Increasing Yield on Dry Fields: Molecular Pathways With Growing Potential

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Summary

Drought stress constitutes one of the major constraints to agriculture all over the world, and its devastating effect is only expected to increase in the following years due to climate change. Concurrently, the increasing food demand in a steadily growing population requires a proportional increase in yield and crop production. In the past, research aimed to increase plant resilience to severe drought stress. However, this often resulted in stunted growth and reduced yield under favorable conditions or moderate drought. Nowadays, drought tolerance research aims to maintain plant growth and yield under drought conditions. Overall, recently deployed strategies to engineer drought tolerance in the lab can be classified into a ‘growth-centered’ strategy, which focuses on keeping growth unaffected by the drought stress, and a ‘drought resilience without growth penalty’ strategy, in which the main aim is still to boost drought resilience, while limiting the side-effects on plant growth. In this review, we put the scope on these two strategies and some molecular players that were successfully engineered to generate drought tolerant plants: abscisic acid, brassinosteroids, cytokinins, ethylene, ROS scavenging genes, strigolactones and aquaporines. We discuss how these pathways participate in growth and stress response regulation under drought. Finally, we present an overview of the current insights and future perspectives in the development of new strategies to improve drought tolerance in the field.

Introduction: Multifaceted Drought and Drought Tolerance

During their life cycle, plants are exposed to a wide variety of environmental stresses such as drought, which is one of the most devastating stresses for crops (Boyer, 1982). Drought stress is defined as a period of reduced fresh water availability that decreases crop growth and is a major cause of income loss in agriculture (Wallander, 2017). Several widely cultivated crops are highly sensitive to drought (e.g. maize, soybean, potatoes and various vegetables), but also second-generation bio-energy plants (e.g. poplar) have high water demands. During the last decade, between a quarter and two-thirds of the US counties were designated as drought disaster area each year (USDA - FSA, 2012-2020), and due to global warming and the continuous decrease in water available for irrigation, drought and its impact on world-wide agriculture are likely to get even worse (McFadden et al., 2019).

Drought stress can occur at different moments during the plants life cycle, which has different consequences (Fuad-Hassan et al., 2008; Araus et al., 2012; Bledsoe et
In general, drought stress occurring during the vegetative growth phase has a negative impact on shoot biomass and triggers changes in root system architecture. In a process called hydrotropism, lateral root angles decrease in order to direct roots deeper in the soil, a process regulated by multiple phytohormones (for a review, see Dietrich, 2018). While roots keep on growing, shoot growth is rapidly reduced upon perception of a decrease in soil water potential. This shoot growth reduction is an adaptive mechanism that evolved to restrict the evaporative surface and redirect the energy from growth towards mechanisms to protect the plant against persisting drought (e.g. thickening of the leaf cuticula). Shoot growth limitation is, thus, an actively regulated process and is not merely the consequence of decreased hydraulics or carbon deficit (reviewed in Muller et al., 2011; Claeys and Inzé, 2013; Dubois and Inzé, 2020). At the cellular level, shoot growth is constrained by active inhibition of both cell division and cell expansion, two processes driving growth of young plants. Several molecular pathways connect drought to cell division arrest, such as the inhibition of positive cell cycle regulators like CYCLIN-DEPENDENT KINASE A (CDKA), CDKB, or CYCLINS (Granier et al., 2000; Skirycz et al., 2011a; Avramova et al., 2015; Kamal et al., 2020), the activation of cell cycle inhibitory proteins from the KIP-RELATED or SIAMESE-RELATED families (Pettkó-Szandtner et al., 2006; Peres et al., 2007; Dubois et al., 2018a) or the activation of cell proliferation-regulating miRNAs in growing Brachypodium distachyon leaves (Bertolini et al., 2018). Initiated within days upon the last watering, cell division arrest was found to be the earliest growth-repressive cellular process induced in young leaves (Dubois et al., 2017). However, a recent study using natural variation in Arabidopsis showed that the extent of cell division reduction is not correlated with the final leaf size decrease under drought. Instead, the degree of cell expansion inhibition is a major determinant of the overall leaf area reduction under drought (Chen et al., 2021). At the molecular level, an increased lignification and the stabilization of growth repressors, like DELLA proteins, restrict cell expansion by inhibiting EXPANSIN proteins and reactive oxygen species (ROS), which both loosen the cell wall under favorable conditions to enable growth (Tenhaken, 2015). When plants perceive drought later during development, in the reproductive phase, seed development is affected. In maize, for example, drought has the most negative effect when it occurs around pollination, as it triggers pollen sterility, tassel necrosis, incomplete fertilisation by increasing the anthesis-silking interval and kernel abortion (Fuad-Hassan et al., 2008; Araus et al., 2012; Bledsoe et al., 2017). In fact, a short period of drought starting during the reproductive stage in maize is eventually more detrimental to yield than a long-term early drought treatment (Verbraeken et al., 2021).
Besides the timing at which drought strikes the plant during development, also drought severity will largely determine the molecular, physiological, and phenotypic outcome of water limitation (Figure 1) (Verslues et al., 2006; Baerenfaller et al., 2012; Lawlor, 2013). When the level of drought stress is mild, plants aim at restoring the balance between water uptake and water loss. Water loss is limited, on the one hand, via stomatal closure and, on the other hand, by decreasing leaf growth. Remarkably, this leaf growth reduction occurs mainly during the day in maize and Arabidopsis, while growth rates recover at night (Caldeira et al., 2014; Dubois et al., 2017). Moreover, the tissue water potential is decreased via accumulation of solutes, like proline, to counteract cell shrinking (Haswell and Verslues, 2015), and root growth is stimulated to increase water uptake (Gupta et al., 2020). There is no extreme cellular damage or high levels of ROS, and carbon and energy are still available to sustain growth at reduced rate. This is called the ‘stress avoidance’ response (Verslues et al., 2006). If the level of drought increases, ‘stress tolerance’ mechanisms can be activated to protect the plants against cellular damage (Verslues et al., 2006). This includes accumulation of protective proteins (e.g. LEA proteins) and detoxification of accumulated ROS. When exposed to prolonged drought stress at a level that will not threaten survival, plants eventually acclimate (Figure 1). In maize, leaf and stem elongation is maintained during a longer period (Nelissen et al., 2018; Verbraeken et al., 2021) and also in Arabidopsis, leaf growth recovers (Tenhaken, 2015). This recovery likely involves the rearrangement of microtubule via MICROCTUBULE-ASSOCIATED STRESS PROTEIN1 (MASP1) (Bhaskara et al., 2017b), but possibly also the upregulation of photosynthesis-related genes (Des Marais et al., 2012) and EXPANSIN genes, reported under prolonged moderate drought (Harb et al., 2010) or osmotic stress (Skirycz and Inzé, 2010). Below ground, the changes in root architecture under drought have also been shown to be a major determinant for shoot biomass maintenance under drought (Klein et al., 2020). Thus, there is not one unique drought response: drought severity is a crucial determinant for the drought response, as the set of genes regulating the drought response at moderate drought level is distinct from those involved in life-threatening stress (Skirycz et al., 2011b). In addition, the response to water limitation is also largely determined by the organ, its developmental stage, and even the cell type (Dinneny et al., 2008; Skirycz et al., 2010; Thatcher et al., 2016; Wang et al., 2019a; Verbraeken et al., 2021).

With the multifaceted drought response, goes a multifaceted “drought tolerance” phenotype. As each of the factors that determines the molecular drought response can also affect the resulting phenotype, it is crucial to precisely define which phenotypic outcome is aimed at when it comes to breeding or engineering ‘drought-tolerant’ plants.
The trade-off between plant growth and stress resilience implies that when stress resilience is boosted, growth could be hampered, and it is debatable whether it is possible to simultaneously increase growth under drought, at mild drought levels, and survival to more severe drought (Figure 1) (Claeys and Inzé, 2013; Huot et al., 2014). For example, it has been shown that numerous transgenic Arabidopsis lines that were reported in literature to survive better to severe drought, do not grow better than wild-type plants when the drought stress is mild (Skirycz et al., 2011b). During many years of drought research in the lab, the importance of this trade-off and of the multiple factors determining the plant response to water restriction have been underestimated, contributing, together with the intrinsically complex drought response, to the limited success by which genes improving growth in model plants under drought in the lab have been translated into improved drought tolerance of field-grown crops (Simmons et al., 2021). In fact, many drought resilient varieties generated in the lab score positive for survival under severe drought, but many of these lines are not optimal due to a poor performance under well-watered or milder drought conditions in terms of final yield.

Nowadays, yield and biomass, which are seen as the most important outcome in the field (Simmons et al., 2021), are also the targeted phenotypic output for research in the lab. This redirected the research objectives (Figure 1) and the way a "drought-tolerant plant" is defined. Therefore, in this manuscript, we define drought tolerance as the capacity to maintain plant biomass and yield under mild to moderate drought stress in soil. Consequently, studies in which survival to severe drought was improved by generating stunted plants with reduced yield, are not included in this review. In addition, whereas studies using in vitro mimics for drought stress (mannitol, sorbitol, PEG, …) are valuable for discovery of new genes involved in low water potential response, we do not discuss them if the results were not validated using drought stress in soil. In the recent years, literature showed two major research strategies that are currently used to maintain yield under drought (Figure 1). First, a growth-centered approach, in which the main aim is to boost growth under moderate drought. This approach holds great potential to increase biomass, as growth is specifically boosted under stress. However, it is unclear how these plants would behave under severe drought (Figure 1). In addition, multiple studies currently aim at increasing resilience to more severe drought but, importantly, they nowadays do monitor that yield and biomass are not negatively affected. In our opinion, these studies might also be beneficial for the engineering of plants tolerant to wider stress spectrum. This second approach could be called ‘resilience without growth penalty’.
In this review, we give a non-exhaustive overview of molecular pathways that are important in yield, growth and/or biomass control under mild or moderate drought stress. As the alteration of nearly every metabolic or regulatory pathway can have a negative effect on plant growth, we focus on several examples of pathways that have the potential to improve plant yield under water-limiting conditions, either in a growth-centered or a ‘resilience without growth penalty’ approach. We explore the molecular pathways behind these success stories to illustrate how they exactly control plant growth or yield under drought stress. Importantly, most of the approaches described here were used in lab-based research and it is still unclear if they could improve drought tolerance in the field, where drought often occurs in combination with other environmental stresses and where plants grow side by side, possibly competing for water.

Increasing Water Use Efficiency and Limiting Growth Reduction via Modulation of Abscisic Acid

In a wide range of land plant species, abscisic acid (ABA) is the major hormone orchestrating responses to water deficit and, consequently, the ABA biosynthesis and signalling pathways are logical targets for the engineering of drought tolerance in plants (Figure 2A). Rapidly upon drought stress, ABA accumulates in the shoot and particularly in guard cells, where a phosphorelay-mediated signalling cascade causes stomatal closure (Hsu et al., 2021). This, in turn, reduces transpiration rates and increases water use efficiency (WUE), e.i. more carbon gain per unit of water. Reduction of stomatal gas exchange limits photosynthesis, which in the long-term hampers plant growth, an effect that is more pronounced in C3 plants than in C4 plants. Together with the ABA-mediated repression of growth-stimulating hormones cytokinins and brassinosteroids (discussed in the next session), this mechanism thus indirectly connects ABA accumulation with leaf growth repression under drought (Figure 2B). Increasing WUE by pushing the ABA pathways has become a major strategy for engineering plants with reduced transpiration, which survive severe drought stress more efficiently. However, as ABA is also linked to growth, this often coincided with reduced plant biomass and yield in Arabidopsis and crops (Yamaguchi-Shinozaki and Shinozaki, 2001; Blum, 2005; Kim et al., 2014a; He et al., 2018). Since dehydration-tolerant plants are not profitable for breeders if they deliver reduced yields when the stress levels are moderate, the greatest challenge in the engineering of ABA-stimulated WUE is the maintenance of plant growth. In the recent years, multiple studies demonstrated that fine-tuning ABA biosynthesis and response can increase WUE while minimizing growth or yield penalty.
Modifying ABA biosynthesis in plants was one of the strategies to achieve increased WUE, but overall this strategy obtained limited success (reviewed in Verslues, 2016) (Figure 3). One of the reasons might be that ABA metabolism is robustly buffering ABA levels and, thus, attempts to increase ABA synthesis in fact resulted in increased levels of ABA degradation and conjugation (Qin and Zeevaart, 2002; Priest et al., 2006). ABA is generated from carotenoids that are cleaved by the rate-limiting enzymes 9-cis-epoxycarotenoid dioxygenases (NCEDs), of which NCED3 is induced under drought stress (Iuchi et al., 2001; Tan et al., 2003; Ruggiero et al., 2004). To find new genetic strategies to increase ABA levels, Kalladan and colleagues (2019) screened natural accessions of Arabidopsis for variations in ABA levels. The low ABA levels in the Shadara accession, which maintains better growth under drought stress (Sharma et al., 2013), are caused by four non-synonymous substitutions in NCED3 compared to the high ABA-accumulating accession Landsberg erecta (Kalladan et al., 2019). Although this still has to be confirmed in crop species, this research using natural variation suggests that slight modulations in NCED3 enzymatic activity, rather than constitutive overexpression, could successfully alter ABA levels. An alternative approach was taken by Brugière and colleagues (2017), which obtained reduced ABA breakdown by overexpressing genes encoding maize XERICO proteins, E3 ubiquitin ligases involved in ABA homeostasis. ZmXERICO-overexpressing maize plants show slightly increased WUE and ABA levels (Brugière et al., 2017) (Figure 3). Consequently, these plants show a growth and yield advantage under drought stress in the field, without negative effects on growth under well-watered conditions (Brugière et al., 2017; Simmons et al., 2021).

Given the difficulties to modify ABA levels, more efforts were put into the modulation of ABA signalling (Figure 2A). ABA signalling is initiated by ABA binding to one of the 14 members of the REGULATORY COMPONENT OF ABA RECEPTORS (RCARs)/PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE (PYL) receptor family (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Fuchs et al., 2014; Tischer et al., 2017). This stabilizes the RCAR/PYL/PYR interaction with clade A protein phosphatases 2C (PP2Cs), inducing a downstream kinase cascade activating ion channels for stomatal closure (Xu et al., 2006; Lee et al., 2009; Ma et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Sato et al., 2009; Geiger et al., 2010; Sirichandra et al., 2010; Brandt et al., 2012; Imes et al., 2013). Overexpressing genes encoding the ABA receptors enhances ABA signalling, WUE and, thus, better resilience under drought. Nevertheless, these plants carry a reduction in plant growth under well-watered or mild drought conditions, as observed in RCAR10-overexpressing poplar or RCAR3-overexpressing rice plants (Papacek et al., 2019; Min et al., 2021). Conversely,
simultaneous mutation of multiple PYLs in rice decreased ABA sensitivity and increased growth and yield under well-watered conditions (Miao et al., 2018). To identify RCAR/PYL/PYR-overexpressing Arabidopsis and rice plants that are more drought-resilient without showing stunted growth, two studies actively screened for lines with normal growth under control conditions (Yang et al., 2016; Bhatnagar et al., 2020). This resulted in the identification of rice overexpressing OsPYL/RCAR7, a gene encoding an ABA receptor that triggers reduced signalling activity compared to other ABA receptors. This reduction in sensitivity could explain why plants overexpressing OsPYL/RCAR7 perform better under drought without a growth trade-off (Bhatnagar et al., 2020). Similarly, overexpressing each RCAR/PYL/PYR in Arabidopsis led to the identification of some receptors of class II, a subclass of this family, as good candidates to decrease stomatal aperture and to increase WUE without yield penalty (Yang et al., 2016). Interestingly, these class II receptors were found to be the ones providing only a moderate ABA response (Tischer et al., 2017). In addition, highly promising results were obtained by overexpressing PYL4 and PYL12 in Arabidopsis (Yang et al., 2016), wheat TapyL4 in Arabidopsis (Mega et al., 2019b) or wheat (Mega et al., 2019a) and poplar PtPYRL1 and PtPYRL5 in poplar (Yu et al., 2017). In all these examples, drought resilience or WUE was increased while minimizing yield penalty (Figure 3). Alternatively, genome editing was recently used to generate small modifications in the OsPYL9 sequence, altering the protein structure and increasing the yield and height of rice plants in drought and well-watered conditions (Usman et al., 2020). Another approach that could hold potential to engineer PYLs without a negative effect on yield under control conditions, could be to modify their phosphorylation sites targeted by the TARGET OF RAPAMYCINE (TOR) kinase, which keeps PYLs inactive under well-watered conditions (Wang et al., 2018b) (Figure 3). Finally, non-GM approaches hold potential to modify the ABA receptor’s activity at the desired moment, particularly with the use of agrochemical molecules that bind to the ABA receptor (reviewed in Hsu et al., 2021).

Downstream of the ABA receptors, clade A PP2Cs have also been explored, for their potential to alter WUE and growth (Figure 2A). In the case of ABA-INSENSITIVE1 (ABI1) and HYPERSENSITIVE TO ABA1 (HAB1) mutants, this resulted in stunted growth because of stomatal closure and impaired water retention, even at low stress levels (Robert et al., 2006; Nishimura et al., 2010). A less explored A-type PP2C, HIGHLY ABA-INDUCED1 (HAI1), is induced by ABA and low water potential, and targets At-HOOK-LIKE10 (AHL10) (Bhaskara et al., 2012; Wong et al., 2019). Interestingly, hai1 and ah110 mutants grow better and yield a higher biomass under moderately low water
potential not threatening plant survival, without growth penalty under control conditions (Wong et al., 2019).

Besides clade A PP2Cs, also clade E GROWTH-REGULATORY PP2Cs (EGRs) can be targeted to achieve better growth under moderate drought, without yield penalty (Bhaskara et al., 2017b) (Figure 2A). EGRs are induced by ABA treatment (Sugimoto et al., 2014) and function downstream of BODYGUARD, a putative regulator of ABA-responsive genes (Wang et al., 2011b). However, the induction of EGRs by low water potential occurs even in the absence of ABA (Bhaskara et al., 2017a), suggesting that this group of PP2Cs, which does not interact with PYLs (Sugimoto et al., 2014), acts independently of ABA. EGRs do not trigger a typical ABA-mediated drought response but, instead, act directly on MASP1, which contributes to microtubule rearrangement and growth when it is phosphorylated (Bhaskara et al., 2017a). Upon drought stress, dephosphorylation of MASP1 by EGRs restraints growth. Consequently, egr2-1 mutants maintain larger rosettes under drought, which was also observed under well-watered conditions (Bhaskara et al., 2017a). In contrast, overexpression of EGRs yields longer petioles and bigger stems (Sugimoto et al., 2014). To our knowledge, the EGR proteins were not studied in crop species so far but engineering the EGR levels in a tissue-specific manner could hold potential to engineer robust crops with better growth both under favorable and drought conditions (Figure 3).

In contrast to the studies performed in the last decades, when ABA sensitivity was boosted to increase resilience without attention to the – often stunted – growth of the generated lines, the recent literature shows that care is now taken to monitor the growth phenotype. Recent studies still focussed on increasing ABA sensitivity to boost WUE while selecting, in second instance, for well-growing plants; these can be categorized as following the ‘drought resilience without yield penalty’ approach (Figure 3). In most cases, this was achieved by precise modulation of the ABA sensitivity, via slight modifications of the ABA receptor sequence, or via overexpression of receptors from a class with low ABA affinity. Although these studies monitor that there is no growth defect when ABA sensitivity is increased, they do not boost plant growth in se. The latter could be achieved by reducing ABA sensitivity, like in the hai1 or ahl10 mutants, which grow better under moderate drought conditions. Alternatively, PP2Cs from the E-clade that are not clearly ABA-dependent, e.g EGRs, were successfully used for engineering better plant growth under moderate low water potential.
Maintaining Vegetative Growth via the Growth-Promoting Hormones Cytokinins and Brassinosteroids

Brassinosteroids (BRs) and cytokinins (CKs) are two key growth regulators that, in recent years, also have shown to exert an effect on plant abiotic stress responses. The role of BRs in the regulation of cell division and cell expansion, two processes particularly important during shoot growth, has been well studied. BRs have been shown to induce the expression of cyclins, such as CYCLIN D3;1 (CYCD3;1), and cell wall-modifying enzymes, such as xyloglucan endotransglycosylases (e.g. TCH4) and EXPANSINS (Mussig et al., 2002; González-García et al., 2011) (Figure 2A). Similarly, BRs signalling activates a large amount of plant growth regulators, such as BES1-INTERACTING MYC-LIKE 1 (BIM1), MYB30 and MYELOBLASTOSIS FAMILY TRANSCRIPTION FACTOR-LIKE 2 (MYBL2) (reviewed in Fridman and Savaldi-Goldstein, 2013). Besides growth, they are also involved in xylem differentiation, photomorphogenesis, reproduction and stress response (Peres et al., 2019; Nolan et al., 2020). BRs are synthetized in the endoplasmic reticulum, transported and eventually perceived in the plasma membrane (Northey et al., 2016). Upon perception, the receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) interacts with RECEPTOR KINASE 1 (BAK1), initiating a phosphorylation cascade. This leads to the activation of the transcription factors BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMSSUPPRESSOR1 (BES1), central mediators of BR responses, and inactivation of BRASSINOSTEROID INSENSITIVE2 (BIN2), a negative regulator of BR signalling (Clouse, 2011; Oh et al., 2012; Yan et al., 2012).

Under drought stress, BR signalling and response are reduced by the antagonistic action of ABA (Figure 2A and 2B). Inhibition of ABI2, negative regulator of ABA signalling that operates through ABA receptors, leads to BIN2 phosphorylation, in turn dampening BR signalling (Wang et al., 2018a). This has a dual consequence, which reflects the complex role of BR during the drought stress response. First, as BR stimulates cell division and growth, the reduced BR response directly limits plant growth. Moreover, BRs interact with ethylene biosynthesis in a way that depends on the BR repression of the ethylene biosynthesis, while, at higher levels, ethylene biosynthesis was post-transcriptionally enhanced (Jiroutova et al., 2018; Tian et al., 2018) (Figure 2B). Because ethylene is a growth inhibitor (see next section), the abolishment of its repression by reduced BR levels under drought could also contribute to growth inhibition. Although the interaction between these two hormones under drought is yet to be studied, it has already been demonstrated under low water potential stress using poly-ethylene
glycol (Wei et al., 2019). Second, as a consequence of the antagonistic pathways between BR and ABA (Ryu et al., 2014), BRs also actively repress the classical, ABA-mediated, response to drought stress (Fàbregas et al., 2018). This dual participation of BR in both growth and ABA-mediated drought resilience is illustrated by the quadruple BR signalling mutant (bri1brl1brl3bak1), which is extremely dwarfed but shows a strong drought avoidance response and, thus, high survival rates. Nevertheless, other studies show that BRs can also positively induce drought genes, some of them also regulated by ABA, making this relation between BR and ABA even more complex (Nolan et al., 2020).

Given the, in general, negative impact of BR on the classical ABA-mediated drought response, one strategy focused on the inhibition of the BR pathway to enhance drought resilience. For example, it was observed that three transcription factors involved in BR signalling, WRKY46, WRKY54, and WRKY70 had both a positive effect in plant growth and a negative effect in the response to drought (Chen et al., 2017). Expectedly, when these transcription factors were silenced, the resilience after a severe drought stress significantly improved, along with a reduction in growth (Chen et al., 2017). A similar response was observed in soybean overexpressing GmMYB14, encoding a transcription factor that negatively regulates BR biosynthesis, which leads to dwarfed drought resilient plants (Chen et al., 2020). Although such lines are not profitable due to their stunted growth, they might still hold potential as a basis for a ‘resilience without growth penalty’ approach. Similar to what was done for ABA, one can imagine that fine-tuning gene expression levels could lessen the effect on dwarfism while maintaining the same levels of drought resilience.

It has been shown that exogenous BR application has a positive effect on plant growth in several crops under water-limiting conditions (Krishna, 2003; Upreti and Murti, 2004; Kagale et al., 2007; Wang et al., 2019c; Huang et al., 2020). Thus, perhaps a more successful approach to maintain growth and yield, is the approach taken by Fàbregas and colleagues (2018) (Figure 3). They overexpressed the vascular BR receptor BRL3 specifically in Arabidopsis roots to promote growth (Fàbregas et al., 2018; Nolan et al., 2020). It would be expected that the enhanced BRL3 activity would antagonize ABA signalling and thus inhibit the drought-induced ABA-mediated response. Instead, overexpressing BRL3 in roots promoted drought tolerance, including a maintained shoot growth advantage under drought conditions. This might result from root architecture modifications for an improved hydrotropic response, increased sugar transport to the root, triggering a drought priming effect, or an increased photosynthetic stability on the
leaves under low water potential conditions. Possibly, restricting the response to the roots, rather than overexpression of BRL3 by the use of constitutive promoters, might have allowed to avoid possible pleiotropic effects on plant growth.

Similar to BRs, CKs are considered to be growth promoting phytohormones. An Isopentenyltransferase (IPT) is considered as the limiting step in catalyzing CK biosynthesis and, upon CK binding in the endoplasmic reticulum to AHK2, 3 and 4 receptors, the signalling cascade is initiated (Higuchi et al., 2004; Nishimura et al., 2004). Illustrating the tight connection between growth regulators, BRs promote the biosynthesis of CK by upregulating IPT expression, while CK upregulates the expression of genes involved in BR biosynthesis and signalling, such as the BRI1 receptor (Li et al., 2021). Typically, IPT expression is reduced under drought, hence limiting CKs biosynthesis (Rivero et al., 2007), which triggers the inhibition of vegetative growth and the onset of senescence (Yang et al., 2002; Kudoyarova et al., 2007; Wang et al., 2011a) (Figure 2A). Another similarity with BR is CK’s antagonistic role with the drought-induced hormone ABA as, for example, lower levels of CK increase the sensitivity to ABA under stress conditions (Nishiyama et al., 2011; Hai et al., 2020) (Figure 2B). Despite this antagonism, CKs can also play a positive role in drought stress adaptation, thus functioning both as positive and negative regulator. CKs mediate drought resilience by modifying root architecture and improving root fitness, influencing the photosynthetic machinery, enhancing antioxidant systems, modulating the water balance and, intriguingly, even directly affecting the expression of drought-responsive genes (reviewed in Hai et al., 2020). Consequently, as CKs affect both the growth and resilience aspect of the drought response, modulating CK signalling or biosynthesis can improve one aspect of this delicate balance, on the detriment of the other.

Therefore, carefully fine-tuning any changes in the CK biosynthesis pathway is essential to engineer plants with minimized yield loss under drought. To overcome the negative effects of a reduction in CK levels on plant growth while maintaining the benefits for drought tolerance, the Arabidopsis CYTOKININ DEHYDROGENASE1 (AtCKX1), encoding a CK degrading enzyme, was expressed under the control of a root-specific promoter (Pospíšilová et al., 2016) (Figure 3). Applying this approach in barley enhanced the response to both mild and severe drought resulting in improved yield under drought. By avoiding the translocation of dehydrogenase AtCKX1 from the root to the shoot, drought tolerance was enhanced in the root without affecting the aerial part, where a deficiency of CKs could have negatively influenced shoot development (Pospíšilová et al., 2016). Another strategy is to reduce CK sensitivity by mutating CKs receptors.
Interestingly, under low water potential conditions, an increase in fresh weight under was observed in the CK receptor mutants ahk3 and ahk4 (Kumar and Verslues, 2015).

Finally, modulating the CK levels can also be used to enhance plant growth under well-watered conditions, while maintaining this advantage under drought conditions. Genetically engineering the pathway to avoid the downregulation of CK biosynthesis under drought resulted in a delayed senescence and, eventually, enhanced drought tolerance in tobacco (Rivero et al., 2007) and rice (Peleg et al., 2011). In Arabidopsis, plants maintaining high levels of CKs under stress retained the expression of plant growth genes, which resulted in delayed drought-induced senescence, a phenotype also named ‘stay-green’ (Golan et al., 2016). This improved the tolerance to drought without compromising plant growth, both under favorable and unfavorable conditions.

Reducing Biosynthesis of and Sensitivity to the Growth-Inhibitory Hormone Ethylene

One of the major hormones that constrains plant growth is the small gaseous hormone ethylene. Synthesized from the amino acid methionine, S-adenosyl-methionine is converted to aminocyclopropane-carboxylic acid (ACC) via ACC-SYNTHASE (ACS) enzymes that control the rate-limiting step of ethylene biosynthesis (Figure 2A). In Arabidopsis, ethylene can subsequently bind to five types of receptors (ETHYLENE RESPONSE1 and 2 (ETR1/2), ETHYLENE RESPONSE SENSORS1 and 2 (ERS1 and 2) and ETHYLENE INSENSITIVE4 (EIN4)), which releases the inhibitory action of the downstream signalling cascade (Clark et al., 1998; Gao et al., 2003; Huang et al., 2003). Many actors of ethylene biosynthesis and signalling are conserved between dicots and monocots (Rzewuski and Sauter, 2008), facilitating the translation from model species into crops to engineer this pathway.

One of the promising molecular strategies to engineer crops that maintain higher yields under moderate drought stress in a growth-centered approach, consisted of reducing the production or detection of this hormone (Figure 3). With reduced ethylene levels or sensitivity, plant growth is stimulated during the vegetative period, plants have a ‘stay-green’ phenotype under drought, and also grain yield is improved. To reduce ethylene production in maize plants, one of the genes encoding an ACS was knocked-out (Young et al., 2004) or targeted via RNAi (Habben et al., 2014). These plants have
a strongly reduced ethylene production, leaves stay green longer under water deficit (Young et al., 2004) and grain yield was increased on dry fields without yield penalty under optimal conditions (Habben et al., 2014). As an alternative approach, ethylene sensitivity can be reduced. This was achieved by targeting the ARGOS proteins in maize, ZmARGOS1 and ZmARGOS8. ZmARGOS1 is an interactor of REVERSION TO ETHYLENE1, which in turn interacts with ethylene receptors to modulate their binding with ethylene (Shi et al., 2016) (Figure 2A). Overexpression of both ZmARGOS1 and ZmARGOS8 reduces ethylene sensitivity, improves maintained growth of Arabidopsis seedlings under moderate drought, and offers increased grain yield in maize grown under water-limiting conditions in the field (Guo et al., 2014; Shi et al., 2015). This yield increase was also observed under favorable growth conditions in the case of ZmARGOS8 overexpression (Shi et al., 2015), but not when overexpressing the stronger ZmARGOS1, which failed to reach wild-type yield levels under well-watered conditions (Guo et al., 2014). More recently, overexpression of ZmARGOS8 via CRISPR-mediated promoter swapping also achieved similar promising results (Shi et al., 2017), validating the use of the ARGOS-overexpressing constructs to increase maize yield under drought, without yield penalty at low drought levels in different genetic backgrounds (Simmons et al., 2021).

The improved growth and yield characteristics of ethylene-desensitized plants under drought conditions illustrate that ethylene likely accumulates in plants perceiving drought, and contributes to the response by reducing shoot growth. Whether ethylene, its precursor ACC, or conjugates are among the mobile signals that are transported from the root, where drought is perceived, to the shoot, is still a matter of debate (Tardieu, 2016). ACC is known to accumulate in the xylem upon water deficit (Pérez-Alfocea et al., 2011), supporting the hypothesis that ACC would be synthesized in roots when a lower water potential is detected and subsequently transported upwards, where it would be converted to ethylene in aerial organs (Skirycz et al., 2011a). Such a mechanism has, to our knowledge, not been formally proven to occur under progressive drought in soil. However, the ‘stay-green’ phenotype and increased grain yield of acs knock-out plants under drought (Young et al., 2004) suggest that ACC biosynthesis occurs under drought stress.

Downstream, multiple molecular pathways can explain the growth shutdown in wild-type plants upon ethylene accumulation in the shoot, in part suppressed in ethylene-desensitized plants (reviewed in Dubois et al., 2018b). Ethylene has direct negative effects on both cell division and cell expansion in leaves, by inhibition of key cell cycle
genes or EXPANSIN genes expression, respectively (Figure 2A). Besides leaf growth, ethylene also orchestrates leaf senescence, a drought-stimulated process of which the engineering can contribute to the ‘stay-green’ phenotype, extending the energy capacity of plants and increasing the yield under drought. In older leaves that are prone to senescence, ethylene downregulates photosynthesis (reviewed in Ceusters and Van de Poel, 2018) and stimulates leaf senescence via the activation of senescence-inducing transcription factors NAP and NAC2 (Kim et al., 2009) and the direct inhibitory action of EIN3 on miR164, which targets NAC2 (Kim et al., 2014b). Consequently, ethylene insensitive mutants etr1-1 and ein2-1 have rosettes that grow for a longer period and display postponed leaf senescence and delayed flowering (Guzmán and Ecker, 1990; Ogawara et al., 2003; Patterson and Bleecker, 2004).

Maize plants with reduced ethylene production (ACS knock-out and RNAi), ethylene-insensitive mutants etr1-1 and ein2-1 and ARGOS-overexpressing maize plants all show delayed senescence and better yield under moderate drought stress (Young et al., 2004; Shi et al., 2015). However, because in these lines the engineering occurred upstream in the ethylene signalling pathway, the overall reduced ethylene sensitivity could have negative effects on plant yield under other environmental conditions, when ethylene has beneficial roles. Ethylene helps the plant to avoid shade and to regulate hyponasty, and is a major actor in the activation of resistance responses to life-threatening stresses, including biotic and severe abiotic stresses. Therefore, it will be crucial to engineer specific aspects of ethylene responses, for example by precisely modulating the level or tissue in which the construct is expressed. For example, whereas the strong ethylene-insensitive ARGOS1-overexpressing maize line has yield reductions under other environmental conditions than drought, including normal growth conditions, the weaker ARGOS8-overexpressing lines – in which ethylene-induced tolerance responses might still be properly activated – do not have this disadvantage under normal field conditions (Shi et al., 2015).

Alleviating the Drought-Induced Oxidative Damage via ROS Scavenging

The plant responses to most types of stresses, including both biotic and abiotic, rely on very complex gene networks in which oxidative signalling is considered a common outcome, involved both in growth regulation and defense (Baxter et al., 2014). Environmental stresses disturb the metabolic balance which, in turn, triggers the production of ROS, one of the earliest cellular responses to abiotic stress in plants (Miller
et al., 2008) (Figure 2A). At moderate levels, ROS molecules have a signalling function, however, the oxidative damage caused by excess of ROS is detrimental to plants, negatively affecting a wide variety of biological processes and eventually resulting in cellular damage and death (Halliwell, 2006; Gill and Tuteja, 2010; Das and Roychoudhury, 2014).

Under moderate drought levels, ROS molecules have a positive drought signalling function (Figure 2A). Induced rapidly upon drought perception (Dubois and Inzé, 2020), ROS molecules act as mutual interactors with the stress hormone ABA in the drought stress response (Cruz de Carvalho, 2008) (Figure 2B). ROS levels are enhanced by ABA in Arabidopsis guard cells, wheat seedlings and maize embryos (Guan et al., 2000; Pei et al., 2000; Zhang et al., 2001; Jiang and Zhang, 2002) while ROS accumulation also plays a role in the regulation of ABA synthesis in wheat under drought stress (Zhao et al., 2001). Under more severe drought, the balance between antioxidant levels and ROS is perturbed, eventually leading to ROS accumulation (Munné-Bosch and Alegre, 2004). Excess accumulation of ROS and consequent oxidative stress under drought stress is directly responsible for a decrease in growth and biomass (Begum et al., 2019). Plants have very complex and diverse scavenging mechanisms to avoid ROS accumulation, involving enzymes like superoxidase dismutase, catalase, ascorbate peroxidase and glutathione peroxidase. Thus, directly engineering the ROS metabolism genes to maintain this equilibrium is one of the strategies to mitigate the effect of water limitation.

One of the approaches to confer drought tolerance is to enhance ROS detoxification and scavenging through priming (Gupta et al., 1993). In maize, H$_2$O$_2$ priming triggered the expression of antioxidant-related genes and lead to better drought tolerance, without a negative effect on plant growth under favorable conditions (Freire de Sousa et al., 2016). Similarly, tuning antioxidant mechanisms using a biotechnological approach can be used to constrain oxidative damage, as successfully illustrated by Fang et al., 2015 (Figure 3). By overexpressing the stress-induced NAC transcription factor-encoding SNAC3 in rice, the tolerance under heat and drought stress was increased while maintaining growth under normal conditions (Fang et al., 2015). Interestingly, ROS homeostasis was maintained by a strong induction of a large number of ROS scavenging genes in an ABA-independent manner. However, the reduction of ROS accumulation in this transgenic line under drought stress remains to be demonstrated. Multiple other examples demonstrated that drought tolerance can be improved without compromising yield by enhancing ROS scavenging in tobacco, sweet pepper, rice and Arabidopsis.
In some cases, like in Arabidopsis overexpressing ABA-RESPONSE ELEMENT BINDING PROTEIN1 (AhAREB1) from peanut (Arachis hypogaea), increased ROS scavenging and drought tolerance goes along with hypersensitivity to and production of endogenous ABA (Li et al., 2013b) (Figure 3). However, whether such perturbation affected the plant growth rate was not measured.

These examples suggest that tuning of the antioxidant mechanism can occur in an ABA-dependent and -independent manner. The latter strategy holds great potential to avoid ABA-mediated stunted growth while maintaining oxidative damage under control, thus, following a ‘resilience without yield penalty’ strategy. Although a large part of the drought-responsive genes overlap between ABA and ROS treatments (Noctor et al., 2014), further studying ROS-induced genes under drought, independent of ABA treatment, could identify candidate genes to reach this goal.

**More Opportunities Coming From Beneath: Arbuscular Mycorrhiza, Strigolactones and Aquaporins**

The majority of land plants establish symbiotic interactions with arbuscular mycorrhizal (AM) fungi. This interaction has a positive effect on plant growth, mineral nutrition and protection against abiotic stresses. In the case of drought stress, AM symbiosis improves the capacity of plants to adapt to drought, by altering plant physiology and gene expression (Ruiz-Lozano, 2006; Aroca et al., 2008; Miransari, 2010). For example, AM can directly enhance drought tolerance by improving water movement into the roots by hyphal water uptake and transport (Augé, 2001). Mycorrhizal plants also produce higher levels of antioxidant enzymes under drought stress, allowing to maintain growth under water deprivation (Ruiz-Lozano, 2003). At the molecular level, the plant–AM interaction begins with the exudation of strigolactones (SLs) to the rhizosphere (Gutjahr and Parniske, 2013; Andreo-Jimenez et al., 2015). Currently, SLs are classified as the newest class of plant hormones, not only responsible for the AM symbiosis initiation ex planta, but also regulating other physiological processes within the plant, like shoot branching, stem secondary growth and leaf senescence (Seto et al., 2012). Moreover, AM colonization and SL production can be stimulated under drought in already colonized tomato and lettuce plants (Ruiz-Lozano et al., 2016). This positive correlation observed between SL production and drought tolerance in AM-colonized plants suggested that under unfavorable conditions SL production is increased as a "call for help" to promote the establishment of AM symbiosis (Ruiz-Lozano et al., 2016).
SLs are carotenoid-derived phytohormones that were shown to be involved in multiple processes of plant development and acclimation to environmental stresses (Dun et al., 2009). Interestingly, some of the SL-mediated processes are part of the drought response, like stomatal closure or leaf senescence (Figure 2A). Recently, it was shown that SLs act as a positive regulator of the plant drought tolerance in an ABA-dependent and -independent manner (Van Ha et al., 2014; Liu et al., 2015). For example, Arabidopsis SL signalling-deficient max2 mutants are hypersensitive to severe drought, because they have more stomata that show slower ABA-induced closure (Van Ha et al., 2014). Besides their connection to ABA, SLs also interact with ethylene and enhance its action, for example, in leaf senescence (Ueda and Kusaba, 2015) (Figure 2B). Finally, a possible interaction between SLs and CK has also been pinpointed (Li et al., 2019). A downregulation of the CK catabolism-related genes upon dehydration was observed in the mutant max2, compared to the wild type (Van Ha et al., 2014), suggesting an antagonistic role between both hormones. Conversely, the CK signalling mutant ahp2,3,5 displayed a lower expression of SL biosynthesis genes than the wild type under drought (Nishiyama et al., 2013), suggesting that under drought SL biosynthesis would be dependent on CK signalling.

Given the indications that SL play a positive role in the drought response, during the last decade, attempts have been made to improve drought tolerance with the use of SL. Several commercial crops showed an improved performance under drought stress conditions after foliar application of SL (Min et al., 2019; Sedaghat et al., 2020), or upon AM symbiosis-induced SL production (Ruiz-Lozano et al., 2016; Ilyas et al., 2021) (Figure 3). However, genetic engineering of the SLs pathway to improve drought tolerance is not straightforward. In fact, in Arabidopsis, the drought tolerance conferred by modulating SL signalling or biosynthesis is a matter of debate, with some studies implying the involvement of SL in the typical drought response (Bu et al., 2014), and others suggesting that this response is SL independent (Van Ha et al., 2014). For example, transgenic maize plants overexpressing AtCXE20, encoding a carboxylase that binds to SLs which was identified both in ABA hypersensitivity and osmotic stress screens in Arabidopsis, exhibited typical features of a SL-deficient phenotype, such as apical dominance and increased tillering (Roesler et al., 2021). Under drought, the ectopic expression of AtCXE20 in maize provided, probably via SL sequestration, a drought-resilient phenotype but carried an impairment in growth (Simmons et al., 2021). Similarly, a negative effect on vegetative growth was observed when increasing drought resilience in rice by inducing SL biosynthesis, which was probably caused by direct induction of ABA signalling (Haider et al., 2018). Remarkably, Islam and colleagues
(2013) demonstrated that Arabidopsis plants expressing *SPOROBOLUS DROUGHT GENE 8, SDG8i*, present in desiccated leaf tissues of resurrection grass, not only showed enhanced drought tolerance, but also better growth under favorable conditions through SL glycosylation acting downstream of ABA (Islam et al., 2013) (Figure 3). In conclusion, SL appear to be involved in drought tolerance, however because of their interactions with ABA and ethylene, which both might explain the reported stunted growth, the role of SL in the drought response must be further studied.

Plant aquaporins (AQPs) are a large family of channel-forming transmembrane proteins expressed in many plant organs. They play a key role in AM symbiosis and are essential players in the uptake and transport of water through the plant (Tyerman et al., 2002) (Figure 2A). AQPs can be found in several isoforms which specifically allow the transport of water or small molecules, and they are tightly involved in plant abiotic stress responses, particularly, in the drought response (Zargar et al., 2017; Shekoofa and Sinclair, 2018). Although their physiological functions in the drought response have been studied across different species, the specific role of each (type of) AQP is not yet well understood. Moreover, AM symbiosis is responsible for changes in the expression of several AQP-encoding genes (Aroca et al., 2007; Li et al., 2013a; Li et al., 2013c), which under drought allows to keep the water status under control (Nehls and Dietz, 2014; Quiroga et al., 2017). However, very diverse responses to mycorrhization were found across different plant species and AQP members (Giovannetti et al., 2012; Chitarra et al., 2016; He et al., 2019) (Figure 2A and 2B). For example, in AM-colonized maize, the regulation of AQP-encoding genes under drought depended on both the watering conditions and the severity of the stress (Bárzana et al., 2014).

Because the AQPs show highly divergent responses to drought stress, it is challenging to pinpoint specific AQPs as an engineering target for drought tolerance. Out of the five AQPs subfamilies, mainly plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs), localized in the plasma and vacuolar membrane, respectively, are involved in water conductance (Maurel et al., 2008). Under drought, most PIPs are downregulated (Jang et al., 2004; Alexandersson et al., 2005), however this is not the case for all AQPs, and even depends on the plant species (Li et al., 2015). For instance, under drought conditions, two very different species can display the opposite *PIP* expression change: the expression of *PIP2* decreased in *Phaseolus vulgaris* (Aroca et al., 2007) but increased in *Jatropha curcas* (Zhang et al., 2007). Similarly, engineering the expression of different PIP genes triggered very variable phenotypic outcomes under drought. For example, in Arabidopsis, an increased drought
tolerance was achieved when ectopically expressing PIPs ThPIP2;5 from *Tamarix hispida* (Wang *et al.*, 2019b) or MaPIP1;1 from banana (Xu *et al.*, 2014) while the opposite effect under drought was observed when GoPIP1 from *Galega orientalis* (Li *et al.*, 2015) or AtPIP1;4 or AtPIP2;5 were overexpressed (Jang *et al.*, 2007). Another approach used to improve plant performance under water deficit conditions is based on the ectopic expression of PIPs of drought-tolerant plants in other, more drought-susceptible species. For example, ectopic overexpression of PIP1 genes from the dehydration tolerant species *Simmondsia chinensis* and *Salicornia bigelovii* successfully enhanced drought tolerance in Arabidopsis and tobacco plants, respectively (Sun *et al.*, 2017; Wang *et al.*, 2019b). However, the opposite effect can also occur, for instance, overexpressing AtPIP1;2 in tobacco plants increased growth under favorable growth conditions but wilted more rapidly under drought stress (Aharon *et al.*, 2003) (Figure 3). Moreover, investigating the physiological function of the most abundant AQP in maize, PIP2;5, highlighted the importance of understanding the contrasting effects of AQP changes at cellular and tissue levels (Ding *et al.*, 2020). Recently, a meta-analysis was performed to assess the complexity of AQP family under drought, concluding that between PIP1 and PIP2 overexpression, PIP2 had a stronger effect on drought-stressed plants. The authors proposed that *Musaceae* and *Poaceae* would be suitable donors for AQP genes to improve drought tolerance (Ren *et al.*, 2021). Finally, TIP proteins were also explored for their potential to affect drought tolerance and plant growth, and similar responses as PIPs have been observed (Pawłowicz *et al.*, 2017; Feng *et al.*, 2018; Kurowska *et al.*, 2019). However, for this family, the high level of redundancy between TIPs might hinder their use (reviewed in Kurowska, 2020). In conclusion, although it is crucial to first elucidate the specific physiological effect and responses to drought of distinct members of the TIPs and PIPs families before their application on crops, some successful examples have shown their potential in the development of plants with beneficial characteristics under drought.

**Concluding Remarks and Future Perspectives**

In the near future, agriculture will have to handle a dual issue to improve food security: the needs of a constantly growing population and the effect of global warming which is expected to reduce crop yield, in particular due to more frequent drought. To fulfil an ever-growing demand of food products and to cope with drought stress, new approaches are not only required to enhance stress resilience, but also to maintain or even enhance yield in favorable as well as mild drought stress conditions. Firstly, it will
be necessary to better understand the plant response to drought, which is a highly entangled multifactorial process that, moreover, depends both on the developmental and spatio-temporal context. The second challenge will be to cope with the intrinsic fitness versus growth balance of plants: plants have a limited amount of energy, which they either use for growth or to implement the stress response. It is expected that this balance cannot be completely overruled, thereby setting boundaries for engineering drought tolerance in plants (Claeys and Inzé, 2013). This imposes a choice to breed either for better stress resilience, or for better growth, the latter one was the chosen strategy in the recent years (Figure 1 and 3).

A first promising strategy that holds potential to improve yield avoiding deleterious effects on stress resilience, or vice-versa, is to target the downstream mechanisms that have direct effects on how cell division and expansion responds to drought stress. Hormones play key roles the regulation of cell division and expansion and are, therefore, crucial targets to engineer. Nowadays, the engineering of hormonal pathways occurs mainly upstream, at the levels of receptors, as was discussed for ABA, ethylene, BRs and CKs (Figure 3). However, the achieved reduction (by ARGOS in the case of ethylene) or increase (by RCAR/PYLs in the case of ABA) in sensitivity to the hormone might also cause negative effects on other developmental aspects or under other environmental stress conditions. Therefore, targeting more downstream mechanisms, such as transcription factors that directly regulate the stress induced effects on growth or tolerance downstream of these hormones, or manipulating the actors directly responsible of the drought response, could result in a more specific engineering without or lessening the negative side-effects. The example of engineering microtubule rearrangements under low water potential that was previously mentioned nicely illustrates this strategy (Bhaskara et al., 2017b). Other methods to identify more potential downstream targets includes, for instance, the study of natural variation. The study of genome-wide associations have allowed partial elucidation of drought tolerance mechanisms in multiple crops (Wang and Qin, 2017). Recently, profiling the physiological aspects involved in several accessions have highlighted key mechanisms adopted by plants to maintain growth under mild drought in Arabidopsis (Chen et al., 2021).

Second, we discussed here multiple diverse molecular pathways that could improve drought tolerance, and it is tempting to combine two or more of them to further stimulate both sides of the fitness versus growth balance, for example via gene stacking. Gene stacking is a promising approach to tackle multigenic and complex traits such as drought tolerance, based on the potential synergistic effect of engineering the expression of more than one gene. These genes could either act on multiple steps of the same
pathway or belong to very different pathways with a synergetic outcome. Recently, Shailani et al., 2020 compiled several successful examples in multiple different crops that achieved an increase in drought tolerance using gene-stacking approaches, for example by targeting the betaine synthesis or the glutamine synthesis pathways in maize and rice, respectively, proving the potential of this technique for future strategies (Shailani et al., 2020). By using this approach, drought tolerance could be enhanced by introducing genes that counteract the typical pleiotropic effect on plant growth of a gene promoting drought tolerance. For the reasons discussed above, it would be beneficial to perform gene stacking on genes that act downstream in either growth-regulating and/or tolerance-related pathway. Particularly as hormonal pathways strongly interact with each other (Figure 2B), gene stacking of upstream hormonal genes might be the cause of undesirable pleiotropic effects.

A third approach, which could be applied in combination with the first two, is to limit undesired side-effects by the use of specific promotors (Martignago et al., 2020). In this approach, the expression of a gene can be directed to a specific tissue, restricting the expression in the rest of the plant, or engineered in a drought-inducible manner, being only expressed when a certain level of stress is reached. For example, avoiding the drought response in the shoot, which usually carries a stronger pleiotropic growth effect, while only expressing a transgenic drought-tolerant gene in the root, has proven to be a successful approach (Jeong et al., 2010; Pospíšilová et al., 2016) (Figure 3). To cite an example of stress-inducible promoters, the ectopic expression of Homeobox Domain 4 (HB4), which is a HD-zip transcription factor from sunflower that decreases the sensitivity to ethylene, in soybean under the native Hahb4 stress-inducible native promotor, led to the development of the first drought tolerant soybean to be commercialized (Waltz, 2015). Interestingly, emerging single-cell RNA-sequencing and spatial transcriptomics techniques could facilitate not only the development of new tissue-specific promotors, but also the engineering of combined tissue-specific and drought-inducible promotors. In this respect, the transcriptomic landscape of leaf cell populations both under favorable and mild drought conditions might be of special interest. Recently our group has unravelled the transcriptomic profile of a young developing leaf under drought at a single-cell resolution (Tenorio-Berrío et al., 2021), which could, ultimately, guide the development of new drought-and-tissue-specific promotors. Altogether, these emerging directions combined with modern breeding techniques have a great potential to restrict the increasing agricultural losses due to global warming, while ensuring food demands of the future.
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Figure 1. Schematic representation of the natural plant responses to drought and lab-used strategies for engineering drought tolerance. (A) Typical early responses to mild drought and subsequent late responses depending on the stress level. Upon growth under favorable conditions (green), mild drought (yellow) can be perceived, initiating the early response including growth inhibition. Subsequently, if the drought persists at mild level (yellow), the plant can acclimate and recover. Instead, if drought gets more severe and unbearable (red), severe symptoms, like wilting, appear. (B) Overview of the classical and modern lab-used research strategies to achieve drought tolerance. For each strategy, the expected crop performance is depicted under three different conditions: favorable conditions and mild or severe drought.
Figure 2. Molecular responses of plants exposed to drought stress. (A) Overview of the six molecular pathways discussed in this review. Upon drought stress, abscisic acid (ABA), ethylene (ETH), reactive oxygen species (ROS) and the arbuscular mycorrhiza (AM)-related processes are induced, while the levels of the growth-promoting hormones cytokinins (CK) and brassinosteroids (BR) are reduced. As each of these pathways can affect either plant growth, or drought tolerance mechanisms, or both, their alteration under drought results in inhibition of plant growth and activation of drought tolerance responses. In panel (A), the connections between the different pathways are limited to the minimum in order to increase clarity. (B) Connections between the different pathways described in (A). Yellow and green labels represent pathways that are induced or reduced under drought stress, respectively. Blue labels highlight processes that react
to drought in a more complex manner, with some aspects or genes of each pathway being induced under drought, others being repressed. Similarly, blue arrows indicate complex regulations, with some aspects positively regulated, others negatively. Plain lines indicate confirmed activating or repressing regulations. Dashed lines represent potential connections not yet confirmed.
Figure 3. Engineering of molecular pathway to improve growth or survival under drought stress. The top panel depicts the engineering of four molecular pathways following a ‘growth-centered’ strategy, thus, with as primary aim to increase growth under drought. In the lower panel, four molecular pathways that were engineered to obtain better survival under drought are depicted. Attention was paid to limit the negative side-effects on plant growth, thus, this approach is called the ‘resilience without yield penalty’ strategy. Straight arrows indicate that ectopic expression (green arrow) or knock-out (red arrow) of the gene was used to reach the aimed drought phenotype. Curled arrows indicate that the overexpression (green arrow) or mutation (red arrow) should be modulated in a specific manner, for example by overexpressing specific variants of the protein or by making targeted mutations.
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