (**Figure 3D-3G**), nor did ABA affected luciferase gene expression induced by the known RAR and PPARγ ligands (**Figure 3D-3G**). Therefore, these results demonstrate that construction of an ABA biosensor based on PPARγ and RAR is not a valid approach.

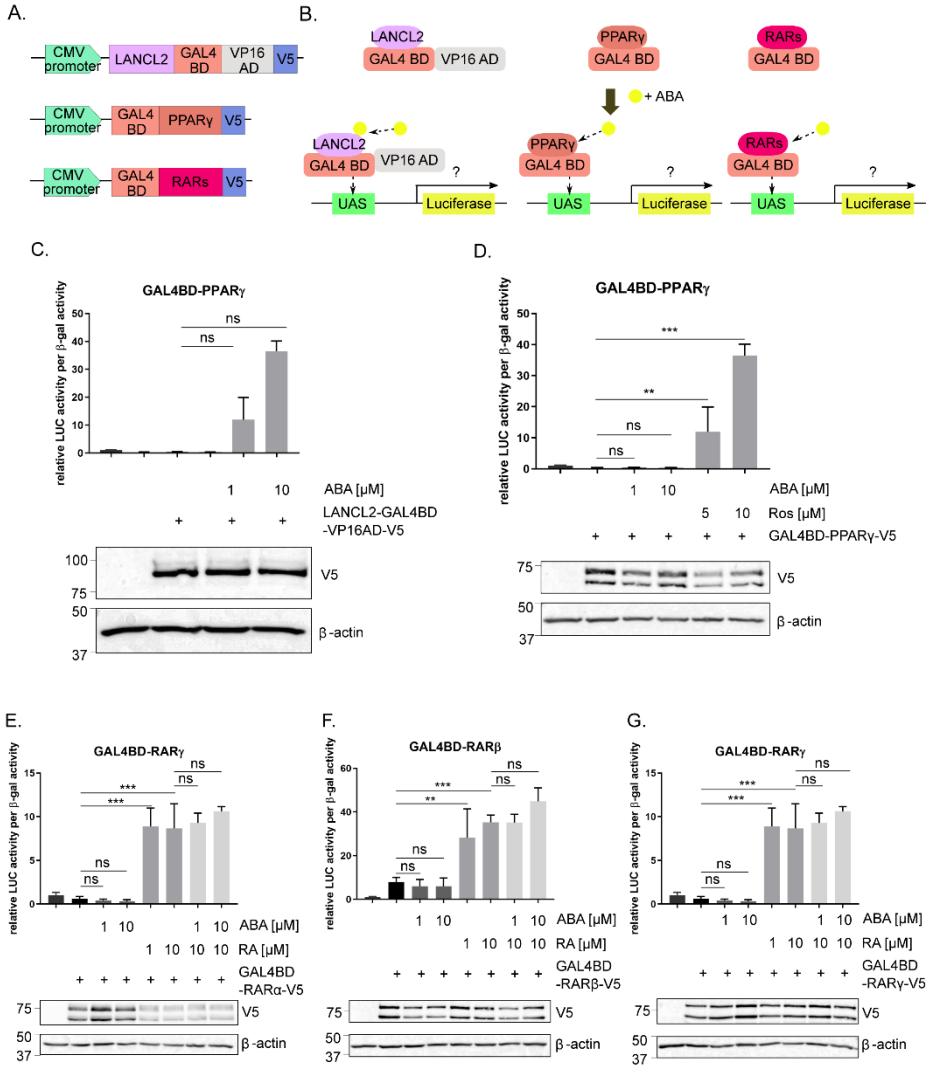


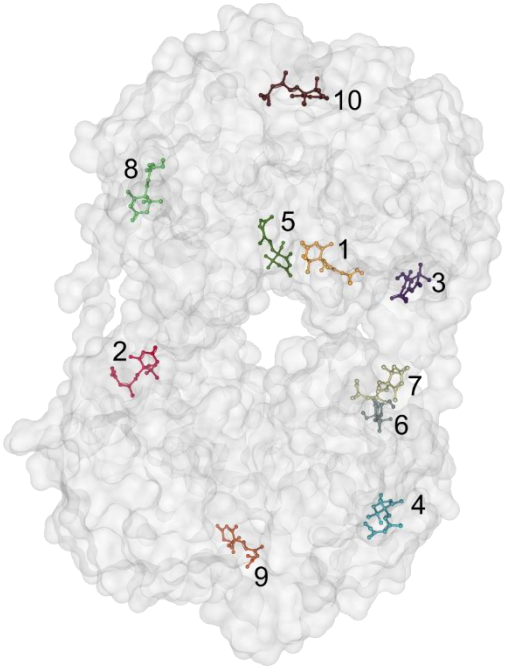
Figure 3. Evaluation of potential human ABA receptors in HEK293T cells (see following page for figure legend).

Figure 3. Evaluation of potential human ABA receptors in HEK293T cells (see previous page for figure)**.**
(A) Overview of the different GAL4DB fusion proteins of potential human ABA receptors. Expression of transgenes encoding these fusion proteins are driven by the CMV promoter. The V5 epitope tag is fused to monitor the receptor protein accumulation via western blotting. (B) Underlying principle of the ABA-inducible luciferase assay based on the proposed role of different human ABA receptors. (C-D) Luciferase activity in HEK293T cells containing the pU22CH-Luc-Zsgreen1 reporter construct. HEK293T cells were co-transfected with the LANCL2-GAL4BD-VP16AD (C) or GAL4BD-PPARγ (D) expression constructs along with pACTβgal (used for normalization of transfection efficiency) and incubated with ABA or rosiglitazone (Ros). Protein accumulation of V5-tagged LANCL2-GAL4BD-VP16AD or GAL4BD-PPARγ was verified by western blot analysis. (E-G) Analysis of RARα- (E), RARβ- (F), RARγ (G)-dependent luciferase gene expression in HEK293T cells containing the pU22CH-Luc-Zsgreen1 reporter construct. HEK293T cells were co-transfected with the corresponding fusion protein GAL4BD-RARs expression constructs along with pACTβgal and incubated with ABA or retinoic acid (RA). Protein accumulation of V5-tagged GAL4BD-RARs was verified by western blot analysis. The lower molecular weight immunoreactive band among two distinct bands might be due to an alternative translation site. Fold induction of luciferase gene expression in cells expressing *LANCL2*, *PPARγ* or *RARs* is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. β-actin was used as a loading control. Error bars represent S.D. Results are the means of four biological replicates and are representative of two independent experiments. Statistical significance was calculated using Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001).

It has been reported that PPARγ is activated indirectly by ABA in a LANCL2-dependent manner [20,24]. To our surprise, in HEK293T cells the PPRE-based reporter gene assay showed that the endogenous level of LANCL2 was sufficient to activate PPARγ activity [24]. Future studies should determine whether the co-overexpression of *LANCL2* will increase the transactivity of PPARγ in the presence of ABA.

It is still unknown how the RAR signaling pathway is implicated in the physiological function of ABA. Our observation that ABA could not activate the biosensor system when using the nuclear receptors RARs suggests a possible role for an additional intermediate signaling cascade that bridges ABA to RARs. One possible intermediate is the retinoid X receptor γ (RXRγ), which acts as an indispensable heterodimer partner for RAR [48,50]. Of interest, increased RXRγ protein accumulation upon ABA administration has been described in U87-MG and A172 cells, and a potential binding site of ABA in the RXRγ ligand-binding domain has been predicted by *in silico* molecular docking (**Figure 4**). Moreover, RXRγ also acts as a heterodimer partner for

PPARγ in regulating gene expression [51,52]. Therefore, we investigated whether the co-expression of *RXRγ* gene leads to the transactivation of the reporter gene upon ABA treatment in HEK293T cells expressing GAL4BD-PPARγ or GAL4DB-RARs. However, ABA treatment did not induce GAL4BD-PPARγ or GAL4DB-RARs (α, β, γ)-dependent luciferase activity in the presence of RXRγ (**Figure 5**), nor did ABA affect luciferase gene expression induced by the known RAR and PPARγ ligands.



ABA	Vina score	Cavity size
1	-7	2872
2	-6.9	1178
3	-6.6	4731
4	-6.3	954
5	-6.3	577
6	-5.8	747
7	-5.8	594
8	-5.6	1761
9	-5.6	845
10	-5.2	601

Figure 4. Graphical presentation of RXRγ ligand-binding domain with predicted docked poses of ABA.
Representation of the ten most favorable interactions ranked with the docking score and the cavity size. RXRγ is depicted in surface representation and ABA is shown as a ball-and-thick stick model. Each predicted interaction is numbered in the surface representation and corresponds with the number in the ABA column. The vina score is a parameter of binding energy, indicating the affinity and stability of interaction. The cavity size is essential as a large interaction interface could provide a high affinity.

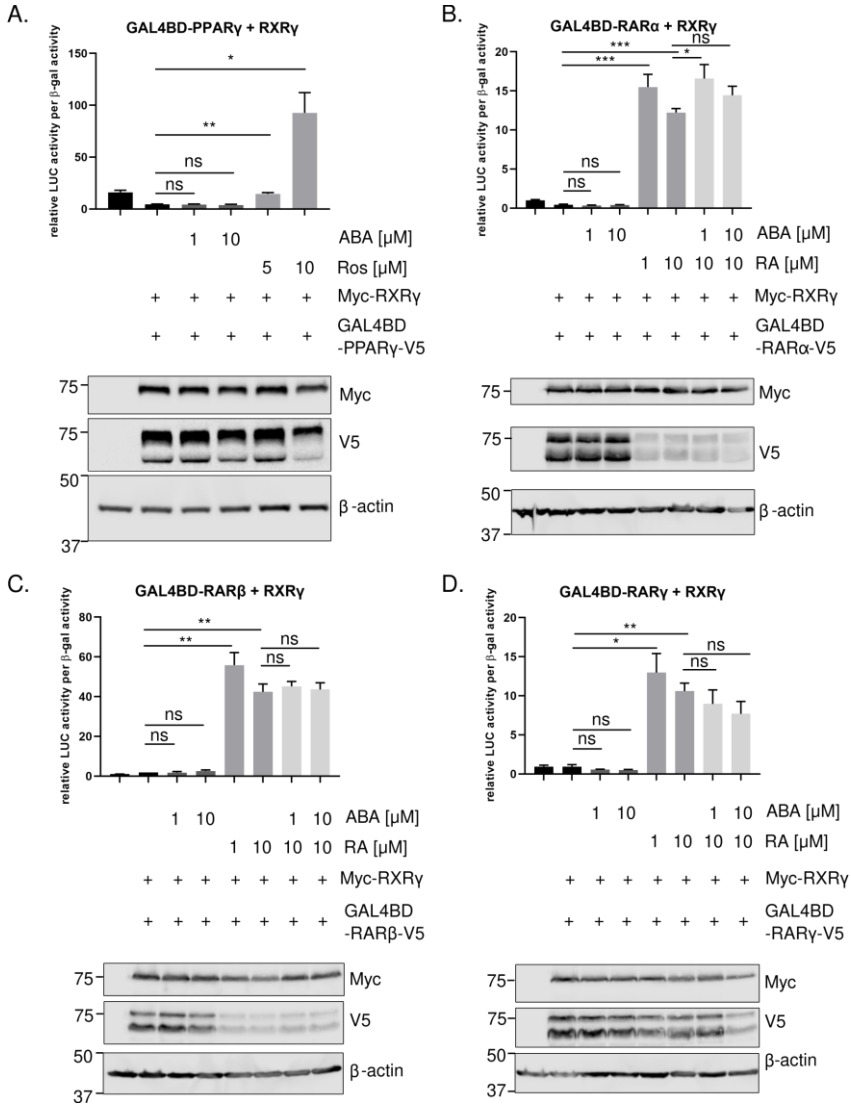


Figure 5. Evaluation of potential human ABA receptors in the presence of RXRγ in HEK293T cells (see following page for figure legend)**.**

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(A-D) Luciferase activity in HEK293T cells containing the pU22CH-Luc-Zsgreen1 reporter construct. HEK293T cells were co-transfected with the GAL4BD-PPARγ and RXRγ (A) expression constructs along with pACTβgal (used for normalization of transfection efficiency) and incubated with ABA or rosiglitazone (Ros). Protein accumulation of Myc-tagged RXRγ and V5-tagged GAL4BD-PPARγ were verified by western blot analysis. (B-D) Analysis of RARα- (B), RARβ- (C), RARγ (D)-dependent luciferase gene expression in HEK293T cells containing the pU22CH-Luc-Zsgreen1 reporter construct. HEK293T cells were co-transfected with the corresponding fusion protein GAL4BD-RARs and RXRγ expression constructs along with pACTβgal and incubated with ABA or retinoic acid (RA). Protein accumulation of Myc-tagged RXRγ and V5-tagged GAL4BD-RARs was verified by western blot analysis. The lower molecular weight immunoreactive band among two distinct bands might be due to an alternative translation site. Fold induction of luciferase gene expression in cells expressing PPARγ or RARs is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. β-actin was used as a loading control. Error bars represent S.D. Results are the means of three biological replicates and are representative of two independent experiments. Each biological replicate refers to independent transfection. Statistical significance was calculated using a one-way ANOVA test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001).

3.4 Applications

Recent advanced knowledge of ABA as a therapeutic/prophylactic agent and a biomarker for the severity of certain disorders such as COPD and glioma prompted the need for a reliable, sensitive, economical, and rapid ABA quantification method. In the present study, we improved the sensitivity of a previously described ABA biosensor system by employing a PYL1H87P or PYL8-ABA-PP2C ternary complex of *Arabidopsis* ABA signaling, which allows the quantification of ABA at levels down to nM concentration in HEK293T cells. Applying three different ABA inactivation strategies using AtCYP707A3, anti-ABA scFv, and AtUGT71C5, validated the selectivity of the ABA biosensor system.

Liang *et al.,* demonstrated the measurement of ABA in serum of mice orally given with ABA (340 mg/kg body weight (BW)) using the original PYL1-based system, which showed its therapeutic application. However, the ABA dose 340 mg/kg BW is relatively high compared to the dose used for the therapeutic/prophylactic treatment [22]. We thus examined whether our improved biosensor could detect ABA levels in mice treated with the dose of ABA close to therapeutic level (100 mg/ kg feed), which was opted for several studies investigating the effect of ABA in a mouse model of colitis [15–17], atherosclerosis [29], and systemic inflammation

(lipopolysaccharide model) [30]. We were able to detect ABA in serum of mice given ABA-supplemented diets for seven days, which was also confirmed by liquid chromatography-mass spectrometry analysis (LC-MS) (**Figure 6A**).

Previously, *Saccharomyces cerevisiae* (TABA) in which the ABA direct biosynthesis pathway from *Botrytis cinerea* was engineered were shown to produce ABA *in vitro* [53]. To analyze the efficacy of TABA to also produce ABA *in vivo*, we measured ABA in serum of mice given TABA-supplemented diets for seven days (1.7×10^8 colony forming unit (CFU)/g feed). However, ABA was not detectable, indicating that TABA supplementation in the diet does not allow to produce ABA *in situ* in the gut, at least not in quantities that it would be available in the serum and exert systemic effects (**Figure 6B**). This example illustrates the potential application of the biosensor system to monitor ABA levels in future pharmacological or pharmacokinetic research. We also envision that the improved ABA biosensor could contribute to large-scale studies that require the quantification of ABA as a biomarker in biological samples in the context of clinical application.

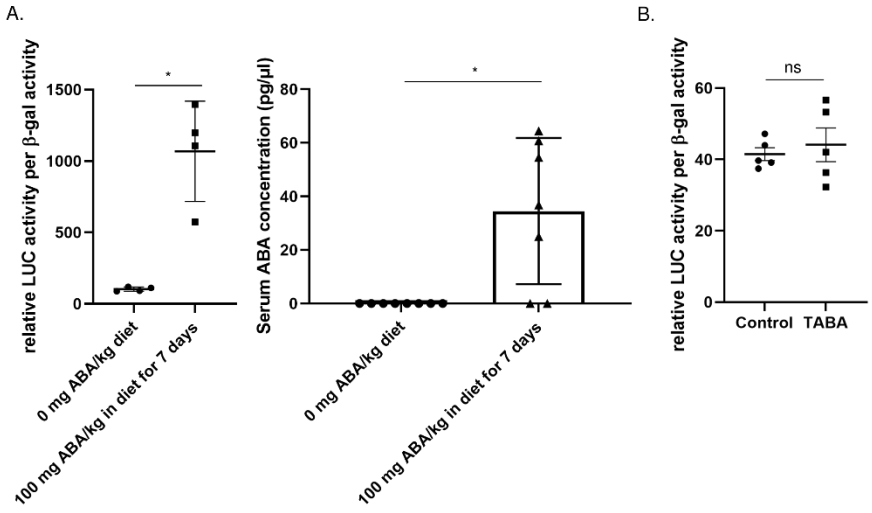

Figure 6. ABA measurement in serum from mice given an ABA-supplemented diet or an ABA-producing *S. cerevisiae* TABA supplemented diet.
(A) ABA measurement in serum from mice given control diets or ABA-supplemented diets (100 mg/kg) for seven days by PYL8-based biosensor and liquid chromatography–mass spectrometry (Vion IMS QTof Mass Spectrometer analysis). **(B)** ABA measurement in serum from mice given with ABA-producing *S. cerevisiae* (TABA)-supplemented diet (1.7×10^8 CFU/g feed). HEK293T cells were transfected with the PYL8 -based biosensor construct along with pU22CH-Luc-Zsgreen1 and pACTβgal and incubated with serum. Fold induction of ABA-induced luciferase gene expression in cells expressing the PYL receptor-based biosensor constructs is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. Error bars represent standard deviation (S.D). Data shown are from one experiment. Each dot represents one mouse. Statistical significance was calculated using Student's t-test **(A)** or Mann-Whitney U test **(B)** (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001).

3.5 Future perspectives

In this study, HEK293T cells were used to validate our engineered ABA biosensor system due to its straightforward transfection with a high efficiency. Along with genetic (*e.g.* small interfering RNA(siRNA) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9)) or pharmacological means, it could also be a powerful tool to screen for molecular and cellular mechanisms that regulate mammalian ABA metabolism also in other cell types. In contrast to ELISA and mass spectrometry, this system will allow a rapid and economic ABA

quantification beneficial for high-throughput screenings without the extraction procedure. Also, our biosensor system could be used like ELISA to quantify ABA levels in culture supernatant of a wide range of cell types under various physiological conditions. For instance, β-cell like rat insulinoma cells (INS-1) are shown to secrete ABA upon glucose treatment, which might induce insulin release and glucose uptake by muscle and adipose tissue [54–56]. To reveal whether endogenous ABA plays a role in interplay between inflammation and glucose or lipid metabolism, one could stimulate β-cells with adipokines (*e.g.,* adiponectin) or inflammatory cytokines (*e.g.,* tumor necrosis factor (TNF), interleukin (IL)-1β, and IL-6) and assess their extracellular ABA levels using our biosensor system in HEK293T cells [57].

Synthetic biology studies have used the PYL1–ABI1 complex formation as they can predictably and orthogonally modulate cellular processes in mammalian cells without disturbing the host's natural biological system [33]. These synthetic biology approaches can also benefit from our improved sensitivity for ABA in the biosensor with different PYL variants. Given that PYL8-based biosensor exhibits a background ABA-independent signal output, a sensitive PYL1(H87P) but showing low background will be plausible. Altogether, our improved ABA biosensor system will help future studies defining the physiological role and metabolism of ABA, which might bring new therapeutic approaches.

4 Material and Methods

4.1 Modification and construction of ABA biosensors

pSv-ABAactDA (pSV40-VP16AD-PYL1cs-HA-IRES-GAL4BD-ABI1-Flag) was a gift from Dr. J. Crabtree (Standford University, USA, Addgene plasmid no. 38247) [32] and was modified to obtain affinity variants. H87P mutation in the *PYL1* gene in pSv-ABAactDA was generated by PCR mutagenesis (see **Table S2** for primers used). *PYL6* CDS was amplified from *Arabidopsis* genomic DNA, and *PYL8* CDS was amplified from *Arabidopsis* cDNA using indicated primers. The PCR products were cloned between *Asc*I and *BamH*I into the pSv-ABAactDA plasmid, from which *PYL1* was removed. All constructs were verified by Sanger sequencing. All plasmids generated in this study were deposited in the BCCM/ GeneCorner plasmid collection (http://www.genecorner.ugent.be/) (**Table S1**).

To generate pLenti6-LANCL2-GAL4BDVP16AD-V5, *LANCL2* was amplified from pET28b-LancL2 (a gift from Jie Chen, the University of Illinois at Urbana-Champaig, USA, Addgene plasmid no. 73407), and the PCR product was subsequently cloned into pDNOR201 by Gateway BP clonase reactions according to manufacturer's instructions (Invitrogen). To generate pLenti6-GAL4BD-PPARγ-V5, pLenti6-GAL4BD-RARα-V5, pLenti6-GAL4BD-RARβ-V5, pLenti6-GAL4BD-RARγ-V5, and pCDNA-Myc-RxRγ, ORFs of *PPARγ, RARα, RARβ, and RARγ*, respectively, cloned in pENTR223 from Human ORFeome library v8.1 were used [58]. These receptors were subsequently transferred into the expression vector pLenti6-V5-puro by Gateway LR clonase reactions (Invitrogen). *GAL4BD-VP16AD* and *GAL4BD* were amplified from pGal4-VP16 using indicated primers (**Table S2**). To generate the final constructs, the *GAL4BD-VP16AD* PCR product was cloned into *XhoI*-opened pLenti6-LANCL2-V5 for C-terminal fusion, and the *GAL4BD* PCR product was cloned into *SpeI*-opened pLenti6-PPARγ-V5, pLenti6- RARα-V5, pLenti6-RARβ-V5, and pLenti6-RARγ-V5 for N-terminal fusion using NEBuilder HiFi DNA Assembly master mix according to manufacturer's instructions (New England BioLabs).

To generate pU22CH-Luc-Zsgreen1 (*i.e.*, 22×UAS), first, pU2CH-Luc-Zsgreen1 (*i.e.*, 2×UAS) was linearized and extended with *SpeI* and *KpnI* by PCR. Second, the 20×UAS sequence was amplified from pENTR-L1-20×UAS-L4 (a gift from S. Stowers, Montana State University, USA, Addgene plasmid no. 32307) using indicated primers (**Table S2**). Finally, the 20×UAS PCR product was subsequently cloned into *SpeI*- and *KpnI*-opened pU2CH-Luc-Zsgreen1, generating pU22CH-Luc-Zsgreen1.

4.2 Construction of vectors for the expression of ABA-catabolic genes or ABA-conjugating genes

HA-tagged mouse codon-optimized ABA-degrading P450 *AtCYP707A3* was synthetically generated and cloned into pUC19 vector (Genscript). pUC19-VSV-mAtCYP707A3-HA-P2A-ZsGreen was digested with *BamHI* and *XhoI* and ligated with *BamHI*- and *XhoI*-opened pENTR3C vector. A Gateway LR clonase reaction was performed to transfer an HA-tagged VSV-mAtCYP707A3 construct into a pCS2 vector according to the manufacturer's instructions (Invitrogen).

Mouse codon-optimized small single-chain antibody gene against ABA with secretion tag (SP) and ER retention tag (KDEL) for intracellular expression was also synthetically generated and cloned into pUC57 vector (Genscript). An SP-scFv-V5-KDEL construct was cloned into *BamHI*- and *XbaI*-opened pENTR3C vector. A Gateway LR clonase reaction was performed to transfer an SP-ScFv-V5-KDEL construct into a pCS2 vector according to the manufacturer's instructions (Invitrogen). *AtUGT71C5* was amplified from *Arabidopsis* genomic DNA using indicated primers (**Table S2**) and cloned into *KpnI*- and *SpeI*-opened pEF6/myc-His vector.

4.3 Cell culture and transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) from Gibco, supplemented with 10 % FCS. HEK293T cells were seeded at a density of 4×10^4 cells in 24-well plates and transfected with a total of 250 ng of plasmid DNA per well using a calcium phosphate method. pACTβgal (a gift from Dr. J. Inoue, Institute of Medical Sciences, Tokyo, Japan) encoding β-galactosidase was co-transfected in each well as an internal control. In addition to a luciferase reporter plasmid and vectors encoding genes of interest, an empty vector was added to maintain equal amounts of DNA in each transfection. Six hours after transfection, the medium was refreshed with or without the addition of ABA (A4906, Sigma, St Louis, MO, USA) and CYP707A inhibitor abscinazole-E3M and incubated for overnight at 37 °C/5 % CO_2. Abscinazole-E3M was a gift from Dr. Y. Todoroki (Shizuoka University, Japan) [59]. As controls to validate the transcriptional activity of GAL4BD-PPARγ and GAL4BD-RARs, Rosiglitazone (R2408, Sigma, St Louis, MO, USA) and Retinoic acid (R2625, Sigma, St Louis, MO, USA) were added to cells at the indicated concentrations when the medium was refreshed 6 hours after the transfection.

4.4 ABA-inducible luciferase assay

The transfected HEK 293T cells were lysed in luciferase lysis buffer (25

mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% Triton X-100). To cell lysates, D-luciferin (E1605, Promega) and CPRG substrate (10884308001, Sigma, St Louis, MO, USA) dissolved in CPRG buffer (60 mM Na_2HPO_4, 10 mM KCl, 1 mM β-mercaptoethanol) were added respectively. Luminescence signals were measured using the GloMax® 96 Microplate Luminometer (Promega), and β-galactosidase activity was quantified using the iMark™ microplate reader (Biorad) at a wavelength of 595 nm. To correct variations in transfection efficiency, luciferase values were normalized by β-galactosidase values.

4.5 Immunoblotting

The accumulation of receptors and ABA-inactivating proteins was confirmed by immunoblotting. 5 × Laemmli buffer (250 mM Tris-HCl, pH 8, 10% SDS, 50% glycerol, 0.005% bromophenol blue, 25% β-mercaptoethanol) was added to cells lysed in RIPA buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors, followed by denaturation for 10 min at 95 °C. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes with 0.45 µm pores (Protran, Perkin Elmer), and probed with the following specific antibodies: anti-HA.11 (MMS-101R-B, Babco), anti-myc (PEPcore), anti-V5-HRP (R96125, Invitrogen), anti-β-actin-HRP (sc-47778, Santa Cruz), and anti-mouse IgG secondary antibody conjugated to HRP (31432, Thermo Fisher Scientific). Western Lightning ECL detection system (Perkin Elmer) was used for protein detection according to the manufacturer's instructions.

4.6 Mice

Five-week-old male wild-type (WT) C57BL/6J mice were purchased from Janvier (Le Genest-St-Isle, France). Mice were bred in the specific pathogen free (SPF) facilities of Pasteur Institute, Lille. Mice were housed for 14 days prior to the experiment for the adaptations. Mice were housed under SPF conditions in individually ventilated cages in accordance with the national guidelines and regulations for the care and use of laboratory animals. Animal protocols were approved by the ethical committee of Ghent University (EC2019-024). Seven-week-old wild-type (WT) C57BL/6J mice were fed with AIN-93G diet (Research Diets, Inc., New Brunswick, NJ, USA) with or without 100 mg/kg of (+)-cis, trans-Abscisic acid (A-050, Goldbio) for seven days. For ABA-producing yeast supplementation, ABA-producing *S. cerevisiae* (TABA) cell pellet was supplemented (1.7×10^8 CFU/g) to conventional mouse diet (V1534-300, Ssniff). TABA was a gift from Dr. V. Siewers (Chalmers

University of Technology, Sweden).To determine ABA levels in serum, blood was collected from the heart ventricle and first incubated at room temperature for 30 min to allow the blood clot, followed by centrifugation at 1,700 x g for 10 minutes to separate the serum from the whole blood.

4.7 Extraction of ABA and detection of ABA by LC-MS (Vion IMS QTof Mass Spectrometer analysis)

To determine ABA levels in serum, blood was collected from the heart ventricle and first incubated at room temperature for 30 min to allow the blood clot, followed by centrifugation at 1,700 x g for 10 min to separate the serum from the whole blood. Next, 200 µl of mice serum was added to 800 µL of methanol. The mixture was vortexed and incubated overnight at -70 °C in an ultrafreezer. The mixture was further centrifuged at 10,600 x g for 15 minutes at 4 °C, and the supernatant was evaporated using a SpeedVac. The dried residue was resuspended in 1 mL of a 50:50 water: cyclohexane solution. The phases were separated by centrifugation at 10,600 x g for 5 minutes. Next, 250 µl of 5% trichloroacetic acid was added to the collected aqueous phase and mixed by vortexing. The mixtures were partitioned three times against 500 µL of ethyl acetate, and the organic layer was collected and evaporated using a SpeedVac. The dried residue was resuspended in 50 µl of ultrapure water and filtered using an AcroPrep Advance 96-filter plate 0.2 µm Supor (Pall Life Sciences) prior to chromatographic separation and quantification by LC-MS/MS.

10 µL of the sample was injected on an ACQUITY UPLC BEH C18 (50 × 2.1 mm, 1.7 µm) column from Waters, and the temperature was maintained at 40 °C. A gradient of two buffers was used for separation: buffer A (99:1:0.1 water:acetonitrile:formic acid, pH 3) and buffer B (99:1:0.1 acetonitrile:water:formic acid, pH 3), as follows: 99% A for 0.1 min decreased to 50% A in 5 min, decreased to 30% from 5 to 7 min, and decreased to 0% from 7 to 10 min. The flow rate was set to 0. 5 mL min−1. The LockSpray ion source was operated in negative electrospray ionization mode under the following specific conditions: capillary voltage, 3.5 kV; reference capillary voltage, 2.5 kV; source temperature, 120 °C; desolvation gas temperature, 600 °C; desolvation gas flow, 1000 L h−1; and cone gas flow, 50 L h−1. The collision energy for the full MS scan was set at 6eV. Mass range was set from 200 to 400 Da, and scan time was set at 1s. Nitrogen (greater than 99.5%) was employed as desolvation and cone gas. Leucine-enkephalin (250 pg µL−1 solubilized in water:acetonitrile 1:1 [v/v], with 0.1% formic acid) was used for the lock mass calibration, with scanning every 2 min at a scan time of 0.1 s. For MRM purposes, precursor m/z and product m/z were set both at 263.13, collision energy was set at 6eV, scan time was set on automatic,

and capillary voltage was set at 3.5kV. Profile data was recorded through Unifi Workstation v2.0 (Waters).

4.8 Statistical analysis

Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the appropriate statistical tests (Student's t-test, Mann-Whitney U test, and the Home-Sidak's multiple comparisons test) were performed to determine a significant difference.

4.9 *In silico* docking of ABA to RXRγ

Potential binding of ABA to the RXRγ ligand-binding domain (PDB ID 2GL8) was predicted using the CB-Dock server, which utilizes the AutoDock Vina algorithm [60]. The top ten candidates were selected based on the vina score, which indicates the affinity and stability of interaction, and the cavity size, which represents the size of the interaction interface.

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Supplementary materials

Table S1. Overview of plasmids used in this study.
Plasmids were deposited in the BCCM/ GeneCorner plasmid collection (http://www.genecorner.ugent.be/).

Plasmid name	Source	BCCM/GeneCorner accession number
pSV-ABAactDA (PYL1)	[32]	LMBP 11162 (Addgene plasmid no. 3

		8247)
pSv-ABAactDA-PYL1H87P	This study	LMBP 11274
pSv-ABAactDA-PYL6	This study	LMBP 12456
pSv-ABAactDA-PYL8	This study	LMBP 11503
pET28b-LanCL2	[61]	LMBP 10610
pENTR223-PPARγ	[58]	LMBP ORF81087-A06
pENTR223-RARα	[58]	LMBP ORF81085-F08
pENTR223-RARβ	[58]	LMBP ORF81093-C07
pENTR223-RARγ	[58]	LMBP ORF81123-C12
pENTR223-RXRγ	[58]	LMBP ORF81086-F08
pDNOR201-LANCL2	This study	LMBP 16292
pLenti6-V5-puro	[62]	LMBP 09590
pGal4-VP16	[63]	LMBP 4708
pLenti6-LANCL2-GAL4BD-VP16AD-V5	This study	LMBP 16363
pLent6-GAL4BD-PPARγ-V5	This study	LMBP 16364
pLenti6-GAL4BD-RARα-V5	This study	LMBP 16365
pLenti6-GAL4BD-RARβ-V5	This study	LMBP 16366
pLenti6-GAL4BD-RARγ-V5	This study	LMBP 16367
pCDNA-Myc-RXRγ	This study	LMBP 13345
pU2CH-Luc-Zsgreen1	This study	LMBP 11219
pENTR-L1-20×UAS-L4	[64]	Addgene plasmid no. 32307
pU22CH-Luc-Zsgreen1	This study	LMBP 11226
pACTβgal	[65]	LMBP 4341
pUC19-BamHI-VSV-mAtCYP707A3-HA-P2A-ZsGreen-XhoI	This study	LMBP 9631
pENTR3C-VSV-mAtCYP707A3-P2A-ZsGreen1	This study	LMBP 9660
pCS2-VSV-mAtCYP707A3-HA	This study	LMBP 9909

pUC57-SP-scFv(ABA)-V5-KDEL	This study	LMBP 10803
pENTR3C-SP-scFv(ABA)-V5-KDEL	This study	LMBP 10805
pCS2-SP-scFv(ABA)-V5-KDEL	This study	LMBP 10807
pCS2-nahG-V5	This study	LMBP 10808
pEF6-AtUGT71C5-myc/his	This study	LMBP 9823
pEF6/Myc-HisA	Invitrogen	LMBP 3964

Table S2. Overview of primers and oligonucleotides used in this study.
Bold text indicates restriction enzyme sites induced by PCR.

	Sequence
PYL1-H87P-F	taggccacagatttacaaacccttcatcaaaagctgtaacg
PYL1-H87P-R	cgttacagcttttgatgaagggtttgtaaatctgtggccta
*Asc*I-PYL6-F	cagtg**ggcgcgcc**caccatcagaaacaggttca
PYL6-HA-*BamH*I-R	cgat**ggatcc**tcaagcgtaatctggaacatcgtatgggtacttatcgttacccgccggta
*Asc*I-PYL8-F	cagtg**ggcgcgcc**atggaagctaacgggattga
PYL8-HA-*BamH*I-R	cgat**ggatcc**tcaagcgtaatctggaacatcgtatgggtagactctcgattctgtcgtgt
attB1-LANCL2-F	ggggacaagtttgtacaaaaaagcaggctatgggcgagaccatgtcaaaga
attB2-LANCL2-R	ggggaccactttgtacaagaaagctgggttttgtcatcgtcatccttagtgtcatccctcttcgaagagtcaagttca
GAL4BD-F-clonez	gactctagaggatccactagtatgaagctactgtct
GAL4BD-R-clonez	attccaccacactggatcgatacagtcaactgt
GAL4BD-VP16AD-F-clonez	gcacagtggcggccgttatgaagctactgtcttct
GAL4BD-VP16AD-R-clonez	ccgcgggccctctagattcccaccgtactcgtcaat
*Spe*I-pU2CH-LucZsGreen-F	caca**actagt**tatgtcgaggtggcgtgtacggtgg
*Kpn*I-pU2CH-LucZsGreen-R	gcct**ggtacc**gactacagtactccgctc
*Kpn*I-20xUAS-F	tagtc**ggtacc**gagcaatgctttttataatgcc
*Xba*I-20xUAS-R	acact**ctaga**gccaactttgtatagaaaagttggg
*Kpn*I-AtUGT71C5-F	acat**ggtacc**atgaagacagcagagctcatattcg

SpeI-AtUGT71C5-R	gtaa**actagt**aaagtgatccccaagaatatctttg

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Chapter 4.2. Assessing the robustness of an abscisic acid biosensor

Seo Woo Kim[1, 2, 3, 4], Yasmine Driege[1, 2], Keylla Bicalho[5], Geert Goeminne[5], Alain Goossens[3, 4], Rudi Beyaert[1, 2], Jens Staal[1, 2, 6]

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium [5]VIB Metabolomics Core Ghent, VIB, Ghent, Belgium
[6]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

Unpublished results

Author contributions

SWK, AG, RB, and JS designed the research and interpreted data. SWK, YD, KB, and GG performed experiments and analyzed the data. SWK wrote the paper with the help of AG, RB, and JS. AG, RB, and JS supervised the project and RB and AG provided resources.

1 Abstract

Background
A reporter gene assay (i.e., biosensor) engineered based on the ABA signaling pathway enabled the characterization of ABA in plants. In **Chapter 4.1** we showed that a biosensor employing a complex consisting of Pyrabactin resistance 1-like 8 (PYL8)-abscisic acid (ABA)-ABA insensitive 1 (ABI1) allowed monitoring ABA levels in mammalian cells. We suggested that this biosensor assay can be utilized for studying ABA metabolism, namely future high-throughput screening to discover inflammatory mediators that potentially changes ABA levels. However, several studies described pitfalls in commonly used reporter gene assays, which may lead to wrong conclusions.

Objective
It is important to assess artifacts of a biosensor assay in a critical way to enhance the utility and credibility of the assay. We here describe a technical pitfall that we discovered when using our newly developed ABA-inducible reporter gene assay in HEK293T cells treated with the cytokine interleukin-1 beta (IL-1β). We observed that IL-1β signaling pathway activation results in increased reporter gene expression.

Methods
The following complementary methods were applied to verify reporter results: (1) metabolomics and (2) ABA-inactivation strategies (anti-ABA scFv).

Key Results & Conclusions
Both metabolomics and ABA-inactivation strategy using anti-ABA scFv pointed that the increased luciferase activation upon IL-1β stimulation is ABA-independent. We observed that IL-1β stimulation increases the accumulation of ABA receptor fusion proteins. Additional experiments with controls (e.g., PYL1(H87P)-based biosensor proteins) suggested that IL-1β signaling might influence the stability of PYL, which boosts the stability of PYL-ABI1, thereby increasing ABA-independent luciferase activation. To circumvent such artifacts, we suggest the following additional approaches in future experiments to screen inflammation- and stress-related signaling pathways that might alter mammalian ABA metabolism: (1) confirmation via metabolomics and (2) use of ABA-inactivation strategies (AtCYP707A3, anti-ABA scFv, and AtUGT71C5).

2 Introduction

Chapter 4.1 showed that a reporter gene assay (i.e., biosensor) employing a ternary complex, composed of Pyrabactin resistance 1-like 8 (PYL8)-abscisic acid (ABA)-ABA Insensitive 1 (ABI1), allowed the quantification of ABA at low nM concentration in HEK293T cells. In addition, we verified ABA inactivation strategies, which confirm the selectivity of the biosensor for ABA. This biosensor assay can be used as a high-throughput screening method for inflammatory mediators that might alter ABA levels, helping to improve our knowledge of ABA metabolism. Noteworthy, previous studies described artifacts in several commonly used reporter gene assays, which may lead to incorrect conclusions [1–3]. Thus, it is essential to recognize potential artifacts of a biosensor assay to avoid wrong conclusions and enhance the utility and credibility of the assay. In this context, we here exemplify a technical concern that we identified when using our newly developed ABA-inducible reporter gene assay in cells treated with the cytokine interleukin-1 beta (IL-1β).

IL-1β is a pro-inflammatory cytokine that mediates host defense in response to infection and injury [4]. IL-1β signaling initiates from the recognition of IL-1β by its cognate receptor IL-1RI [4]. This allows the recruitment of the adaptor protein myeloid differentiation primary response protein 88 (Myd88) [5], resulting in the recruitment and activation of a series of signaling proteins including mitogen-activated protein kinases and ultimately leading to the activation of the transcription factor NF-κB [6]. Targeting IL-1β or NF-κB signaling pathways showed to be effective in resolving chronic and acute inflammation, autoimmune diseases, and metabolic diseases [6–8]. Considering the anti-inflammatory role of ABA in a broad range of inflammation diseases [9–17], we wanted to analyze whether mammalian cells alter their ABA biosynthesis or catabolism in response to IL-1β-mediated inflammation. However, we observed that IL-1β signaling pathway activation in HEK293T cells leads to ABA-independent reporter gene expression. This chapter describes our experiments in detail and suggests additional experimental set-ups and approaches to circumvent such artifacts.

3 Results and discussion

3.1 IL-1β signaling results in ABA-independent reporter gene activity

To investigate whether IL-1β signal transduction regulates ABA metabolism, we first treated HEK293T cells with IL-1β or stimulated the IL-1β pathway by overexpressing Myd88, and measured ABA-inducible luciferase activity using a PYL8-based ABA biosensor, pSv-ABAactDA (PYL8). IL-1β stimulation or *Myd88* overexpression resulted in increased luciferase activation (**Figure 1A and 1B**), indicating that IL-1β-mediated signaling might influence ABA synthesis or catabolism in HEK293T cells. To verify the increase of ABA levels in IL-1β-stimulated or Myd88-overexpressing cells, we then wanted to confirm the increase of ABA levels in IL-1β-stimulated or Myd88-overexpressing cells via mass spectrometry (MS). First, we extrapolated the intracellular ABA concentration in IL-1β-stimulated cells by generating an ABA standard calibration curve ranging from 2 nM to 100 nM ABA. This was however a rough assumption as an actual level will be lower than the extrapolated value given an ABA-independent dimerization of PYL8 and protein phosphatases 2C (PP2C) [18]. This way, we anticipated that ABA levels in IL-1β-stimulated cells might be approximately < 43.9 nM (**Figure 1C**). Taking into account the detection limit (3 pg) of the VION IMS Q-TOF mass spectrometer (will be shown in **Chapter 4.3**) and the ABA-independent PYL8-ABI1 formation, we analyzed 1.2×10^6 cells for ABA via MS, which exceeds the minimum required number of cells (10,000 ×). However, we could not detect the peak corresponding to ABA in IL-1β-stimulated or Myd88-overexpressing HEK293T cells (**Figure 1D and 1E**). It is possible that other components of HEK293T cell extracts derive the matrix effect, which hindered the detection of ABA. For instance, co-eluting metabolites might have changed the ionization efficiency of ABA analysis [19]. Alternatively, the ABA signal detected in the biosensor assay reflects an artifact.

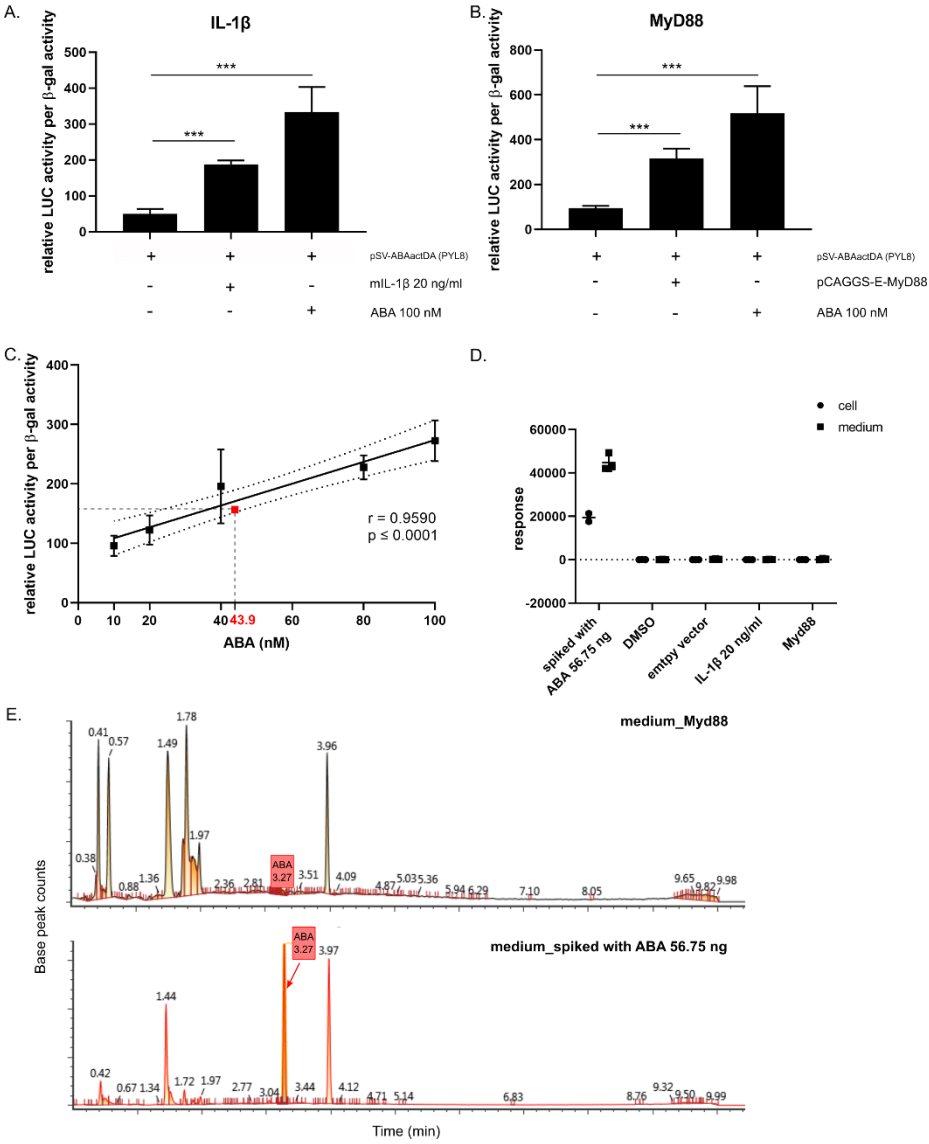

Figure 1. Determination of ABA levels in IL-1β-stimulated or Myd88-overexpressing HEK293T cells (see following page for figure legend)**.**

Figure 1. Determination of ABA levels in IL-1β-stimulated or Myd88-overexpressing HEK293T cells (see previous page for figure)**.**
(A) ABA-inducible luciferase activity in HEK293T cells stimulated with IL-1β (20 ng/mL). HEK293T cells were transfected with the PYL8-based biosensor construct pSv-ABAactDA (PYL8), pU22ch-Luc-Zsgreen1, and pACTβgal. Six hours post-transfection, cells were incubated in the absence or presence of 100 nM ABA or 20 ng/mL IL-1β. **(B)** ABA-dependent luciferase activity in HEK293T cells overexpressing Myd88. HEK293T cells were transfected with the indicated construct along with PYL8-based biosensor construct pSv-ABAactDA (PYL8) along with pU22ch-Luc-Zsgreen1 and pACTβgal. Six hours post-transfection, cells were incubated in the absence or presence of 100 nM ABA. **(C)** Determination of ABA levels in HEK293T cells stimulated with IL-1β (20 ng/mL). An ABA standard calibration curve was generated with HEK293T cells treated with ABA ranging from 0 nM to 100 nM. HEK293T cells were transfected with the PYL8-based biosensor construct pSv-ABAactDA (PYL8) along with pU22ch-Luc-Zsgreen1 and pACTβgal. Six hours post-transfection, cells were incubated with ABA at the indicated concentration. Relative luciferase activity is shown as fold induction relative to cells only transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. **(D)** Response to ABA (peak area at *m/z* 263.1290) in medium extracts and cell extracts of HEK293T cells stimulated with IL-1β or HEK293T cells overexpressing Myd88. HEK293T cells treated with DMSO and HEK293T cells overexpressing empty vector were negative controls. HEK293T cells treated with 56.75 ng ABA positive controls. **(E)** Representative TIC VION chromatograms of medium extracts spiked with 56.75 ng ABA and medium extracts from HEK293T cells overexpressing Myd88. Statistical significance was calculated using a Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001). R-value and P-value in (C) were calculated using simple linear regression analysis. Error bars represent standard deviation. Results shown are the means of at least three biological replicates and the representative of three independent experiments (A, B) or one experiment (C, D, and E).

3.2 Co-expression of *anti-ABA scFv* does not lower luciferase enzyme activity in IL-1β-stimulated or Myd88-overexpressing HEK293T cells

To further investigate if luciferase activation in our biosensor assay is ABA-dependent or not, we analyzed the effect of endoplasmic reticulum (ER)-targeted anti-ABA single-chain Fv (scFv). As described in **Chapter 4.1**, co-expression of *anti-ABA scFv* reduces luciferase gene expression in ABA-treated cells (**Figure 2**). We therefore co-expressed anti-ABA scFv along with a PYL8-based ABA biosensor, pSvABAactDA (PYL8), and measured IL-1β- or Myd88- induced luciferase activation. However, anti-ABA scFv did not decrease IL-1β- or Myd88- induced luciferase activation (**Figure 2**), indicating that IL-1β signaling led to luciferase reporter gene activation in an ABA-independent manner.

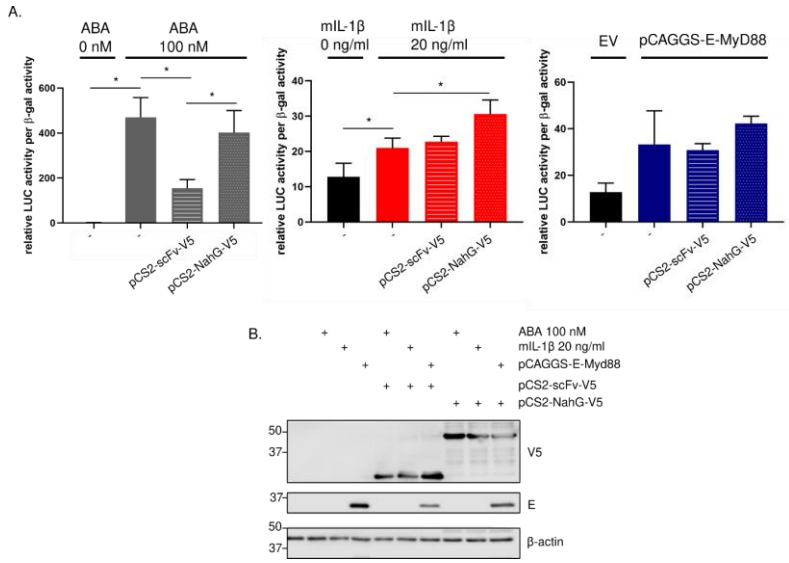

Figure 2. IL-1β- or Myd88-induced ABA-dependent luciferase activation is not decreased upon ABA depletion by overexpression of *anti-ABA scFv.* **(A)** HEK293T cells were transfected with the indicated construct along with pSv-ABAactDA (PYL8), pU22CH-Luc-Zsgreen1 and pACTβgal, and incubated in the presence or absence of 100 nM ABA or 20 ng/mL IL-1β. Fold induction of ABA-induced luciferase gene expression in cells expressing the pSv-ABAactDA (PYL8) is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. Error bars represent standard deviation. Statistical significance was calculated using a Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001). **(B)** Western blot analysis of protein accumulations of V5-tagged scFv and NahG and E-tagged Myd88. The results shown are the means of three biological replicates and represent two independent experiments.

3.3 IL-1β stimulation increases the accumulation of ABA receptor fusion proteins

MS analysis and the ABA inactivation strategy using anti-ABA scFv revealed that the increased luciferase activation upon IL-1β stimulation is ABA-independent. Given that the luciferase activation is driven by the PYL/ABI1 interaction, recruiting the VP16 activation domain (VP16AD) to the GAL4-dependent promoter, we hypothesized that IL-1β stimulation may directly perturb three elements during this process: (1) ABA receptor fusion proteins expressed from pSV-ABAactDA or (2) the luciferase reporter proteins expressed from pU22ch-Luc-Zsgreen1 or (3) PYL8-ABI1 complex. We therefore compared the abundance of VP16AD-PYL1-HA and VP16AD-PYL8-HA fusion proteins in IL-1β-stimulated cells to those in untreated cells. Here, it should be mentioned

that the fusion protein GAL4BD-ABI1-Flag was not detectable by anti-Flag immunoblotting, possibly due to the lower efficiency of IRES-dependent expression of the second cistron compared to the cap-dependent first cistron expression (VP16AD-PYL-HA) in a bicistronic vector [20]. Therefore, we determined the abundance of VP16AD-PYL-HA as being representative for the expression of genes coding for ABA receptor fusion proteins, which are under the control of the same SV40 promoter. Immunoblotting with anti-HA revealed that the abundance of VP16AD-PYL1-HA and VP16AD-PYL8-HA fusion proteins is enhanced upon IL-1β stimulation (**Figure 3A**). Furthermore, we observed a correlation between luciferase activity and the amount of transfected pSV-ABAactDA (**Figure 3B**), demonstrating that the increased luciferase activity upon IL-1β stimulation may be a consequence of the enhanced abundance of the ABA receptor fusion proteins.

IL-1β stimulation might have increased the abundance of VP16AD-PYL-HA via the following mechanisms. First, at the transcription level, the strength of the SV40 promoter element or ribosomal binding site might have been affected. Second, VP16AD-PYL-HA might have been stabilized. To challenge these mechanisms, we cloned the ABA receptor in a different backbone vector, pLenti6-ABAactDA (PYL8), bearing a CMV promoter. We stimulated with IL-1β or co-expressed Myd88 in cells expressing pLenti6-ABAactDA (PYL8), determined the protein abundance of VP16AD-PYL-HA, and measured luciferase activation. Similar to our experiments with the other expression construct, protein levels of VP16AD-PYL8-HA increased upon IL-1β stimulation or *Myd88* overexpression (**Figure 3C**). However, in a confounding manner, the luciferase activity did not increase (**Figure 3C**). This confounding result might be due to the competition between the CMV promoter in the pLenti6 vector and the minimal CMV promoter (minCMV) in pU22ch-Luc-Zsgreen1 in tethering identical intracellular proteins that form a transcription pre-initiation complex [21]. Furthermore, it is noteworthy that the CMV promoter was previously described to be affected by IL-1β signaling [22,23], TNF-α-induced NF-κB activation [22], or the overexpression of *MAP/ERK kinase kinase 1* (*Mekk1*) and JNK-Jun/Fos signaling [23]. Deletion analysis of the CMV promoter revealed that a cyclic AMP response element (CRE) site mediates Mekk1-induced CMV promoter activation [23]. A CRE site, TGACGTCA, was found to be similar to the DNA binding site of transcription factor Activated Protein-1 (TGA G/C TCA), which is activated by downstream targets of activated Mekk1 [23,24]. This suggests that the increase in CMV-driven accumulation of VP161AD-PYL8-HA upon IL-1β stimulation might also reflect CRE site activation (**Figure 3C**). In conclusion, the possible competition between the CMV promoter in the pLenti6 and the minCMV

in pU22ch-Luc-Zsgreen1 and the effect of IL-1β signaling on the CMV promoter implicate that future studies on ABA-dependent reporter gene activation by IL-1β and other inflammatory stimuli should use a distinct expression vector bearing a different type of promoter than the CMV promoter.

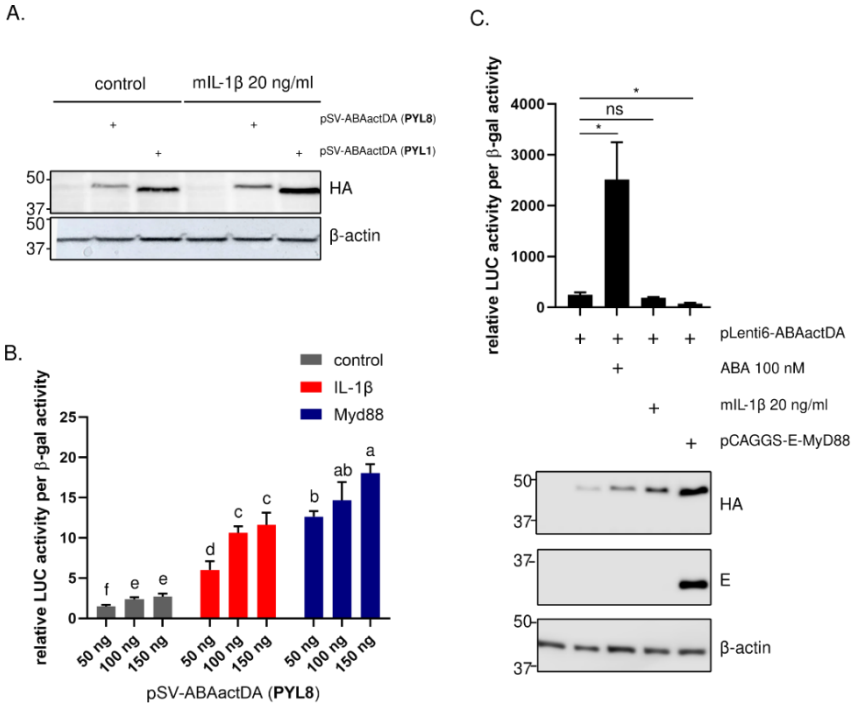

Figure 3. The activation of IL-1β signaling increases the accumulation of VP16AD-PYL8-HA (see following page for figure legend)**.**

Figure 3. The activation of IL-1β signaling increases the accumulation of VP16AD-PYL8-HA (see previous page for figure)**.**
(A) HEK293T cells were transfected with the indicated construct along with pU22ch-Luc-Zsgreen1 and pACTβgal. Six hours post-transfection cells were incubated in the absence or presence of 20 ng/mL IL-1β. A protein level of HA-tagged VP16AD-PYL was verified by western blot analysis. **(B)** HEK293T cells were transfected with the indicated constructs pSv-ABAactDA (PYL8), pU22ch-Luc-Zsgreen1, pACTβgal along with or without pCAGGS-E-Myd88. Six hours post-transfection cells were incubated in the absence or presence of 20 ng/mL IL-1β. **(C)** HEK293T cells were transfected with the indicated constructs along with pU22ch-Luc-Zsgreen1 and pACTβgal. Six hours post-transfection cells were incubated in the absence or presence of 20 ng/mL IL-1β or 100 nM ABA. A protein level of HA-tagged VP16AD-PYL and E-tagged Myd88 were verified by western blot analysis. β-actin was used as a western blot loading control. Fold induction of ABA-induced luciferase gene expression in cells expressing the pSv-ABAactDA (PYL8) is compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. The results shown are the means of three biological replicates and represent two independent experiments. Error bars represent standard deviation. Statistical significance was calculated using a Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001).

3.4 The PYL-ABI1 complex is indispensable in IL-1β-induced luciferase activation

As mentioned above, another possible reason for the ABA-independent luciferase activation in response to IL-1β is a direct effect of IL-1β signaling on luciferase gene expression. In this context, we first analyzed if the PYL-ABA-ABI1 complex is indispensable in IL-1β-induced luciferase activity (**Figure 4**). Therefore, we examined luciferase activity upon IL-1β stimulation or *Myd88* overexpression in ABA receptor-deficient cells transfected with empty vector and cells that express receptor fusion protein exhibiting lower ABA binding affinity (PYL1 or PYL1H87P). In the absence of ABA, IL-1β signaling did not induce luciferase gene expression in cells not expressing receptor fusion proteins (**Figure 4**). Likewise, IL-1β signaling did not drive luciferase gene expression in cells expressing PYL1- or PYL1H87P-based biosensor fusion proteins (**Figure 4**). On the contrary, in the presence of ABA, luciferase gene expression increased upon IL-1β stimulation in cells expressing PYL1- or PYL1H87P-based biosensor proteins (**Figure 4**). This suggests that only after the transcription begins, IL-1β signaling might upregulate the transcription rate of the luciferase gene.

One possible mechanism underlying the boosted transcription rate is that IL-1β signaling directly upregulates the transactivation of GAL4VP16 in a PYL-ABA-ABI1-independent manner. In this context, we

further analyzed whether IL-1β stimulation upregulates GAL4VP16-transactivated luciferase gene expression in the absence of a PYL-ABA-ABI1 ternary complex. Therefore, we transfected HEK293T cells with a plasmid encoding the fusion protein GAL4VP16 (pSV40-GAL4VP16) in the presence or absence of IL-1β. As expected, expression of *GAL4VP16* induced luciferase gene expression, but this was not boosted upon IL-1β stimulation (**Figure 4**). This suggests that IL-1β signaling does not upregulate the transactivation capacity of GAL4VP16 but instead affects a PYL-ABA-ABI1 ternary complex.

The above findings may also answer the question we brought up in section 3.2: Does IL-1β stimulation enhances the expression level of the VP16AD-PYL gene by positively regulating the strength of the SV40 promoter? Indeed, while genes encoding both VP16AD-PYL and GAL4VP16 are controlled by the same SV40 promoter, IL-1β stimulation increased accumulation of VP16AD-PYL but not GAL4VP16-transactivated luciferase activity, excluding an effect of IL-1β signaling on SV40 promoter strength. This points to the fact that IL-1β signaling might positively regulate the stability of PYL.

The third hypothesis for the ABA-independent luciferase activation is an effect of IL-1β signaling on the PYL8-ABI1 complex. As mentioned in **Chapter 4.1**, a yeast two-hybrid assay showed that the interaction of PYL8 and another PP2C member Hypersensitive to ABA 1 (HAB1) occurred in the absence of ABA [18], suggesting the spontaneous formation of PYL8-ABI1 complex. It is thus possible that the formation or stability of spontaneous PYL8-ABI1 complex was affected by IL-1β signaling, resulting in increased luciferase activation. Interestingly, on the contrary, PYL1 does not interact with HAB1 in the absence of ABA [25], indicating that PYL1-ABI1 formation is ABA-dependent. In our observation, the increase of luciferase activity in cells expressing PYL1-based biosensor proteins was ABA-dependent (**Figure 4**), suggesting that IL-1β signaling does not induce the formation of PYL and ABI1 but might affect stability of PYL-ABI1 complex (PYL8-ABI1 or PYL1-ABA-ABI1). Collectively, IL-1β signaling might influence the stability of PYL, which boosts the stability of PYL-ABI1, thereby increasing ABA-independent luciferase activation.

The stability of proteins and protein-protein interactions can be regulated by post-translational modifications [26,27]. It would be interesting to determine if IL-1β stimulation has an impact on the receptor proteins' stability, thereby increasing the strength of a PYL-ABI1 complex interaction. Future studies using another chemical modulator of PYL, agrochemical Mandipropamid, might help to clarify this [28]. PYR1 mutant, called PYR1^{MANDI}, of which the ABA binding

pocket was altered, showed nanomolar sensitivity to Mandipropamid *in vitro* and *in vivo* but did not show responsiveness to ABA [29,30]. As PYR1 does not interact with HAB1 in the absence of ABA [25], employing PYR^{MANDI} and ABI1 in the GAL4VP16/UAS system might reveal whether IL-1β stimulation stabilizes ABA-independent PYL-ABI1 complex formation.

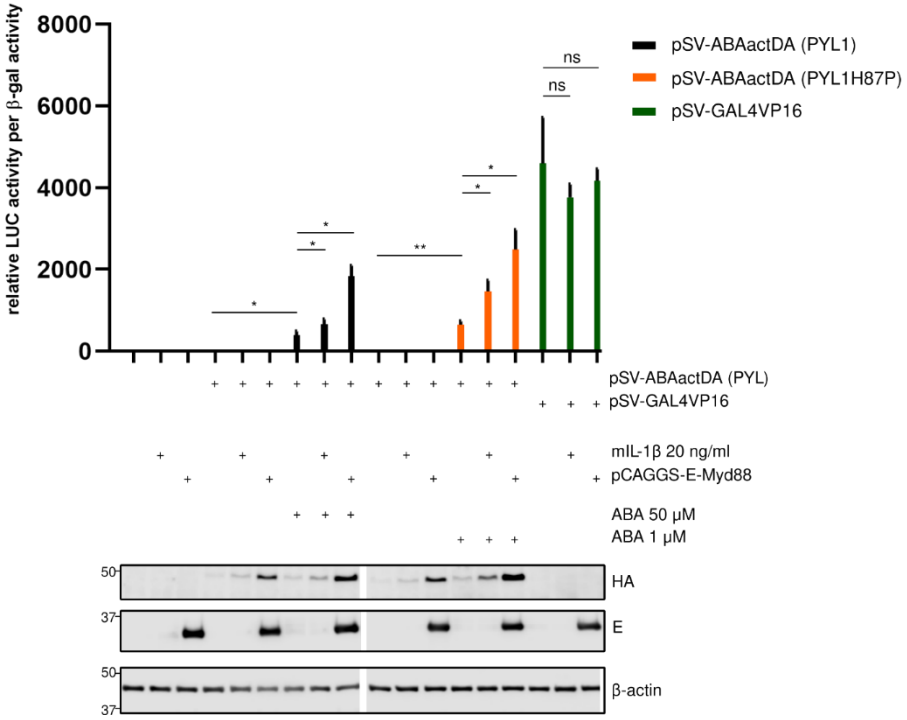

Figure 4. The PYL-ABA-ABI1 complex is dispensable in IL-1β- or Myd88-induced ABA-dependent gene expression.
Determination of ABA-dependent luciferase activity in HEK293T cells stimulated with IL-1β or HEK293T cells overexpressing Myd88, using PYL1 or PYL1H87P-based biosensors. HEK293T cells were transfected with the indicated construct along with an emtpy vector or the ABA biosensor construct Sv-ABAactDA (PYL1 or PYL1H87P) **or** pGAL4VP16, pU22ch-Luc-Zsgreen1, and pACTβgal. Six hours post-transfection cells were incubated in the absence or presence of 20 ng/mL IL-1β or ABA at the indicated concentration. A protein level of HA-tagged VP16AD-PYL and E-tagged Myd88 were verified by western blot analysis. β-actin was used as a western blot loading control. Fold induction of ABA-induced luciferase gene expression in cells expressing the PYL receptor-based biosensor constructs was compared to the average background level of cells transfected with the empty vector. Luciferase values were normalized by β-galactosidase values. Error bars represent standard deviation. The results shown represent two independent experiments (A, B). Statistical significance was calculated using a Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001).

4 Conclusions

The present study illustrates our ABA biosensor system, as exemplified with an artifact that occurred while screening for inflammatory mediators that alter ABA metabolism. Our observation illustrate a risk of misinterpretation of ABA-inducible reporter gene assay results. Therefore, as described in this thesis, we suggest the following additional approaches and controls to improve the credibility and reliability of future experiments to screen inflammation- and stress-related signaling pathways that might alter mammalian ABA metabolism: (1) confirmation via metabolomics and (2) use of ABA-inactivation strategies (AtCYP707A3, anti-ABA scFv, and AtUGT71C5).

5 Materials and methods

5.1 Cell culture and transfection

HEK293T cells were cultured and transfected as described in **Chapter 4.1**, section 4.3. We additionally used plasmids pCAGGS-E-Myd88, pGAL4VP16, pLenti6-VP16AD-PYL8-HA-IRES-GAL4BD-ABI1-Flag, and pIgKconaLUC (LMBP 3249), which contains an NF-κB–driven luciferase reporter gene. pCAGGS-E-Myd88(LMBP 6654) and pGAL4VP16(LMBP 4708) are available at the GeneCorner plasmid collection (https://genecorner.ugent.be) along with the detailed sequence and cloning information. pLenti6-VP16AD-PYL8-HA-IRES-GAL4BD-ABI1-Flag was a gift from Femke Van Gaever (VIB-UGent Center for Inflammation Research). pNFconluc was a gift from Dr. Alain Israel (Institut Pasteur, Paris, France). Six hours after the transfection, the medium was refreshed with or without mIL-1β or ABA as described in **Chapter 4.1**. mIL-1β (1 mg/mL) was a gift from the VIB protein service facility.

5.2 Luciferase reporter assay

Methods are described in **Chapter 4.1**, section 4.4.

5.3 Extraction of ABA and detection of ABA by LC-MS (Vion IMS QTof Mass Spectrometer analysis)

For mass spectrometry, HEK293T cells were cultured in Opti-MEM™ (11058021, Thermo Fisher). HEK 293T cells were seeded at a density of 1.2×10^6 cells in 100mm plates and transfected with a total of 10 μg of plasmid per plate using a calcium phosphate method or stimulated with 20 ng/mL mIL-1β or treated with 56.75 ng of ABA. After 16 hours, medium and cells were subjected to metabolite extraction. To ensure transfection, NF-κB activation upon IL-1β stimulation, ABA treatment in HEK293T cells cultured in reduced serum media Opti-MEM™, the ABA-inducible luciferase assay, and NF-κB luciferase assay were performed (**Figure S1**). For medium extraction, 45 mL of 80 % of cold methanol was added to 5 mL of medium, and the mixture was incubated at -70 °C in an ultra freezer overnight. For cell extraction, cells were scraped using a scraper in 2 mL of 80 % of cold methanol. Scraped cells in 80 % methanol were homogenized using Precellys 24 with 2.8mm Zirconium oxide beads (Bertin technologies, Rockville, US) and incubated at -70 °C in an ultra freezer overnight. The incubated medium and cell extracts were further centrifuged at 10,600 x g for 10 min at 4 °C to remove debris, and the supernatant was evaporated using a SpeedVac. Next, nonpolar compounds were removed by cyclohexane/water

partition. 2 mL of a 50:50 water: cyclohexane solution was added to the dried residue, and the aqueous phase was collected. The collected phase was next dried, and the dried residue was resuspended in 50 µL of ultrapure water and filtered using an AcroPrep Advance 96-filter plate 0.2 µm Supor (Pall Life Sciences) prior to chromatographic separation and quantification by VION. VION analysis was performed as described in **Chapter 4.1**, section 4.7

5.4 Immunoblotting

Methods are described in **Chapter 4.1**, section 4.5. We additionally used antibodies anti-E (ab3397, Abcam) and anti-rabbit IgG secondary antibody conjugated to HRP (31464, Thermo Fisher Scientific).

5.5 Statistical analysis

Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the appropriate statistical test (Student's t-test) was performed to determine a significant difference.

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Supplementary materials

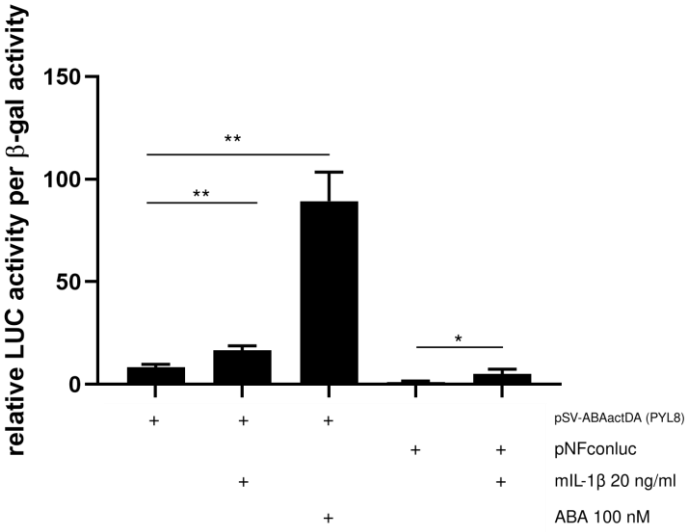

Figure S1. NF-κB activation of HEK293T cells stimulated with IL-1β. HEK293T cells were cultured in Opti-MEM™, transiently transfected with the indicated plasmids, and incubated in the absence or presence of 100 nM ABA or 20 ng/mL IL-1β.

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Chapter 4.3. Exploration of transgenic mice overexpressing *Arabidopsis CYP707A3* and *ABH2*

Seo Woo Kim[1, 2, 3, 4], Jacob Pollier [3,4], Yasmine Driege[1, 2], Keylla Bicalho[5], Geert Goeminne[5], Alain Goossens[3, 4], Rudi Beyaert[1, 2], Jens Staal[1, 2, 6]

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[5]VIB Metabolomics Core Ghent, VIB, Ghent, Belgium
[6]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

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Author contributions

SWK, AG, RB, and JS designed the research and interpreted data. SWK, YD, KB, and GG performed experiments and analyzed the data. SWK wrote the paper with the help of AG, RB, and JS. AG, RB, and JS supervised the project and RB and AG provided resources.

1 Abstract

Background
In recent years, the anti-inflammatory profile of ABA treatment in animal models and humans has attracted wide attention. Although these previous studies point to the potential involvement of endogenous ABA in immune homeostasis, the physiological relevance of endogenous ABA remains largely unknown. A bottleneck in unraveling the role of endogenous ABA is its unknown biosynthesis pathway and poorly understood physiological and molecular mechanisms underlying its function. In **Chapter 4.1**, we showed that the overexpression of *Arabidopsis CYP707A3* results in decreased ABA levels in HEK293T cells, suggesting that depletion of ABA in animals using this ABA inactivation pathway could be a strategy.

Objective
This chapter aims to reveal the immunoregulatory role of endogenous ABA. We ask whether the depletion of ABA could alter immune responses or initiate or exacerbate the development of inflammatory diseases.

Methods
We generated transgenic mice overexpressing *Arabidopsis CYP707A3*, which encodes an enzyme converting ABA to phaseic acid (PA). Given the ABA-like biological activities of PA shown in animals, we also co-expressed *Arabidopsis* phaseic acid reductase *ABH2* encoding an enzyme that converts PA to dihydrophaseic acid (DHPA). We characterized the immunological change of transgenic mice overexpressing *Arabidopsis CYP707A3* and *ABH2* (hereafter called *CYP-ABH2*) by assessing the percentage of splenic T cells, B cells, natural killer cells, and dendritic cells in using flow cytometry analysis. To analyze its ABA-degrading activity, we profiled ABA in mice tissues (brain and brown adipose tissues) using mass spectrometry (MS)-based metabolomics.

Key Results & Conclusions
Due to technical challenges in detecting ABA in mice tissues by MS, we could not verify the conversion of ABA to DHPA in transgenic *CYP-ABH2* mice. The challenge to detect ABA might mainly attribute to the low level of ABA. Also, transgenic *CYP-ABH2* mice show a normal splenic immune cell subsets population. Possibly, ABA depletion only has an effect under inflammatory conditions associated with the development of metabolic disorders such as colitis and type 2 diabetes in mice. However, ABA depletion in *CYP-ABH2* mice will first need to be confirmed before challenging them with disease models. Therefore,

future development of a reliable and stable quantification method for ABA in mice remains necessary.

2 Introduction

ABA administration in humans or animal models showed beneficial effects of ABA against metabolic syndrome and its comorbid diseases [1–11]. These previous observations reflect the potential contribution of endogenous ABA in immune homeostasis and the pathogenesis of metabolic syndrome. However, not much is known about the physiological functions of endogenous ABA. One obstacle that makes it challenging to reveal them is its unknown biosynthetic pathway. Consequently, biochemical or genetic approaches that can alter ABA levels in animal cells could not be established. We suggest that exploiting ABA inactivation pathways in other organisms like plants could be an alternative strategy.

In *Arabidopsis*, one catabolic pathway to inactivate ABA is through oxidation triggered by the ABA 8'-hydroxylases, which convert ABA to 8'OH-ABA. 8'OH-ABA is then spontaneously isomerized to phaseic acid (PA). ABA 8'-hydroxylases are encoded by *CYP707As*. Overexpression and knockout studies revealed that AtCYP707A3 is responsible for the ABA catabolism in vegetative tissues of *Arabidopsis* [12]. Furthermore, in line with the enzymatic activity of AtCYP707A3 shown in insect cells [13], in **Chapter 4.1** we reported that the overexpression of *Arabidopsis CYP707A3* results in decreased ABA levels in HEK293T cells. This indicates that the heterologous expression of *CYP707A3* in animals might be a useful strategy to determine whether ABA depletion could alter immune responses or initiate or exacerbate the development of diseases relevant to metabolic syndrome. We thus generated and immunologically characterized transgenic mice overexpressing *AtCYP707A3*. Additionally, we also overexpressed *Arabidopsis* phaseic acid reductase ABH2 to convert the AtCYP707A3 ABA degradation product phaseic acid (PA) to dihydrophaseic acid (DHPA) because PA was shown before to have ABA-like biological activities in mice brains [14].

3 Results and discussion

3.1 Transgenic *CYP-ABH2* mice are viable and healthy

To study the functional role of ABA, we generated transgenic mice that overexpress transgenes *AtCYP707A3* and *AtABH2* linked by P2A (hereafter called *CYP-ABH2*), using the Piggybac transposon system. First, the expression of *CYP-ABH2* was inactivated by positioning a lox-stop-lox (LSL) sequence upstream of the coding sequences. This was to maintain the mouse line in the event of a lethal phenotype caused by ABA depletion (**Figure 1A**). To excise the polyA stop cassette thereafter, we crossed *LSL-CYP-ABH2*$^{+/-}$ mice with Sox2Cre deleter, which express Cre recombinase under the control of the mouse *Sox2* (SRY-box containing gene 2) promoter. Sox2Cre mice provide epiblast-specific Cre-mediated LoxP recombination, thereby activating the transgene expression in the embryo. This resulted in *Sox2Cre*$^{+/-}$;*CYP-ABH2*$^{+/-}$ mice that express *CYP-ABH2* under the CAG promoter (**Figure 1B**). *Sox2Cre*$^{+/-}$;*CYP-ABH2*$^{+/-}$ mice are viable and do not show a lethal phenotype from birth up to 15 weeks old; therefore, we crossed *Sox2Cre*$^{+/-}$;*CYP-ABH2*$^{+/-}$ mice with WT mice, allowing Cre-independent *CYP-ABH2* expression in mice. This generated heterozygous transgenic mice that express *CYP-ABH2* in the absence of Cre (*Sox2Cre*$^{-/-}$;*CYP-ABH2*$^{+/-}$, hereafter called *CYP-ABH2* mice). *CYP-ABH2* mice were healthy and did not show any phenotype under basal conditions. Protein analysis of the spleen confirmed the expression of *AtCYP707A3* and *ABH2* in *CYP-ABH2* mice (**Figure 1C**). We detected a HA-immunoreactive band with a size around 50 kDa corresponding to the predicted size (52 kDa) of AtCYP707A3, a V5-immunoreactive band with a size around 37 kDa corresponding to the predicted size (39 kDa) of ABH2, and a V5-immunoreactive band with a size around 100 kDa corresponding to the predicted size (94 kDa) of AtCYP707A3-P2A-AtABH2, which were all absent in wild-type.

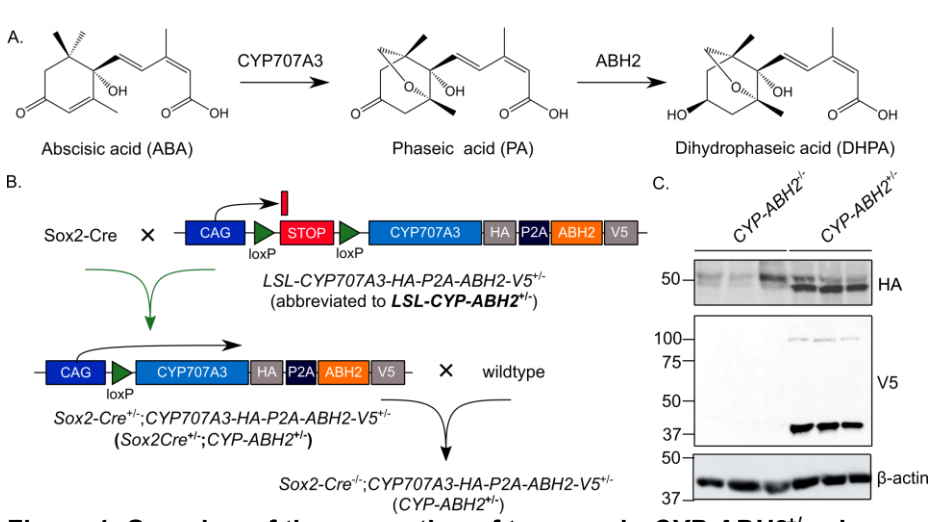


Figure 1. Overview of the generation of transgenic *CYP-ABH2*$^{+/-}$ mice. **(A)** Outline of the catabolism of abscisic acid (ABA) and phaseic acid (PA) by AtCYP707A3 and AtABH2. **(B)** Mating scheme to generate transgenic mice expressing *CYP707A3* and *ABH2*. Transgenic mice expressing *lox-stop-lox (LSL)-CYP707A3-P2A-ABH2* (abbreviated to *LSL-CYP-ABH2*$^{+/-}$) were bred with female Sox2-Cre mice. The resulting progeny, *Sox2Cre*$^{+/-}$; *CYP-ABH2*$^{+/-}$ was bred with wild-type mice. This resulted in *Sox2-Cre*$^{-/-}$;*CYP707A3-P2A-ABH2*$^{+/-}$ (abbreviated to *CYP-ABH2*$^{+/-}$). **(C)** Representative Western blot showing AtCYP707A3-HA, AtABH2-V5, and AtCYP707A3-HA-P2A-AtABH2-V5 protein levels in spleen lysates of wild-type *CYP-ABH2*$^{-/-}$ and *CYP-ABH2*$^{+/-}$ mice.

3.2 Transgenic *CYP-ABH2* mice show a normal population of splenic immune cell subsets

Even though *CYP-ABH2* mice did not show overt differences or spontaneously developed diseases, previous studies showing a potential role of ABA in the complex interplay between host immune regulation and metabolism [4,5,11] prompted us to study whether the expression of *CYP-ABH2* alters the immunophenotype of 10-14 weeks old mice. The lineage, maturation, and activation status of different immune cells were examined through flow cytometry by staining cells with fluorescently labeled antibodies specifically binding to different cell surface molecules and intracellular molecules (**Chapter 3.2 Figure 1**).

3.2.1 T cells

Previous findings showed that alteration in T cell subsets contributes to the alleviating effect of ABA in experimental atherosclerosis and colitis in mice [4,5,11]. Dietary ABA supplementation was shown to inhibit the infiltration of helper T cells ($CD4^+$) in the aortic root of atherosclerosis-

prone apolipoprotein E-deficient (Apoe$^{-/-}$) mice [11]. Also, ABA administration enhanced the frequencies of cytotoxic T-lymphocyte–associated antigen 4 (CTLA4)-expressing helper T cells (CD4^{+}CTLA4^{+}) in blood and helper T cells (CD4^{+}) in the mesenteric lymph node in mice with experimental colitis [4,5]. Therefore, we studied whether the expression of *CYP-ABH2* affects the frequency of T cell subsets (**Figure 2A**). The percentage of helper T cells (CD4^{+}) and cytotoxic T cells (CD8^{+}) in the spleen were comparable in WT and *CYP-ABH2* mice. Also, the frequencies of activated T cells (CD44^{+}CD4^{+} or CD44^{+}CD8^{+}) and Treg cells (CD4^{+}CD25^{+}FoxP3^{+}) were similar. We also found comparable frequencies of CTLA4- and tumor necrosis factor receptor 2 (TNFR2)-producing activated T cells and regulatory T cells (Tregs), which are cell surface receptors. CTLA4 is a negative regulator of T cell immune function, suppressing activation of autoreactive T cells [15]. TNFR2 is a receptor of cytokine TNF that regulates the suppressive activity of Tregs [16]. Finally, the frequencies of naïve, effector memory (EM), and central memory (CM) T cells populations were also comparable (**Figure 2B**).

Moreover, we determined whether *CYP-ABH2* affects cytokine production by T cells (**Figure 2C**). Splenocytes from WT and *CYP-ABH2* mice were stimulated with PMA/Ionomycin/Brefeldin A, which efficiently induced TNF, IL-2, and IFNγ cytokine production in activated CD4 and CD8 T cells. However, we did not observe any effect of expression of *CYP-ABH2* on the percentage of cytokine- (i.e., TNF, IL-2, IFNγ, IL-4, IL-7, IL-10, and granzyme B) producing cells.

Here our findings show that the expression of *CYP-ABH2* does not drive any abnormalities in the immunophenotype of splenic T cells in mice under basal conditions. This implies that basal ABA levels might not participate in regulating T cell activation and differentiation in healthy mice. However, ABA might still coordinate T cell subsets in inflammatory conditions. Therefore, future experiments should focus on preclinical models of inflammatory disease in *CYP-ABH2* mice to further reveal the relevance of ABA in T-cell mediated immunity.

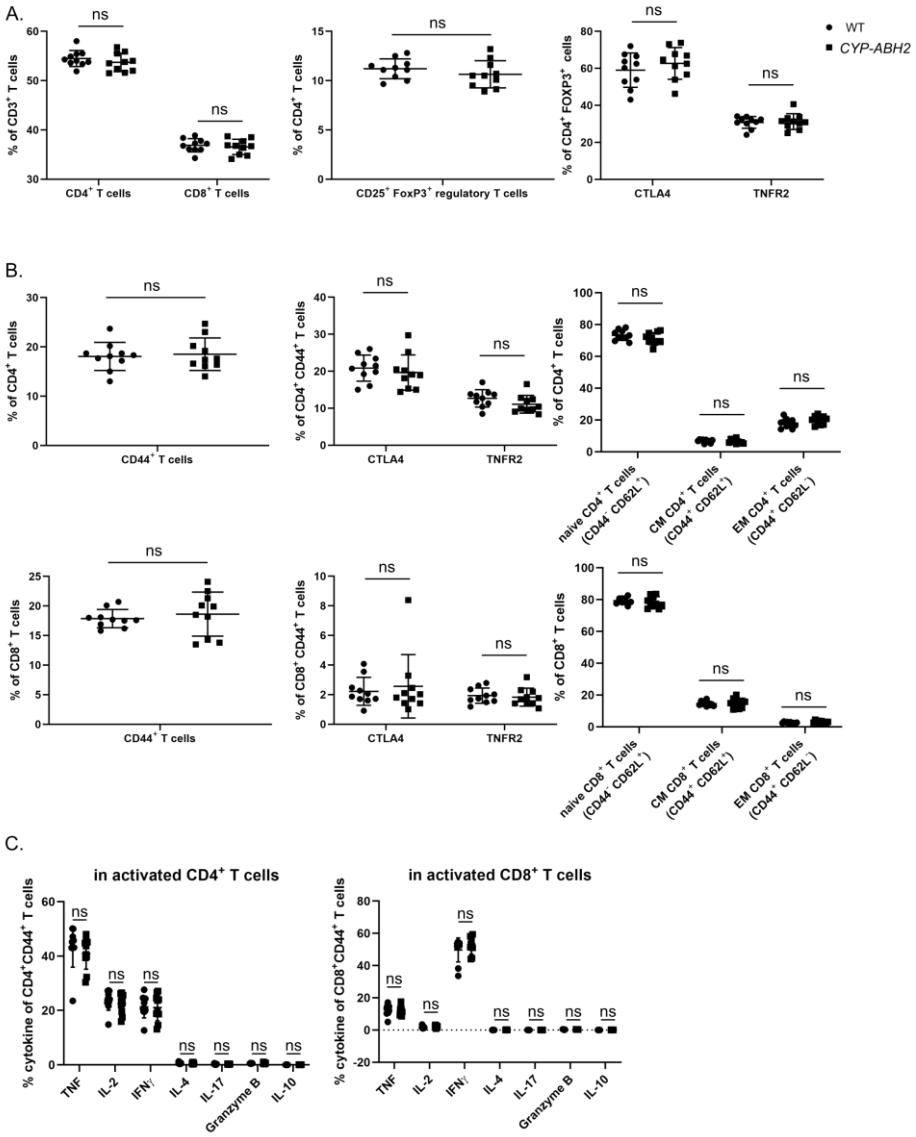


Figure 2. *CYP-ABH2* mice have normal T cell development and activation
(see following page for figure legend).

Figure 2. *CYP-ABH2* mice have normal T cell development and activation (see previous page for figure)**.**
(A) Percentages of splenic helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), Tregs ($CD4^+CD25^+FoxP3^+$), and CTLA4- or TNFR2- expressing Tregs ($CTLA4^+$ or $TNFR2^+$ Tregs). **(B)** Percentages of activated T cells ($CD44^+CD4^+$ or $CD44^+CD8^+$), CTLA4- or TNFR2- expressing activated T cells ($CTLA4^+$ or $TNFR2^+$ activated T cells), and activation stages (naïve ($CD44^-CD62L^+$), central memory (CM;$CD44^+CD62L^+$) and effector memory (EM; $CD44^+CD62L^-$) T cells) of $CD4^+$ and $CD8^+$ T cells. **(C)** Intracellular TNF, IL-2, IFNγ, IL-4, IL-17, Granzyme B, and IL-10 cytokine production in activated $CD4^+$ and $CD8^+$ T cells. Error bars represent SD. Statistical differences were determined using the Student's t-test, (not significant (ns)). Data shown are from one experiment (n=10).

3.2.2 B cells

Next, we assessed the frequencies of follicular B cells ($CD19^+$ $CD21/CD35^+$ $CD23^+$), marginal zone ($CD19^+$ $CD21/CD35^+$ $CD23^-$) B cells, and Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$). Here we found again comparable frequencies in all of these subsets between WT and *CYP-ABH2* mice (**Figure 3A**), which shows that the expression of *CYP-ABH2* does not induce abnormalities in B cell immunophenotypes under basal conditions.

So far, a direct link between ABA and immune responses involving B cells has not been revealed. B cells and Tfh are generally known to regulate the pathophysiology of autoimmune disorders. However, imbalance in B cells or Tfh cells was also shown to correlate with the development of metabolic disorders and their comorbidities [17–19]. Therefore, it would be interesting to investigate whether *CYP-ABH2* mice have an altered B cell or Tfh homeostasis when subjected to experimental mouse models for metabolic syndrome-related disorders.

3.2.3 NK cells

Interestingly, NK cells were shown to be involved in the development of metabolic syndrome and related disorders like colitis [20,21], obesity [22,23], and atherosclerosis [24,25]. ABA administration was shown to ameliorate disease progression in mouse models of these metabolic disorders. However, the activity and number of NK cells in the peripheral lymphoid tissues of mice supplemented with dietary ABA were not determined or described in previous studies [3,4,11]. We here asked if the expression of *CYP-ABH2* altered the frequencies of NK cells ($NK1.1^+$) and their production of pro-inflammatory cytokines TNF and IFNγ. However, we did not observe altered frequencies of NK cells or TNF- or IFNγ- producing NK cells (**Figure 3B**), which indicates that the expression of *CYP-ABH2* also did not drive abnormalities in NK cell

immunophenotypes under basal conditions. Therefore, it would be interesting to see whether the expression of *CYP-ABH2* influences NK cell development or function during the pathogenesis of metabolic disorders in experimental models.

3.2.4 DCs

Previous studies on the role of ABA in atherosclerosis *in vitro* and *in vivo* suggested that humans might produce ABA to suppress the development of the atherosclerotic lesion, and monocytes activated by ABA were shown to be involved in this context [11,26]. We thus studied if the absence of ABA in mice could alter the percentage of monocyte or moDCs in spleen; however, the frequencies of monocytes ($CD11b^+Ly6c^+MHCII^-$) and moDCs ($CD11b^+Ly6c^-MHCII^+$) in WT and *CYP-ABH2* mice were similar **(Figure 3C**). Also, the percentage of DC subsets (cDC1 and cDC2), characterized by CD11c, MHCII, SIRPα, and CD8 presence, were comparable. Similar to other immune cell subsets mentioned above, the absence of ABA might not affect the differentiation of DCs in healthy mice. Therefore, future experiments should induce inflammatory diseases such as atherosclerosis in *CYP-ABH2* mice to determine whether monocytes and DCs might be involved in the preventive role of ABA in the development of atherosclerosis.

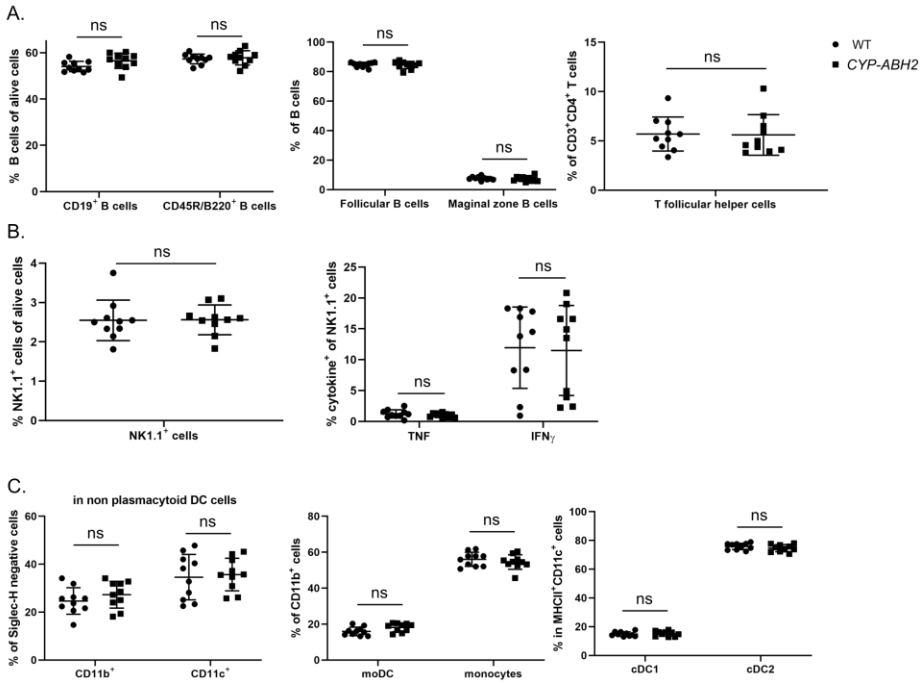


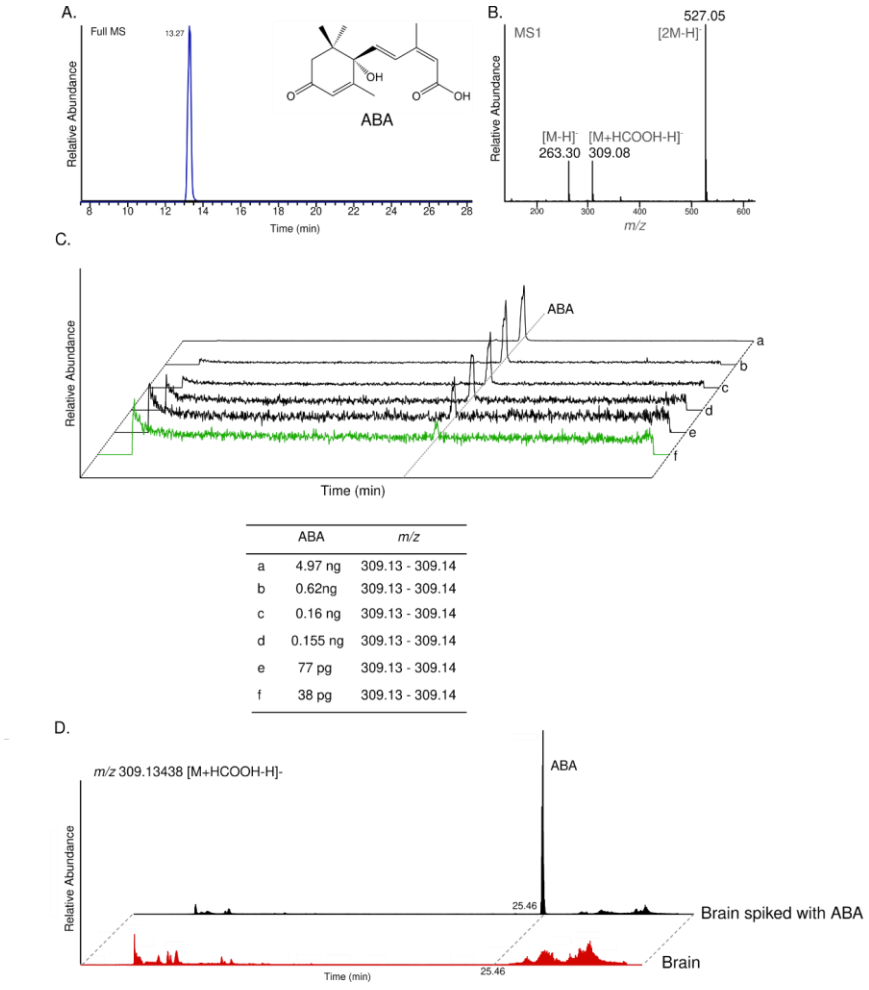
Figure 3. *CYP-ABH2* mice have a normal population of B cell, NK cell, and non-plasmacytoid dendritic cell subsets.
(A) Percentages of B cells (CD45R/B220$^+$ or CD19$^+$) in spleen and the frequencies of follicular (CD19$^+$ CD21/CD35$^+$ CD23$^+$), marginal zone (CD19$^+$ CD21/CD35$^+$ CD23$^-$) B cells, and T follicular helper cells (CD4$^+$CXCR5$^+$PD-1$^+$). **(B)** Percentages of NK cells (NK1.1+) and TNF- or IFNγ- expressing NK cells (TNF$^+$ NK1.1$^+$ or IFNγ$^+$ NK1.1$^+$) **(C)** Percentages of non-plasmacytoid DC subsets (conventional DC1 and DC2 abbreviated as cDC1 and cDC2), monocyte, and monocyte-derived dendritic cells (moDCs). cDC1 cells were distinguished by assessing CD11c$^+$MHCII$^+$SIRPα$^-$CD8$^+$ cells, cDC2s by CD11c$^+$MHCII$^+$SIRPα$^+$CD8$^-$, monocytes by CD11b$^+$Ly6c$^+$MHCII$^-$, and moDCs by CD11b$^+$Ly6c$^-$MHCII$^+$. Error bars represent SD. Statistical differences were determined using the Student's t-test, (not significant (ns)). Data shown are from one experiment (n=10).

3.3 ABA depletion in transgenic *CYP-ABH2* mice could not be confirmed in mouse tissues

As discussed above, the expression of *CYP-ABH2* does not drive abnormalities in T cell, B cell, NK cell, and DC immunophenotypes in mice. This suggests that the absence of ABA might not have an impact under basal conditions, and that disease models should be implemented on *CYP-ABH2* mice in future experiments to reveal a potential immunoregulatory role of endogenous ABA. However, solid conclusions

can also only be drawn after first confirming ABA depletion in *CYP-ABH2* mice using mass spectrometry (MS).

As the brain was previously described to contain a higher amount of ABA (180 ± 37 ng/ 100g of fresh weight (FW) of tissue) in comparison to other tissues such as the liver (57 ± 16 ng/ 100g FW) and kidney (37 ± 7 ng/ 100g FW) in pigs [27], we first aimed to detect ABA in mouse brain. In the group of prof. Alain Goossens, two mass spectrometric methods were established, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography–Fourier transform mass spectrometry (LC-FTMS). ABA measurement by GC-MS was however incompetent due to the derivatization process that causes tautomerization, therefore, we opted for LC-FTMS. As a limit of detection (LOD) for ABA was determined to be between 38 pg to 77 pg (**Figure 4A-4C**), we speculated that brain tissues pooled from four mice might contain ABA at a higher concentration than LOD. However, we could not obtain a peak for ABA from the pooled brain sample (**Figure 4D**). Therefore, the pooled brain sample might have contained ABA at a much lower level than expected due to the species difference in ABA metabolism between pig and mouse. Another cause could be ion suppression derived from the tissue matrix, reducing the detection response for ABA in LC-FTMS.



	ABA	*m/z*
a	4.97 ng	309.13 - 309.14
b	0.62ng	309.13 - 309.14
c	0.16 ng	309.13 - 309.14
d	0.155 ng	309.13 - 309.14
e	77 pg	309.13 - 309.14
f	38 pg	309.13 - 309.14

Figure 4. Measurement of ABA in brain using LC-FTMS.
(A) Total ion chromatogram (TIC) LC-FTMS chromatograms of ABA standard. **(B)** Mass fragmentation of a peak at 13.27 min. **(C)** Extracted ion chromatogram (EIC) (*m/z* = 309.13-309.14) LC-FTMS chromatograms of a dilution series of ABA standards ranging from 38 pg (green) to 4.97 ng. In measuring low concentrations of ABA, LC-FTMS yielded higher abundance of formic acid adduct [M+HCOOH-H]- ions (*m/z* = 309.13), which was used to screen for the presence of ABA. **(D)** EIC (*m/z* = 309.13-309.14) LC-FTMS chromatograms of extract of brain spiked with ABA standard (black) or four-pooled brains (red).

Next, we opted for brown adipose tissue (BAT) for profiling ABA and optimized the sample preparation method based on the protocol obtained from rof. Elena Zocchi (University of Genova School of Medicine, Italy), who is specialized in the role of ABA in human physiology. BAT was opted since it contains high levels of ABA [28] (prof.

Elena Zocchi, personal communication). Also, we switched from LC-FTMS to a high resolution VION IMS Q-TOF mass spectrometer (VION) equipped in the VIB metabolomics core Gent since it reaches lower LOD (3 pg) for ABA (**Figure 5A**). To begin with, we crosschecked whether each step of the sample extraction procedure might cause a considerable loss of ABA (**Figure 5B**). Sample preparation begins with the overnight incubation of homogenized tissue in 80% methanol at -70 °C (here referred to as 'methanol'). Next, the mixture was dried and subjected to water/cyclohexane partition to eliminate apolar metabolites in samples (here referred to as 'cyclohexane'). To improve the purity of samples further, trichloroacetic acid (TCA) was added to the subsequent aqueous phase and subjected to water/ethyl acetate partition (here referred to as 'TCA-ethylacetate'). The addition of TCA protonates ABA, which consequently allows ABA to be transferred into the ethyl acetate phase. As a result, the cyclohexane step did not cause the massive loss of ABA. However, the TCA-ethylacetate step did cause the massive loss of ABA (**Figure 5B**). Therefore, we abandoned the TCA-ethylacetate step in the sample preparation for profiling ABA in BAT. We analyzed the samples extracted from one to four-pooled BATs and observed a clear peak of ABA (**Figure 5C**). Here, we observed that the addition of biological samples to ABA standard decreased the response for ABA, indicating the negative effect of a tissue matrix.

To confirm the reliability of our detection of ABA in BATs, we repeated the measurement of ABA in BATs (**Figure 5D**). Given the high pressure on the machine, possibly from the non-polar compounds in BATs, we opted to use C18 solid-phase extraction (SPE) columns instead of the water/cyclohexane partition to remove non-polar impurities better (here referred to as 'SPE'). However, as a result, we could not obtain a peak for ABA from BATs, while ABA was detected in BATs from mice fed with an ABA-supplemented diet (100mg/kg). The sample matrix compounds retained in the SPE columns were also analyzed as a backup, but a peak for ABA was not observed.

Therefore, we could not develop a reliable MS method to quantify ABA in mice tissues. The challenge to detect ABA in mouse tissues might mainly attribute to the low level of ABA. Although the performance of MS is controlled by routine instrumental maintenance, many factors can affect the stability, resolution, and sensitivity of MS [29,30]. The different performances of MS per individual analysis do not substantially impact detecting metabolite exhibiting high level. However, this could negatively affect detecting the low ABA contents in mice tissues. This might reflect why we were able to detect a peak for ABA in BATs at the first attempt (**Figure 5C**) but not at the second attempt (**Figure 5D**). Due to this bottleneck, we could not verify the metabolic conversion of ABA to DHPA

in transgenic *CYP-ABH2* mice.

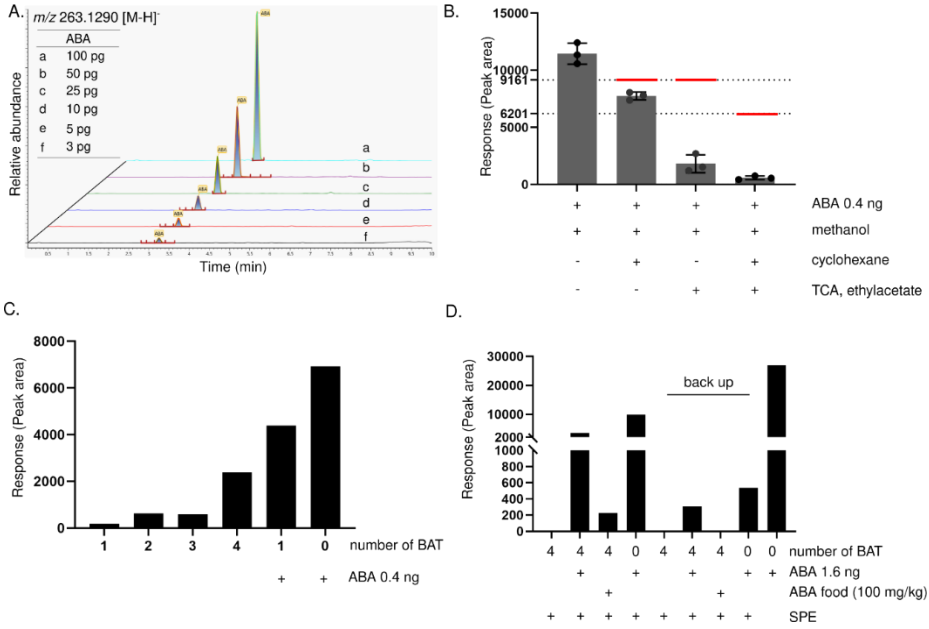


Figure 5. Measurement of ABA in BAT using VION.
(A) EIC (*m/z* = 263.1290) VION chromatograms of a dilution series of ABA standards ranging from 3 pg to 100 pg. (B) Response to ABA (peak area at m/z 263.1290) of 0.4 ng of ABA standards extracted with distinct sample preparation methods. 'Methanol' indicates the overnight incubation of extract at -70 °C. 'Cyclohexane' indicates the subsequent cyclohexane/water partition. 'TCA, ethylacetate' indicates the addition of trichloroacetic acid (TCA) to the extracted aqueous phase (resulting from cyclohexane/water partition), followed by the water/ethylacetate partition. (C) Response to ABA (peak area at *m/z* 263.1290) in one or two or three or four-pooled BATs. 0.4 ng of ABA standard and a BAT spiked with 0.4 ng of ABA were analyzed as controls. Nonpolar compounds were eliminated from extracts by cyclohexane/water partition (cyclohexane). (D) Response to ABA (peak area at *m/z* 263.1290) in four-pooled BATs. Four-pooled BATs from mice fed with an ABA-supplemented diet (100mg of ABA/ kg of diet), four-pooled BATs spiked with 1.6 ng of ABA and 1.6 ng of ABA were analyzed as controls. Nonpolar compounds were eliminated from extracts using C18 solid-phase extraction (SPE) columns.

In addition, we attempted to quantify ABA in serum from WT and *CYP-ABH2* mice given control or an ABA-supplemented diet (100 mg/kg) in a single experiment using our PYL8-based optimized biosensor (**Figure 6**). However, this preliminary experiment was inconclusive as the level of ABA in serum from untreated mice was near the detection limit. Furthermore, comparing ABA levels in serum of untreated and ABA-

treated mice was not conclusive as the feeding time is uncontrollable in the ad libitum feeding system. To overcome this limitation, future studies should increase the number of mice per group, measure different tissue types such as brown adipose tissue, measure ABA levels at multiple time-points, and employ other feeding systems like oral-gavage and intraperitoneal injection.

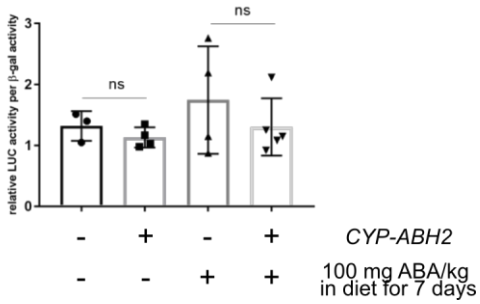


Figure 6. ABA measurement in serum from WT and transgenic (TG) *CYP-ABH2* mice given a control (MOCK) diet or an ABA-supplemented diet (100 mg/kg) for seven days by PYL8-based biosensor.
HEK293T cells were transfected with the PYL8-based biosensor construct along with pU22CH-Luc-Zsgreen1 and pACTβgal and incubated with serum. Fold induction of ABA-induced luciferase activity in cells treated with serum is compared to the average background level of untreated cells. Luciferase values were normalized by β-galactosidase values. Error bars represent standard deviation (S.D). Data shown are from one experiment. Each dot represents one mouse. Statistical significance was calculated using Student's t-test (*P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001).

In conclusion, we were unable to show an effect of *CYP-ABH2* expression on the health of mice and immune homeostasis under basal conditions. Possibly, ABA depletion only has an effect under inflammatory conditions associated with the development of metabolic disorders such as colitis and type 2 diabetes in mice. However, ABA depletion in *CYP-ABH2* mice will first need to be confirmed before challenging them with disease models. Unfortunately, we could not verify ABA depletion in *CYP-ABH2* mice due to the low level of ABA in the brain and BAT, resulting in difficulties in developing a reliable and stable quantification method for ABA in mice.

4 Materials and methods

4.1 Generation of pPB-CAG-LoxP-STOP-LoxP-AtCYP707A3-HA-P2A-ABH2-V5 plasmid

P2A-linked mouse codon-optimized *AtCYP707A3* and *ABH2* was cloned into Mammalian LSL Conditional Gene Expression PiggyBac Vector (pPB-LoxP-Stop-LoxP) by Vectorbuilder (Cyagen Biosciences, Santa Ana, CA, USA). The vector ID of pPB-LSL-AtCYP707A3-HA-P2A-AtABH2-V5 is VB170726-1238phy, which can be used to retrieve detailed information about the vector on www.vectorbuilder.com. Plasmid of the cloned gene was deposited in the BCCM/GeneCorner plasmid collection (LMBP 10705) along with detailed descriptions of cloning strategy and plasmid sequence (http://bccm.belspo.be/about-us/bccm-genecorner).

4.2 Mice

Mice were bred and maintained under specific pathogen-free conditions and housed in individually ventilated cages following the national and institutional guidelines for the care and use of laboratory animals. Transgenic mice that express *Lox-STOP-Lox (LSL)-AtCYP707A3-HA-P2A-ABH2-V5* (*LSL-CYP-ABH2*) were first generated by using the PiggyBac transposon technology (Cyagen Bioscience, Santa Ana, CA, USA). In brief, Piggybac transgenic plasmid pPB-CAG-LSL-AtCYP707A3-HA-P2A-ABH2-V5 was injected into the pronucleus of fertilized eggs. These eggs were implanted into surrogate mothers, and offspring pups were genotyped by PCR to identify those carrying the transgene. The mice produced by these surrogate mothers are defined as founders, and pups from two founders (#8 and #9) were genotyped by PCR.

Next, to induce cre excision of the LSL cassette, transgenic mice expressing *LSL-AtCYP707A3-HA-P2A-ABH2-V5* were bred with Sox2-Cre transgenic mouse strains (C57BL6/J background). This generated transgenic mice that express *AtCYP707A3-HA-P2A-AtABH2-V5 transgene* (*Sox2-Cre*$^{+/-}$;*CYP707A3-HA-P2A-ABH2-V5*$^{+/-}$). Then, crossing these transgenic mice with wild-type mice on the C57BL6/J background yielded *Sox2-Cre*$^{-/-}$;*AtCYP707A3-HA-P2A-AtABH2-V5*$^{+/-}$ transgenic mice (here referred as *CYP-ABH2* mice). As controls, littermates *Sox2-Cre*$^{-/-}$;*AtCYP707A3-HA-P2A-AtABH2-V5*$^{-/-}$ or *Sox2-Cre*$^{+/-}$;*AtCYP707A3-HA-P2A-AtABH2-V5*$^{-/-}$ were used (here referred to as wild-type (WT)).

Five-week-old male wild-type (WT) C57BL/6J mice were purchased from Janvier (Le Genest-St-Isle, France). Mice were bred in the SPF

facilities of Pasteur Institute, Lille. Mice were housed for 14 days prior to the experiment for the adaptations. Mice were housed under SPF conditions in individually ventilated cages in accordance with the national guidelines and regulations for the care and use of laboratory animals. Animal protocols were approved by the ethical committee of Ghent University (EC2019-024). Seven-week-old wild-type (WT) C57BL/6J mice were fed with AIN-93G diet (Research Diets, Inc., New Brunswick, NJ, USA) with or without 100 mg/kg of (+)-cis, trans-Abscisic acid (A-050, Goldbio) for 7 days prior to euthanasia.

4.3 Genotyping

To extract genomic DNA of *CYP-ABH2* mice or littermate control mice, toes or tails were clipped and lysed in 100 µL of 50 mM NaOH at 95 °C for 1 hour while shaking (850-1100 rpm). 10 µL of 1.5 M Tris-HCl (pH 8.8) was added to the lysate, and 1-2 µL of genomic DNA was used per PCR reaction. Genotyping was performed by PCR with Taq master mix (Highqu, Kraichtal, Germany) using the following conditions: one cycle at 95 °C 5 min, 35 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 1 min and a final extension step at 72°C for 5 min. The primers used to detect the *CYP-ABH2* transgene were Forward_3 5-CGGAATCGAGGAGATCGTGG-3, and Reverse_3 5-TCAGATGCTCAAGGGGCTTC-3, which amplified a 280 bp PCR product.

4.4 Western blotting

After sacrifice, spleens were harvested and immediately frozen and stored at -70 °C. To make protein lysates, tissues were homogenized and lysed using Precellys 24 with 2.8mm Zirconium oxide beads (Bertin technologies, Rockville, US) in RIPA buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors. Debris was removed by a centrifuge at 14000 rpm for 10 min at 4 °C. Pierce™ BCA Protein Assay Kit (23225, ThermoFisher Scientific) was used to determine protein concentration. 5 × Laemmli buffer (250 mM Tris-HCl, pH 8, 10% SDS, 50% glycerol, 0.005% bromophenol blue, 25% β-mercaptoethanol) was added to the samples lysed in RIPA buffer, followed by denaturation for 10 min at 95 °C. Equal amounts of proteins were loaded and separated by 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes with 0.45 µm pores (Protran, Perkin Elmer), and probed with the following specific antibodies: anti-HA.11 (MMS-101R-B, Babco), anti-V5-HRP (R96125, Invitrogen), anti-β-actin-HRP (sc-47778, Santa Cruz), and anti-mouse IgG secondary antibody conjugated to HRP (31432, Thermo Fisher Scientific). Western

Lightning ECL detection system (Perkin Elmer) was used for protein detection according to the manufacturer's instructions.

4.5 Metabolite extraction

4.5.1 Brain

After sacrifice, brain tissues were immediately frozen and stored at -70 °C. Tissues were ground using a pestle and mortar. 80 % of methanol was added to the ground tissue, and the mixture was incubated overnight at -70 °C in an ultra freezer. The mixture was further centrifuged at 10,600 x g for 15 min at 4 °C, and the supernatant was evaporated using a SpeedVac. The dried residue was resuspended in 1 mL of a 50:50 water: cyclohexane solution. The phases were separated by centrifugation at 10,600 x g for 5 min. The aqueous phase was collected and evaporated using a SpeedVac. The dried residue was resuspended in 50 µL of ultrapure water before chromatographic separation and quantification by LC-FTMS.

4.5.2 Brown adipose tissue

After sacrifice, interscapular brown adipose tissues were immediately frozen and stored at -70 °C. Tissues were homogenized lysed using Precellys 24 with 2.8mm Zirconium oxide beads (Bertin technologies, Rockville, US) in 200 µL of phosphate-buffered saline buffer at a slightly basic pH (8.0). Debris was removed by a centrifugation step at 10,600 x g for 10 min at 4 °C. Next, 800 µL of methanol was added to the supernatant and mixed by vortexing, followed by the overnight incubation at -70 °C in an ultra freezer. The mixture was further centrifuged at 10,600 x g for 15 min at 4 °C, and the supernatant was evaporated using a SpeedVac. Next, nonpolar compounds were removed by cyclohexane/water partition or C18 solid-phase extraction columns (abbreviated to SPE columns, Thermo Scientific™, HyperSep™ C18 Cartridges). For cyclohexane/water partition, 1 mL of a 50:50 water: cyclohexane solution was added to the dried residue. The phases were separated by centrifugation at 10,600 x g for 5 minutes, and the aqueous phase was collected. For using SPE columns, the dried residue was resuspended in 0.5 mL of 50 % methanol, and the following sequence of steps was performed: washing with 3 mL 100 % methanol, 3 mL 50 % methanol, application of the sample, elution with 1.5 mL 50 % methanol, elution of back-ups with 1.5 mL 100 % methanol. Next, the collected sample from the cyclohexane/water partition or the SPE column was evaporated using a SpeedVac. Next, the dried residue was resuspended in 50 µL of ultrapure water and filtered using an

AcroPrep Advance 96-filter plate 0.2 µm Supor (Pall Life Sciences) prior to chromatographic separation and quantification by VION.

For an additional clean-up step using trichloroacetic acid and ethyl acetate after cyclohexane/water partition, 250 µL of 5% trichloroacetic acid was added to the collected aqueous phase. The mixtures were vortexed and partitioned three times against 500 µL of ethyl acetate, and the organic layer was collected and evaporated using a SpeedVac. The dried residue was resuspended in 50 µL of ultrapure water and filtered using an AcroPrep Advance 96-filter plate 0.2 µm Supor (Pall Life Sciences) prior to chromatographic separation and quantification by VION.

4.6 Profiling of ABA by LC-FTMS

10 µL of the sample was injected in an Acquity UPLC BEH C18 column (2.1x 150 mm, 1.7 µm) mounted on an LC system consisting of an Accela pump and autosampler (Thermo Electron Corporation, Waltham, MA, USA) coupled to a LTQ FT Ultra (Thermo Electron Corporation) via an electrospray ionization source operated in negative mode. The following gradient was run using acidified (0.1% (v/v) formic acid) solvents A (water/acetonitrile, 99:1 v/v) and B (acetonitrile/water; 99:1, v/v): time 0 min, 5% B; 30 min, 45% B; 33 min, 100% B. Negative ionization was obtained with the following parameter values: capillary temperature 300 °C, sheath gas 30 (arbitrary units), aux. gas 10 (arbitrary units) and spray voltage 5 kV. Full MS spectra between m/z 120 and 1,400 were recorded. MSn spectra (MS2 and two dependent MS3 scan events, in which the two most abundant daughter ions were fragmented) were generated from the most abundant ion of each full MS scan. The collision energy was set at 35 eV.

4.7 Profiling of ABA by VION

Methods are described in **Chapter 4.2**, section 5.3.

4.8 Flow cytometry

Frequencies of splenic T cells, NK cells, B cells, DCs in spleens were analyzed as described in **Chapter 3.2**, section 4.7.2.

4.9 Luciferase reporter assay

Methods are described in **Chapter 4.1**, section 4.4.

4.10 Statistical analysis

Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the appropriate statistical test (Student's t-test; equal variances t-test) has been performed to determine a significant difference.

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Chapter 5. Investigation on the prophylactic effect of ABA in dextran sodium sulfate-induced acute colitis in mice

Chapter 5. Investigation on the prophylactic effect of ABA in dextran sodium sulfate-induced acute colitis in mice

Seo Woo Kim[1, 2, 3, 4], Yasmine Driege[1, 2], Mira Haegman[1, 2], Keylla Bicalho[5], Geert Goeminne[5], Alain Goossens[3, 4], Rudi Beyaert[1, 2], Jens Staal[1, 2, 6]

[1]Center for Inflammation Research, VIB, Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[3]Center for Plant Systems Biology, VIB, Ghent, Belgium
[4]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium
[5]VIB Metabolomics Core Ghent, VIB, Ghent, Belgium
[6]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium

Unpublished results

Author contributions

SWK, AG, RB, and JS designed the research and interpreted data. SWK, YD, KB, GG, and MH, performed experiments. SWK, KB, and GG analyzed the data. SWK wrote the paper with the help of AG, RB, and JS. AG, RB, and JS supervised the project and RB and AG provided resources.

1 Abstract

Background
Colitis is an idiopathic inflammatory bowel disease (IBD) characterized by diarrhea, rectal bleeding, abdominal pain, and weight loss. Dietary ABA was shown to exert a prophylactic effect in dextran sodium sulfate (DSS)-induced colitis in mice in a PPAR γ-dependent manner. Also, the value of the proposed ABA receptor lanthionine synthetase C-like protein 2 (LANCL2) was appreciated as a therapeutic target to develop a drug candidate against colitis. This raises a fundamental question of whether the endogenous ABA is involved in the modulation of immune and inflammatory responses in the context of IBD.

Objective
To understand a possible function of endogenous ABA in the context of IBD, we initially wanted to investigate disease development in ABA-depleted mice using the DSS model of colitis. In line with previous studies showing that dietary ABA protects against DSS-induced colitis in mice, we expect that the prophylactic role of ABA in DSS-induced colitis is abrogated in transgenic *CYP-ABH2* mice. The DSS-induced colitis model is widely used because of its simplicity and similarity to the disease development of IBD in humans. However, the pathogenesis of this model can be easily varied depending on environmental and genetic factors. We thus first aimed to validate the published results showing a prophylactic effect of ABA in the DSS-induced colitis model by monitoring disease development in ABA-administered WT mice with DSS-induced colitis.

Methods
C57BL/6J mice were administered with ABA-supplemented diets for 35 days and treated with DSS 3%, 2.25%, 2%, and 1.5% in their drinking water to induce acute colitis. Mice were monitored daily for clinical signs of colitis by determining body weight loss, stool consistency, and occult or gross blood loss per rectum (rectal bleeding).

Key Results & Conclusions
In consistent with the previous reports from the Bassaganya-Riera J group, we did not observe the preventive effect of ABA against DSS-induced colitis in mice. The reasons for the controversial findings are unclear yet. The efficacy of ABA-supplemented diets might have decreased due to the loss of ABA during the food manufacturing process, resulting in a too low dose of ABA in mice receiving the ABA containing diet. Also, different gut microbiota composition of mice might have decreased its efficacy. Future research remains necessary to investigate the acceptable range of ABA levels to exert its efficacy in

mice and the impact of ABA on the gut microbiome. This subsequently will form a solid basis for future experiments to reveal the physiological role of ABA.

2 Introduction

Colitis is an idiopathic inflammatory bowel disease (IBD) resulting from immunological abnormalities caused by a complex interplay of genetic factors, environmental risks, and gut microbiota [1,2]. IBD is comprised of two major disorders: ulcerative colitis (UC) and Crohn's disease (CD) and is characterized by symptoms like diarrhea, rectal bleeding, weight loss, fatigue, and abdominal pain [3]. Moreover, IBD could eventually bring other comorbid conditions such as colon cancer, cardiovascular disease, and venous thromboembolism [4]. The prevalence and incidence of IBD are increasing, predominantly in newly-industrialized and Western countries, which imposes a significant economic impact on their health care systems [5,6]. As about 0.2% of the European population is diagnosed with IBD and the speed of its prevalence continues to rise, improving the trajectories of early diagnosis, prevention, and remediation of this disease is in demand [5,6].

In the pathogenesis of IBD, the imbalance of immune homeostasis is triggered by epithelial damage and the infiltration of diverse immune cells into the lamina propria [7]. During the inflammation response, pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β, interferon (IFN)-γ, and IL- 23 are generated in the activated lamina propria cells [7]. Given that the progression of IBD often requires surgeries and hospitalization [8,9], therapeutic agents that control disease symptoms and progression early in the disease course have been developed [1,9]. Currently available therapeutic options are aminosalicylates, corticosteroids, anti-TNF blockers, anti-integrin blockers, anti-IL-12 inhibitors, and anti-IL-23 inhibitors [1,9]. Also, novel approaches like microbiome targeted therapies and stem-cell therapy have emerged, showing promise in IBD treatment [10–12]. Considering the adverse effects of each type of treatment (e.g., osteoporosis and venous thromboembolism induced by the treatment of corticosteroids [13]) and patients' divergent clinical goals and complications, choosing the most appropriate and effective therapy for each patient is paramount [14].

Interestingly, ABA showed its potential as an agent to prevent and ameliorate colitis [15–17]. Long-term (35 days) pretreatment of dietary ABA exerted protective effects in dextran sodium sulfate (DSS)-induced colitis in mice by increasing the number of regulatory T (Treg) cells in mesenteric lymph nodes (MLN) and the colon, through a PPAR γ-dependent mechanism [15–17]. In the DSS-induced colitis mouse model, sulfated polysaccharides with variable molecular weights damage the colonic epithelial monolayer and lead to the subsequent invasion of commensal bacteria and their antigens into the intestinal

mucosal barrier, which induces acute inflammation in the colon as well as the impairment of mucosal barrier function [18]. The subsequent clinical symptoms such as perianal soiling, rectal bleeding, diarrhea, and piloerection were alleviated upon dietary ABA supplementation. In addition, a study using T-cell specific PPAR γ-deficient mice reported that ABA treatment also reduced the DSS-elevated colonic mRNA expression of adhesion molecules such as *E-selectin*, *vascular cell adhesion protein 1* (*vcam1*), and *mucosal vascular addressin cell adhesion molecule 1* (*madcam1*), pro-inflammatory cytokine *il6,* and *matrix metalloproteinase 9* (*mmp9*) in a T-cell PPAR γ-dependent manner [15,16]. Subsequently, drug development targeting the proposed ABA receptor lanthionine synthetase C–like protein 2 (LANCL2) has resulted in a drug candidate (BT-11) with promising effects against colitis [19–23].

Considering the protective effect of ABA against IBD by controlling immune regulatory mediators, we asked whether endogenous ABA has functional roles in the modulation of immune and inflammatory responses in the context of IBD. Moreover, before using ABA as a prophylactic against IBD, a better understanding of the physiological relevance of ABA in the pathogenesis of IBD will be essential. As described in **Chapter 4**, to understand ABA's physiological roles, we made use of *Arabidopsis* ABA 8'-hydroxylase CYP707A3, which plays a key role in ABA catabolism in *Arabidopsis thaliana.* We generated transgenic mice overexpressing *Arabidopsis thaliana CYP707A3* and *ABH2* to degrade ABA and the AtCYP707A3 ABA degradation product phaseic acid (referred to as transgenic *CYP-ABH2* mice onwards). In line with previous studies showing that dietary ABA protects against DSS-induced colitis in mice [15–17], we initially planned to investigate whether the preventive role of ABA in DSS-induced colitis is abrogated in transgenic *CYP-ABH2* mice.

One of the cornerstones for these initial research plans is the reproducibility of published scientific results showing a prophylactic effect of ABA in the DSS-induced colitis model. The DSS-induced colitis murine model has multiple advantages because of its similarity to the disease development of IBD in humans and its ease of use. However, environmental factors and the genetic background of mice can easily introduce variability, making the model convoluted [24–27]. This required us to check if the observation of the prophylactic effect of ABA in a mouse model of DSS-induced colitis was reproducible in our specific pathogen-free (SPF) animal facility prior to the employment of the corresponding model on the transgenic *CYP-ABH2* mice. In this chapter, we thus first aimed to validate the published results showing a prophylactic effect of ABA in the DSS-induced colitis model by

monitoring disease development in ABA-administered WT mice with DSS-induced colitis.

3 Results and Discussion

3.1 Oral administration of ABA in diet did not protect against DSS-induced colitis in mice

Mice were administered with ABA-supplemented diets for 35 days and treated with DSS 3% and 2.25 % in their drinking water to induce acute colitis. In contrast to the previous studies from the Bassaganya-Riera J group, which showed a high degree of protection by ABA pretreatment against DSS-induced colitis in mice, in our experiments ABA did not have a protective effect against the 3 % DSS-induced colitis (**Figure 1A**) or only offered slight protection to mice given 2.25 % DSS (**Figure 1B**). The stool consistency and weight loss contributed to the small degree of protection provided by dietary ABA administration in mice given 2.25% DSS water (**Figure S1B**). On the contrary, the previous study from the Bassaganya-Riera J group revealed no significant difference in the average daily weight loss between the mice fed with the control and ABA-supplemented diet during the treatment of 2.5 % DSS in their drinking water [15]. In this previous study scores with regard to other parameters (i.e., rectal bleeding, stool consistency, perianal soiling, and piloerection) contributed to the alleviated severity of DSS-induced colitis in mice fed with ABA-supplemented diets [15]. Albeit contradicting results were obtained between our study and the previously reported study, the slight reduction of disease severity in mice fed with dietary ABA suggests that further modification of the experimental set-up might be necessary to observe a higher preventive effect of ABA in DSS-induced colitis. The differences in disease outcome in the previously published studies and our results do however highlight that the dramatic differences previously reported are not robust and not easily reproduced in an independent setting.

While in the study from the Bassaganya-Riera J group the protective effect of ABA started on the third day of drinking 2.5 % DSS solution water [15], our study revealed a difference in disease severity starting at day 2 (**Figure 1B**). We thus determined whether a better protective effect of ABA could be shown in mice exposed to milder colitis induced by lower concentrations of DSS 2 % and 1.5 %. However, an ABA-supplemented diet for 35 days did not protect against either 2 % or 1.5 % DSS-induced colitis in mice (**Figure 1C, 1D**). Thus, the DSS-induced colitis model was shown to be an inappropriate disease model to employ on transgenic *CYP-ABH2* mice to investigate their sensitivity to ABA treatment and to study the physiological role of endogenous ABA in the pathogenesis of IBD.

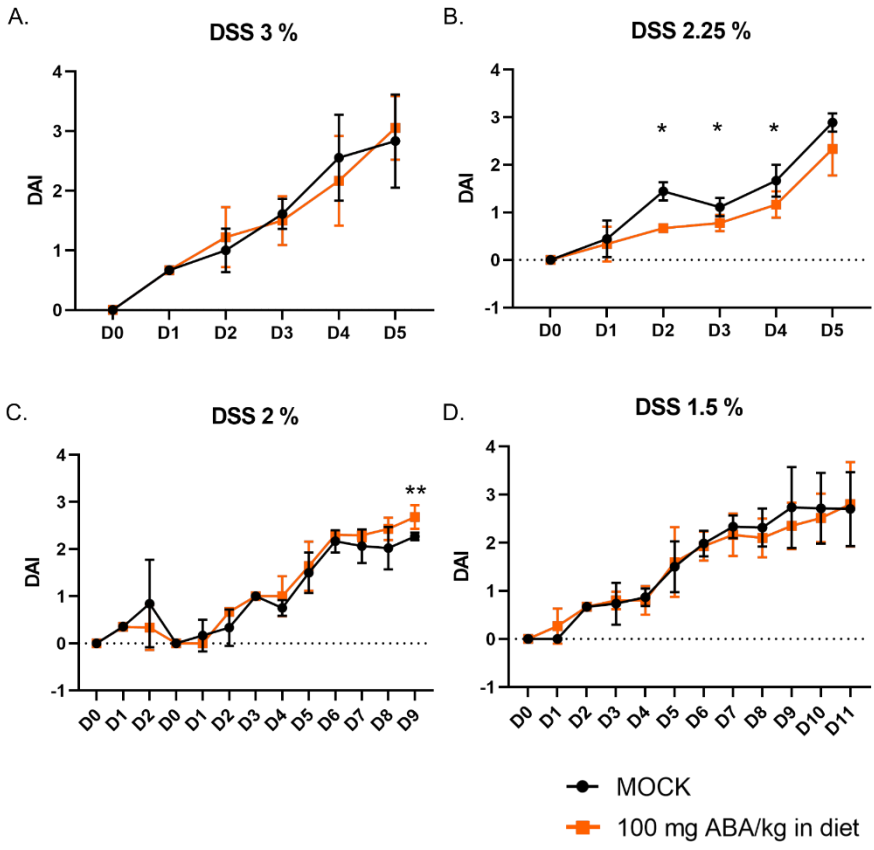

Figure 1. Disease activity index (DAI) during DSS treatment in mice given control diets and ABA-supplemented diets (100 mg/kg).
(A) Disease activity index (DAI) during 3 % DSS. Mice were given with the control diets (MOCK, n=6) or ABA-supplemented diets (n=6) for 35 days, followed by five days of 3 % DSS. (B) DAI during 2.25 % DSS. Mice were given the control diets (MOCK, n=3) or ABA-supplemented diets (n=6) for 35 days, followed by five days of 2.25 % DSS. (C) DAI during 2 % DSS. Mice were given with the control diets (MOCK, n=4) or ABA-supplemented diets (n=6) for 35 days, followed by nine days of 2 % DSS. (D) DAI during 1.5 % DSS. Mice were given with the control diets (MOCK, n=5) or ABA-supplemented diets (n=5) for 35 days, followed by 11 days of 1.5 % DSS. Daily DAI reflects the clinical severity of colitis based on weight loss, stool consistency, and rectal bleeding. Error bars represent ±SD. Statistical differences were determined using the Student's t-test. * P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

Several reasons might explain the discrepancy between the study of the Bassaganya-Riera J group and our observations. The first might be a too low dose of ABA in mice receiving the ABA containing diet, which

might have decreased its efficacy against DSS-induced colitis. For this study, the manufacturer, on our request, produced AIN-93 purified diets containing 100 mg of ABA per kg. However, the actual concentration of ABA measured by mass spectrometry was approximately 14.8 mg/kg (**Figure 2**). It should be noted that the sample preparation procedure for the metabolomics was not optimal and caused the loss of ABA, as the control samples containing 10 µg of ABA in methanol were quantified as 3.18 µg of ABA after sample preparation steps. Considering this loss of ABA during the sample preparation procedure, the concentration of ABA in diets was speculated to be 46.5 mg/kg. This indicates that the loss of ABA occurred during the food manufacturing process, although the sensitivity of ABA to light and heat was considered (**Table 1**). A previous study from the Bassaganya-Riera J group evaluated the efficacy of different concentrations of ABA in AIN-93 diets (0, 100, 200, 400, and 800 mg/kg diet) in a mouse model of obesity and type 2 diabetes, which all exerted the antidiabetic effects by improving glucose tolerance after the 35 days of pretreatment [28]. Therefore, the lowest effective oral dose of ABA (100 mg/kg diet) was selected for their follow-up studies investigating the effect of ABA in a mouse model of colitis [15–17], atherosclerosis [29], and systemic inflammation (lipopolysaccharide model) [30]. In these previous studies, the actual concentration of ABA in diets and the level of ABA in blood or tissues of ABA-administered mice were not determined, implicating that the minimal dose of ABA in diets or the acceptable range of ABA levels to exert its efficacy in mice is unclear. Therefore in future studies, considering no adverse effect observed in mice treated with a higher dose of ABA (200, 400, and 800 mg/kg diet) [28], diets that are manufactured to reach a higher dose of ABA could be utilized. In parallel, the actual concentration of ABA in diets and the adequate ABA levels in the blood should be verified.

Table 1. Overview of speculation of ABA concentration in diets.
LC-MS analysis speculates the actual ABA concentration in AIN-93 diets (100 mg/kg). The blue text represents the speculated loss ratio that occurred during the food manufacturing, and the red text represents the speculated loss ratio that occurred during the sample extraction.

Sample	Expected conc. (or amount)	Actual conc. (or amount)	Loss	Potential reasons for the loss
AIN-93 diets	100 mg/kg	14.8 mg/kg	6.75×	sample preparation, food manufacturing
Synthetic ABA	10 μg	3.18 μg	3.14×	sample preparation
Speculation ↓				
AIN-93 diets	100 mg/kg	46.5 mg/kg	2.15×	food manufacturing

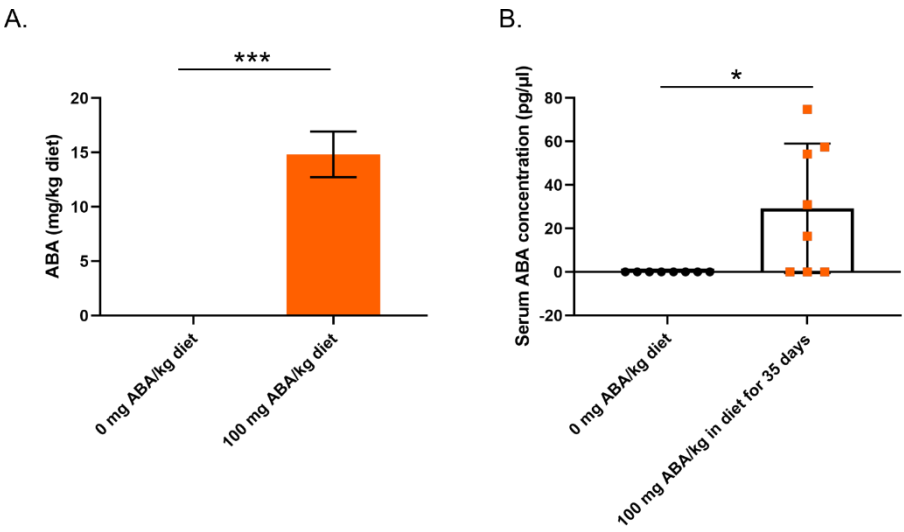

Figure 2. ABA quantification of ABA-supplemented diets and serum from mice given ABA-supplemented diets.
(A) ABA quantification of control food diets and ABA-supplemented diets (100 mg/kg) by LC-MS (B) ABA quantification of serum from mice given control diets or ABA-supplemented diets (100 mg/kg) for 35 days by LC-MS. Statistical differences were determined using the Student's t-test. * P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

The other reason for the controversial findings might be the different gut microbiota compositions of mice. Different gut microbiome compositions might come from the hygienic conditions of vivarium where the mice were maintained or the commercial vendors where the mice were bred. The screening of our SPF facility showed the presence of *Helicobacter ganmani*, which was previously reported to exert a protective effect against DSS-induced colitis and therefore might have influenced the development of colitis of mice in our facility [31].

Previous studies indicated that the interaction between the gut microbiota and non-antibiotic drugs could be bidirectional, as non-antibiotic drugs could influence the gut microbiota diversity [32–34], and the gut microbiota could also reciprocally impact the efficacy of drugs [35,36]. For instance, an altered gut microbiome contributed to the beneficial effect of metformin, which is the first-line drug for patients with type 2 diabetes (T2D) [37]. Contrariwise, the efficacy and tolerance of metformin in T2D patients were reported to rely on the composition of the gut microbiome [38]. Interestingly, decreased bacterial diversity was shown in IBD patients and DSS-treated mice, and restoring the normal microbiota diversity by treating probiotics or targeting a key modulator in inflammation pathways (e.g., Mucosa-associated lymphoid tissue lymphoma translocation protein 1) could alleviate the disease [39,40]. Yet, whether the treatment of ABA in animals could impact their gut microbiome is still enigmatic. The plant rhizosphere has many features that are common with the animal gut, and many host-commensal interactions show many commonalities [41]. In plants, ABA was suggested to be a direct source of carbon for some rhizobacteria, which establishes a strong symbiotic relationship with plants in the zone of soil surrounding the roots (rhizosphere) [42]. For instance, a *Rhodococcus* sp. and a *Novoshingobium* sp. could metabolize ABA and elicit an impact on plant growth [42]. The treatment of *Rhodococcus qingshengii* to plants was suggested to be a promising phytoremediation strategy to decontaminate metal-polluted soil by regulating ABA-mediated heavy metal transporters [43]. In addition, some plant growth-promoting rhizobacteria such as *Bacillus subtilis* in wheat plants [44], *Azospirillum brasilense* in *Arabidopsis* [45], and *Bacillus licheniformis* and *Pseudomonas fluorescens* in grape plants [46] impact the ABA levels in plants and alter their abiotic stress responses. Thus, the direct and indirect interaction between ABA and plant rhizosphere points to the potential impact of ABA on the diversity of gut microbiota, which might have subsequently allowed the protection against DSS-induced murine colitis in the observation of the Bassaganya-Riera J group. As similar to plant rhizobacteria, it may be possible that certain gut bacteria directly consume ABA as a source and subsequently increase their richness in

the microbial community or indirectly affect the host ABA metabolism. An additional potential mechanism is that ABA directly influences the gut mycobiota by inhibition of chorismate mutase [47]**.** Since some fungi are able to produce ABA, it is likely that there is biological variation among fungi regarding this inhibition mechanism. Gut mycobiota is recently emerging as an overlooked but important player in the development of gastrointestinal diseases like IBD and irritable bowel syndrome [48,49]**.** It will be further of interest to determine if ABA administration restores the gut microbial dysbiosis in DSS-treated mice. If so, it is likely that the bacteria, which play a role in alleviating DSS-induced colitis are absent in mice of our facility. The other possibility would be the presence of the bacteria limiting the anti-inflammatory efficacy of ABA (e.g. ABA-mediated activation of T-cell PPAR *γ*) in the commensal gut bacterial community.

In conclusion, the present study could not show the prophylactic effect of ABA against DSS-induced colitis in mice, which is inconsistent with the previous reports from the Bassaganya-Riera J group [15–17]. The absence of other studies showing the efficacy of ABA in a mouse model of colitis or IBD patients indicates a clear need for careful interpretation and validation of their reports. The potential reasons driving the discrepancy between the report of the Bassaganya-Riera J group and our observation is unclear yet, which brought open questions to be addressed. To counteract the potential loss of ABA during the food manufacturing process, a future experiment using a higher dose of ABA in diets will be necessary. In-depth characterization of the efficacy of ABA and investigating its impact on the gut microbiome will also be informative, which subsequently will provide an interesting basis for future research elucidating the physiological role of ABA.

4 Materials and methods

4.1 Mice

Five-week-old male wild-type (WT) C57BL/6J mice were purchased from Janvier (Le Genest-St-Isle, France). Mice were bred in the SPF facilities of Pasteur Institute, Lille. Mice were housed for 14 days prior to the experiment for the adaptations. Mice were housed under SPF conditions in individually ventilated cages in accordance with the national guidelines and regulations for the care and use of laboratory animals. Animal protocols were approved by the ethical committee of Ghent University (EC2019-024).

4.2 Dietary ABA supplementation and DSS-induced colitis

Seven-week-old wild-type (WT) C57BL/6J mice were fed with AIN-93G diet (Research Diets, Inc., New Brunswick, NJ, USA) with or without 100 mg/kg of (+)-cis, trans-Abscisic acid (A-050, Goldbio) for 35 days prior to and during the induction of colitis. Acute colitis was induced in mice by adding 1.5 % or 2 % or 2.25 % or 3 % DSS (MW = 36,000-50,000, MP Biomedicals) to their drinking water for 11 or 9 or 5 days, respectively. Mice were monitored daily for clinical signs of colitis by examining body weight loss, stool consistency, and occult or gross blood loss per rectum (rectal bleeding), using a previously reported clinical scoring [50]. Fecal blood was inspected by Hemooccult SENSA (Beckman Coulter) test. The baseline clinical score was determined on day 0. For the weight loss score, no weight loss from the baseline was scored as 0, weight loss of 1-5 % as 1, 5-10 % as 2, 10-20 % as 3, and >20 % as 4. For stool consistency, well-formed stools were scored as 0, pastry and semi-formed stools were scored as 2, and liquid stools were scored as 4. For rectal bleeding, negative hemoccult was scored as 0, positive hemoccult was scored as 2, and gross rectal bleeding was scored as 4. Total clinical score from 0 to 4 was created by calculating the average of three scores. On day 5 or 9 or 11, mice were euthanized by CO_2 narcosis.

4.3 Extraction of ABA and detection of ABA by LC-MS/MS

To determine ABA levels in serum, blood was collected from the heart ventricle and first incubated at room temperature for 30 min to allow the blood clot, followed by centrifugation at 1,700 x g for 10 min to separate the serum from the whole blood. Next, 200 µL of mice serum was added to 800 µL of methanol. The mixture was vortexed and incubated overnight at -70 °C in an ultrafreezer. The mixture was further centrifuged at 10,600 x g for 15 min at 4 °C, and the supernatant was evaporated using a SpeedVac. The dried residue was resuspended in 1 mL of a 50:50 water: cyclohexane solution. The phases were separated

by centrifugation at 10,600 x g for 5 min. Next, 250 µL of 5% trichloroacetic acid was added to the collected aqueous phase and mixed by vortexing. The mixtures were partitioned three times against 500 µL of ethyl acetate, and the organic layer was collected and evaporated using a SpeedVac. The dried residue was resuspended in 50 µl of ultrapure water and filtered using an AcroPrep Advance 96-filter plate 0.2 µm Supor (Pall Life Sciences) prior to chromatographic separation and quantification by LC-MS/MS. For the determination of ABA levels in mouse diets, food samples were homogenized using Precellys 24 (Bertin technologies with CK26 beads) in 200 µL of phosphate-buffered saline buffer at a slightly basic pH (8.0). Debris was removed by a centrifugation step at 10,600 x g for 10 min at 4 °C. Next, 800 µL of methanol was added to the supernatant and mixed by vortexing, followed by the overnight incubation at -70 °C in an ultrafreezer. The mixture was further centrifuged at 10,600 x g for 15 min at 4 °C, and the supernatant was evaporated using a SpeedVac. The dried residue was resuspended in 1 mL of a 50:50 water: cyclohexane solution. The phases were separated by centrifugation at 10,600 x g for 5 min. The aqueous phase was collected and evaporated using a SpeedVac, and the dried residue was resuspended in 50 µL of ultrapure water and filtered using an AcroPrep Advance 96-filter plate 0.2 µm Supor (Pall Life Sciences) prior to chromatographic separation and quantification by LC-MS/MS. For both of serum and food analyses, 10 µg of ABA standard were spiked to the 1 mL of methanol and analyzed as control samples. To create the calibration curve, the three replicates of ABA stock solution (0.01 mg/mL) were diluted to generate dilution series of ABA in the range of 3pg/µL -10,000pg/µL. Peak areas of three replicate measurements at each concentration were determined.

4.4 Vion IMS QTof Mass Spectrometer analysis

Methods are described in **Chapter 4.1**, section 4.7

4.5 Statistical analysis

Statistical calculations were performed using SigmaPlot 12.0 (Jandel Scientific, San Jose, CA, United States) software. After testing data for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), the appropriate statistical test (Student's t-test) has been performed to determine a significant difference.

Supplementary materials

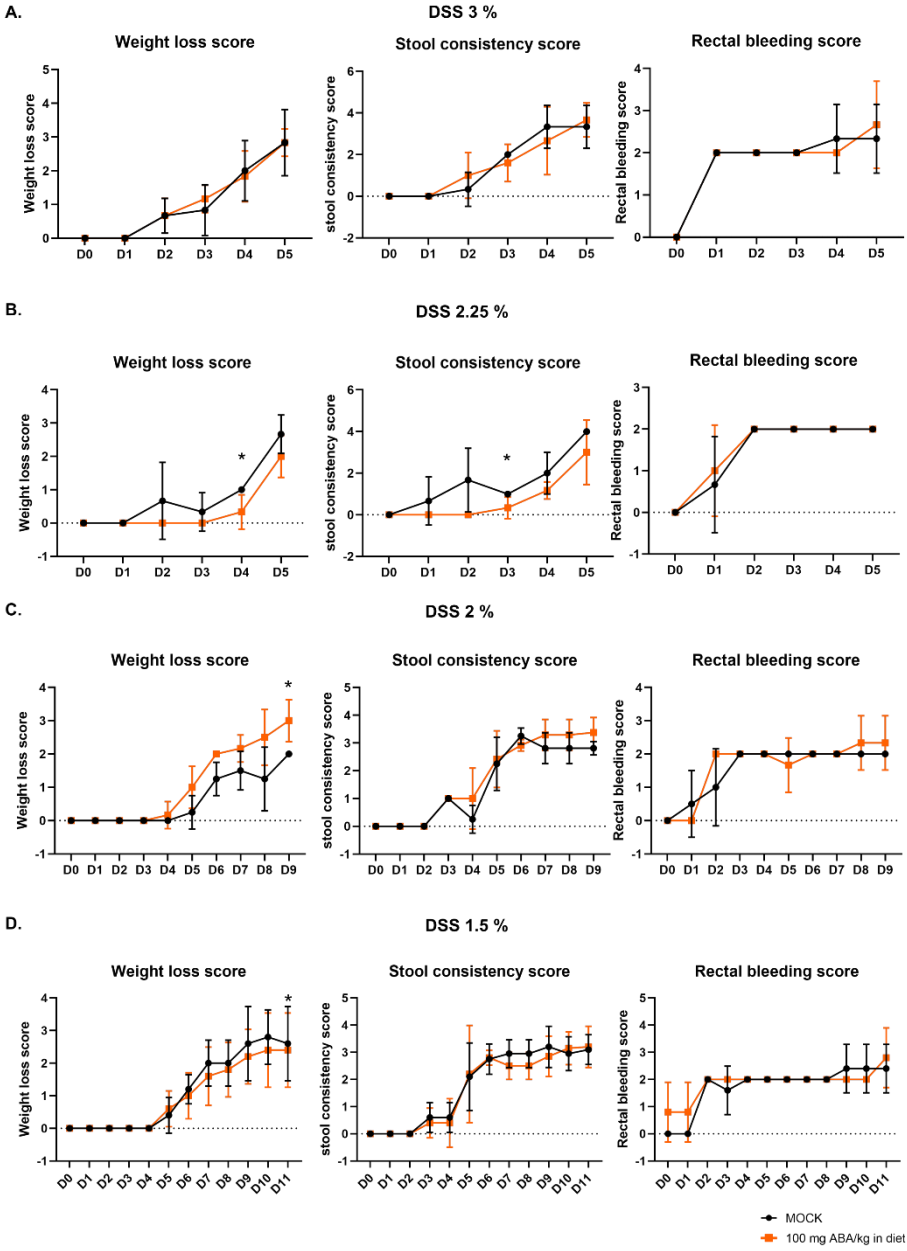

Figure S1. The severity of weight loss, stool consistency, and rectal bleeding during DSS treatment in mice given the control diets and ABA-supplemented diets (100 mg/kg) (see following page for figure legend).

Figure S1. The severity of weight loss, stool consistency, and rectal bleeding during DSS treatment in mice given the control diets and ABA-supplemented diets (100 mg/kg) (see previous page for figure)**.**
(A) The severity of weight loss, stool consistency, and rectal bleeding during 3% DSS. Mice were given the control diets (MOCK, n=6) or ABA-supplemented (n=6) for 35 days, followed by five days of 3% DSS. (B) The severity of weight loss, stool consistency, and rectal bleeding during 2.25% DSS. Mice were given the control diets (MOCK, n=3) or ABA-supplemented (n=6) for 35 days followed by five days of 2.25% DSS. (C) The severity of weight loss, stool consistency, and rectal bleeding during 2% DSS. Mice were given the control diets (MOCK, n=4) or ABA-supplemented (n=6) for 35 days, followed by nine days of 2% DSS. (D) The severity of weight loss, stool consistency, and rectal bleeding during 1.5% DSS. Mice were given the control diets (MOCK, n=5) or ABA-supplemented (n=5) for 35 days, followed by 11 days of 1.5% DSS. The baseline clinical score was determined on day 0. For the weight loss score, no weight loss from baseline was scored as 0, weight loss of 1-5% as 1, 5-10% as 2, 10-20% as 3, and >20% as 4. For stool consistency, well-formed stools were scored as 0, pastry and semi-formed stools were scored as 2, and liquid stools were scored as 4. For rectal bleeding, negative hemoccult was scored as 0, positive hemoccult was scored as 2, and gross rectal bleeding was scored as 4. Error bars represent ±SD. * P-value ≤ 0.05, **P-value ≤ 0.01 and ***P-value ≤ 0.001.

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PART IV: DISCUSSION AND PERSPECTIVES

General discussion and future perspectives

1 Phytohormones in animals – a riddle, wrapped in a mystery, inside an enigma

Anti-inflammatory profiles of salicylic acid (SA) and abscisic acid (ABA) in several human and animal models of inflammation-associated diseases has lead to the proposal of using these phytohormones as nutraceuticals against metabolic syndrome and its comorbidities. Given their inflammation-modulating functions, the discovery of endogenous SA and ABA biosynthesis brought forward a fundamental question: "Do endogenous SA and ABA play a prominent role as immune regulators?"

To unravel the physiological relevance of endogenous SA, we first investigated which tissues could produce or accumulate SA (**Aim 1.1**). As we profiled SA in tissues of mice under normal feeding conditions, this SA content also includes exogenous SA derived from diet and the gut microbiome. Nonetheless, discovering SA-accumulating tissues could reveal the site of action and potential functions of endogenous SA (**Chapter 3.1**). We observed relatively higher SA levels in the thymus and brown adipose tissues (BAT) compared to other tissues such as liver and white adipose tissues (WAT) in mice under normal feeding conditions (**Figure 1**). Moreover, SA treatment slightly altered thymic T cell development, which prompted us to investigate whether endogenous SA plays a role in T cell ontogeny.

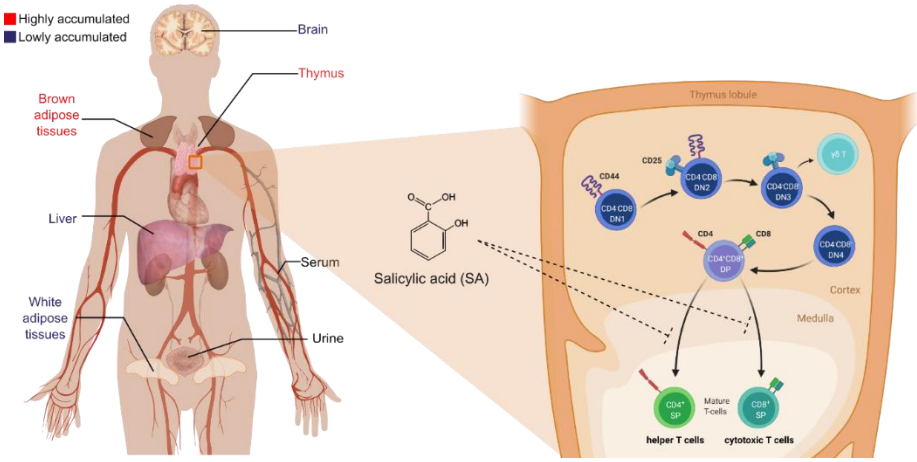

Figure 1. Suggested model for salicylic acid (SA) accumulation in animals under normal feeding conditions and its functional roles.
SA was previously shown to be secreted in human serum and urine **[1]**. Our study showed that under normal mouse feeding conditions, SA accumulates in thymus and brown adipose tissues at relatively higher levels than in white adipose tissues, brain, and liver. Furthermore, we observed that administration of high-dose SA alters thymic T cell development by slightly interfering with the differentiation of $CD4^+CD8^+$ DP T cells into $CD4^+$SP and $CD8^+$SP T cells. This finding raises the question of whether endogenous or accumulated SA would also affect thymic T cell ontogeny. As a summary, the source of SA accumulation and secretion in animals and humans is indicated in the human body diagram. Human body diagrams are under Public Domain licensing and were obtained from Wikimedia Commons, the free media repository.

However, given that human diets are enriched with plant metabolites, one could bring up the following questions: (1) "Would continuous uptake of dietary SA not be sufficient to contribute to T cell ontogeny?" and thus (2) "Is the endogenous production of SA essential?" Lacking a transgenic SA-deficient mouse model, we were unable to answer these questions. To corroborate this, we aimed to generate transgenic mice depleting SA by overexpressing bacterial salicylate hydroxylase NahG (**Aim 1.2; Chapter 3.2**). The reason for employing NahG was SA's unknown biosynthesis pathways. Moreover, this mouse model would be the most informative compared to mutants defective in SA biosynthesis as it will also deplete exogenous SA. However, because we could not demonstrate that NahG is active *in vivo* (**Chapter 3.2**), it remains unclear whether SA depletion would result in impaired thymic T cell development or an impaired immune system (**Figure 1**).

Likewise, we aimed to generate transgenic mice depleting ABA by overexpressing *Arabidopsis CYP707A3* and *ABH2* (*CYP-ABH2*) (**Aim 2.2; Chapter 4.3**). However, due to technical difficulties in measuring

ABA in mice, we did not succeed in demonstrating ABA depletion in transgenic *CYP-ABH2* mice and could thus not reveal whether the absence of ABA would abrogate immune homeostasis *in vivo*. Nonetheless, recent findings point to the role of endogenous ABA in modulating cellular and molecular processes during inflammatory responses (**Table 1**). In plants, ABA increases the resistance of plants against various kinds of stresses such as drought and salinity [2]. Stress stimuli initiate an ABA signaling pathway that tightly regulates molecular processes, allowing plants to adapt and survive [2]. Likewise, the altered ABA levels in diverse animal and human cells in response to different inflammation stimuli could reflect that endogenous ABA might act like an animal stress hormone (**Table 1**). For instance, endogenous ABA was proposed to suppress the pathogenesis of atherosclerosis [3,4]. Therefore, future research should focus more on the interplay between ABA metabolism and inflammation.

It would be interesting to do a broad screen for cellular mechanisms that regulate mammalian ABA metabolism. To implement this, we aimed to develop a rapid, economical, and reliable quantification method for ABA (**Aim 2.1; Chapter 4.1 and 4.2**). We succeeded in generating a highly sensitive ABA biosensor based on the ABA-dependent interaction between *Arabidopsis* monomeric PYL ABA-receptor, PYL1(H87P) or PYL8, and the co-receptor ABI1 (**Figure 2**). The selectivity of this biosensor system was confirmed by the following ABA inactivation strategies: 1) a major *Arabidopsis* ABA 8'-hydroxylase AtCYP707A3, 2) capturing ABA by expressing anti-ABA single-chain Fv antibodies (scFv) anchored to endoplasmic reticulum (ER), and 3) *Arabidopsis* UDP-glucosyltransferase71C5 (AtUGT71C5) (**Figure 2**). These observations in turn support the validity of our transgenic approaches that can help decipher endogenous ABA functions.

In addition, we evaluated the potential use of previously proposed mammalian ABA receptors, LANCL2, PPARγ, and RAR (α, β, γ) in the GAL4-VP16/UAS system [5–9] (**Figure 2**). However, we observed ABA-independent reporter gene expression in the LANCL2-GAL4BD-VP16AD/UAS system due to the nuclear localization sequence in GAL4BD, which abrogated the membrane anchoring of LANCL2. Also, ABA treatment did not induce GAL4BD-PPARγ or GAL4DB-RARs (α, β, γ)-dependent luciferase activity. Therefore, we demonstrated that engineering an ABA biosensor based on LANCL2, PPARγ and RAR (α, β, γ) is not a valid approach.

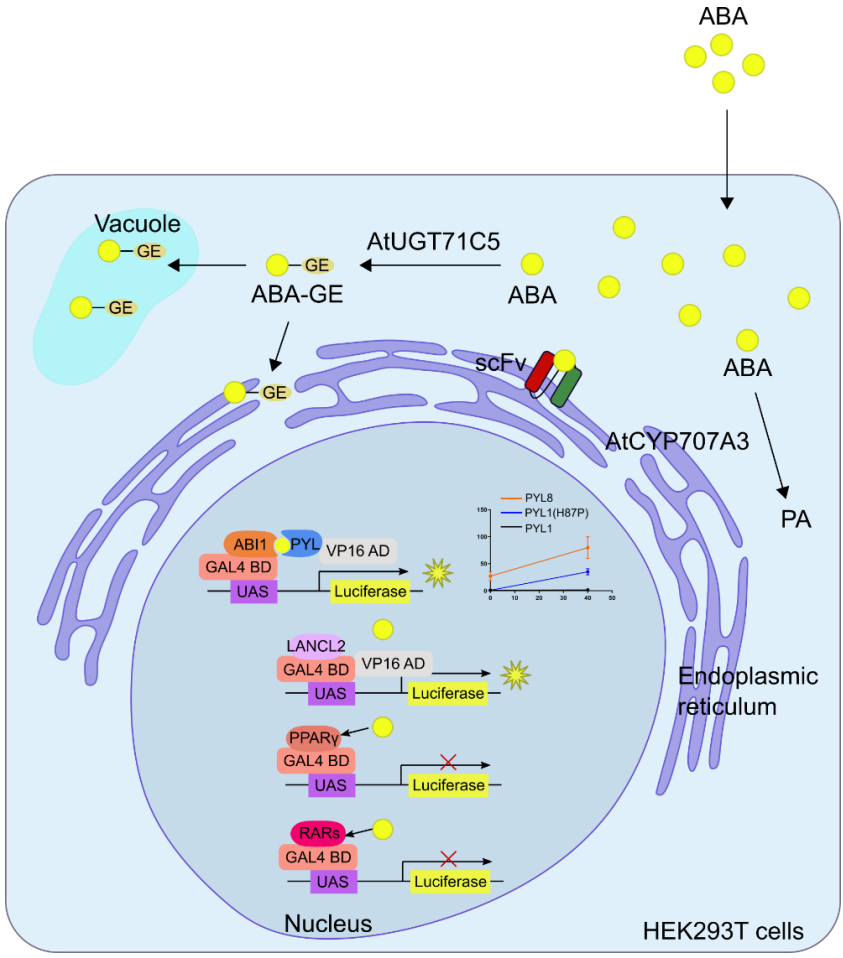

Figure 2. Schematic representation of the ABA biosensors and ABA-inactivation strategies that we generated and evaluated.
In our first approach to engineer an ABA biosensor, we increased the sensitivity of the previously described ABA biosensor system based on the interaction between the plant ABA receptor PYL1 and co-receptor ABI1 by replacing PYL1 with other monomeric PYLs (PYL1 (H87P) and PYL8). In the second approach, we demonstrated that engineering of the ABA biosensor based on LANCL2-GAL4BD-VP16AD or GAL4BD-PPARγ or GAL4DB-RARs (α, β, γ) is not a valid approach. We observed ABA-independent reporter gene expression in the LANCL2-GAL4BD-VP16AD/UAS system. Also, luciferase gene expression was not induced by GAL4BD-PPARγ or GAL4DB-RARs (α, β, γ) upon ABA treatment. Three ABA-inactivation strategies confirmed the selectivity of the improved ABA biosensor. First, the C-8' position of ABA is hydroxylated by cytochrome P450s (CYP), AtCYP707A3. In an alternative catabolic strategy, ABA-glucosyltransferase produces ABA-glucose ester (ABA-GE) from ABA, an inactive form of ABA stored in vacuoles. Lastly, ABA is captured by anti-ABA single-chain Fv antibodies (scFv) in the endoplasmic reticulum (ER).

For the development of a cellular ABA biosensor system, we used HEK293T cells because of their straightforward and highltransfection efficiency. However, the same approach could be used in other cell types too, and previously reported ABA-producing cell types would be plausible options (**Table 1**). To reveal specific signals regulating ABA metabolism, one can stimulate such cells expressing ABA biosensor proteins with a wide range of reagents including pleiotropic-pro-inflammatory cytokines (*e.g.*, Interleukin (IL)-1β, IL-6, and tumor necrosis factor receptor; TNF), chemokines (*e.g.,* monocyte chemoattractant protein; MCP-1), anti-inflammatory cytokines (*e.g.,* IL-4, IL-10, IL-11, and IL-13), lipopolysaccharide (LPS), phorbol myristate acetate (PMA)/ionomycin, and lectins [10]. RAW264.7 cells can be an interesting cellular model, given the inhibitory effect of ABA against LPS-induced inflammatory responses and the increased level of ABA in response to quartz particles in these cells [11,12]. Besides LPS stimulation, one can also induce downstream signaling of LPS activation by overexpressing signaling components such as tumor necrosis factor receptor (TNFR) associated factor 6 (TRAF6) and TANK-binding kinase TBK1 in the ABA biosensor expressing cells, and reveal which specific signaling pathways and transcription factors are involved in ABA biosynthesis in RAW264.7 cells. Noteworthy, as we observed that the treatment of HEK293T cells with IL-1β induced ABA-independent luciferase activation (**Chapter 4.2**), we emphasize the importance of validating the result using mass spectrometry (MS), ABA ELISA, and ABA-inactivation strategies.

The ABA biosensor assay that we developed can not only be used to detect ABA that is present in the supernatant of different cells cultured *in vitro*, it can also be used to determine the *in vivo* bio-distribution of ABA under basal conditions or inflammation using bioluminescence imaging (**Chapter 4.1**) [13]. Determining ABA levels in various mouse tissues or cells in disease models where dietary ABA was previously shown to exert preventive effects (*e.g*., type 2 diabetes and neuroinflammation) or under conditions that were reported to increase blood ABA levels (*e.g*., chronic obstructive pulmonary disease; COPD and atherosclerosis) [3,14–18] would be informative. This bioluminescent mouse model can also be useful to validate the ABA-depleting activity of AtCYP707A3 in transgenic *CYP-ABH2* mice (**Chapter 4.3**).

However, to spot changes in ABA metabolism, the future challenge remains to lower the detection limit of the biosensor further. In several studies describing endogenous production of ABA, ABA ELISA was used to measure intracellular and extracellular ABA levels [11,18]. To our knowledge, the most sensitive ELISA available on the market is the

Phytodetek® competitive ABA ELISA kit (Agdia, Indiana, USA) which displays a detection range between 0.032 nM and 100 nM. To make our cell-based ABA biosensor system competitive regarding its sensitiveness, further optimization will be required to lower the detection limit. In this context, there is still room for further improvement. For example, one could employ a set of orthogonal coiled-coil peptide heterodimer pairs, designed by Lebar *et al.* [19], which could be integrated to regulate the interaction between GAL4BD-ABI1 and PYL-VP16AD. Splitting GAL4BD-ABI1 into GAL4BD with concatenated peptide repeats (10 × P3) and ABI1 with its partner peptide (P4) increased the efficiency of recruiting ABI1-PYL-VP16AD complex [19]. Integrating the [10 × P3]-P4 pair could thus boost the amplitude of the luminescence output signal. Also, altering VP16AD to another transcriptional activator like VP64-p65-Rta (VPR) could improve the transcriptional activation of the target gene [20]. These improvements will allow us to spot small differences close to the detection limit.

Contrary to ABA, the physiological relevance of endogenous SA in inflammation has not been reported so far. Similar to the suggested strategy for ABA, a broad *in vitro* or *in vivo* screening for molecular and cellular mechanisms that influence SA metabolism will be a plausible start. A cell-based assay employing the *Arabidopsis* SA receptor non-expressor of pathogenesis-related genes 1 (NPR1) could be used [21,22]. In *Arabidopsis*, NPR1 is located in the cytoplasm as an oligomer, but in the presence of SA, NPR converts to a monomer and then translocates to the nucleus, which induces various SA-dependent transcriptional events. The nuclear localization of ectopic NPR1 upon SA treatment was previously described in HEK293T cells, supporting the rationale for building NPR1-based SA biosensors [23]. Noteworthy here, however, the GAL4/VP16-UAS system might not be the best option due to the nuclear localization sequence in GAL4BD. Transcription activator-like effector (TALE)- and clustered regularly interspaced short palindromic repeats (CRISPR)-based transcription activation systems might be better candidates. If not, the above mentioned [10 × P3]-P4 could be integrated to split GAL4BD and NPR1 and build GAL4BD-[10× P3] and NPR1-P4-VPR [19]. Upon SA treatment, SA-bound NPR1 will locate into the nucleus, and the interaction between [10× P3] and P4 will result in the reporter gene activation. Challenging the mice with experimental SA-relevant disease models for T2D or cardiovascular disease ($APO^{-/-}$) and imaging the altered levels of SA *in vivo* would be most interesting.

Table 1. Overview of ABA levels in different types of cell cultures.
The data represent the approximate ABA fold change in response to stimuli relative to the basal ABA level.

Cell	Type	Stimuli	*vs.* control (intra cellular)	*vs.* control (extra cellular; supernatant)	Ref.
granulocyte	Primary human	heat shock	3×		[18]
		phorbol 12-myristate 13-acetate (PMA)	2×		
		UV	2.5×	1.75×	[24]
monocyte	Primar human	heat shock	1×	3.5×	[3]
		thrombin-activated platelets (PLT+T)	1.5×	9×	
		MCP-1	2.5×	2.5×	
keratinocytes	NCTC 2544	UV	4×	3.5×	[24]
macrophage	RAW264.7	quartz particles (1h)	1.5×	5×	[11]
	BAL			10×	
microglia	N9	PMA	1×	3×	[25]
		LPS	3-4×	3×	
		LPS+β-amyloid		5×	
		formyl-methionyl-leucyl-phenylalanine (f-MLP)		5.5×	
fibroblast	Primary human (healthy)	UV	1-1.5×		[26]
	Primary human (systemic sclerosis)		2×		
β-cell	Primary human	glucose	2×	25×	[27, 28]
	INS-1		2×	10×	
	RIN-m		2×	20×	

One path we did not pursue in this PhD is the generation of ABA- or SA-insensitive mutants by targeting their receptors. For instance, plants less sensitive to ABA or SA were generated by mutating *PYL* or *NPR* and utilized to reveal their roles in abiotic or biotic stress resistance [29–32]. Lantionine synthetase C-like protein 2 (LANCL2), a peripheral membrane receptor, was reported as a mammalian ABA receptor [7,33]. LANCL2 originally resides in the plasma membrane by N-terminal glycine myristoylation but translocates to the nucleus upon ABA binding [7]. The dissociation constant (K_d) of endogenous LANCL2 for ABA in HeLa cells transfected with CD38 is low (~8 nM), whereas the K_d shown for ABA binding to human recombinant LANCL2 is relatively high (110 µM), indicating that the post-translational modification of LANCL2 such as myristoylation or the interaction with additional proteins is possibly involved in the high-affinity binding of ABA to native LANCL2 [34–36]. In previous studies, *lancl2* knockdown cells and mice were shown to be

less sensitive to exogenous ABA [16,28,33,34,37]. However, the low remaining ABA responsiveness in LANCL2-deficient muscle cells and adipocytes suggests the presence of another ABA receptor than LANCL2 [28,33,37]. For instance, also LANCL1 was shown to bind ABA [38]. LANCL2$^{-/-}$ mice seem to express a higher level of LANCL1 in skeletal muscle than wild-type mice [38]. LANCL1 was shown to be redundant with LANCL2 in ABA-induced glucose uptake and respiration in L6 myoblasts [38]. However, *lancl1-3* triple knock-out mice do not develop any overt phenotype [39,40]. This could indicate that ABA-insensitive mice do not have an impaired immune system. Alternatively, it could also reflect the presence and role of additional receptors. Plant ABA receptors Pyrabactin resistance 1 (PYR1)/PYR1-Like (PYL)/Regulatory components of aba receptors (RCAR) are homologous of human StAR-related lipid-transfer (START) domain proteins [41]. The mammalian START domain forms a hydrophobic pocket binding to sterols. The START protein family consists of 15 proteins, and each member contains the START domain with other distinct domains that direct their protein localization and activity [42]. It would thus be interesting to determine whether these human START proteins directly bind to ABA. Recombinant proteins of these 15 START domain family proteins could be produced, and their ABA-binding features can be determined using existing techniques such as surface plasmon resonance (SPR) spectroscopy [43], isothermal calorimetry (ITC) [44], and thermoshift assays [45]. Alternatively, the discovery of novel ABA-binding proteins using a proteome-wide approach, such as limited proteolysis (LiP) with mass spectrometry (MS), might be beneficial [46,47]. This way, on top of discovering ABA-binding receptors, ABA binding proteins involved in its metabolism could be discovered.

In contrast to ABA, a proteome wide-screening for SA-binding proteins in HEK293 cell lysates using photoaffinity labeling combined with MS revealed many direct target proteins of SA, namely high mobility group box protein (HMGB1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glycolytic enzymes alpha-enolase (ENO1), and pyruvate kinase isozyme M2 (PKM2) [48,49]. These proteins are involved in essential physiological functions [50–53]. HMGB1 and GAPDH knock-out mice develop lethal phenotypes [54,55]. Therefore, the generation of SA-insensitive mutants by targeting these proteins needs to be considered. We thus suggest to generate knock-in mice bearing a mutation in the SA-binding site of these proteins. For instance, mutation of Arg24 and Lys28 of HMGB1's box A domain would be options as they were shown to be responsible for its SA binding [56].

Other interesting pieces that we did not address in this PhD are the ABA and SA biosynthesis pathways. In **Chapter 1**, we suggested that

homologs of *Botrytis cinerea* ABA biosynthetic genes are unlikely to be responsible for human ABA production. Nevertheless, to corroborate this, one could engineer fungal ABA biosynthesis in yeast, given its well-established tools and disciplines to steer metabolic flux by introducing foreign genes [57,58]. Subsequently, replacing the *Botrytis cinerea* genes one by one with human homolog genes could identify biosynthetic genes [59]. Otto *et al.* successfully produced ABA in yeast by expressing *B. cinerea* genes *BcABA1*, *BcABA2*, *BcABA3*, and *BcABA4* in *Saccharomyces cerevisiae* [60]. *BcABA1 and 2* encode cytochrome P450 monooxygenase (CYP); therefore, investigating 57 human genes encoding P450 would be a plausible start. Lievens *et al.* demonstrated a phylogenetic tree of BcABA1, BcABA2, and human P450 proteins [59]. Human CYP proteins that are relatively closer to BcABA1 and 2, such as HsCYP20A1 and HsCYP39A, might be options [59].

An alternative approach to unravel ABA biosynthesis would be a genome-wide screening for ABA biosynthetic genes using CRISPR-based activators [61] or an ORF expression library [62] in HEK293T cells expressing our ABA biosensor system. Among a collection of genes in the library, overexpression of endogenous human ABA biosynthetic genes will increase reporter gene activation. Cells expressing abundant reporter gene expression (*e.g.,* Green Fluorescent Protein; GFP) can be subsequently sorted and identified. To implement this, an ABA influx or efflux should be blocked to screen ABA-producing cells among a pool of cells. In this way, ABA-producing cells will not secrete and non-ABA-producing cells will not take up extracellular ABA and induce GFP accumulation. However, ABA transport in HEK293T cells should be identified in advance. The only known ABA transporters are anion exchange proteins (AE) [63,64]. AE1 (SLC4A1) regulates ABA uptake and eventually leads to ATP release from erythrocytes in a LANCL2 and adenylate cyclase-dependent manner [64]. AE2 (SLC4A2) was shown to be responsible for the ABA influx in human nucleated cells [63]. As AE2 is the most widely expressed AE, one could thus use an inhibitor for AE2 like 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) to determine which transporter is involved [65,66]. Noteworthy, this genome-wide screening has a potential risk, considering the artifact discussed in **Chapter 4.2**. Simultaneous cellular or genetic manipulation can easily cause spontaneous activation of the reporter gene. One could complement this by carrying out both loss-of-function and gain-of-function studies using CRISPR-based activator and repressor libraries [61]. Mapping ABA biosynthetic genes by matching results from both screenings might rule out inconsequential candidates. In addition, validation of screening results by an independent ABA measurement

method such as MS will be necessary.

Paterson *et al*. reported a $^{13}C_6$-BA precursor feeding study where a $^{13}C_6$ BA load ingested by patients (who were fasting and underwent major colorectal surgery) increased the excretion of $^{13}C_6$-labelled urinary salicylic acid and salicyluric acid [1]. Hence, a benzoic acid 2-hydroxylase (BA2H) was claimed to produce SA from BA, which might derive from milk-based diets and food preservatives in humans. The BA2H enzyme activity was already confirmed in tobacco, soybean, and rice, but the corresponding genes still need to be identified [67–69]. This prompted an idea to identify BA-interacting proteins via a proteome-wide approach, using LiP-MS. To verify BA2H activity in animals, we performed the BA2H enzyme assay. However, HeLa cells, HEK293T cells, and primary splenocytes did not convert BA-α-^{13}C to SA-α-^{13}C (**Chapter 3 Addendum**). Therefore, it is doubtful whether BA is an authentic precursor. An alternative approach should be taken using similar strategies suggested for ABA. One could carry out a genome-wide screening employing the above-mentioned NPR-based biosensor (GAL4BD-[10× P3] & NPR1-P4-VPR). Furthermore, in order to prevent SA influx or efflux and sort/identify SA-producing cells, inhibitors for the putative SA transporter could be used, namely inhibitors of organic anion transport [70], MCT transport [71], and organic anion transporting polypeptide [72–74].

2 Lessons for future research on phytohormones

Based on the findings and challenges we faced in our novel investigation on the role of endogenous SA and ABA, we would like to suggest a workflow for future studies on ABA, SA, and other phytohormones, namely indole-3-acetic acid (IAA) and cytokinins (CK). The ameliorating effect of IAA in HFD-induced NAFLD and anti-cancer properties of CK suggest the involvement of IAA and CK in the interplay between inflammation and metabolism [75–81].

2.1 A cell-based bioassay coupled with MS

One of the biggest challenges in this PhD was developing a reliable quantification method for ABA. Nonetheless, the high-resolution of the ABA biosensor and the reliability of MS compensated for each of their respective drawbacks, allowing us to validate the ABA-inactivation strategies and evaluate possible artifacts in the cell-based reporter assay. We thus suggest setting up two independent quantification methods that go hand in hand towards a detection platform enabling high-throughput and reliable measurements.

For IAA detection, we propose employing the IAA-inducible reporter system engineered by Zhao *et al*. [82]. This system utilized orthogonal *Arabidopsis* auxin signaling components: transport inhibitor response 1 (TIR1) and Aux/IAA transcriptional repressor in the GAL4/VP16-UAS system [82,83]. In the presence of auxin, TIR1 brings an E3 ubiquitin ligase complex (Skp-Cullin-F-box containing protein complex; SCF complex), which subsequently degrades the transcriptional repressor, thereby releasing auxin response factors [82,84]. In this biosensor system, given the nature of SCF box in catalyzing ubiquitination, the TIR mutant was mutated (E7K/E10K) to be unable to bind to SCF complex [82]. In the presence of IAA, the heterodimerization of the TIR1 mutant and the transcriptional repressor induces proximity of GAL4BD and VP16, which leads to the reporter gene activation and enables the quantification of IAA in mammalian cells [82].

A mammalian CK biosensor has not been developed yet. A well-known CK signal transduction pathway in plants is the histidyl-to-aspartyl system (His-Asp phosphorelay), a multi-component signaling system conserved between plants, bacteria, and fungi, but not in animals [85–87]. Because of phosphorelay features, employing this pathway to develop a mammalian CK biosensor would be rather complicated and require several signaling components. A CK-dependent homodimerization of *Arabidopsis* histidine kinases 4 (AHK4) could be applied, which previously was shown to allow CK detection in an electrochemical biosensor [88,89]. However, one could expect a

drawback of this system with the possibility of forming a GAL4BD-AHK4 homodimer, which should be further investigated in the future. A CK biosensor was previously engineered in yeast employing *Malus domestica* CHASE Histidine Kinase receptor 2 (MdCHK2). This apple tree CHK2 perceives the broadest range of CK like free-bases, ribosides, glucosides, and methylthio-cytokinins [90]. The CK-dependent growth assay was developed by replacing yeast transmembrane histidine phosphotransfer kinase and osmosensor (SLN1) with MdCHK2 in *S. cerevisiae*. However, as this system used the endogenous yeast osmostress signaling pathway, other downstream signaling components should be integrated to apply this system to mammalian cells. Cell-based assays to detect IAA or CK will benefit high-throughput metabolite measurements. However, reflecting on our findings showing possible artifacts leading to ABA-independent reporter gene expression (**Chapter 4.2**), it should be accompanied with MS.

2.2 Phytohormone-deficient mutants

Another bottleneck that hindered the discovery of functional roles of endogenous SA and ABA was the lack of a transgenic SA or ABA-deficient or –insensitive mouse model. Based on the experience and perspective we developed during our study, we discuss the possible genetic models that will augment understanding the role of endogenous IAA and CK.

IAA was shown to be produced in several animal tissues such as liver, kidney, and brain [91–93] and massively secreted in human patients with neuromuscular diseases, phenylketonuria, diabetes mellitus, liver injury, and cancer [75,91,94]. IAA in animals was shown to be synthesized from tryptophan (Trp) via two pathways: 1) the tryptamine (TAM) pathway or 2) the indole-3-pyruvic acid (IPA) pathway (**Chapter 1**). Recent studies identified that *Il4i1* encoding L-amino acid oxidase (LAAO) is responsible for the conversion of Trp to indole-3-pyruvic acid (IPA), which spontaneously converts into Indole-3-acetaldehyde (I3A), IAA, and Indole-3-lactic acid (ILA) [95,96]. Indole derivatives, including IAA, act via the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor [97]. Therefore, one could target IPA or AHR pathways to produce IAA-deficient or insensitive mouse mutants. However, given the involvement of other various Trp-derived indoles and AHR ligands, these mutants will not solely show the consequence of IAA -deficiency or -insensitiveness. Moreover, dietary Trp could be metabolized by the human host and by commensal gut microbiota to produce AHR ligands [98,99]. Therefore, inhibition of IAA biosynthesis *in vivo* would not exclude the dietary and gut microbiome source, suggesting employing a plant or bacterial heterologous IAA-degradation

pathway. For instance, IAA-amino acid conjugate synthase (GH3), UGT, IAA-methyltransferase, dioxygenase could be options [100–105].

CKs are active in various modified forms like CK ribosides and free-based CKs (**Chapter 1**), making it complicated to generate CK-deficient or insensitive mutants. **Chapter 1** described the putative human CK N6-isopentenyl adenosine biosynthesis via a tRNA isopentenyl transferase 1 (TRIT1) that transfers an isopentenyl residue to tRNA-adenine 37 [106–109]. N^6-isopentenyl adenosine 5'-diphosphate can be released from prenylated-tRNA through tRNA-degradation and might be converted to iPA consequently. TRIT1 is shown to regulate mitochondrial diseases and lung carcinogenesis [107–109]. However, this might be due to the deficiency of the intermediate tRNA added with isopentenyl residue, not iPA. In addition, mammalian adenosine receptors (AR), A3R, and A2AR are involved in CK signaling [110–112]. Before utilizing these receptors to generate CK-insensitive mutants, CK-binding sites remain to be identified. Therefore, plant and fungal enzymes responsible for CK homeostasis could be employed to manipulate CK levels *in vivo* and *in vitro*, such as cytokinin oxidase [80,113].

A major issue in characterizing the role of SA was the maintained SA levels in blood and mice tissues of interest, albeit NahG was expressed. Moreover, although we opted for AtCYP707A3 to degrade ABA *in vivo*, we observed that the efficiency of AtCYP707A3 in lowering ABA levels was lower than anti-ABA scFv, which could be improved by pairing NADPH-cytochrome P450 reductase [114]. Our experience emphasizes the importance of evaluating different heterologous metabolic pathways *in vitro* before producing transgenic mouse lines, which demands high cost and labor. Therefore, the enzymatic activity at the physiologically relevant metabolite level and in animal model-, tissue-, and cell-types of interest must be confirmed in advance. Also, the risk of artifacts driven by the consequent (by-)enzymatic reaction product supports the importance of evaluating at least two different metabolic strategies [115].

2.3 Conclusions and future perspectives

Collectively, we could not reveal the role of endogenous SA and ABA due to several technical difficulties. However, reflecting on these challenges could contribute to future phytohormone-related research (**Figure 3A**). The workflow of a phytohormone research project should start with establishing two independent detection methods that allow rapid, high-throughput, and reliable phytohormone measurement (**Figure 3B**). Employing a cell-based bioassay along with genetic or pharmacological means would allow to screen for biosynthetic genes and relevant molecular and cellular mechanisms (**Figure 3C**). Also, the

activity of enzymes for lowering metabolite levels could be evaluated. We emphasize the importance of evaluating their enzyme activity at physiologically-relevant metabolite levels. In addition, their animal-, tissue- and cell-type specificity should be taken into account. Subsequently, the verified metabolic engineering strategies could be translated into *in vivo* models (**Figure 3D**). Flow cytometry-mediated immunophenotyping of various immune cells could be implemented to assess the impact of metabolic engineering on the immune response. Moreover, generating luciferase reporter mice might not only be beneficial to verify transgenic mice but also to discover relevant inflammatory stimuli and disease models (**Figure 3E**). Nonetheless, we stress the complementary use of MS-based metabolomics when deciphering the result of screenings performed with reporter assays.

Another lesson we learned during this study is that we still lack the knowledge to establish the proposed tools and approaches (**Figure 3**), namely phytohormone -metabolic genes (biosynthesis/catabolism), -responsive genes, and -receptors. Therefore, future studies could use a 'multi-omics' approach to get a bigger picture of which genes are involved in regulating endogenous phytohormone metabolism [116]. The rationale behind this approach is also supported by the number of public gene expression data sets deposited in the NCBI GEO archive [117]. For instance, the number of datasets associated with ABA in animals is only 15 (from one study [8]), whereas transcriptomics implemented in *Arabidopsis* relevant to ABA reaches 499. First, to understand phytohormone metabolism, we could integrate multiple transcriptomics and proteomics data of cells or tissues under inflammatory conditions that were shown to increase or decrease endogenous phytohormone production. In this way, we could augment our understanding of which pathways are driving or affected by the metabolism of a respective molecule. The challenge here would be different diseases' etiology and cell physiology. It will thus be important to integrate as much data as possible to identify a relevant pathway. Second, as mentioned above, discovering phytohormone-binding proteins via a proteomics approach is advantageous. This will reveal phytohormone receptors and enzymes that catalyze anabolism or catabolism. From a biomedical perspective, finding a novel binding proteins could also reveal additional mechanisms of action. Third, upon identification of anabolic and catabolic enzymes, metabolomics or lipidomics could be integrated to determine metabolic flux diverted from steering respective phytohormone metabolic pathways, which might reveal their role in immunometabolism. Also, reversely, similar to the profiling of SA in different tissues of mice (**Chapter 3.1**), a metabolome-wide screening of urine or blood of patients with different inflammatory

diseases could be integrated into the first transcriptomics approach. In conclusion, we believe that the knowledge delivered in this PhD provides a proof-of-concept and lays the foundation for future phytohormone research.

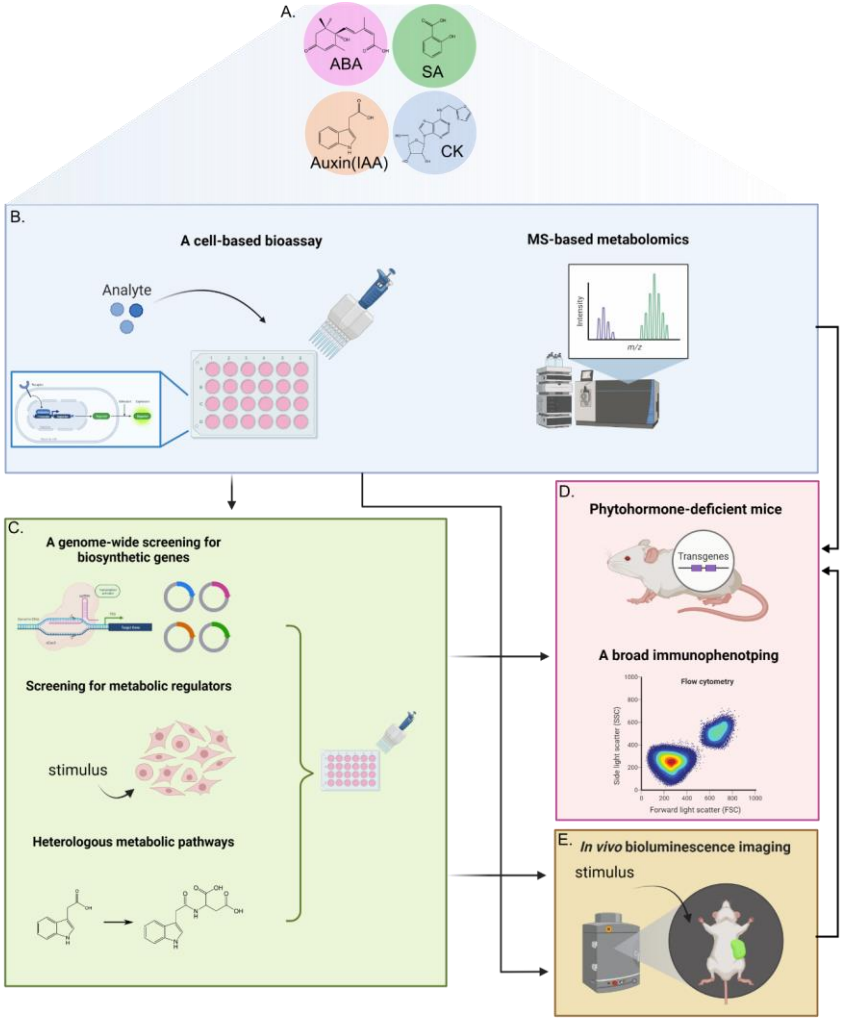

Figure 3. A proof-of-concept towards the characterization of phytohormones.
Based on the experience we gained and the challenges we faced during this PhD, we suggest a proof-of-concept for studying the function and metabolism of endogenous phytohormones **(A)**. In the first stage, two independent quantification methods are fundamental, such as a cell-based reporter assay and MS-based metabolomics **(B)**. A cell-based assay could screen biosynthetic genes and relevant-molecular and cellular mechanisms. Also, heterologous metabolic pathways could be evaluated **(C)**. The verified metabolic strategies could be translated into *in vivo* models. The immunophenotype of transgenic mice could be analyzed by flow cytometry **(D).** Also, luciferase reporter mice for *in vivo* monitoring of the metabolite levels might be beneficial to discovering relevant inflammatory stimuli and to verifying transgenic phytohormone-deficient mice **(E)**. This figure was created with BioRender.

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PART V: ADDENDUM

Curriculum Vitae

Curriculum Vitae

Seo Woo Kim

Personal
04.02.1992
South Korean

Contact
seowki92@gmail.com
+32 465358489
Reigerstraat 25 9000 Ghent, Belgium

Experience & Skills

2017-present

Doctoral researcher at VIB-UGent Center for Inflammation Research and VIB-UGent Center for Plant Systems Biology, Belgium

Funded by the VIB International PhD program

- Investigation of the roles of phytohormones in immunometabolism and the potential of plant-based nutraceuticals
- Interdisciplinary research in collaboration with VIB-UGent research teams
- Development and qualification of biosensors for a biomarker in inflammation-driven diseases

Acquired competences

- Organization, execution, and troubleshooting of experiments
- Managing expectations of collaborators
- Working in an international environment
- Writing of experimental protocols, scientific papers, and literature reviews
- Effective oral presentations

- Guidance of master students and bachelor students

2015-2017 — Research assistant at Crop Biotech Institute, Kyung Hee University, South Korea

- Study on the expression patterns of circadian rhythm genes and daily level of melatonin in athletes and sedentary university students
- Investigation of the mutual interaction between water deficiency response and diurnal regulation in rice

2015 — Teaching assistant for the undergraduate Biochemistry laboratory course at Kyung Hee University, South Korea

Education

2022 (expected) — Doctor of Science, Biochemistry and Biotechnology, Ghent University, Belgium

2017 — Master of Science, Plant Biotechnology, Graduate School of Biotechnology, Kyung Hee University, South Korea

2015 — Bachelor of Science, Genetic Engineering, Life Sciences, Kyung Hee University, South Korea

- Exchange student program at the Institute of Technology, Linköping University, Sweden (2013)

Scientific Expertise

Immunology & cell biology — Cell lines and primary cell culture, transfections, lentiviral transductions, flow cytometry, mouse handling, intraperitoneal injections and oral gavage in mice, maintaining a mouse colony, mouse necroscopy and preparation of tissues for histology and metabolite profiling

Molecular biology	gene expression analysis, SDS-PAGE, western blotting, cloning, cell-based reporter assays, microbiology techniques
Data analysis	FlowJo, GraphPad Prism
Certificate	laboratory animal science (Felasa C attest)
Published papers	Kim, S.W., Alci, K., Van Gaever, F., Driege, Y., Goossens, A., Beyaert, R., Staal, J., 2022. Engineering a highly sensitive biosensor for abscisic acid in mammalian cells (manuscript in preparation). Kim, S.W., Goossens, A., Libert, C., Van Immerseel, F., Staal, J. and Beyaert, R., 2020. Phytohormones: Multifunctional nutraceuticals against metabolic syndrome and comorbid diseases. Biochemical pharmacology, 175, p.113866. Song, Y., Choi, G., Jang, L., Kim, S.W., Jung, K.H. and Park, H., 2018. Circadian rhythm gene expression and daily melatonin levels vary in athletes and sedentary males. Biological Rhythm Research, 49(2), pp.237-245. Kim, S.W., Lee, S.K., Jeong, H.J., An, G., Jeon, J.S. and Jung, K.H., 2017. Crosstalk between diurnal rhythm and water stress reveals an altered primary carbon flux into soluble sugars in drought-treated rice leaves. Scientific reports, 7(1), pp.1-18. Kim, S.W., Jeong, H.J. and Jung, K.H., 2015. Integrating omics analysis of salt stress-responsive genes in rice. Genes & Genomics, 37(8), pp.645-655.

Additional Information

Personality	strong problem solving attitude, critical thinking, independent, structured, eager to learn
Languages	English (professional working proficiency)

	Korean (native proficiency)
	Dutch (Elementary proficiency, A1 level)
IT skills	MS office, Inkscape, Zotero
Hobbies	sports, sign language, dance

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