Plant growth under suboptimal water conditions: early responses and methods to study them

Marieke Dubois[1,2] and Dirk Inzé[1,2*]

[1]Ghent University, Department of Plant Biotechnology and Bioinformatics, Ghent, Belgium.

[2]VIB Center for Plant Systems Biology, Ghent, Belgium.

* correspondence: dirk.inze@psb.vib-ugent.be

E-mails: marieke.dubois@psb.vib-ugent.be; dirk.inze@psb.vib-ugent.be

Corresponding author:

Dirk Inzé

VIB Center for Plant Systems Biology

Ghent University, Department of Plant Biotechnology

Technologiepark 71

B-9052 Ghent (Belgium)

Tel.: +32 9 3313800; Fax: +32 9 3313809

Abstract

Drought stress forms a major environmental constraint during the life cycle of plants, often decreasing plant yield and in extreme cases threatening survival. The molecular and physiological responses induced by drought have therefore been the topic of extensive research during the last decades. Because soil-based approaches to study drought responses are often inconvenient due to low throughput and insufficient control of the conditions, osmotic stress assays in plates were developed to mimic drought. Addition of compounds such as poly-ethylene glycol, mannitol, sorbitol, or NaCl to controlled growth media has become increasingly popular since it offers the advantage of accurate control of stress level and onset. These osmotic stress assays enabled the discovery of very early stress responses, occurring within seconds or minutes following osmotic stress exposure. In this review, we construct a detailed timeline of early responses to osmotic stress, with a focus on how they initiate plant growth arrest. We further discuss the specific responses triggered by different types and severities of osmotic stress. Finally, we compare short-term plant responses under osmotic stress vs. in-soil drought and discuss the advantages, disadvantages and future of these plate-based proxies for drought.

Keywords: Drought, Mannitol, Osmotic stress, PEG, Poly-ethylene glycol, Salt, Signaling, Sorbitol, Stress response

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ERF, ETHYLENE RESPONSE FACTOR; GA**,** gibberellic acid; JA, jasmonic acid**;** nHRSC, non-hydraulic root-sourced signal; PIPs, plasma membrane intrinsic proteins; PEG, polyethylene glycol (PEG); RBOHs, RESPIRATORY BURST OXIDASE HOMOLOGs; ROS, reactive oxygen species; SOS, Salt Overly Sensitive.

From soil-based drought to osmotic stress in plates: when, how and why

About half a century ago, the drastically increasing world population and the upcoming effects of global warming started to become a point of concern (Broecker, 1975). It was clear that drought stress would form a major constraint for worldwide agriculture and drought stress responses in plants formed an important research area (extensively reviewed first in Hsiao and Acevedo (1974), Supp. Fig. 1). Drought stress experiments were performed in soil either by progressive drying of the soil or by transplantation to pots with reduced moisture levels (Saunier *et al.*, 1968; Boyer, 1971). These methods were, however, found inappropriate because it was impossible to exactly control the stress levels and, most importantly, because they were unsuitable to grow large numbers of plants. Therefore, during the seventies and eighties, alternative approaches to study drought response were elaborated, and alternative setups to mimic drought stress were introduced first on cell cultures and not much later on plants (Kaufmann and Eckard, 1971). By adding certain compounds to the artificial growth medium the water potential was lowered, making it harder for the plants to take up water from the substrate (Heyser and Nabors, 1981; Nguyen and Lamant, 1989; Claes *et al.*, 1990). Among them, sodium chloride (NaCl) is most commonly used, followed by polyethylene glycol (PEG), mannitol and sorbitol (Supp. Fig. 1)(Verslues *et al.*, 2006; Claeys *et al.*, 2014b). Although these compounds have very different chemical properties and elicit specific responses in plants (discussed in a later section), they share the property of triggering osmotic adjustment of the plant's cells and are therefore commonly categorized as osmotic stress, together with drought or freezing stress. In this review, these four compounds commonly used to mimic drought stress will be referred to as 'osmotic stress compounds'. When they are used as addition to the growth medium for sterile culture of plants (e.g. whole plants grown in petri plates with MS medium with sorbitol), we also use the term '*in vitro* osmotic stress assays'.

Mannitol and sorbitol are non-metabolizable sugar analogs sharing a common chemical structure that only differs in steric C-atom conformation. Because of their low molecular weight and their behavior as ideal solutes, mannitol, sorbitol and salt offer the advantage of being easily and equally dissolved in the growth medium. PEG doesn't have this advantage and has to infiltrate solidified medium through diffusion (for a detailed procotol, we refer to the work of (Verslues *et al.*, 2006)). PEG is a very commonly used compound that was proven to be particularly useful for applying stable, long-term low water potential stress (Verslues *et al.*, 2006; Claeys *et al.*, 2014b). Multiple studies report a homogeneous PEG-induced stress response in plant roots grown on vertical petri plates, while a higher variability was observed, in our hands, when growing plants on horizontal plates with PEG (Skirycz et al., 2010). The reason behind this variability is unclear; one explanation could be that PEG entered the roots via small lesions caused by growth on horizontal plates. Importantly, PEG also offers the advantage of triggering cellular responses that are similar to drought stress. Drought stress triggers cytorrhysis; shrinking of cell wall and protoplast simultaneously due to passive export of water molecules (Haswell and Verslues, 2015). High-molecular weight PEG (PEG6000 or higher) molecules do not penetrate the cell wall and also

trigger cytorrhysis (Kaufmann and Eckard, 1971; Heyser and Nabors, 1981). In contrast, small molecules of mannitol, sorbitol, or salt enter the cell wall, potentially triggering plasmolysis (shrinking of the protoplast only, which detaches from the cell wall). Moreover, mannitol was reported to activate mannitol-specific downstream responses, unrelated to drought stress (Trontin *et al.*, 2014) – this will be discussed in more detail in a later section.

Finally, NaCl is often used in parallel to drought. Salt triggers a dual stress, composed of an osmotic component which might mimic drought, but also of an ionic stress component caused by high levels of Na^+. The two components of salt stress can be separated in time (Munns, 2002; Verslues *et al.*, 2006; Munns and Tester, 2008; Rajendran *et al.*, 2009; Isayenkov and Maathuis, 2019). Immediately upon stress exposure, the osmotic component is the main inducer of stress responses within hours, triggering stomatal closure and growth inhibition, as described later in more detail. After several days or weeks, the Na^+-ions taken up by the plants cause toxicity by inhibiting enzymatic activities and triggering the uptake of other toxic positive ions such as Li^+ and K^+ (Xiong and Zhu, 2002). However, this might depend on the concentration of salt stress used. Under moderate osmotic stress (f.e. 100mM salt, corresponding to -0.7MPa), Na^+ is taken up by the plants (Zhao *et al.*, 2010) and root growth is more affected than on non-ionic PEG stress at the same osmotic equivalent (Verslues *et al.*, 2006). However, when salt concentrations are lower (f.e. 50mM salt – a basal stress level reducing plant growth), the growth of salt-stressed plants is equally affected as that of sorbitol-stressed plants after 15 days of stress (non-ionic, at osmotic equivalent level)(Claeys *et al.*, 2014b). This suggests that very mild salt stress levels do not trigger Na^+-toxicity, or much later, and that plants evolved to deal with the basal salt stress levels in the field.

As compared to soil-based drought assays, *in vitro* setups are easy to use and suitable to expose large amounts of plants simultaneously to stress, as well as to precisely control the stress levels, onset and duration. Because *in vitro* assays can be used to apply a stable stress level during a long period of the plant's development, they are particularly useful for the study of stress acclimation responses. When no exogenous sugars are supplied via the growth medium, the long-term responses of plants grown on osmotic stress not triggering plasmolysis (e.g. via the use of PEG), are comparable to the responses of drought-stressed plants. Another advantage of *in vitro* setups is the possibility to apply stress at a well-chosen moment and study the response within minutes or hours after stress application, further referred to as short-term stress assays. For this purpose, young seedlings can either be grown in liquid medium or hydroponics cultures to which the compound can be added at the preferred moment. Alternatively, seedlings can be grown on solid control medium overlaid with a nylon mesh, which can be transferred to medium supplemented with the osmoticum at the desired time point (Verslues *et al.*, 2006).

The possibility to precisely control the stress onset offers two major benefits. First, the exact time point at which the stress will be applied can be chosen. As the response to stress in different plant organs has been shown to highly depend on the cell type and developmental stage of the tissue (Dinneny *et al.*,

2008; Skirycz *et al.*, 2010; Thatcher *et al.*, 2016; Wang *et al.*, 2019), it might be suitable to expose plants to stress at a particular moment during development. Second, because the moment of stress exposure is precisely known, the short-term stress response can be followed by harvesting the tissue of interest after hours, minutes, or even seconds upon stress exposure. These short-term responses comprise large changes on proteome, phosphoproteome and transcriptome level that are highly dynamic and can be masked on longer term, when new steady state levels are reached. Therefore, the study of short-term responses can reveal new candidate genes or processes that are overlooked at later time points, but that could still be important in the establishment of the plant's stress response.

Here, we combined the available osmotic stress studies that investigated this short-term response to construct a timeline of the response of *Arabidopsis thaliana* to osmotic stress. Ideally, an individual timeline should be constructed per type of stress, organ, developmental stage and per level of stress, as all of these factors were shown to differently affect the stress response (Dinneny *et al.*, 2008; Skirycz *et al.*, 2010; Verelst *et al.*, 2013; Claeys *et al.*, 2014b; Ma *et al.*, 2014). However, since too few studies are available to enable such an analysis, we focus on the different types and levels of stresses but mention the specificity of the response, when reported.

Control of stress onset enables the unraveling of short-term stress responses

Continuous detection of water potential in roots: a role for osmosensors?

In order to adapt properly to continuous environmental microchanges, plants developed mechanisms tracking the status of their surroundings. Just like phytochromes permanently sense the light quality around the aerial parts, it is reasonable to think that receptors in root cells monitor the water availability (Haswell and Verslues, 2015). One of these so-called osmosensors that has attracted scientists' attention during years is the histidine kinase protein AtHK1, a receptor – not a cytokinin receptor, in contrast to other members of its family – that acts upstream of a two-component His-kinase transduction pathway. AtHK1 was identified as the first putative plant osmosensor because it complements the salt-sensitive phenotype of yeast strains mutated in osmosensors like *sln1* (Urao *et al.*, 1999). In Arabidopsis, depletion of AtHK1 was found to render plants more sensitive to severe, life-threatening drought stress in soil, whereas plants overexpressing the putative osmosensor showed increased survival rates under drought (Tran *et al.*, 2007; Wohlbach *et al.*, 2008). Moreover, AtHK1 acts upstream of the abscisic acid (ABA) response and is itself induced transcriptionally in roots by osmotic stress, making it a plausible candidate for connecting the soil water status to the drought stress response (Tran *et al.*, 2007; Wohlbach *et al.*, 2008). However, the mutant is not affected in drought-induced stomatal closure, neither in solutes nor ABA accumulation triggered by lower water potential (Wohlbach *et al.*, 2008; Kumar *et al.*, 2013). In fact, it is more likely that an increased stomatal density, and not a loss of putative osmosensor activity, is at the origin of the drought-hypersensitive phenotype (Kumar *et al.*, 2013). Although it remains intriguing that AtHK1 and its poplar ortholog HK1b have similar molecular roles as yeast osmosensors (Urao *et al.*,

1999; Héricourt *et al.*, 2016), they might not be critical for osmosensing in plants, not in roots, neither in leaves (Kumar *et al.*, 2013; Sussmilch *et al.*, 2017).

Besides histidine kinases that have demonstrated osmosensor function in other kingdoms, integrin-type proteins can also act as stress-sensing proteins. Integrin proteins are transmembrane receptors that can elicit stress-response pathways, particularly in mammalian cells. In Arabidopsis, a protein with a small integrin-like domain, At14a-like1 (AFL1), is emerging as a new putative osmosensor. AFL1 is a transmembrane protein that is strongly induced by low water potential and interacts with PDI5 and NAI2, which are two known regulators of growth and accumulation of proline, a central molecule in drought and osmotic stress response (Kumar *et al.*, 2015). At the plasmamembrane, AFL1 could participate in the initiation of clathrin-mediated endocytosis described in the next section. A prominent role for AFL1 in osmotic and drought stress response is further supported by its strong down-regulation in salt-hypersentitive Arabidopsis mutants and an improved root growth under moderate osmotic stress levels in plants overexpressing *AFL1* (Kumar *et al.*, 2015; Huang *et al.*, 2018). Although the presence and mode-of-action of a true osmosensor at the origin of the drought stress response still remains to be formerly demonstrated in plants, the above-mentioned osmotic stress-induced transmembrane proteins that impact plant growth under stress can be considered robust candidates.

Endocytosis and Ca^{2+} initiate the response within seconds

When roots are exposed to environments with a water potential lower than the one of the root cells, water is passively exported out of cells (Fig. 1a) (Zonia and Munnik, 2007). This lowers the intracellular turgor pressure, initiates a hydraulic signal (see later), and has three direct molecular consequences. First, the surface-to-volume ratio of cells is increased, generating a surplus of plasma membrane which is internalized by clathrin-mediated and clathrin-independent endocytosis (Fig. 1b) (Leshem *et al.*, 2007; Baral *et al.*, 2015a; Baral *et al.*, 2015b; Zwiewka *et al.*, 2015). As the putative osmosensor, AFL1 is localized at foci near clathrin-mediated vesicle formation, and it is possible that it stimulates this process (Kumar *et al.*, 2019). Vesicle internalization has been observed under both ionic and non-ionic osmotic stresses even under relatively mild concentrations (75 mM mannitol or sorbitol, 50 mM KCl, 100 mM NaCl) and occurs within ten minutes of stress exposure (Zwiewka *et al.*, 2015). Consistently, mutants defective in clathrin components or in regulators of vesicle trafficking are unable to adapt their growth when exposed to osmotic stress (Leshem *et al.*, 2007; Zwiewka *et al.*, 2015).

A second and central response triggered by the decreased cellular water potential is the accumulation of free cytosolic Ca^{2+} molecules (Fig. 1c). The accumulation of Ca^{2+} has been reported to occur under salt, mannitol, sorbitol and PEG-mediated osmotic stress and occurs in waves of which the first peak was observed within five seconds following stress exposure (Kiegle *et al.*, 2000; Yuan *et al.*, 2014; Stephan *et al.*, 2016). The Ca^{2+} peak is compound-specific (see later) and only lasts for about one minute but repetitive peaks can trigger sustained signals in specific root cell types (Kiegle *et al.*, 2000).

While the observation that Ca^{2+} accumulates in response to stress is more than forty years old (Kaufmann and Eckard, 1971), the question about its upstream effectors is very timely (Isayenkov and Maathuis, 2019). Cytosolic Ca^{2+} increase can be mediated via Ca^{2+} release from cellular compartments such as vacuole or ER, or from Ca^{2+} import from the apoplast (Dodd *et al.*, 2010). Several calcium-channels with mechano-sensivity have been studied and form good candidates as upstream effectors of Ca^{2+} accumulation. For example, a forward genetics screens enabled the identification of OSCA1 (OSmolality-induced CA^{2+}-increase) and OSCA3, Ca^{2+}-gated osmosensors which stimulate ion influx channels for further Ca^{2+} accumulation (Fig. 1c)(Yuan *et al.*, 2014; Zhang *et al.*, 2018). They have a domain for interaction with membranes, possibly mediating mechano-sensitivity, and form a pore, although it is still unclear whether this pore is for Ca^{2+} transport (Jojoa-Cruz *et al.*, 2018; Murthy *et al.*, 2018; Maity *et al.*, 2019). Consistently, in *osca1* mutants, the Ca^{2+} wave is weaker resulting in impaired osmotic stress responses. Calcium can also be transported via cyclic nucleotide-gated channels (CNGC), and the levels of cyclic guanosine monophosphate (cGMP) increase upon osmotic stress. Chemical inhibition of cGMP disables the Ca^{2+} accumulation under salt, suggesting that CNGCs might participate in the Ca^{2+} peak, although this might be a salt-specific effect (Fig. 1c)(Donaldson *et al.*, 2004). A similar strategy, with chemical inhibitors, was used to demonstrate that Na^+/H^+-antiport systems could participate in the Ca^{2+} release under salt stress in *Populus callus* (Sun *et al.*, 2010). Under cold stress, a stress that can cause osmotic stress when it becomes severe, two mechanosensitive calcium channels, MCA1 and MCA2, mediate the Ca^{2+} peak (Mori *et al.*, 2018), but their involvement in the sorbitol-induced osmotic stress response remains questionable (Kurusu *et al.*, 2013; Stephan *et al.*, 2016). Instead, two plastid-localized K^+ exchange antiporters (KEA) are necessary for Ca^{2+} release under salt and sorbitol stress, and seedlings defective in KEA proteins show altered sensitivity to salt stress (Fig. 1c)(Kunz *et al.*, 2014; Stephan *et al.*, 2016). Finally, other membrane-localized proteins that do not function directly as channels can also promote calcium uptake. As such, the receptor-like kinase FERONIA senses changes in cell wall integrity upon root bending or osmotic stress and participates in establishing the Ca^{2+} peak, although this mechanisms might be specific to high salt stress (Shih *et al.*, 2014; Feng *et al.*, 2018).

Finally, changes in cytoskeleton dynamics were reported to be another process initiated within minutes upon stress application and were observed for salt, PEG and mannitol, although for the latter it has not been reported in root cells (Wang *et al.*, 2007; Yu *et al.*, 2018). In Arabidopsis root epidermal cells, the actin and microtubule cytoskeleton is highly dynamic and filaments continuously polymerize, assemble and elongate. Upon exposure of roots to high salt stress, actin depolymerization and bundle fragmentation is stimulated within ten minutes of stress exposure (Liu *et al.*, 2012), probably to adapt the cytoskeleton to the new cell shape under higher osmotic pressure (Fig. 1e). Accordingly, a mutant affected in microtubule organization was found to be hypersensitive to osmotic stress (Bhaskara *et al.*, 2017b). The precise molecular mechanisms triggering these changes are poorly understood, but evidence suggests that microtubule disassembly can, in turn, also stimulate an increase in Ca^{2+} levels (Wang *et al.*,

2011). Altogether, different early osmotic stress-responsive mechanisms converge towards fast but transient waves of intracellular Ca^{2+}.

Reactive oxygen species (ROS) peak within minutes following osmotic stress

During the rapid and transient Ca^{2+} peak, Ca^{2+} molecules can be recognized and bound by different families of proteins such as calmodulin and the calmodulin-binding transcription factors (CAMTA) to further elicit transcriptional responses. Alternatively, they can initiate phosphorylation cascades by binding Ca^{2+}-dependent protein kinases (CDPKs) and calmodulin-dependent kinases (CCaMKs) (Fig. 1d) (Kaplan *et al.*, 2006; Finkler *et al.*, 2007; Dodd *et al.*, 2010). Among the multiple downstream effects of Ca^{2+}-induced signaling, one notable example observed under salt stress is the calcium-induced activity of RSA1, which directly binds Ca^{2+} and RITF1, a transcription factor that triggers *SOS1* transcription (Fig. 1d) (Guan *et al.*, 2013). Ca^{2+} also binds the calcineurin-like B protein CBL4/SOS3 which causes activation of the kinase SOS2 that, in turn, phosphorylates SOS1. In these salt-stress specific examples, Ca^{2+} thus contributes to stress avoidance by stimulating SOS1-mediated Na^+ export to prevent toxicity (reviewed in Julkowska and Testerink (2015) and Isayenkov and Maathuis (2019)). Other examples include Ca^{2+}-mediated inhibition of non-specific cation channels or, in aerial plant organs, stomatal closure (Geiger *et al.*, 2010; reviewed in Daszkowska-Golec and Szarejko, 2013).

Another important downstream effect of Ca^{2+} accumulation is the stimulation of ROS by post-translational activation of ROS-producing enzymes, RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs). Some RBOH proteins possess EF-hand motifs at the N-terminus through which they might bind Ca^{2+} and thereby be activated (Fig. 1f) (Canton and Grinstein, 2014; Navathe *et al.*, 2019), while other RBOHs have phosphorylation sites targeted by CCaMKs (Fig. 1g) (Kadota *et al.*, 2015). Although less characterized at the molecular level, several lines of evidence also suggest that the RBOH enzymes are stimulated by stress-induced endocytosis and actin depolymerization (Fig. 1h) (Wang *et al.*, 2011; Hao *et al.*, 2014; Tian *et al.*, 2015). Following short-term stress exposure, RBOHs produce O_2^- in mitochondria, peroxisomes and the apoplast of root cells (Gill and Tuteja, 2010), which is further converted to H_2O_2 (Fig. 1i) (Suzuki *et al.*, 2011). Conversely, ROS like OH-radicals can activate ANNEXIN-type channels that further stimulate Ca^{2+} import upon salt stress (Laohavisit *et al.*, 2012; Laohavisit *et al.*, 2013).

Each RBOH produces ROS in a different context; RBOH-D and E are responsible for ROS production under osmotic stress (Xie *et al.*, 2011; Ma *et al.*, 2012). While in high concentrations ROS can damage proteins, H_2O_2 acts as stress signaling molecule at low levels (Foyer and Noctor, 2009). Therefore, plants affected in ROS production, e.g. upon mutation of the important RBOH-D enzyme, are more sensitive to salt stress (Huang *et al.*, 2019). Short-term accumulation of ROS has now been reported in multiple species upon exposure to different types of osmotic stress, although the evidence for a ROS burst under PEG and mannitol is rather limited or contradictory (Leshem *et al.*, 2007; Si *et al.*, 2010; Tamás *et al.*, 2010; Uzilday *et al.*, 2014; Ben Rejeb *et al.*, 2015a). Overall, studies that performed ROS measurements

in different species report a fast but transient ROS wave ranging from minutes to 12 h following stress exposure.

Root-to-shoot-transported molecules propagate the signal upwards within one hour

Osmotic stress triggers molecular and phenotypic changes locally at root level, but the effects in the shoot are even more pronounced (Claeys *et al.*, 2014b; Khadka *et al.*, 2019). Shoot responses to osmotic stress reduce growth, redirect energy metabolism and minimize water loss, requiring a fast and mobile signal from the root to the shoot. Whether this signal has a hydraulic origin or is a non-hydraulic root-sourced signal (nHRSC), such as a hormone-related molecule, is still under debate. Root conductivity is affected within minutes by osmotic stress through ROS-induced internalization of the aquaporins via clathrin-mediated endocytosis (Fig. 1j) (Boursiac *et al.*, 2008; Ueda *et al.*, 2016; Kapilan *et al.*, 2018). As a result, water transport is reduced, further sustaining the hydraulic signal along the root up to the shoot, where this decrease in water potential is perceived (reviewed in Rodrigues *et al.*, 2019). However, several lines of evidence suggest that root-to-shoot signaling following stress still occurs when the water potential is maintained by watering parts of the roots or by adjusting the osmotic potential, thereby strongly suggesting the presence of nHRSCs (Davies and Zhang, 1991; Nonami *et al.*, 1997; Tang and Boyer, 2002; Parent *et al.*, 2010; Bonhomme *et al.*, 2012; Batool *et al.*, 2019a; Batool *et al.*, 2019b). The current view is that the earliest response to mild osmotic stress is dominated by nHRSCs, whereas the longer-term response, or when the stress is more severe, is sustained by hydraulic signals (Schachtman and Goodger, 2008; Pérez-Alfocea *et al.*, 2011; Batool *et al.*, 2019a; Batool *et al.*, 2019b). In the context of the rapid stress response presented here, only nHRSCs will be discussed.

One criterium for candidate early nHRSCs is that biosynthesis or accumulation in the roots, xylem-mediated upwards transport and accumulation in the shoot occur in subsequent order. As the first responses in shoots are captured between ten minutes and one hour upon exposure of the root to stress (see next section), this series of steps is expected to be extremely fast. It can thus be considered unlikely that the earliest signals are synthesized by enzymes that are regulated at the transcriptional level; post-translational activation or release of stored/conjugated molecules is more plausible. A first good candidate nHRSCs is ROS, as post-translational mechanisms activate ROS biosynthesis within minutes upon osmotic stress. Transmitted through a wave, ROS induce RBOH activity in adjacent cells, which in turn produce ROS (Fig. 1k) (Miller *et al.*, 2009; Mittler *et al.*, 2011). Such waves can reach a speed of 8 cm per minute, and could trigger responses in leaves of small seedlings within minutes. Along with ROS, the other secondary signaling molecule, Ca^{2+}, would also be capable of long-distance transport, as was observed under salt stress (Choi *et al.*, 2014; Choi *et al.*, 2016). Whether a root-to-shoot Ca^{2+} wave also takes place under other types of osmotic stress, is still unclear.

Another good candidate for root-to-shoot signaling under osmotic stress is the precursor of ethylene gas, 1-aminocyclopropane-1-carboxylic acid (ACC), or a soluble ACC-conjugate. ROS can activate

a phosphorylation cascade involving MKK9, MPK3 and MPK6 (Yuasa *et al.*, 2001; Liu *et al.*, 2010; Chang *et al.*, 2012; Tsugama *et al.*, 2012). MPK3/6 in turn phosphorylate and activate ACC-SYNTHASE2 (ACS) and ACS6, regulating the rate-limiting step of ACC biosynthesis (Fig. 1l), although the precise involvement of ROS in this process and the occurrence under osmotic stress are unclear (Liu and Zhang, 2004; Xu *et al.*, 2014). Osmotic stress and drought have previously been found to increase the ACC concentration in the xylem in several – but not all – studies exploring this, leaving the question whether ACC could act as a root-to-shoot signal still open (Pérez-Alfocea *et al.*, 2011; Marino *et al.*, 2017). The earliest ethylene-related transcriptional responses in shoots were detected 30-45 min following biotic or osmotic stress (see further), suggesting that ACC would be transported within the first half hour of stress (Fig. 1m)(Hasegawa *et al.*, 2011; Dubois *et al.*, 2015).

Third, jasmonic acid (JA) is emerging as a candidate to either function as or trigger the root-to-shoot signal. Several genes encoding JA biosynthesis enzymes can be induced by Ca^{2+}, and also ROS might be involved in this process (Kang *et al.*, 2006; Hu *et al.*, 2009). JA levels are increased in Arabidopsis, wheat and eucalyptus roots following exposure to drought (Du *et al.*, 2013; Correia *et al.*, 2014; Liu *et al.*, 2015), and JA-responsive genes are commonly found among drought-induced transcriptomic changes (Baerenfaller *et al.*, 2012; Clauw *et al.*, 2016; Dubois *et al.*, 2017). However, a closer look at the timing of JA accumulation could question its possible function in early root-to-shoot signaling. The accumulation of JA (60 min) precedes the induction of its biosynthesis genes (90 min), pointing towards a feedback mechanism rather than a causal effect (Ellouzi *et al.*, 2014). Moreover, the induction of JA biosynthesis genes upon Ca^{2+} accumulation occurs transcriptionally, further questioning the hypothesis of JA as a very early signal.

Fourth, the central drought-responsive hormone ABA has historically grown as the most obvious candidate for root-to-shoot signaling, although recent pieces of evidence lead to question this (reviewed by Pérez-Alfocea *et al.*, 2011; McAdam *et al.*, 2016a; McAdam *et al.*, 2016b). Increased ABA production upon osmotic stress and drought has been observed extensively in multiple species (reviewed in Waadt *et al.*, 2014; Batool *et al.*, 2019a; Batool *et al.*, 2019b; Brunetti *et al.*, 2019; Khadka *et al.*, 2019; Takahashi and Shinozaki, 2019). ABA levels are increased in roots and in the xylem sap, most likely as a result of increased transcription of *NCED3*, the gene encoding the rate-limiting ABA biosynthesis enzyme (Brunetti *et al.*, 2019). As transcriptional induction takes time, there would be a delay in ABA accumulation for root-to-shoot signaling, which is in line with the recent view on this process. In this model (reviewed in McAdam *et al.*, 2016b; Takahashi and Shinozaki, 2019), the ABA signal in the shoot is not a direct consequence of fast root-to-shoot ABA transport, but a combined effect of ABA biosynthesis in the shoot and bidirectional ABA transport. Indeed, ABA was recently shown to accumulate more rapidly in the shoot (10 min) than in the roots (Yuan *et al.*, 2014; Liu *et al.*, 2015), thus, the increased levels of ABA in the xylem (Pérez-Alfocea *et al.*, 2011; Correia *et al.*, 2014) might result from re-circulation of leaf-sourced ABA rather than from early stress-responsive root-to-shoot transport (Zeevaart and Boyer, 1984;

Schachtman and Goodger, 2008)(Fig. 1n). It is speculated that ABA biosynthesis in the shoot might result from a JA-mediated signal or from the hydraulic signal, as in some species the level of ABA is not increasing when the hydraulic signal is dampened (de Ollas *et al.*, 2015; McAdam *et al.*, 2016b; Marino *et al.*, 2017; Takahashi and Shinozaki, 2019).

Besides these putative root-to-shoot signals, other molecules deserve additional research in order to determine their possible role in this process, including cytokinin, salicylic acid, malate, acetylcholine, sulfate, gibberellic acid (GA) and peptides of the CLE family (Gaion *et al.*, 2018; Nelissen *et al.*, 2018; Takahashi and Shinozaki, 2019). The latter consists of small, cell non-autonomous peptides that can be responsive to drought stress and trigger drought-related responses such as stomatal closure (CLE9 and CLE10) (Kucukoglu and Nilsson, 2015; Qian *et al.*, 2018; Zhang *et al.*, 2018). Interestingly, *CLE25* expression is induced in the roots upon dehydration stress and exogenous CLE25 treatment of the root induces ABA synthesis in the shoot (Takahashi *et al.*, 2018). Whether the CLE25 peptide itself is transported from root to shoot and whether this takes place upon short-term or mild osmotic stress is a future area to be explored.

Within the first hour, leaves close stomata and initiate growth arrest

Following stress exposure of Arabidopsis roots, the stress signal reaches the shoot and triggers responses that are known to be highly dependent on the organ, the developmental stage of each leaf, the tissue, and even the cell type (Dinneny *et al.*, 2008; Skirycz *et al.*, 2010; Verelst *et al.*, 2013). In the shoot, rapid physiological responses such as stomatal closure were captured within ~10 minutes following treatment of roots with PEG or sorbitol (Fig. 1r) (Yuan *et al.*, 2014). At the molecular level, differential phosphorylation of ~70 proteins was measured in leaves within 30 minutes upon mild mannitol-induced stress (Nikonorova *et al.*, 2018). Other phosphoproteomics studies detected extensive differential phosphorylation upon shorter stress exposure (up to 5 minutes), or investigated phosphoproteome changes by using other osmotic stress compounds, which helped to characterize important stress-responsive phosphorylation pathways, such as the PP2C-SnRK2 described below (Xue *et al.*, 2013; Stecker *et al.*, 2014; Bhaskara *et al.*, 2017a; Wong *et al.*, 2019). Unfortunately, the latter studies lack organ-level resolution necessary to draw conclusions about the timing at which these phosphoproteome changes occur in the shoot. Specifically in shoots, transcriptomics and proteomics analyses found notable osmotic stress-induced changes within 30~60 minutes following osmotic stress (Skirycz *et al.*, 2011a; Nikonorova *et al.*, 2018). Overall, these studies situate the short-term stress response in aerial plant tissues of Arabidopsis around 10~60 minutes upon stress sensing by the roots.

In fully-grown leaves, ABA is the central hormone controlling the response to *in vitro* osmotic stress, but also to drought, cold, heavy metal, or UV stress (reviewed in Vishwakarma *et al.* (2017)). Whereas stomatal closure is an immediate ABA-regulated stress response, the accumulation of osmolytes and ROS is initiated by ABA for a long-lasting response (see details in next section). In short, ABA binds to

a family of PYL/PYR (PYRABACTIN-RESISTANCE(-LIKE)) soluble receptors, thereby changing their conformation and causing them bind to PROTEIN-PHOSPHATASE 2C (PP2C) (Fig. 1r)(reviewed in Joshi-Saha *et al.*, 2011; Agurla *et al.*, 2018). PP2C is then unable to inhibit its downstream targets, kinases of the SnRK2 (SNF-RELATED KINASE2) family, of which OST1 (OPEN STOMATA1) is crucial in stomatal closure (Fujii *et al.*, 2011; Fujita *et al.*, 2013). The released kinases are free to phosphorylate on the one hand K^+ influx channels, thereby inhibiting them, and on the other hand anions efflux channels, activating them, which results in fast stomatal closure (Fig. 1r). Additionally, transcription factors of the ABA-RESPONSIVE ELEMENT BINDING family (AREBs) are targeted by SnRK2 kinases as well as by Ca^{2+}-dependent protein kinases, further controlling the ABA-dependent downstream stress response (Fujita *et al.*, 2011).

In young Arabidopsis leaves, 57 transcripts respond within 1.5 h of stress, or even earlier (Skirycz *et al.*, 2011a; Dubois *et al.*, 2015; Van den Broeck *et al.*, 2017; Nikonorova *et al.*, 2018). The majority of these genes is involved in ethylene response and, consistently, osmotic stress was shown to trigger an increase in ACC levels in young seedlings after 1 h (Fig. 1m) (Skirycz *et al.*, 2011a). The role of ACC in young leaves of plants exposed to stress is summarized by the "pause-and-stop model" (Skirycz *et al.*, 2011a; reviewed in Dubois *et al.*, 2018b). Within hours following stress, the accumulation of ACC triggers phosphorylation and inactivation of CYCLIN-DEPENDENT KINASE A (CDKA), causing a cell cycle "pause" (Fig. 1o) (Skirycz *et al.*, 2011a). This subsequently triggers a permanent cell cycle exit possibly controlled by two distinct mechanisms. First, ETHYLENE RESPONSE FACTORs (ERFs), such as ERF6, activate GA degradation by inducing the *GA2-OXIDASE6* gene, thereby stabilizing DELLA proteins, which further push cells into differentiation (Fig. 1p) (Claeys *et al.*, 2012; Dubois *et al.*, 2013; reviewed in Claeys *et al.*, 2014a). This ERF-mediated pathway can strongly impact Arabidopsis leaf growth and is therefore tightly controlled and fine-tuned by a densely connected stress-responsive network (Van den Broeck *et al.*, 2017). Second, inactivation of CDKA might cause stabilization of cell cycle inhibitory proteins like SMR1, transcriptionally and post-translationally induced by drought stress as well as by ACC (Dubois *et al.*, 2018a). In the end, these parallel mechanisms cause a reduction in leaf growth that was observed 24 h following exposure to mild osmotic stress (Fig. 1q) (Skirycz *et al.*, 2011a).

Upon initial burst of stress signals, a new steady state is established in root and shoot

After the initial response, levels of ACC decrease between 4 and 16 h following stress (Fig. 1w) (Ellouzi *et al.*, 2014). Accordingly, transcript levels of some *ERFs* return to pre-stress levels after 48h, even when the stress persists (Van den Broeck *et al.*, 2017). In contrast, ABA levels continue to increase progressively (Fig. 1x) (Ellouzi *et al.*, 2014), in accordance with previous reports that ABA would play an increasingly important role when the stress signal persists (Schachtman and Goodger, 2008). As a result of increased ABA accumulation, *RBOH* genes are transcriptionally activated through the ABA-responsive NTL4 (NAC WITH TRANSMEMBRANE MOTIF-LIKE4) transcription factor or via ERF74, maintaining further ROS production (Fig. 1y) (Lee *et al.*, 2012; Yao *et al.*, 2017). *RHOB-D* is transcriptionally induced in Arabidopsis

roots from 3 h upon stress initiation onwards (Suzuki *et al.*, 2011) and H_2O_2 levels continue to increase until 10 to 24 h, depending on the species, before they reach a plateau (Si *et al.*, 2010; Ellouzi *et al.*, 2014; Niu *et al.*, 2018). The H_2O_2 accumulation further maintains endocytosis and internalization of plasma membrane-localized aquaporins (plasma membrane intrinsic proteins, PIPs)(Fig. 1j) (Boursiac *et al.*, 2008). Eventually, root growth is inhibited between 5 to 10 h depending on the type of stress, and root water content decreases after 16 h, and is reduced by half after 72 h (Fig. 1z) (Verslues *et al.*, 2006; Ellouzi *et al.*, 2014). Under salt stress, upon this initial period of growth reduction, the primary root growth recovers via ABA-mediated inhibition of ethylene (Geng *et al.*, 2013), while the lateral root emergence remains inhibited (Duan *et al.*, 2013; Dinneny, 2015). These mechanisms redirect the energy towards the deep-growing primary root.

Above-ground, within hours following stress, closure of stomata enables a reduction in leaf transpiration but also limits CO_2 uptake, thereby lowering photosynthesis, and chlorophyll contents decrease on middle-long term (4 h) (Fig. 1s) (Ellouzi *et al.*, 2014). Also within 4 h and likely as a result of the ABA increase, H_2O_2 levels are induced and further increase progressively (Fig. 1t) (Ellouzi *et al.*, 2014). Both ROS and ABA can contribute to the biosynthesis of proline: ROS triggers NADPH-mediated biosynthesis of proline, and ABA up-regulates proline biosynthesis genes (Fig. 1x) (Ábrahám *et al.*, 2003; Ben Rejeb *et al.*, 2015b). It has recently been demonstrated that the transcription of *P5CS1*, encoding the rate-limiting enzyme in proline biosynthesis, is impaired in ABA-deficient and ABA-insensitive mutants upon phosphate starvation (Aleksza *et al.*, 2017). This suggest that ABA might be necessary for proline biosynthesis in some conditions, but not sufficient for proline accumulation under osmotic stress (Sharma and Verslues, 2010). Proline accumulates in the leaves with a similar timing as ROS and ABA (Fig. 1u)(Ellouzi *et al.*, 2014; Ben Rejeb *et al.*, 2015b). As proline biosynthesis and catabolism affect the NAPDH/NADP ratio, proline metabolism is crucial for maintaining the redox status under stress (Sharma *et al.*, 2011) and proline participates in ROS scavenging. As a highly soluble molecule, proline also acts as an osmolyte to counteract the effects of reduced water potential of the medium. However, proline by itself is not crucial for stress tolerance, and studies in Arabidopsis mutants, accesssions, and other plant species have demonstrated that, although often correlated, higher proline accumulation is not necessarily linked to improved osmotic stress or drought tolerance (Sharma *et al.*, 2011; Kesari *et al.*, 2012; Bandurska *et al.*, 2017) ; reviewed in (Kavi Kishor and Sreenivasulu, 2014; Bhaskara *et al.*, 2015). Via the rapid induction of above-described mechanisms, leaf water content and osmotic potential are only mildly affected during the first two days of osmotic stress, but eventually show a clear reduction 72 h following exposure to severe salt stress, and likely longer when the stress is milder (Fig. 1v) (Ellouzi *et al.*, 2014; Ben Rejeb *et al.*, 2015a).

Compound-specific early responses

While all types of osmotic stress share a common feature in lowering the water potential of the growth medium, some compounds are known to elicit specific responses (Table 1). Although cytosolic Ca^{2+} accumulation occurs under multiple types of (osmotic) stresses, it contributes to the establishment of stress-specific responses. The specificity is achieved through the Ca^{2+} signature, determined by the speed, amplitude, frequency and duration of the Ca^{2+} peak (Chinnusamy *et al.*, 2004). For example, in the root endodermis, mannitol induces a low primary Ca^{2+} peak (1.1 µM) but with a long duration (50 s), while salt stress induces a higher primary peak (1.8 µM) that lasts a shorter time (30 to 40 s) (Kiegle *et al.*, 2000). The speed with which the peak occurs correlates mainly with the severity of the stress (Zhu *et al.*, 2013), and the combination of each profile across the different cell types forms an additional characteristic contributing to the specificity (Kiegle *et al.*, 2000). More downstream, RBOHs D and E have been shown to be induced only by salt and not by non-ionic osmotic stress: the ionic component is determinant for fast ROS production (Leshem *et al.*, 2007), and one of the direct effects of ROS in cucumber is the exclusion of the toxic Na^{+} enzyme out of the cell (Niu *et al.*, 2018). As different RBOH enzymes are induced depending on the type of environmental stress, the specificity of the Ca^{2+} signature could be determinant in this process (Canton and Grinstein, 2014; Kadota *et al.*, 2015). Another specific effect of salt-induced Ca^{2+} accumulation is the activation of the well-characterized Salt Overly Sensitive (SOS) pathway, as was detailed in an earlier section (Chinnusamy *et al.*, 2004).

Besides specificity achieved by the Ca^{2+} signature, plant-encoded receptors can be involved in the recognition of osmotic stress compounds. As such, a compound-specific response pathway was reported for mannitol through the action of two putative mannitol receptors, EGM1 and EGM2 (Enhanced Growth on Mannitol) (Trontin *et al.*, 2014). Consequently, the growth of mutants lacking one of these EGMs is less affected by mannitol. Whether EGM proteins are upstream of the above-described leaf responses is a matter of debate; they might act only under rather high mannitol concentration or under long-term mannitol-induced stress (Dubois *et al.*, 2015). It is nevertheless clear that mild levels of mannitol (25 mM for 24 h, Skirycz *et al.*, 2011a) trigger a response very different from mild salt levels (50 mM for 48 h, Shen *et al.*, 2014), as there is no correlation between the transcriptomic changes caused by both stresses in growing shoot tissue ($CC_{Spearmann}$ = -0.38). Accordingly, genes identified as regulators of growth under mild mannitol-mediated stress, such as *ERF6*, are not affected by mild salt stress, and consequently the corresponding mutants, which grow better than the wild type on low concentrations of mannitol, do not perform better under mild salt stress (Dubois *et al.*, 2013).

Altogether, these compound-specific early responses can cause downstream phenotypic effects that are different depending on the compound (Claeys *et al.*, 2014b). They have been reported in Arabidopsis and rice, and are important to be taken into account (Hazman *et al.*, 2016). When osmotic stress is used as a user-friendly alternative to drought, compound-specific effects should be avoided. In this respect, PEG-imposed osmotic stress is a good option for gene discovery, as no PEG-specific responses were reported so far. Alternatively, proteins/pathways that were originally identified via the

use of mannitol or salt could be validated by verifying their functionality (e.g. via mutant phenotyping) under PEG-based osmotic stress or even soil-based drought, as has been done, for example, for the characterization of SnRK2 kinase and for the validation of some of the above-mentioned ERF genes (Fujii *et al.*, 2011; Dubois *et al.*, 2017; Van den Broeck *et al.*, 2017).

Importance of short-term stress assays

During the 20th century, "short-term" responses to sudden osmotic stress were mainly studied after two to three days following stress exposure, without taking into account earlier time points (Kaufmann and Eckard, 1971; Kalantari *et al.*, 2000). Since then, numerous studies reported time-course measurements following stress exposure with a range and resolution varying depending on the type of experiment (Gamboa *et al.*, 2013; Luo *et al.*, 2013; Bu *et al.*, 2014; Yuan *et al.*, 2014; Zhao *et al.*, 2015). Time-course analyses made it possible to distinguish two types of responses. On the one hand, certain processes are induced shortly upon stress and further maintained or enhanced over time, such as proline and ABA accumulation, contributing to their widely accepted role as main stress response factors (Zeller *et al.*, 2009). On the other hand, several processes are only transiently induced following stress. For example, the very short-term Ca^{2+} signal that initiates a large part of the response becomes fully undetectable within minutes following stress initiation (Yuan *et al.*, 2014). Also ROS-mediated stress signaling, induced within minutes and peaking after hours upon stress, is decreasing back to control levels after on average 24 h as a result of the activation of multiple ROS-scavenging enzymes (Fig. 1t and y) (Ben Rejeb *et al.*, 2015b). Finally, the ACC accumulation, orchestrating the short-term growth-inhibitory response of growing Arabidopsis leaves, is also just a transient signal which decreases back to control levels after days following stress exposure (Fig. 1m) (Kalantari *et al.*, 2000; Ellouzi *et al.*, 2014). This is most likely achieved by the ABA-mediated repression of the ethylene pathway (Takahashi and Shinozaki, 2019).

Overall, at transcriptome level, on average about one-fourth of the genes differentially expressed at the most early time points (1-3 h) following osmotic stress, is no longer affected at later time points (10-24 h) (Kreps *et al.*, 2002; Matsui *et al.*, 2008; Zeller *et al.*, 2009; Skirycz *et al.*, 2011a). A similar trend was observed in a proteomics study following mannitol treatment: after 4 h of stress, only approximately one-third of the earliest (30 min) mannitol-responsive proteins remained differentially accumulated in the same way, one-third disappeared, while one-third even showed an opposite trend (Nikonorova *et al.*, 2018).

As these responses disappear over time even though the stress is maintained, their importance in long-term plant acclimation to osmotic stress could be questioned. Interestingly, altering the expression of genes that are involved specifically in short-term response can have an impact on the long-term stress sensitivity. For example, compromising the (very transient) Ca^{2+} increase upon stress in the *osca1* mutant affects the growth of roots after 10 days of osmotic stress (Yuan *et al.*, 2014). Similarly, whereas the transcript level of some osmotic stress-responsive transcription factors, like *ERF2*, *ERF8*, and others, is

only transiently increased, mutants in these genes do show a long-term phenotypic effect upon osmotic stress – a phenotype that is, interestingly, also conserved upon mild drought stress in soil (Dubois *et al.*, 2017; Van den Broeck *et al.*, 2017). These examples show that genes that are transiently induced upon osmotic stress can be important in the long-term stress response and underscore the importance of sampling at early time points, especially when the aim is to study the early stress avoidance mechanisms of plants.

Importance of controlling the stress level

Besides the exact control of the stress onset, osmotic stress setups give the possibility to expose plants to a wide range of stress levels by varying the concentrations of the osmotic compound. Overall, *in vitro* osmotic stress research mainly focused on rather severe stress levels (Claeys *et al.*, 2014b). High levels of stress (>25 mM mannitol, >100 mM sorbitol, >50 mM salt) trigger huge transcriptional responses and easily visible and measurable phenotypic effects such as bleaching, alterations in leaf shape, inhibition of root growth, or germination defects, and are therefore used in the majority of stress studies (Table 1). However, more sensitive traits are affected by much milder stress, inhibiting only growth, while other symptoms remain absent (Claeys *et al.*, 2014b). Arabidopsis leaf growth is already affected by very low concentrations of stress, making it the most sensitive macroscopic phenotype to detect if plants feel the stress. Also at the molecular level, plants react according to the stress level to balance growth and survival and, consequently, different mechanisms control growth under moderate stress and survival to life-threatening, severe stress (for a review, see Claeys and Inzé, 2013). Most likely, different stress levels do not only quantitatively affect gene expression by increasing/decreasing the number of differentially expressed genes or their expression level, but rather trigger specific responses qualitatively depending on stress severity.

Added value of osmotic stress setups for future drought stress research

In vitro osmotic stress setups are used as convenient alternatives to mimic drought stress, but how comparable are they? When Arabidopsis seedlings are exposed to *in vitro* osmotic stress for days or weeks, their phenotype and long-term physiological acclimation resembles the one of drought stressed plants: stomata are closed, osmolytes accumulate, plants are smaller and, depending on the stress level, the photosynthesis rate is reduced and more severe symptoms can become visible. For the study of these longer-term physiological responses, *in vitro* osmotic compounds that do not trigger compound-specific responses and that are applied without exogenous sugars, at a relevant level of severity, form a good alternative to in soil drought (Fig. 2) (Verslues et al., 2006).

Similar phenotypic or physiological responses could be the result of different early signaling events, involving different regulators. When comparing short-term osmotic stress responses with short-term drought stress signaling (see next section about the feasibility of such measurements), three tendencies are clear. First, high levels of osmotic stress (> 100 mM NaCl, > 200 mM mannitol and > 20%

PEG6000) induce short-term transcriptome responses that are comparable between different compounds (comparison between the dataset of Kreps *et al.* (2002) and Matsui *et al.* (2008)) and that correlate with severe dehydration responses ($CC_{Spearman}$ = 0.74)(Matsui *et al.*, 2008). As a consequence, many mutants with an increased tolerance to severe osmotic stress also survive better when grown in soil and exposed to severe dehydration stress (Gamboa *et al.*, 2013; Lü *et al.*, 2013; Song *et al.*, 2013; Xiao *et al.*, 2013; Cai *et al.*, 2014; Cho *et al.*, 2014; Kim *et al.*, 2014; Li *et al.*, 2014; Liu *et al.*, 2014; Qin *et al.*, 2014; Cai *et al.*, 2015; Zhao *et al.*, 2015; Liu *et al.*, 2019). These mutants often carry mutations in genes involved in early, general stress response pathways such as ROS- and Ca^{2+}-signaling (Fig. 2). Secondly, the overlap in short-term responses between mild osmotic stress imposed by PEG and mild progressive drought is significant. About 25% of the genes up- or down-regulated in young seedlings upon 4 days of low water potential stress using PEG (-0.7 MPa) are similarly affected in young leaves of seedlings exposed to 4 days of progressive drought in soil, with genes involved in water deprivation, ABA, and anthocyanin metabolism in the overlap (Supp. Fig. 2)(Dubois *et al.*, 2017; Wong *et al.*, 2019). Consistently, mutants with similar phenotypes under progressive drought and mild PEG-induced osmotic stress are found, e.g. mutants in E-CLADE GROWTH-REGULATING PP2C (EGR) and MICROTUBULE-ASSOCIATED STRESS PROTEIN1 (MASP1), both involved in microtubule re-organization, one of the earliest responses to stress (Bhaskara *et al.*, 2017b). Third, the correlation between osmotic stress and drought is less obvious when mannitol, a plasmolysis-inducing compound, is used. Transcriptome datasets from mild mannitol-induced osmotic stress and progressive drought stress, both on very young Arabidopsis leaves that were similarly affected at phenotypic level, do not correlate ($CC_{Spearmann}$ = -0.19) (Skirycz *et al.*, 2011a; Clauw *et al.*, 2015). Moreover, in young leaves, the central regulators of leaf growth under mild mannitol-mediated osmotic stress, such as ERF6, are not responsive to drought stress in the soil (Fig. 2). However, recent studies using mild to moderate drought stress in the soil highlighted a role for ethylene, GA and JA in growing Arabidopsis leaves (Baerenfaller *et al.*, 2012; Dubois *et al.*, 2017). It can therefore be speculated that, upon drought, (other) transcription factors from the ethylene-responsive family act on the DELLA proteins to regulate leaf growth, as happens *in vitro*. For example, the *ERF8* gene is induced in young leaves by mannitol and by progressive drought, and the mutant has the same phenotype under both osmotic stress and drought (Dubois *et al.*, 2017; Van den Broeck *et al.*, 2017). Altogether, these examples show that the general response mechanisms between mild osmotic stress and mild drought might be conserved, regarding hormonal pathways or gene families, but that the identity of the regulators involved in specific processes might be different (Fig. 2).

These comparisons show that *in vitro* osmotic stress assays can be useful tools for future studies of both long-term and short-term stress responses. Given the compound-specific responses initiated by mannitol and salt, PEG is currently the most suitable compound, not triggering plasmolysis and enabling a stable stress level. When used properly (medium without sugar and on vertical plates with high agar concentration, as described in Verslues *et al.,* 2006), PEG homogeneously decreases the growth of plant

roots. Because, in our opinion, PEG might be less suitable for studying rosette growth on horizontal plates (see introductory section), sorbitol might be a better compound for this purpose. As an ideal solute, sorbitol dissolves equally in solid *in vitro* growth medium, however, it should be kept in mind that this compound can cause plasmolysis of root cells. Importantly, upon gene discovery using one of the osmotic compounds, it is in our opinion critical to validate the genes of interest by using another osmotic compound or, even more relevant, by the use of actual drought stress, in soil.

Gene discovery and validation in soil-based drought assays... a feasible challenge

Most commonly, early osmotic stress responses are being unraveled *in vitro*. The validation of the findings under actual drought is straight-forward when studying very general stress responses such as Ca^{2+}, ABA or ROS signaling, but can be very challenging when aiming at validating specific responses to milder levels of stress. An obvious reason why some osmotic stress responses cannot be translated is likely because soil is intrinsically different from artificial growth medium: along the same root, local differences in soil composition or soil humidity can initiate different responses in the closest root cells (Fig. 2). These micro-differences around the root can become increasingly important upon soil drying, as the soil within a pot does not dry homogeneously. Another plausible reason for this limited success is that some less general *in vitro* responses per definition depend on the precise context, being either (i) the developmental stage of the studied organ, (ii) the stress level or (iii) the duration of the stress. When care is not taken to maintain as far as possible this context during the in soil validation, the specific gene or pathway that is to be validated, might not be detected. Indeed, also in soil-based drought stress assays, the plants' developmental stage and the severity of the drought stress are determinant for the observed response (Skirycz *et al.*, 2011b; Ma *et al.*, 2014) and mutants surviving better under severe stress are not performing better when measuring rosette growth under milder drought (Skirycz *et al.*, 2011b).

To increase the probability of validating a certain *in vitro*-identified process under drought, controling stress levels and duration is of crucial importance, particularly when studying short-term stress responses – it, nevertheless, forms a considerable challenge. In progressive drying soil setups, which are more comparable to natural drought situations than sudden dehydration assays, the stress level can easily be confounded with the duration of stress exposure, as stress levels and duration increase simultaneously (Fig. 2). Moreover, when soil is dried out progressively, it is unclear at which level of soil humidity the stress response will be activated, and different processes are likely to be induced when different thresholds in soil humidity are reached.

To capture short-term drought responses during non-lethal drought stress, the following setup can be used. First, seedlings should be grown under controlled well-watered conditions, in sufficiently large pots, and the soil humidity of each pot should be measured and adapted daily, manually or via the use of automated drought platforms, as detailed in Granier *et al.* (2006) and Skirycz *et al.* (2011b). The well-watered treatment should be maintained until the seedling or organ of interest almost reaches the

desired developmental stage (e.g. before leaf emergence if the aim is to study leaf growth, before flowering if one aims to study flowers, etc) (Fig. 2). At this time point, progressive drying of the soil can start and should be, from the start, accompanied by a detailed time-course analysis with high-resolution phenotypic, physiological and transcript- or protein-level measurements. From the moment drought triggers small phenotypic changes, the soil humidity during the drought treatment should be kept constant. The time-course experiment can then still be continued for several days, without confusion between stress duration and stress severity. If one aims to study very young Arabidopsis seedlings upon moderate drought, another technique is to transfer young seedlings directly to pots with dry soil (Clauw *et al.*, 2016). This technique is easily applicable for young seedlings (with short roots), although special care should be taken to not stress the seedlings during the transfer itself. These approaches can be followed by expression analysis to validate the responsiveness of the gene of interest to drought, and by phenotypic measurements of wild-type and the mutant (Fig. 2).

Finally, because *in-vitro*-to-drought translation of specific genes or pathways appears to be difficult, e.g. regarding the shoot growth inhibition, another relevant approach is to perform gene discovery directly under soil-based drought. The setup described here-above can be combined with transcriptomics, proteomics or phosphoproteomics to discover new regulators or effectors of short-term drought responses. In such large-scale studies, the challenge is to pinpoint genes linked to the studied phenotype. Genes involved in classical drought responses, such as ABA, ROS or proline, are omni-present in drought stress datasets, even when the drought is mild (Baerenfaller *et al.*, 2012; Clauw *et al.*, 2015; Clauw *et al.*, 2016; Dubois *et al.*, 2017). Nevertheless, these processes are not necessarily linked to the studied phenotype. Possible approaches to distinguish key phenotype-related genes from the overall drought response include the comparison of the genome-wide response in wild-type vs. a mutant affected in the studied phenotype, or in multiple accessions differently affected in it. Alternatively, genes/proteins that are not related to the phenotype can be filtered out if they show the same differential response in a context not related to the phenotype (e.g. a different organ, or the same organ in a different developmental stage). Finally, if the phenotype is quantifiable the dynamics of the phenotype and the transcriptome/proteome can be integrated to find possible positive (correlating with phenotype) or negative (anti-correlating) regulators. Most likely, mathematical modeling approaches will be necessary to get insights in these extremely complex responses.

Conclusions and perspectives

Over the last decades, *in vitro* osmotic stress setups have evolved as reliable and user-friendly alternative to drought for imposing a lower water potential to plants. As compared to drought, plate-based methods offer the possibility to apply a stable stress level for a long period, enabling the study of long-term acclimation mechanisms. Additionally, by exposing plants to *in vitro* osmotic stress only at a desired stage during development and subsequently measuring the response at multiple time points upon stress, the

short-term phenotypic effects and pathways governing it can be deciphered. Unraveling these short-term responses is of great importance to understand, and in the future possibly improve, the plants' response to drought: they form the connection between the drought perception and the long-term drought acclimation. Consequently, mutants in early responses such as Ca^{2+} or ROS signaling, are more sensitive to osmotic stress on the long term, underscoring the importance of these short-term responses. These early signaling processes subsequently initiate a trigger from the root to the shoot, causing organ-specific responses in the aerial part within the first hour upon stress exposure.

In soil-based drought stress experiments, capturing these early responses is a challenge, particularly for the discovery of new genes involved in specific phenotypic drought responses. Nevertheless, some processes uncovered using *in vitro* stress setups were successfully translated using in soil drought. This is particularly true for the general stress responses, such as ABA and Ca^{2+} signaling, or osmolyte accumulation. On the contrary, the mechanisms controlling very specific responses, such as the inhibition of the growth of young leaves under mild stress levels, appeared to be less translatable. Therefore, we can conclude that osmotic stress assays are great tools for the identification of early and late stress-responsive genes, but that it is crucial to validate the responsiveness and function of the identified genes in a broader range of stresses, either by using more than one, preferable not plasmolysis-inducing, osmotic compound or by using drought stress in soil. In this case, special care should be taken to develop a drought assay that is comparable to the *in vitro* stress in terms of stress severity and timing.

As detailed in this review, a large number of stress-responsive pathways have been identified, but the precise molecular regulation of many of them forms an exciting area for future research. As such, the first process involved in this response, the stress sensing, is a fascinating and not yet understood mechanism. However, doubts can be raised as whether stress-sensing mechanisms can be unraveled by the use of *in vitro* mimics, as drying soil likely gives a different effect on the root surface than osmotic compound-containing, but still humid, *in vitro* medium. Similarly, how this stress-sensing mechanisms subsequently trigger cellular Ca^{2+} accumulation also remains elusive, although much progress has been booked during the last years. Another open question is the exact identity of the root-to-shoot signal under drought, even though many candidates were proposed. In this respect, the possibility that more than one signal is transported from root to shoot in order to initiate specific shoot responses, should not be excluded.

Acknowledgements

We thank Dr. Annick Bleys for carefully reading and preparing this manuscript for publication, and present and former members of the Systems Biology of Yield group for helpful discussions. We also would like to acknowledge the anonymous reviewers for their useful suggestions that helped to make this review more complete and attractive. This work was supported by Ghent University ('Bijzonder

Onderzoeksfonds Methusalem Project' no. BOF08/01M00408). Marieke Dubois is a post-doctoral fellow of Flanders Research Foundation (FWO no. 12Q7919N).

References

Ábrahám E, Rigó G, Székely G, Nagy R, Koncz C, Szabados L. 2003. Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in *Arabidopsis*. Plant Molecular Biology **51,** 363-372.

Agurla S, Gahir S, Munemasa S, Murata Y, Raghavendra AS. 2018. Mechanism of stomatal closure in plants exposed to drought and cold stress. Advances in Experimental Medicine and Biology **1081,** 215-232.

Aleksza D, Horváth GV, Sándor G, Szabados L. 2017. Proline accumulation is regulated by transcription factors associated with phosphate starvation. Plant Physiology **175,** 555-567.

Baerenfaller K, Massonnet C, Walsh S, *et al.* 2012. Systems-based analysis of Arabidopsis leaf growth reveals adaptation to water deficit. Molecular Systems Biology **8,** 606.

Bandurska H, Niedziela J, Pietrowska-Borek M, Nuc K, Chadzinikolau T, Radzikowska D. 2017. Regulation of proline biosynthesis and resistance to drought stress in two barley (*Hordeum vulgare* L.) genotypes of different origin. Plant Physiology and Biochemistry **118,** 427-437.

Baral A, Irani NG, Fujimoto M, Nakano A, Mayor S, Mathew MK. 2015a. Salt-induced remodeling of spatially restricted clathrin-independent endocytic pathways in Arabidopsis root. Plant Cell **27,** 1297-1315.

Baral A, Shruthi KS, Mathew MK. 2015b. Vesicular trafficking and salinity responses in plants. IUBMB Life **67,** 677-686.

Batool A, Akram NA, Cheng Z-G, Lv G-C, Ashraf M, Afzal M, Xiong J-L, Wang J-Y, Xiong Y-C. 2019a. Physiological and biochemical responses of two spring wheat genotypes to non-hydraulic root-to-shoot signalling of partial and full root-zone drought stress. Plant Physiology and Biochemistry **139,** 11-20.

Batool A, Cheng Z-G, Akram NA, Lv G-C, Xiong J-L, Zhu Y, Ashraf M, Xiong Y-C. 2019b. Partial and full root-zone drought stresses account for differentiate root-sourced signal and yield formation in primitive wheat. Plant Methods **15,** 75.

Ben Rejeb K, Benzarti M, Debez A, Bailly C, Savouré A, Abdelly C. 2015a. NADPH oxidase-dependent H_2O_2 production is required for salt-induced antioxidant defense in *Arabidopsis thaliana*. Journal of Plant Physiology **174,** 5-15.

Ben Rejeb K, Lefebvre-De Vos D, Le Disquet I, Leprince A-S, Bordenave M, Maldiney R, Jdey A, Abdelly C, Savouré A. 2015b. Hydrogen peroxide produced by NADPH oxidases increases proline accumulation during salt or mannitol stress in *Arabidopsis thaliana*. New Phytologist **208,** 1138-1148.

Bhaskara GB, Nguyen TT, Yang T-H, Verslues PE. 2017a. Comparative analysis of phosphoproteome remodeling after short term water stress and ABA treatments versus longer term water stress acclimation. Frontiers in Plant Science **8,** 523.

Bhaskara GB, Wen T-N, Nguyen TT, Verslues PE. 2017b. Protein phosphatase 2Cs and *Microtubule-Associated Stress Protein 1* control microtubule stability, plant growth, and drought response. Plant Cell **29,** 169-191.

Bhaskara GB, Yang T-H, Verslues PE. 2015. Dynamic proline metabolism: importance and regulation in water limited environments. Frontiers in Plant Science **6,** 484.

Bonhomme L, Valot B, Tardieu F, Zivy M. 2012. Phosphoproteome dynamics upon changes in plant water status reveal early events associated with rapid growth adjustment in maize leaves. Molecular & Cellular Proteomics **11,** 957-972.

Boursiac Y, Prak S, Boudet J, Postaire O, Luu D-T, Tournaire-Roux C, Santoni V, Maurel C. 2008. The response of Arabidopsis root water transport to a challenging environment implicates reactive oxygen species- and phosphorylation-dependent internalization of aquaporins. Plant Signaling & Behavior **3,** 1096-1098.

Boyer JS. 1971. Nonstomatal inhibition of photosynthesis in sunflower at low leaf water potentials and high light intensities. Plant Physiology **48,** 532-536.

Broecker WS. 1975. Climatic change: are we on the brink of a pronounced global warming? Science **189,** 460-463.

Brunetti C, Gori A, Marino G, *et al.* 2019. Dynamic changes in ABA content in water-stressed *Populus nigra*: effects on carbon fixation and soluble carbohydrates. Annals of Botany doi: 10.1093/aob/mcz005.

Bu Q, Lv T, Shen H, *et al.* 2014. Regulation of drought tolerance by the F-box protein MAX2 in Arabidopsis. Plant Physiology **164,** 424-439.

Cai G, Wang G, Wang L, Liu Y, Pan J, Li D. 2014. A maize mitogen-activated protein kinase kinase, ZmMKK1, positively regulated the salt and drought tolerance in transgenic *Arabidopsis*. Journal of Plant Physiology **171,** 1003-1016.

Cai W, Liu W, Wang W-S, Fu ZW, Han T-T, Lu Y-T. 2015. Overexpression of rat neurons nitric oxide synthase in rice enhances drought and salt tolerance. PLoS ONE **10,** e0131599.

Canton J, Grinstein S. 2014. Priming and activation of NADPH oxidases in plants and animals. Trends in Immunology **35,** 405-407.

Chang R, Jang CJH, Branco-Price C, Nghiem P, Bailey-Serres J. 2012. Transient MPK6 activation in response to oxygen deprivation and reoxygenation is mediated by mitochondria and aids seedling survival in *Arabidopsis*. Plant Molecular Biology **78,** 109-122.
Chinnusamy V, Schumaker K, Zhu J-K. 2004. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. Journal of Experimental Botany **55,** 225-236.
Cho HY, Lee C, Hwang S-G, Park YC, Lim HL, Jang CS. 2014. Overexpression of the *OsChI1* gene, encoding a putative laccase precursor, increases tolerance to drought and salinity stress in transgenic Gene **552,** 98-105.
Choi W-G, Hilleary R, Swanson SJ, Kim S-H, Gilroy S. 2016. Rapid, long-distance electrical and calcium signaling in plants. Annual Review of Plant Biology **67,** 287-307.
Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S. 2014. Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proceedings of the National Academy of Sciences of the United States of America **111,** 6497-6502.
Claes B, Dekeyser R, Villarroel R, Van den Bulcke M, Bauw G, Van Montagu M, Caplan A. 1990. Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. Plant Cell **2,** 19-27.
Claeys H, De Bodt S, Inzé D. 2014a. Gibberellins and DELLAs: central nodes in growth regulatory networks. Trends in Plant Science **19,** 231-239.
Claeys H, Inzé D. 2013. The agony of choice: how plants balance growth and survival under water-limiting conditions. Plant Physiology **162,** 1768-1779.
Claeys H, Skirycz A, Maleux K, Inzé D. 2012. DELLA signaling mediates stress-induced cell differentiation in Arabidopsis leaves through modulation of anaphase-promoting complex/cyclosome activity. Plant Physiology **159,** 739-747.
Claeys H, Van Landeghem S, Dubois M, Maleux K, Inzé D. 2014b. What is stress? dose-response effects in commonly used in vitro stress assays. Plant Physiology **165,** 519-527.
Clauw P, Coppens F, De Beuf K*, et al.* 2015. Leaf responses to mild drought stress in natural variants of Arabidopsis. Plant Physiology **167,** 800-816.
Clauw P, Coppens F, Korte A*, et al.* 2016. Leaf growth response to mild drought: natural variation in Arabidopsis sheds light on trait architecture. Plant Cell **28,** 2417-2434.
Correia B, Pintó-Marijuan M, Castro BB, Brossa R, López-Carbonell M, Pinto G. 2014. Hormonal dynamics during recovery from drought in two *Eucalyptus globulus* genotypes: from root to leaf. Plant Physiology and Biochemistry **82,** 151-160.
Daszkowska-Golec A, Szarejko I. 2013. The molecular basis of ABA-mediated plant response to drought. In: Vahdati K, ed. Abiotic Stress - Plant Responses and Applications in Agriculture. London, UK: IntechOpen, 103-133.
Davies WJ, Zhang J. 1991. Root signals and the regulation of growth and development of plants in drying soil. Annual Review of Plant Physiology and Plant Molecular Biology **42,** 55-76.
de Ollas C, Arbona V, Gómez-Cadenas A. 2015. Jasmonoyl isoleucine accumulation is needed for abscisic acid build-up in roots of Arabidopsis under water stress conditions. Plant, Cell & Environment **38,** 2157-2170.
Dinneny JR. 2015. Traversing organizational scales in plant salt-stress responses. Current Opinion in Plant Biology **23,** 70-75.
Dinneny JR, Long TA, Wang JY*, et al.* 2008. Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. Science **320,** 942-945.
Dodd AN, Kudla J, Sanders D. 2010. The language of calcium signaling. Annual Review of Plant Biology **61,** 593-620.
Donaldson L, Ludidi N, Knight MR, Gehring C, Denby K. 2004. Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. FEBS Letters **569,** 317-320.
Du H, Liu H, Xiong L. 2013. Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. Frontiers in Plant Science **4,** 397.
Duan LN, Dietrich D, Ng CH, Chan PMY, Bhalerao R, Bennett MJ, Dinneny JR. 2013. Endodermal ABA signaling promotes lateral root quiescence during salt stress in *Arabidopsis* seedlings. Plant Cell **25,** 324-341.
Dubois M, Claeys H, Van den Broeck L, Inzé D. 2017. Time of day determines Arabidopsis transcriptome and growth dynamics under mild drought. Plant, Cell & Environment **40,** 180-189.
Dubois M, Selden K, Bediée A*, et al.* 2018a. SIAMESE-RELATED1 is regulated posttranslationally and participates in repression of leaf growth. Plant Physiology **176,** 2834-2850.
Dubois M, Skirycz A, Claeys H*, et al.* 2013. ETHYLENE RESPONSE FACTOR6 acts as a central regulator of leaf growth under water-limiting conditions in Arabidopsis. Plant Physiology **162,** 319-332.
Dubois M, Van den Broeck L, Claeys H, Van Vlierberghe K, Matsui M, Inzé D. 2015. The ETHYLENE RESPONSE FACTORS ERF6 and ERF11 antagonistically regulate mannitol-induced growth inhibition in Arabidopsis. Plant Physiology **169,** 166-179.
Dubois M, Van den Broeck L, Inzé D. 2018b. The pivotal role of ethylene in plant growth. Trends in Plant Science **23,** 311-323.

Ellouzi H, Ben Hamed K, Hernández I, Cela J, Müller M, Magné C, Abdelly C, Munné-Bosch S. 2014. A comparative study of the early osmotic, ionic, redox and hormonal signaling response in leaves and roots of two halophytes and a glycophyte to salinity. Planta **240,** 1299-1317.
Feng W, Kita D, Peaucelle A, *et al.* 2018. The FERONIA receptor kinase maintains cell-wall integrity during salt stress through Ca^{2+} signaling. Current Biology **28,** 666-675.
Finkler A, Ashery-Padan R, Fromm H. 2007. CAMTAs: calmodulin-binding transcription activators from plants to human. FEBS Letters **581,** 3893-3898.
Foyer CH, Noctor G. 2009. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxidants & Redox Signaling **11,** 861-905.
Fujii H, Verslues PE, Zhu J-K. 2011. *Arabidopsis* decuple mutant reveals the importance of SnRK2 kinases in osmotic stress responses in vivo. Proceedings of the National Academy of Sciences of the United States of America **108,** 1717-1722.
Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K. 2011. ABA-mediated transcriptional regulation in response to osmotic stress in plants. Journal of Plant Research **124,** 509-525.
Fujita Y, Yoshida T, Yamaguchi-Shinozaki K. 2013. Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. Physiologia Plantarum **147,** 15-27.
Gaion LA, Monteiro CC, Cruz FJR, Rossatto DR, López-Díaz I, Carrera E, Lima JE, Peres LEP, Carvalho RF. 2018. Constitutive gibberellin response in grafted tomato modulates root-to-shoot signaling under drought stress. Journal of Plant Physiology **221,** 11-21.
Gamboa MC, Baltierra F, Leon G, Krauskopf E. 2013. Drought and salt tolerance enhancement of transgenic *Arabidopsis* by overexpression of the vacuolar pyrophosphatase 1 (EVP1) gene from *Eucalyptus globulus*. Plant Physiology and Biochemistry **73,** 99-105.
Geiger D, Scherzer S, Mumm P, *et al.* 2010. Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca^{2+} affinities. Proceedings of the National Academy of Sciences of the United States of America **107,** 8023-8028.
Geng Y, Wu R, Wee CW, Xie F, Wei X, Chan PMY, Tham C, Duan L, Dinneny JR. 2013. A spatio-temporal understanding of growth regulation during the salt stress response in *Arabidopsis*. Plant Cell **25,** 2132-2154.
Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry **48,** 909-930.
Granier C, Aguirrezabal L, Chenu K, *et al.* 2006. PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in Arabidopsis thaliana permitted the identification of an accession with low sensitivity to soil water deficit. New Phytol **169,** 623-635.
Guan Q, Wu J, Yue X, Zhang Y, Zhu J. 2013. A nuclear calcium-sensing pathway is critical for gene regulation and salt stress tolerance in *Arabidopsis*. PLoS Genetics **9,** e1003755.
Hao H, Fan L, Chen T, Li R, Li X, He Q, Botella MA, Lin J. 2014. Clathrin and membrane microdomains cooperatively regulate RbohD dynamics and activity in Arabidopsis. Plant Cell **26,** 1729-1745.
Hasegawa S, Sogabe Y, Asano T, *et al.* 2011. Gene expression analysis of wounding-induced root-to-shoot communication in *Arabidopsis thaliana*. Plant, Cell & Environment **34,** 705-716.
Haswell ES, Verslues PE. 2015. The ongoing search for the molecular basis of plant osmosensing. Journal of General Physiology **145,** 389-394.
Hazman M, Hause B, Eiche E, Riemann M, Nick P. 2016. Different forms of osmotic stress evoke qualitatively different responses in rice. Journal of Plant Physiology **202,** 45-56.
Héricourt F, Chefdor F, Djeghdir I, *et al.* 2016. Functional divergence of poplar histidine-aspartate kinase HK1 paralogs in response to osmotic stress. International Journal of Molecular Sciences **17,** 2061.
Heyser JW, Nabors MW. 1981. Growth, water content, and solute accumulation of two tobacco cell lines cultured on sodium chloride, dextran, and polyethylene glycol. Plant Physiology **68,** 1454-1459.
Hsiao TC, Acevedo E. 1974. Plant responses to water deficits, water-use efficiency, and drought resistance. Agricultural Meteorology **14,** 59-84.
Hu X, Li W, Chen Q, Yang Y. 2009. Early signal transduction linking the synthesis of jasmonic acid in plant. Plant Signaling & Behavior **4,** 696-697.
Huang K-C, Lin W-C, Cheng W-H. 2018. Salt hypersensitive mutant 9, a nucleolar APUM23 protein, is essential for salt sensitivity in association with the ABA signaling pathway in *Arabidopsis*. BMC Plant Biology **18,** 40.
Huang Y, Cao H, Yang L, *et al.* 2019. Tissue-specific respiratory burst oxidase homolog-dependent H_2O_2 signaling to the plasma membrane H^+-ATPase confers potassium uptake and salinity tolerance in Cucurbitaceae. Journal of Experimental Botany doi: 10.1093/jxb/erz328.
Isayenkov SV, Maathuis FJM. 2019. Plant salinity stress: many unanswered questions remain. Frontiers in Plant Science **10,** 80.
Jojoa-Cruz S, Saotome K, Murthy SE, Tsui CCA, Sansom MSP, Patapoutian A, Ward AB. 2018. Cryo-EM structure of the mechanically activated ion channel OSCA1.2. eLife **7,** e41845.

Joshi-Saha A, Valon C, Leung J. 2011. A brand new START: abscisic acid perception and transduction in the guard cell. Science Signaling **4,** re4.
Julkowska MM, Testerink C. 2015. Tuning plant signaling and growth to survive salt. Trends Plant Sci **20,** 586-594.
Kadota Y, Shirasu K, Zipfel C. 2015. Regulation of the NADPH oxidase RBOHD during plant immunity. Plant & Cell Physiology **56,** 1472-1480.
Kalantari KM, Smith AR, Hall MA. 2000. The effect of water stress on 1-(malonylamino)cyclopropane-1-carboxylic acid concentration in plant tissues. Plant Growth Regulation **31,** 183-193.
Kang S, Kim HB, Lee H, Choi JY, Heu S, Oh CJ, Kwon SI, An CS. 2006. Overexpression in *Arabidopsis* of a plasma membrane-targeting glutamate receptor from small radish increases glutamate-mediated Ca^{2+} influx and delays fungal infection. Molecules and Cells **21,** 418-427.
Kapilan R, Vaziri M, Zwiazek JJ. 2018. Regulation of aquaporins in plants under stress. Biological Research **51,** 4.
Kaplan B, Davydov O, Knight H, Galon Y, Knight MR, Fluhr R, Fromm H. 2006. Rapid transcriptome changes induced by cytosolic Ca^{2+} transients reveal ABRE-related sequences as Ca^{2+}-responsive *cis* elements in *Arabidopsis*. Plant Cell **18,** 2733-2748.
Kaufmann MR, Eckard AN. 1971. Evaluation of water stress control with polyethylene glycols by analysis of guttation. Plant Physiology **47,** 453-456.
Kavi Kishor PB, Sreenivasulu N. 2014. Is proline accumulation *per se* correlated with stress tolerance or is proline homeostasis a more critical issue? Plant, Cell & Environment **37,** 300-311.
Kesari R, Lasky JR, Villamor JG, Des Marais DL, Chen Y-J, Liu T-W, Lin W, Juenger TE, Verslues PE. 2012. Intron-mediated alternative splicing of *Arabidopsis P5CS1* and its association with natural variation in proline and climate adaptation. Proceedings of the National Academy of Sciences of the United States of America **109,** 9197-9202.
Khadka VS, Vaughn K, Xie J, Swaminathan P, Ma Q, Cramer GR, Fennell AY. 2019. Transcriptomic response is more sensitive to water deficit in shoots than roots of *Vitis riparia* (Michx.). BMC Plant Biology **19,** 72.
Kiegle E, Moore CA, Haseloff J, Tester MA, Knight MR. 2000. Cell-type-specific calcium responses to drought, salt and cold in the *Arabidopsis* root. Plant Journal **23,** 267-278.
Kim H, Lee K, Hwang H*, et al.* 2014. Overexpression of *PYL5* in rice enhances drought tolerance, inhibits growth, and modulates gene expression. Journal of Experimental Botany **65,** 453-464.
Kreps JA, Wu Y, Chang H-S, Zhu T, Wang X, Harper JF. 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiology **130,** 2129-2141.
Kucukoglu M, Nilsson O. 2015. CLE peptide signaling in plants - the power of moving around. Physiol Plant **155,** 74-87.
Kumar MN, Bau Y-C, Longkumer T, Verslues PE. 2019. Low water potential and At14a-Like1 (AFL1) effects on endocytosis and actin filament organization. Plant Physiology **179,** 1594-1607.
Kumar MN, Hsieh Y-F, Verslues PE. 2015. At14a-Like1 participates in membrane-associated mechanisms promoting growth during drought in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the United States of America **112,** 10545-10550.
Kumar MN, Jane W-N, Verslues PE. 2013. Role of the putative osmosensor Arabidopsis *histidine kinase1* in dehydration avoidance and low-water-potential response. Plant Physiology **161,** 942-953.
Kunz H-H, Gierth M, Herdean A, Satoh-Cruz M, Kramer DM, Spetea C, Schroeder JI. 2014. Plastidial transporters KEA1, -2, and -3 are essential for chloroplast osmoregulation, integrity, and pH regulation in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **111,** 7480-7485.
Kurusu T, Kuchitsu K, Nakano M, Nakayama Y, Iida H. 2013. Plant mechanosensing and Ca^{2+} transport. Trends in Plant Science **18,** 227-233.
Laohavisit A, Richards SL, Shabala L*, et al.* 2013. Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein annexin1. Plant Physiology **163,** 253-262.
Laohavisit A, Shang Z, Rubio L*, et al.* 2012. *Arabidopsis* annexin1 mediates the radical-activated plasma membrane Ca^{2+}- and K^{+}-permeable conductance in root cells. Plant Cell **24,** 1522-1533.
Lee S, Seo PJ, Lee H-J, Park C-M. 2012. A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis. Plant Journal **70,** 831-844.
Leshem Y, Seri L, Levine A. 2007. Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. Plant Journal **51,** 185-197.
Li X-l, Yang X, Hu Y-x, Yu X-d, Li Q-l. 2014. A novel NAC transcription factor from *Suaeda liaotungensis* K. enhanced transgenic *Arabidopsis* drought, salt, and cold stress tolerance. Plant Cell Reports **33,** 767-778.
Liu C, Li J, Zhu P, Yu J, Hou J, Wang C, Long D, Yu M, Zhao A. 2019. Mulberry EIL3 confers salt and drought tolerances and modulates ethylene biosynthetic gene expression. PeerJ **7,** e6391.
Liu H, Sultan MARF, Liu Xl, Zhang J, Yu F, Zhao Hx. 2015. Physiological and comparative proteomic analysis reveals different drought responses in roots and leaves of drought-tolerant wild wheat (*Triticum boeoticum*). PLoS ONE **10,** e0121852.

Liu SG, Zhu DZ, Chen GH, Gao X-Q, Zhang XS. 2012. Disrupted actin dynamics trigger an increment in the reactive oxygen species levels in the *Arabidopsis* root under salt stress. Plant Cell Reports **31,** 1219-1226.
Liu W, Tai H, Li S, Gao W, Zhao M, Xie C, Li W-X. 2014. *bHLH122* is important for drought and osmotic stress resistance in *Arabidopsis* and in the repression of ABA catabolism. New Phytologist **201,** 1192-1204.
Liu X-M, Kim KE, Kim K-C*, et al.* 2010. Cadmium activates *Arabidopsis* MPK3 and MPK6 via accumulation of reactive oxygen species. Phytochemistry **71,** 614-618.
Liu Y, Zhang S. 2004. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell **16,** 3386-3399.
Lü P, Kang M, Jiang X, Dai F, Gao J, Zhang C. 2013. *RhEXPA4*, a rose expansin gene, modulates leaf growth and confers drought and salt tolerance to *Arabidopsis*. Planta **237,** 1547-1559.
Luo X, Bai X, Sun X*, et al.* 2013. Expression of wild soybean WRKY20 in *Arabidopsis* enhances drought tolerance and regulates ABA signalling. Journal of Experimental Botany **64,** 2155-2169.
Ma L, Zhang H, Sun L, Jiao Y, Zhang G, Miao C, Hao F. 2012. NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na^+/K^+ homeostasis in *Arabidopsis* under salt stress. Journal of Experimental Botany **63,** 305-317.
Ma X, Sukiran NL, Ma H, Su Z. 2014. Moderate drought causes dramatic floral transcriptomic reprogramming to ensure successful reproductive development in Arabidopsis. BMC Plant Biology **14,** 164.
Maity K, Heumann JM, McGrath AP*, et al.* 2019. Cryo-EM structure of OSCA1.2 from *Oryza sativa* elucidates the mechanical basis of potential membrane hyperosmolality gating. Proceedings of the National Academy of Sciences of the United States of America **116,** 14309-14318.
Marino G, Brunetti C, Tattini M, Romano A, Biasioli F, Tognetti R, Loreto F, Ferrini F, Centritto M. 2017. Dissecting the role of isoprene and stress-related hormones (ABA and ethylene) in *Populus nigra* exposed to unequal root zone water stress. Tree Physiology **37,** 1637-1647.
Matsui A, Ishida J, Morosawa T*, et al.* 2008. *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant & Cell Physiology **49,** 1135-1149.
McAdam SAM, Brodribb TJ, Ross JJ. 2016a. Shoot-derived abscisic acid promotes root growth. Plant, Cell & Environment **39,** 652-659.
McAdam SAM, Manzi M, Ross JJ, Brodribb TJ, Gómez-Cadenas A. 2016b. Uprooting an abscisic acid paradigm: shoots are the primary source. Plant Signaling & Behavior **11,** e1169359.
Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, Dangl JL, Mittler R. 2009. The plant NADPH xxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Science Signaling **2,** ra45.
Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F. 2011. ROS signaling: the new wave? Trends in Plant Science **16,** 300-309.
Mori K, Renhu N, Naito M, Nakamura A, Shiba H, Yamamoto T, Suzaki T, Iida H, Miura K. 2018. Ca^{2+}-permeable mechanosensitive channels MCA1 and MCA2 mediate cold-induced cytosolic Ca^{2+} increase and cold tolerance in Arabidopsis. Scientific Reports **8,** 550.
Munns R. 2002. Comparative physiology of salt and water stress. Plant, Cell & Environment **25,** 239-250.
Munns R, Tester M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Biology **59,** 651-681.
Murthy SE, Dubin AE, Whitwam T, Jojoa-Cruz S, Cahalan SM, Mousavi SAR, Ward AB, Patapoutian A. 2018. OSCA/TMEM63 are an evolutionarily conserved family of mechanically activated ion channels. eLife **7,** e41844.
Navathe S, Singh S, Singh VK, Chand R, Mishra VK, Joshi AK. 2019. Genome-wide mining of respiratory burst homologs and its expression in response to biotic and abiotic stresses in *Triticum aestivum*. Genes & Genomics **41,** 1027-1043.
Nelissen H, Sun XH, Rymen B, *et al.* 2018. The reduction in maize leaf growth under mild drought affects the transition between cell division and cell expansion and cannot be restored by elevated gibberellic acid levels. Plant Biotechnology Journal **16,** 615-627.
Nguyen A, Lamant A. 1989. Variation in growth and osmotic regulation of roots of water-stressed maritime pine (*Pinus pinaster* Ait.) provenances. Tree Physiology **5,** 123-133.
Nikonorova N, Van den Broeck L, Zhu S, van de Cotte B, Dubois M, Gevaert K, Inzé D, De Smet I. 2018. Early mannitol-triggered changes in the Arabidopsis leaf (phospho)proteome reveal growth regulators. Journal of Experimental Botany **69,** 4591-4607.
Niu M, Huang Y, Sun S, Sun J, Cao H, Shabala S, Bie Z. 2018. Root respiratory burst oxidase homologue-dependent H_2O_2 production confers salt tolerance on a grafted cucumber by controlling Na^+ exclusion and stomatal closure. Journal of Experimental Botany **69,** 3465-3476.
Nonami H, Wu Y, Boyer JS. 1997. Decreased growth-induced water potential (a primary cause of growth inhibition at low water potentials). Plant Physiology **114,** 501-509.

Parent B, Suard B, Serraj R, Tardieu F. 2010. Rice leaf growth and water potential are resilient to evaporative demand and soil water deficit once the effects of root system are neutralized. Plant, Cell & Environment **33,** 1256-1267.
Pérez-Alfocea F, Ghanem ME, Gómez-Cadenas A, Dodd IC. 2011. *Omics* of root-to-shoot signaling under salt stress and water deficit. OMICS **15,** 893-901.
Qian P, Song W, Yokoo T, Minobe A, Wang G, Ishida T, Sawa S, Chai J, Kakimoto T. 2018. The CLE9/10 secretory peptide regulates stomatal and vascular development through distinct receptors. Nature Plants **4,** 1071-1081.
Qin L-X, Li Y, Li D-D, Xu W-L, Zheng Y, Li X-B. 2014. *Arabidopsis* drought-induced protein Di19-3 participates in plant response to drought and high salinity stresses. Plant Molecular Biology **86,** 609-625.
Rajendran K, Tester M, Roy SJ. 2009. Quantifying the three main components of salinity tolerance in cereals. Plant Cell and Environment **32,** 237-249.
Rodrigues J, Inzé D, Nelissen H, Saibo NJM. 2019. Source–sink regulation in crops under water deficit. Trends in Plant Science **24,** 652-663.
Saunier RE, Hull HM, Ehrenreich JH. 1968. Aspects of the drought tolerance in Creosotebush (Larrea divaricata). Plant Physiology **43,** 401-404.
Schachtman DP, Goodger JQD. 2008. Chemical root to shoot signaling under drought. Trends in Plant Science **13,** 281-287.
Sharma S, Verslues PE. 2010. Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant, Cell & Environment **33,** 1838-1851.
Sharma S, Villamor JG, Verslues PE. 2011. Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. Plant Physiology **157,** 292-304.
Shen X, Wang Z, Song X, Xu J, Jiang C, Zhao Y, Ma C, Zhang H. 2014. Transcriptomic profiling revealed an important role of cell wall remodeling and ethylene signaling pathway during salt acclimation in *Arabidopsis*. Plant Molecular Biology **86,** 303-317.
Shih H-W, Miller ND, Dai C, Spalding EP, Monshausen GB. 2014. The receptor-like kinase FERONIA is required for mechanical signal transduction in *Arabidopsis* seedlings. Current Biology **24,** 1887-1892.
Si Y, Dane F, Rashotte A, Kang K, Singh NK. 2010. Cloning and expression analysis of the *Ccrboh* gene encoding respiratory burst oxidase in *Citrullus colocynthis* and grafting onto *Citrullus lanatus* (watermelon). Journal of Experimental Botany **61,** 1635-1642.
Skirycz A, Claeys H, De Bodt S, *et al.* 2011a. Pause-and-stop: the effects of osmotic stress on cell proliferation during early leaf development in *Arabidopsis* and a role for ethylene signaling in cell cycle arrest. Plant Cell **23,** 1876-1888.
Skirycz A, De Bodt S, Obata T, *et al.* 2010. Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress. Plant Physiology **152,** 226-244.
Skirycz A, Vandenbroucke K, Clauw P, *et al.* 2011b. Survival and growth of *Arabidopsis* plants given limited water are not equal. Nature Biotechnology **29,** 212-214.
Song JB, Gao S, Sun D, Li H, Shu XX, Yang ZM. 2013. miR394 and LCR are involved in Arabidopsis salt and drought stress responses in an abscisic acid-dependent manner. BMC Plant Biology **13,** 210.
Stecker KE, Minkoff BB, Sussman MR. 2014. Phosphoproteomic analyses reveal early signaling events in the osmotic stress response. Plant Physiology **165,** 1171-1187.
Stephan AB, Kunz H-H, Yang E, Schroeder JI. 2016. Rapid hyperosmotic-induced Ca^{2+} responses in *Arabidopsis thaliana* exhibit sensory potentiation and involvement of plastidial KEA transporters. Proceedings of the National Academy of Sciences of the United States of America **113,** E5242-E5249.
Sun J, Wang M-J, Ding M-Q, *et al.* 2010. H_2O_2 and cytosolic Ca^{2+} signals triggered by the PM H^+-coupled transport system mediate K^+/Na^+ homeostasis in NaCl-stressed *Populus euphratica* cells. Plant, Cell & Environment **33,** 943-958.
Sussmilch FC, Brodribb TJ, McAdam SAM. 2017. Up-regulation of *NCED3* and ABA biosynthesis occur within minutes of a decrease in leaf turgor but *AHK1* is not required. Journal of Experimental Botany **68,** 2913-2918.
Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R. 2011. Respiratory burst oxidases: the engines of ROS signaling. Current Opinion in Plant Biology **14,** 691-699.
Takahashi F, Shinozaki K. 2019. Long-distance signaling in plant stress response. Current Opinion in Plant Biology **47,** 106-111.
Takahashi F, Suzuki T, Osakabe Y, Betsuyaku S, Kondo Y, Dohmae N, Fukuda H, Yamaguchi-Shinozaki K, Shinozaki K. 2018. A small peptide modulates stomatal control via abscisic acid in long-distance signalling. Nature **556,** 235-238.
Tamás L, Mistrík I, Huttová J, Halusková L, Valentovičová K, Zelinová V. 2010. Role of reactive oxygen species-generating enzymes and hydrogen peroxide during cadmium, mercury and osmotic stresses in barley root tip. Planta **231,** 221-231.

Tang A-C, Boyer JS. 2002. Growth-induced water potentials and the growth of maize leaves. Journal of Experimental Botany **53,** 489-503.
Thatcher SR, Danilevskaya ON, Meng X, Beatty M, Zastrow-Hayes G, Harris C, Van Allen B, Habben J, Li B. 2016. Genome-wide analysis of alternative splicing during development and drought stress in maize. Plant Physiology **170,** 586-599.
Tian M, Lou L, Liu L*, et al.* 2015. The RING finger E3 ligase STRF1 is involved in membrane trafficking and modulates salt-stress response in *Arabidopsis thaliana*. Plant Journal **82,** 81-92.
Tran L-SP, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, Yamaguchi-Shinozaki K. 2007. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **104,** 20623-20628.
Trontin C, Kiani S, Corwin JA, Hématy K, Yansouni J, Kliebenstein DJ, Loudet O. 2014. A pair of receptor-like kinases is responsible for natural variation in shoot growth response to mannitol treatment in *Arabidopsis thaliana*. Plant Journal **78,** 121-133.
Tsugama D, Liu S, Takano T. 2012. Drought-induced activation and rehydration-induced inactivation of MPK6 in Arabidopsis. Biochemical and Biophysical Research Communications **426,** 626-629.
Ueda M, Tsutsumi N, Fujimoto M. 2016. Salt stress induces internalization of plasma membrane aquaporin into the vacuole in *Arabidopsis thaliana*. Biochemical and Biophysical Research Communications **474,** 742-746.
Urao T, Yakubov B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T, Shinozaki K. 1999. A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. Plant Cell **11,** 1743-1754.
Uzilday B, Turkan I, Ozgur R, Sekmen AH. 2014. Strategies of ROS regulation and antioxidant defense during transition from C_3 to C_4 photosynthesis in the genus Flaveria under PEG-induced osmotic stress. Journal of Plant Physiology **171,** 65-75.
Van den Broeck L, Dubois M, Vermeersch M, Storme V, Matsui M, Inzé D. 2017. From network to phenotype: the dynamic wiring of an Arabidopsis transcriptional network induced by osmotic stress. Molecular Systems Biology **13,** 961.
van der Weele CM, Spollen WG, Sharp RE, Baskin TI. 2000. Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. Journal of Experimental Botany **51,** 1555-1562.
Verelst W, Bertolini E, De Bodt S, Vandepoele K, Demeulenaere M, Pè ME, Inzé D. 2013. Molecular and physiological analysis of growth-limiting drought stress in *Brachypodium distachyon* leaves. Molecular Plant **6,** 311-322.
Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu J-K. 2006. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant Journal **45,** 523-539.
Vishwakarma K, Upadhyay N, Kumar N, *et al.* 2017. Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Frontiers in Plant Science **8,** 161.
Waadt R, Hitomi K, Nishimura N, Hitomi C, Adams SR, Getzoff ED, Schroeder JI. 2014. FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. eLife **3,** e01739.
Wang B, Liu C, Zhang D, He C, Zhang J, Li Z. 2019. Effects of maize organ-specific drought stress response on yields from transcriptome analysis. BMC Plant Biology **19,** 335.
Wang C, Li J, Yuan M. 2007. Salt tolerance requires cortical microtubule reorganization in Arabidopsis. Plant & Cell Physiology **48,** 1534-1547.
Wang C, Zhang L-J, Huang R-D. 2011. Cytoskeleton and plant salt stress tolerance. Plant Signaling & Behavior **6,** 29-31.
Wohlbach DJ, Quirino BF, Sussman MR. 2008. Analysis of the *Arabidopsis histidine* kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. Plant Cell **20,** 1101-1117.
Wong MM, Bhaskara GB, Wen T-N, Lin W-D, Nguyen TT, Chong GL, Verslues PE. 2019. Phosphoproteomics of *Arabidopsis* Highly ABA-Induced1 identifies AT-Hook-Like10 phosphorylation required for stress growth regulation. Proceedings of the National Academy of Sciences of the United States of America **116,** 2354-2363.
Xiao Y, Huang X, Shen Y, Huang Z. 2013. A novel wheat α-amylase inhibitor gene, *TaHPS*, significantly improves the salt and drought tolerance of transgenic *Arabidopsis*. Physiologia Plantarum **148,** 273-283.
Xie Y-J, Xu S, Han B*, et al.* 2011. Evidence of Arabidopsis salt acclimation induced by up-regulation of *HY1* and the regulatory role of RbohD-derived reactive oxygen species synthesis. Plant Journal **66,** 280-292.
Xiong L, Zhu J-K. 2002. Molecular and genetic aspects of plant responses to osmotic stress. Plant, Cell & Environment **25,** 131-139.
Xu J, Xie J, Yan C, Zou X, Ren D, Zhang S. 2014. A chemical genetic approach demonstrates that MPK3/MPK6 activation and NADPH oxidase-mediated oxidative burst are two independent signaling events in plant immunity. Plant Journal **77,** 222-234.

Xue L, Wang P, Wang L, Renzi E, Radivojac P, Tang H, Arnold R, Zhu J-K, Tao WA. 2013. Quantitative measurement of phosphoproteome response to osmotic stress in *Arabidopsis* based on Library-Assisted eXtracted Ion Chromatogram (LAXIC). Molecular & Cellular Proteomics **12,** 2354-2369.
Yao Y, He RJ, Xie QL*, et al.* 2017. *ETHYLENE RESPONSE FACTOR 74* (*ERF74*) plays an essential role in controlling a respiratory burst oxidase homolog D (RbohD)-dependent mechanism in response to different stresses in Arabidopsis. New Phytologist **213,** 1667-1681.
Yu Q, Ren J-J, Kong L-J, Wang X-L. 2018. Actin filaments regulate the adhesion between the plasma membrane and the cell wall of tobacco guard cells. Protoplasma **255,** 235-245.
Yuan F, Yang H, Xue Y*, et al.* 2014. OSCA1 mediates osmotic-stress-evoked Ca^{2+} increases vital for osmosensing in *Arabidopsis*. Nature **514,** 367-371.
Yuasa T, Ichimura K, Mizoguchi T, Shinozaki K. 2001. Oxidative stress activates ATMPK6, an Arabidopsis homologue of MAP kinase. Plant & Cell Physiology **42,** 1012-1016.
Zeevaart JAD, Boyer GL. 1984. Accumulation and transport of abscisic acid and its metabolites in *Ricinus* and *Xanthium*. Plant Physiology **74,** 934-939.
Zeller G, Henz SR, Widmer CK, Sachsenberg T, Rätsch G, Weigel D, Laubinger S. 2009. Stress-induced changes in the *Arabidopsis thaliana* transcriptome analyzed using whole-genome tiling arrays. Plant Journal **58,** 1068-1082.
Zhang M, Wang D, Kang Y, Wu J-X, Yao F, Pan C, Yan Z, Song C, Chen L. 2018. Structure of the mechanosensitive OSCA channels. Nature Structural & Molecular Biology **25,** 850-858.
Zhao K-F, Song J, Fan H, Zhou S, Zhao M. 2010. Growth response to ionic and osmotic stress of NaCl in salt-tolerant and salt-sensitive maize. Journal of Integrative Plant Biology **52,** 468-475.
Zhao X, Xu M, Wei R, Liu Y. 2015. Expression of OsCAS (Calcium-Sensing Receptor) in an *Arabidopsis* mutant increases drought tolerance. PLoS ONE **10,** e0131272.
Zhu X, Feng Y, Liang G, Liu N, Zhu J-K. 2013. Aequorin-based luminescence imaging reveals stimulus- and tissue-specific Ca^{2+} dynamics in *Arabidopsis* plants. Molecular Plant **6,** 444-455.
Zonia L, Munnik T. 2007. Life under pressure: hydrostatic pressure in cell growth and function. Trends in Plant Science **12,** 90-97.
Zwiewka M, Nodzyński T, Robert S, Vanneste S, Friml J. 2015. Osmotic stress modulates the balance between exocytosis and clathrin-mediated endocytosis in *Arabidopsis thaliana*. Molecular Plant **8,** 1175-1187.

TABLE

Table 1. Characteristics of commonly used osmotic stress compounds. Advantages and disadvantages of each compound are reported. For each osmoticum, the minimal concentration to reduce root and rosette size by half is given, as well as the concentration inducing severe stress symptoms, such as bleaching or anthocyanin accumulation in at least 20% of the plants (van der Weele *et al.*, 2000; Verslues *et al.*, 2006; Claeys *et al.*, 2014b).

		(Dis)-advantages of the compound	Root length -50%	Rosette area/weight -50%	Severe symptoms (>20%)	References
PEG	+	No compound-specific pathway reported	-1.2 MPa	~-0.8 MPa	< -1.7 MPa	van der Weele *et al.*, 2000 Verslues *et al.*, 2006
	+	Commonly used, demonstrated relevance				
	-	Less convenient for preparation solid medium				
SORBITOL	+	No compound-specific pathway reported	150 mM (-0.36MPa)	100 mM (-0.24MPa)	300 mM (-0.72MPa)	Claeys *et al.*, 2014
	-	Not commonly used, lack of information				
MANNITOL	+/-	Combined effect of biotic and osmotic stress	150 mM (-0.36MPa)	25 mM (-0.6MPa)	>300 mM (-0.72MPa)	Claeys *et al.*, 2014
	-	Possible compound-specific response				
SALT	+/-	Combined effect of (non)-ionic osmotic stress	75 mM (-0.36MPa)	50 mM (-0.24MPa)	75 mM (-0.36MPa)	Claeys *et al.*, 2014
	-	SOS-mediated compound-specific pathway				

FIGURE LEGENDS

Fig. 1. Overview of the osmotic stress responses over time following stress. Osmotic stress decreases the cellular water potential (a), which triggers internalization of the plasma membrane (b) and the increase of cytosolic Ca^{2+} levels (c). Ca^{2+}-triggered cascades (orange) comprise activation of Ca^{2+}-dependent kinases and transcription factors (d). Stress also triggers cytoskeleton changes (e). Ca^{2+}, cytoskeleton changes and membrane internalization are involved in the activation of RBOH enzymes (f, g and h). RBOH enzymes produce ROS like hydrogen peroxide (H_20_2, red) (i), which rapidly induce internalization of plasma membrane intrinsic proteins (PIPs) (j). The ROS signal is transduced to the shoot through a ROS-wave in which RBOH enzymes are subsequently activated (k). ROS can also activate ACC-biosynthesis enzymes (l). This triggers the accumulation of ACC, which is possible transported to the shoot and triggers an ethylene response in growing leaves within 1h (m). This response causes leaf growth arrest and is denominated the "pause-and-stop" model, as leaf growth is first transiently arrested through CDKA phosphorylation (o) and later stopped by acting on the GA/DELLA pathway (p). As a result, leaf growth is inhibited within 24 h. In fully-grown leaves, ABA triggers closure of the stomata (r), which on longer term blocks photosynthesis (s). ABA also triggers further ROS generation in roots and leaves (n), which activates proline biosynthesis (u). ROS detoxifying mechanisms are induced to buffer the increasing ROS levels in shoot and root (t and y). Further dehydration also reduces leaf and root water content (v and z). After days of stress, ACC levels decrease again (w), while ABA and proline levels remain high (x). Full lines indicate activation/inhibition, dashed lines transport, and dotted lines evolution over time. See main text for abbreviations and references.

Fig. 2. Comparison between osmotic stress assays and soil-based drought experiments. Even before the stress onset, osmotic stress assays in petri plates impose a very different environment to plants compared to soil-based experiments. In *in vitro*-performed experiments, plants are grown on very homogeneous growth medium, while in soil the local structure of the soil can be very different along one root. To impose osmotic stress, *in vitro* assays offer the advantage of a precise control of stress onset and of stress level. The imposed stress is homogeneous along the root and the moment at the moment at which the root feels the stress is clear. In contrast, when watering of soil is withheld, it is unclear when the plants start to perceive drought and at which moment the short-term responses should be captured. Consequently, short-term responses observed *in vitro* are hard to translate in soil, but the general response including Ca^{2+}, ROS, and ABA signaling seems conserved. After a longer period of days or weeks upon osmotic stress or drought, the responses are more comparable in terms of phenotype and molecular acclimation.

Figure 1

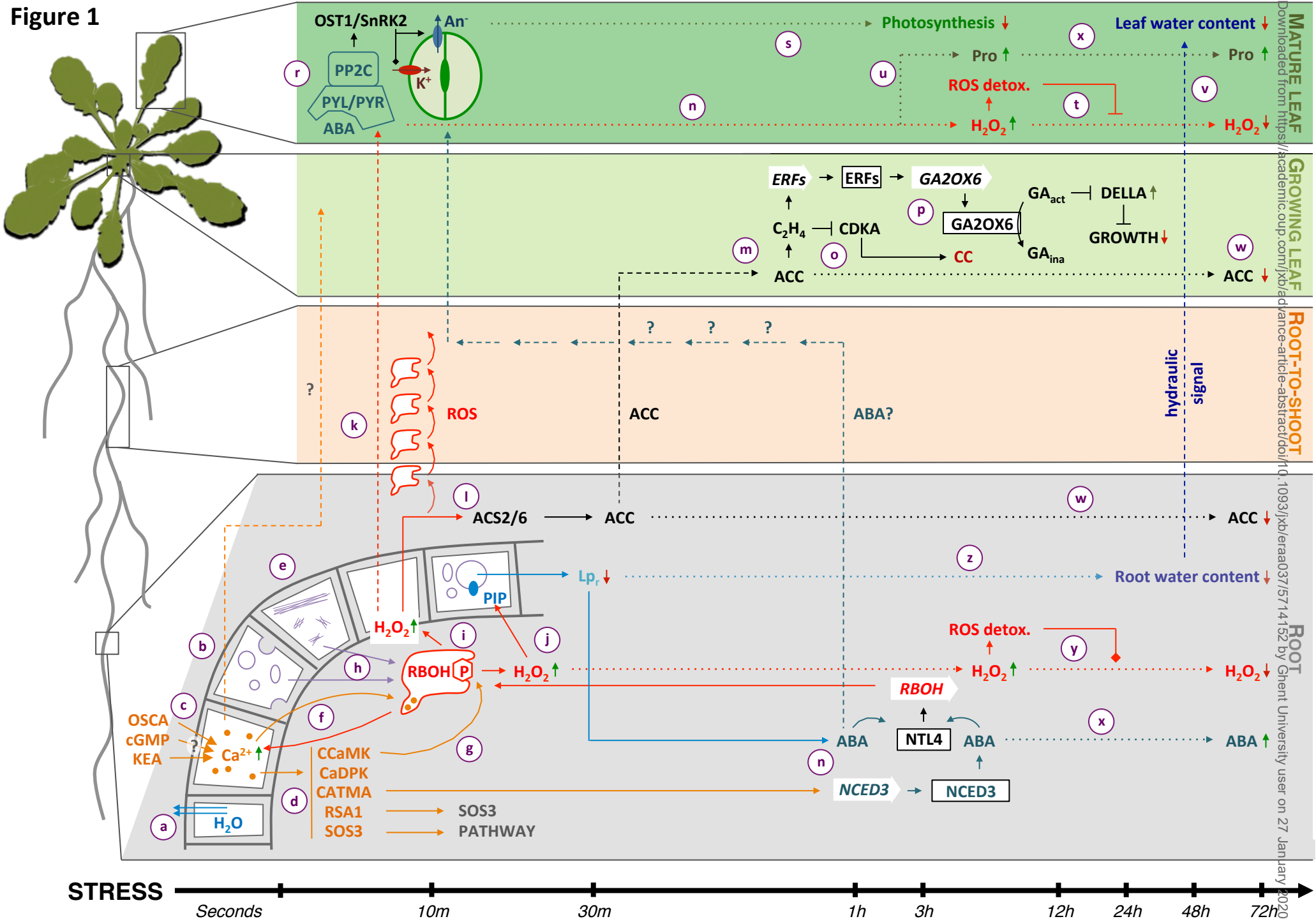

Figure 2