Biotechnology of water and salinity stress tolerance

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Drought and salinity are among the environmental factors that constrain agricultural productivity most dramatically. Classical breeding programs aiming to improve stress tolerance have been hampered by the multigenic nature of the trait and the seemingly scarce natural genetic variability in crop plants. Novel genetic determinants governing the function of stomata and improving the performance of plants under water shortage have been identified and show promise of application in crops. Moreover, receptors of the stress hormone abscisic acid have been characterized and their interplay with key regulatory components is being understood. A critical factor of salinity tolerance in plants is the ability to exclude Na^+ from the shoot, and the modification of specific Na^+ transport processes has yielded enhanced salinity tolerance.

Introduction

Environmental factors are the primary cause of crop failure, causing average yield losses of more than 60% for major crops worldwide [1]. The abiotic stresses that plants encounter most frequently and that adversely affect growth are drought, salinity, flooding, and low or high temperature. Environmental clues trigger physiological and molecular responses enabling the plant to prevent or minimize exposure to stressful conditions, or to acclimate and overcome the unavoidable hurdle [2]. Among the adaptations aimed to drought-avoidance are growing deeper roots in pursuit of receding groundwater or to entirely escape dry periods by completing the life cycle while water is available. On the other hand, molecular responses that are associated with the acclimatization to water and salinity stress are multigenic and often interrelated, thus hindering the dissection of signaling processes and the identification of major determinants of stress tolerance. Transcriptomic, proteomic, and molecular genetics approaches have identified many stress-related genes, which are generally classified into two major groups [2]. One group is involved in signaling cascades, transcriptional control, and the degradation of transcripts or proteins, whereas members of the other group function in membrane protection, osmoprotection, as antioxidants and as reactive oxygen species (ROS) scavengers. Simultaneously, metabolic pathways are adjusted to regain homeostasis in a changing environment.

The word *drought* is often used as a way of expressing water deficit imposed by withholding irrigation, but drought is in fact an ecophysiological term that describes a prolonged period of abnormally low rainfall. Hence, I will rather use the more precise terms *water deficit*, that is when the quantity of water available to the plant is insufficient to meet basic needs, and *desiccation* when the relative water content (RWC) in plant tissues falls drastically. Values of RWC around 85–95% are found in well-hydrated tissues. A RWC lower than the critical mark of 50% typically results in plant death, with the notable exceptions of mature orthodox seeds and xerophytic plant species [1]. Water deficit can be induced by many environmental conditions and it is often associated with concurrent stresses, like salinity and high or low temperatures. Indeed, salinity stress and water deficit are intimately related. Salts dissolved in the soil solution reduce the water potential (i.e. diminish water availability to the plant) and water uptake by roots is thermodynamically hampered. Within minutes of being exposed to high salt concentrations plant growth is impaired, a response due to hyperosmolarity of the soil solution [3•]. Mild osmotic stress leads rapidly to growth inhibition of shoots, whereas roots may continue to elongate, seeking water from deeper soil layers. In the short term, osmotic adjustment is the primary issue. The subsequent accumulation of salts in the cells and the apoplast become critical mainly in plants exposed to long-term salinity (days or weeks) and results in necrosis of the tissues, particularly in old leaves that cannot dilute the incoming salts by cell expansion. Ion-specific stress maladies are superimposed on those caused by dehydration. Molecular responses to water and salt stress are largely identical except for the ionic component [2,3•]. For most species, Na^+ appears to reach a toxic concentration before other ions do, and thus most studies have concentrated on the control of Na^+ homeostasis [4]. Maintenance of appropriate intracellular K^+/Na^+ balance is critical for metabolic function as Na^+ cytotoxicity is largely due to competition with K^+ for binding sites in enzymes essential for cellular

functions [5]. Another consequence is the production of ROS, which then in turn affects cellular structures and metabolism negatively [2].

Although considerable progress was made to increase and secure crop yield through conventional breeding, the goal of improving the resistance of crops to abiotic stresses has seen limited success because of the complex, multigenic nature of the traits and the narrow genetic variation in the gene pools of major crops. Numerous genes and proteins have been shown to affect the tolerance to environmental stress in an array of plant species, which together compose a complex puzzle with a myriad of individual elements and crisscrossing signal transduction pathways that are not easily integrated into a holistic model. The use of hardy wild relatives of crop plants as a source of genetic determinants of stress tolerance is time-consuming and labor-intensive. Further, undesirable genes are often transferred in combination with desirable ones. Classical breeding approaches have revealed that stress tolerance traits are dispersed in various quantitative trait loci (QTLs), which make genetic selection of these traits difficult, albeit a few success stories regarding QTLs related to salinity will be described herein. The use of molecular markers and the development of powerful bioinformatics tools have led to the identification of genes with major contributions to Na^+ and K^+ homeostasis using the QTL approach.

This review focuses on recent advances following molecular genetics approaches in model plants. I will present our current understanding of Na^+ transport in plants, arguably the most relevant ion in salinity tolerance and which is one of the most extensively studied processes regarding salt stress. Recent developments in abscisic acid (ABA) perception, improving resistance to water deficit by means of controlling the function of stomata, and achieving the survival of higher plants to severe desiccation will also be highlighted and discussed.

Water stress and desiccation

Stomatal pores regulate gas exchange for photosynthesis and the loss of water by transpiration. Plants fine-tune the opening and closing of their stomata to optimize the conflicting needs of gas exchange while limiting water loss through evapo-transpiration. The engineering of stomatal closure as a means to reduce water loss is an attractive approach to improve the performance of plants under water limitation, thereby meeting the pressing need of developing crops with higher water use efficiency (WUE, or biomass production per unit of water used). In principle, the trade-off of diminished gas exchange, with the predictable decline of photosynthetic activity, should be taken into account when designing plants with reduced water loss through enhanced stomata closure. For instance, the ABA-hypersensitive *era1* and *cbp20* mutants of *Arabidopsis*, which retain more water when subjected to limited water supply because of restricted stomatal exchange, could not compete favorably with wild-type plants in mixed populations growing in the same pots [6•]. Roots of neighboring wild-type plants competed for water in the soil around the mutants, whose water potential declined following that of their wild-type neighbors. Thus, reduced transpiration may be necessary but not sufficient to keep plants hydrated in a progressively drying environment [6•]. However, there is substantial natural genetic variation of WUE among species and genotypes within a species, and the underlying mechanisms that regulate transpiration are just beginning to be understood [7•]. Transpiration rates and net carbon dioxide (CO_2) assimilation show different slopes in response to changes in stomatal conductance, and small reductions in stomatal transpiration that minimize the negative impact on CO_2 assimilation translate into improved WUE. Current evidence suggests that plants have physiological mechanisms for improving WUE that are under genetic control [7•]. Moreover, transpiration occurs not only through stomata, but also across the cuticle and the boundary layer. Because of the differential diffusion properties of water and CO_2 through these pathways, it is feasible that WUE could be improved by decreasing transpiration without a concomitant reduction in CO_2 uptake [7•].

Many signaling components have been identified that are involved in the control of stomatal aperture, including second messengers, protein kinases and phosphatases, phospholipases, and constituents of the machinery controlling RNA metabolism [8]. Recently, several transcription factors have been implicated in the regulation of stomatal movements in *Arabidopsis*, adding an additional level of regulation to the signaling network that controls stomatal aperture. The transcription factors AtMYB60 and AtMYB61 are expressed in guard cells, and they play opposite roles in the response of stomata to environmental signals [9,10]. The expression of AtMYB60 is negatively modulated during water stress whereas light, which promotes stomatal aperture, increases AtMYB60 gene expression. A null *myb60* mutation reduced stomatal opening and wilting under water stress conditions. However, stomatal closure induced by ABA or dark was unaffected by the *myb60* mutation. In contrast to AtMYB60, AtMYB61 is expressed only in the dark, under conditions when stomata are usually closed. Stomata in the *myb61* mutant were more open than in the wild-type plant. Neither ABA nor water stress affected AtMYB61 expression, suggesting that this transcription factor does not mediate ABA-induced reductions in stomatal aperture. Hence, these MYB transcription factors are primarily involved in light-induced opening and dark-induced closure. Ectopic overexpression of another MYB protein of *Arabidopsis*, MYB44, which is normally expressed in the vasculature and in guard cells, downregulated an array of 2C-type protein phosphatases that are well-known

negative regulators of ABA signaling (see below) [11]. Consequently, transgenic plants expressing MYB44 had a faster ABA-induced stomatal closure response than wild-type plants. The *MYB44* gene was itself upregulated by dehydration, low temperature, and salinity, thereby enhancing ABA signal relay.

A mutant with improved resistance to water shortage and to oxidative stress, *enhanced drought tolerance 1*, was isolated in a gain-of-function mutant screen in *Arabidopsis* [12]. The *edt1* plants showed an array of stress-related traits, including a more extensive root system with deeper primary roots and more lateral roots than the wild-type plants, and higher levels of ABA, proline, and superoxide dismutase. The more developed root system of the mutant improved accessibility to water. In addition, the *edt1* plants showed 30% reduction in stomatal density per area unit, which was presumably a consequence of larger and fewer cells in the epidermis of the mutant. Surprisingly, although the rate of transpiration was lower in the mutant, the rate of photosynthesis was higher compared to the wild type. Consequently, the WUE was greater in the mutant than in the wild-type plant. The increased photosynthesis rate of the mutant is unexpected, as reduced stomatal density is believed to decrease CO_2 exchange. The phenotypes of *edt1* were brought about by the T-DNA activation tagging of the gene *HDG11* encoding a putative homeodomain-START transcription factor. Overexpression of *HDG11* in transgenic tobacco recapitulated water stress tolerance associated with modified root architecture and reduced leaf stomatal density. Targets that HDG11 may directly or indirectly regulate included effectors of ABA synthesis and signal relay (NCED3, LOS5/ABA3, CIPK3, and ABI3), the proline biosynthetic enzyme P5CS, Ca^{2+} and K^+ transporters (CAX3 and KAT1), and the transcriptional regulators ERECTA, which is known to affect stomatal density, RGAL, a RGA-like DELLA protein that is a negative regulator of GA signaling, and IAA28, which negatively regulates lateral root formation [12]. The *HDG11* gene did not respond itself to stress treatments and the knockout mutant had no discernible phenotype.

The *HARDY* (*HRD*) gene encoding an AP2/ERF-like transcription factor was also identified as a gain-of-function mutation in *Arabidopsis*. The *hardy* mutant had increased mesophyll cell layers, produced a denser root network, and showed enhanced tolerance to water deficit and salinity [13]. The mechanism through which HARDY regulates these processes is not known, albeit higher efficiency could be related to the increased number of photosynthetic mesophyll cells in thicker leaves. Overexpression of *HRD* in rice reduced transpiration while increasing WUE [13]. Adaptation to water deficit is often associated not only with stomatal control of water use but also with rooting depth. The HDG11 and HARDY proteins link both phenomena molecularly, suggesting that these proteins may have evolved as master switches directing the evolution of plant species better suited to survive drought.

The *Arabidopsis abo1* mutant was isolated in a genetic screen for mutants with altered water stress responses. This drought-resistant mutant shows hypersensitive seedling growth and enhanced stomatal closing in response to ABA [14]. The *abo1* mutation mapped in gene *ELO2*, encoding the largest subunit of Elongator, a multifunctional complex with roles in transcription elongation, secretion, and tRNA modification. Alike the *edt1* mutant, *abo1* had a reduced density of functional stomata compared to wild-type plants. Some pairs of guard cells did not form normal stomata or formed stomata with very small pores, albeit the total number of guard cells that formed stomata with or without pores in the *abo1* plant was almost the same as that in the wild-type genotype. Thus, the *abo1* mutation appears to affect only the development of guard cells and their adjacent pavement cells, not the division and differentiation of their precursor cells. Moreover, *abo1* affected the stomatal sensitivity to ABA, unlike other stomatal developmental mutants that do not show defects in ABA sensitivity.

Additional transcription factors affecting water relations are NF-YA5 and NF-YB1. The Nuclear Factor Y (NF-Y) complex is composed of three subunits: NF-YA, NF-YB, and NF-YC. Initially, a heterodimer is formed in the cytoplasm between subunits NF-YB and NF-YC. This dimer then translocates to the nucleus, where the third subunit NF-YA, which provides the DNA sequence-specific interaction, is recruited to generate the mature, heterotrimeric NF-Y transcription factor [15]. Mature NF-Y binds promoters with the core pentamer nucleotide sequence CCAAT, and this can result in either positive or negative transcriptional regulation. In animals and yeast, each subunit of NF-Y is encoded by a single gene, whereas the *Arabidopsis* genome encodes 10 NF-YAs, 13 NF-YBs, and 13 NF-YCs [16]. Transgenic *Arabidopsis* overexpressing NF-YB1 did not wilt as much as the wild-type plants and maintained higher photosynthetic rates under water deprivation. Its ortholog gene in maize, ZmNF-YB2, also led to enhanced drought resistance in this crop [17••]. Simulated drought conditions reduced maize yield by more than 50% in control lines, while the best-performing transgenic maize line produced up to 50% more than control plants. A number of stress-related parameters, including chlorophyll content, stomatal conductance, leaf temperature, reduced wilting, and maintenance of photosynthesis, were all improved in the transgenics. Overexpression of another NF-Y subunit, NF-YA5, also reduced drought susceptibility and stomatal aperture in *Arabidopsis*, while *nfya5* mutants had the opposite phenotype [18]. Messenger RNA of gene *NF-YA5* was upregulated by water stress in an ABA-dependent manner. This transcript is a target for miRNA169,

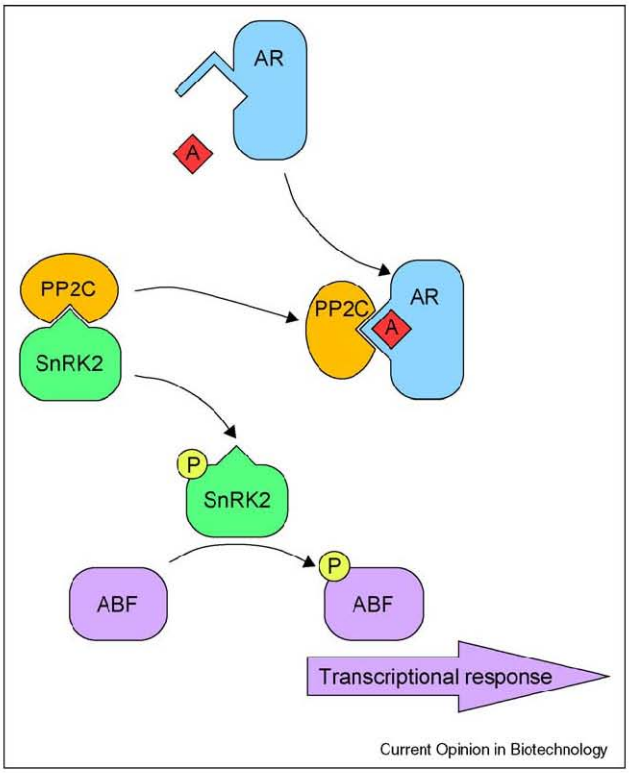
which is itself downregulated by ABA. Similar to *nfya5* loss-of-function, overexpression of miRNA169 rendered plants more susceptible to water stress. However, promoter:β-glucuronidase (GUS) analysis suggested that part of *NF-YA5* induction occurred at the transcriptional level. Thus, *NF-YA5* is regulated both transcriptionally and post-transcriptionally, with the downregulation of miR169 by water stress contributing to greater expression of *NF-YA5* in these conditions. Microarray analysis implicated *NF-YA5* in the expression of a surprisingly low number of stress-responsive genes. In turn, the regulon under the control of subunit NF-YB1 did not significantly overlap with that of CBF4, a drought-induced transcription factor, or with the ABA-response regulon [17••]. The candidate target genes of NF-YB1 do not have obvious associations with stress tolerance, and some of them appear to be related to polysaccharide metabolism. The lack of substantial overlap between the target genes of *NF-YA5* and *NF-YB1* indicates that the two transcription factors may be governing separate gene regulons. There might be a multiplicity of NF-Y dependent regulons as a result of combinatorial formation of trimeric NF-Y complexes. We can reasonably expect that NF-YC subunit(s) will eventually be discovered to complete defined trimeric NF-Y complex. Together, these data suggest that NF-Y proteins are components of a previously unrecognized transcription-regulated response pathways(s) to water stress, although the precise mechanism by which NF-Y proteins improve the performance of plants under water shortages remains unknown.

When strategies to reduce water loss fail, the drying plant is left with the last resource of withstanding desiccation. In the plant kingdom, only resurrection plants and orthodox seeds are able to withstand drastic water loses (water potentials $\Phi \leq -100$ MPa) and survive desiccation. Recently, Jordano's lab reported that young seedlings of transgenic tobacco ectopically expressing the sunflower, seed-specific transcription factor HaHSFA9 (heat stress factor A9) could withstand severe dehydration [19•]. Fast-drying procedures were used achieving losses of ≈98% of total water content (to $\Phi \approx -40$ MPa). True leaves efficiently survived dehydration but most roots did not recover, which limited survival of the whole seedlings. These results represent a first step toward transferring tolerance to severe dehydration from seeds to vegetative tissues of homohydric (nonresurrection) plants. A crucial difference from previous studies might be the use of a transcription factor, HaHSFA9, which is specifically involved in the activation of a genetic program expressed only in seeds. Gain-of-function in transgenic tobacco has shown that the genetic program activated by HaHSFA9 contributes to seed longevity and to embryo desiccation tolerance [19•]. The HaHSFA9 program does not include late embryogenesis abundant (*LEA*) genes among target genes, but instead a subset of cytosolic small HSP of class I (*sHSP CI*), which differ from similar genes activated by heat or drought in vegetative tissues [19•,20]. However, there could be a similar specificity for *LEA* genes in connection with desiccation tolerance. The accumulation in vegetative tissues of a resurrection plant of *LEA* mRNAs that might correspond to seed-specific genes in *Arabidopsis* has also been reported [21]. These observations suggest that genetic programs involved in desiccation tolerance are embryo-specific in most plants, except in resurrection plants. Such programs can be reinduced in vegetative tissues by osmotic stress and ABA, but only early during seed germination, and then are shutdown in nonresurrection plants. During germination, embryonic transcription factors such as ABI5 and ABI3 are progressively diluted, degraded and/or repressed [22]. In *Arabidopsis*, *AtHSFA9* (the putative *HaHSFA9* ortholog gene) controls a similar genetic program and is a direct target of ABI3 [23]. Additional transcription factors may become limiting after germination. Moreover, the transcription factor(s) specifically involved in the activation of the subset of seed-specific LEA genes is (are) unknown. If they were identified, their ectopic overexpression might also lead to improved vegetative tolerance to severe dehydration.

ABA perception and response

ABA is an important phytohormone regulating seed dormancy, germination, seedling growth, and plant transpiration. Multiple sites of ABA perception, inside and outside of the cell surface, have been biochemically detected [8]. In the last few years, several candidate receptors (FCA, CHLH, and GCR2) have been proposed based on their purported capacity to bind ABA *in vitro*. However, the report on the FCA receptor has been retracted and data on GCR2 and CHLH have been questioned based on their localization, lack of specificity for ABA stereoisomers, and inability to genetically interact with previously defined, key components of the ABA signaling pathway [24,25]. The latest addition to this list are GTG1 and GTG2, two membrane proteins with homology to G-protein-coupled receptors (GPCRs) that interact with the sole *Arabidopsis* G protein α subunit GPA1, although they also have intrinsic GTP-binding and GTPase activity [26]. Evidence was provided that GTGs specifically bind the biologically active (+)-ABA stereoisomer with apparent dissociation constant of ca. 20 nM. However, the stoichiometry of binding was very low — only 1% of the GTG present in the *in vitro* assay actually bound ABA. Mutants lacking GTGs had reduced ABA-induced gene expression and displayed low sensitivity to ABA in seed germination, early seedling growth, and ABA-induced stomatal closure assays. Together, these are credible hallmarks substantiating the involvement of GTGs in ABA perception at the plasma membrane. On the basis of biochemical and genetic evidence these authors also suggested that GPA1 would negatively regulate ABA signaling by promoting the GTP-bound conformation of GTG, which exhibits weaker ABA binding.

Figure 1



Current model of ABA receptor signaling in *Arabidopsis*. In the absence of ABA (*diamond A*), the protein phosphatase PP2C binds to and counteracts the phosphorylation of the activation loop in the protein kinase SnRK2, keeping it inactive. This interaction is constant in the absence of ABA. The locking of ABA into the receptor protein PYR/PYL/RCAR (*AR*) exposes the gating loop of the receptor protein and creates a binding surface for the active site of PP2C, thereby relieving SnRK2 from inhibition. Next, the activated SnRK2 phosphorylates downstream targets, including ABA-responsive transcription factors (ABF) to start transcriptional responses.

A recent and exciting development in the field has been the discovery of novel (and likely *bona fide*) ABA receptor proteins. The protein phosphatases 2C (PP2C) ABI1 and ABI2 (*ABA-I*nsensitive) have long been known as negative regulators of ABA signaling (the *abi1-1* and *abi2-1* alleles isolated initially were single dominant mutations that rendered the mutant plants highly insensitive to exogenous ABA). In a yeast two-hybrid screen for plant proteins that interact with ABI2, Ma *et al.* [27] identified members of the RCAR protein family (regulatory component of ABA receptor). RCAR1 and related proteins bind ABA and block the phosphatase activity of PP2Cs in an ABA-dependent manner (Figure 1). Furthermore, the ABA affinity of the RCAR1–ABI2 protein complex was much higher than that of RCAR1 alone, which is consistent with a heterodimeric receptor complex or the generation of a highly stable ternary complex that effectively titrates ABA from the medium, as suggested from the analysis of ABA binding by PYL5 (similar to RCAR1, see below) in the presence of the PP2C family member HAB1 [28]. The dominant mutation *abi1-1* abolished the interaction with RCAR1 and conferred insensitivity to ABA. Transgenic plants overexpressing RCAR1 were hypersensitive to ABA, while reducing the expression of RCAR1 by RNA interference counteracted the ABA response. RCAR1 belongs to a protein family with 14 members. Other RCARs also mediated ABA-dependent regulation of ABI1 and ABI2, consistent with a combinatorial assembly of receptor complexes. Simultaneously, in a chemical genetic screen using pyrabactin, a ABA agonist that inhibits seed germination, Park *et al.* [29••] isolated several *pyr1* (pyrabactin resistance 1) allelic mutants. PYR1 belongs to the same protein family than RCARs. These authors showed that pyrabactin and ABA promoted the interaction of PYR1 with PP2Cs and the enzymatic inhibition of the protein phosphatases. Mutants of PYR1 with reduced pyrabactin binding also reduced ABA-induced PYR1–PP2C interactions, as did the dominant *abi1-1* and *abi2-1* mutations causing ABA insensitivity. Triple and quadruple *pyr1* and *pyr1*-like (*pyl*) mutants were insensitive to ABA. PYR/PYL/RCAR proteins specifically interact with members of group A of the PP2Cs (ABI1, ABI2, HAB1, and PP2CA/AHG3) that negatively regulate ABA responses [28]. The redundant PYR/PYL/RCAR and PP2C gene families, together with the combinatorial nature of their interaction explain why these ABA receptors have eluded classical genetic screens for so long.

Proteins PYR/PYL/RCAR contain an START domain comprising a conserved hydrophobic ligand-binding pocket that can bind ABA. The crystal structure of PYR/PYL proteins bound to ABA has been resolved [30–32] and shows that PYR/PYL consists of a dimer in which one of the subunits is bound to ABA. In the ligand-bound subunit, the loops surrounding the entry to the binding cavity fold over the ABA molecule as a gate and latch, enclosing it inside (Figure 1). The same loops in the unbound form adopt an open conformation. Moreover, the ABA-induced conformational change involving the loops surrounding the entry to the ABA binding cavity creates a surface that enables the receptor to dock into the PP2C active site and inhibit the phosphatase by locking the entry of substrate proteins [32].

In addition to PYR/PYL/RCAR, PP2Cs are known to physically interact with SnRK2s, a group of ABA-activated protein kinases that are positive regulators of ABA signaling [33–35]. PP2Cs efficiently inhibited SnRK2s via dephosphorylation of Ser residues in the activation loop [36,37]. SnRK2 kinases phosphorylate and activate the transcription factors of the ABFs/AREBs family relying ABA signals [38]. The ABFs bind to ABA-responsive promoter elements (ABREs) to induce ABA-dependent gene expression [39]. These signaling intermediaries

have now been shown to act in concert with the PYR/PYL/RCAR receptors [40]. Coexpression of PYR1, ABI1, SnRK2.6/OST1, and the transcription factor ABF2/AREB1 into *Arabidopsis* protoplasts resulted in ABA-responsive gene expression of the reporter construct *RD29B::LUC*. The dominant *abi1-1* mutation disrupted the interaction between ABI1 and PYR1 and precluded ABA-dependent expression of *RD29B::LUC*. All of the tested PYR/PYLs were operative in this test, an indication that every PYR/PYLs is likely to function as ABA receptor. ABA-dependent phosphorylation of ABF2 could be recapitulated in the test tube with purified proteins, demonstrating that these components are sufficient for ABA signal output. In the presence of ABA, PYR/PYL impaired the interaction of several PP2Cs with SnRK2.6, relieving the kinase from inhibition and leading to phosphorylated ABF2. Without ABA, PYR1 could not reverse the inhibitory effect of ABI1 on SnRK2.6. These results have been compiled into a model (Figure 1) that posits that SnRK2 kinases are kept inactive by the PP2Cs through physical interaction and dephosphorylation. In the presence of ABA, the PYR/PYL receptor proteins bind and sequester PP2C phosphatases, thereby relieving the SnRK2 kinases, which in turn phosphorylate ABA-responsive transcription factors. The activity or expression of the signal intermediaries in this surprisingly compact core regulatory module may be modified by other elements previously identified as being involved in ABA responses.

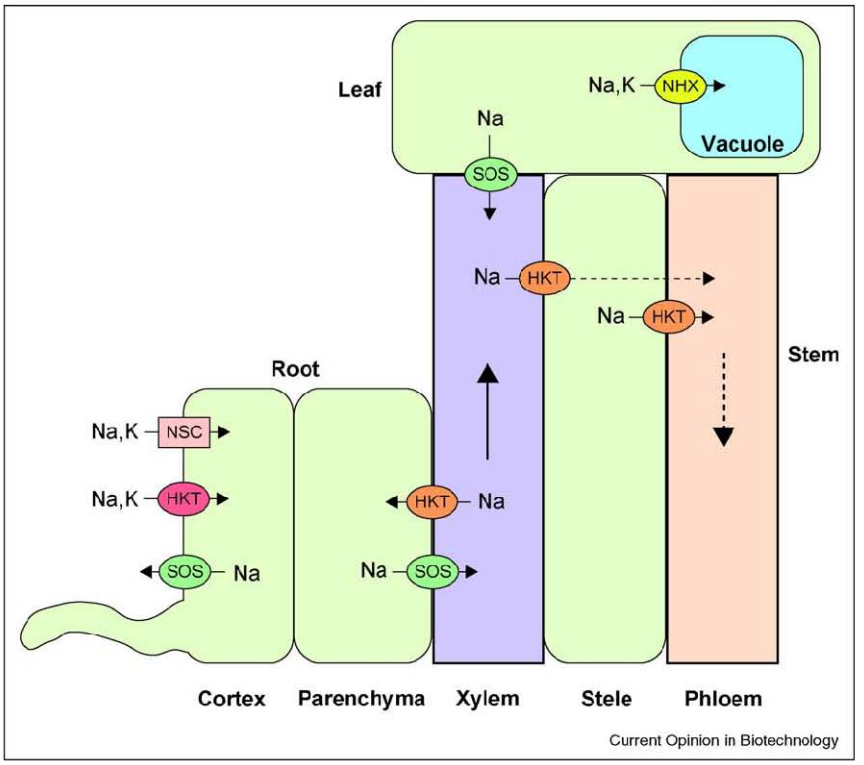
Salinity

The excess of salts in the soil solution poses an additional challenge to the plant besides the impediment of water uptake. Na^+ and other ions taken up by roots are transported to shoots in the transpiration stream, where they accumulate over time [3•]. Elevated concentrations of salts are built in the apoplast, and eventually inside the cell, as water evaporates. The accumulation of ions in plant tissues results in progressive damage. These ionic specific stress effects are superimposed on those caused by hyperosmolarity [3•]. Sodium has a strong inhibitory effect on K^+ uptake by cells, presumably by interfering with transporters in the root plasma membrane such as K^+-selective ion channels and the HAK/KUP transporters that mediate high-affinity and low-affinity K^+ transport at the plasmalemma and tonoplast [41,42]. On the other hand, members of the HKT gene family are Na^+-specific transporters (although they were initially described as high-affinity K^+ transporters and hence their name) that mediate either preferential Na^+ transport or Na^+–K^+ symport, partly depending on whether the specific transporter has a highly conserved serine (subfamily 1) or glycine (subfamily 2) residue in the first pore loop of the protein, and on the extracellular Na^+/K^+ ratio [43••,44]. Generally, HKT members of subfamily 1 have a relatively higher Na^+-to-K^+ selectivity than subfamily 2 HKT transporters. Electrophysiological evidence suggests that weakly voltage-dependent nonselective cation channels (NSCCs) may constitute a major pathway for passive Na^+ entry into the roots at high soil NaCl concentrations, but their molecular identity remains elusive [4,45]. Several members of the CNGCs (cyclic-nucleotide-gated channels) family of *Arabidopsis* (AtCNGC1, AtCNGC3, and AtCNGC4) are permeable to K^+ and Na^+ [45]. However, mutations that cause a major reduction in Na^+ uptake by roots have not yet been found in any plant species, with the limited exceptions of HKTs of subfamily 2 in cereals (see below). Thus, the underlying Na^+ transporters or regulators remain to be unequivocally identified.

In *Arabidopsis*, loss-of-function of the only one *HKT1;1* gene encoding a Na^+-selective transporter caused the accumulation of Na^+ in leaves but reduced Na^+ concentrations in roots, with little effect on the net uptake of Na^+ by the plant [46–48]. *AtHKT1;1* is preferentially expressed in the vasculature, where it is thought to regulate the Na^+ distribution between roots and shoots [43••,48]. Two complementary functions for AtHKT1;1 have been proposed (Figure 2). The phloem recirculation model posits that Na^+ is loaded into shoot phloem cells by AtHKT1;1 and then transferred to roots via the downward stream of phloem, preventing Na^+ overaccumulation in shoots [49]. However, there seems to be little (10% or less) retranslocation of Na^+ from leaves via the phloem relative to the amount imported in the transpiration stream via the xylem [4,50,51]. On the other hand, AtHKT1;1 is generally accepted to mediate the retrieval of Na^+ from the xylem sap, thereby restricting the amount of Na^+ reaching the photosynthetic tissues [48,51]. These two Na^+ transport processes could be functionally linked to achieve basipetal translocation of Na^+ because ions that were unloaded by xylem parenchyma cells might be transported into the phloem via symplastic diffusion (Figure 2). Engineered expression of *AtHKT1;1* in the root pericycle of *Arabidopsis* enhanced inward Na^+-transport in the targeted cells, reduced root-to-shoot transfer of Na^+ and improved salt tolerance [52••]. Notwithstanding these results, ion profiling of shoot tissue from 12 different *Arabidopsis* accessions revealed two coastal salt-tolerant ecotypes that accumulated *higher* shoot levels of Na^+ than other accessions [53]. Reciprocal grafting experiments suggested that reduced *AtHKT1;1* expression in the roots of the salt-tolerant accessions was responsible for elevated shoot Na^+. However, it remains unclear whether the reduced activity of AtHKT1;1 was the sole basis for enhanced tolerance or there were other processes at play that could also contribute to salt tolerance linked to enhanced Na^+ accumulation such as improved capacity for Na^+ sequestration in vacuoles [54].

Similar studies in cereals have shown that natural variation in the activity or expression of HKT transporters may be a genetic resource for enhanced NaCl tolerance.

Figure 2



Schematic model for the function of SOS1 and HKT proteins in achieving Na^+ exclusion in plants subjected to salinity stress. Nonselective cation channels (NSC), and possibly other cation transporters (not shown) are thought to mediate Na^+ influx in root cells. The Na^+/H^+ antiporter SOS1 extrudes Na^+ at the root–soil interface, thereby reducing the net uptake of Na^+. At the xylem parenchyma cells, SOS1 loads Na^+ into xylem sap, a process that requires active transport, whereas HKT1-like proteins mediate the reverse flux and unload Na^+ from xylem vessels to prevent Na^+ overaccumulation in photosynthetic tissues. The opposite transport fluxes mediated by the SOS1 and HKT1-like systems must be finely coordinated to achieve the adequate partition of Na^+ between organs. To translocate Na^+ back to roots, ions unloaded from the xylem may be transported into the phloem via symplastic diffusion (dashed line) [48] or facilitated by additional HKT1-like proteins [49]. HKT proteins of subfamily 2, depicted with a redder color than HKT1-like proteins in the vasculature, mediate Na^+ entry in roots under low external concentrations of Na^+ and K^+, yet they are not likely to play a significant role in the salinity tolerance of plants [43••]. Thick arrows in the xylem and phloem indicate the flow of Na^+ in the sap. The dashed arrow symbolizes the reduced basipetal flow of Na^+ via phloem compared to the acropetal flux via xylem. Incoming Na^+ in shoots is stored in the large central vacuole by tonoplast localized NHX exchangers.

QTLs analyses showed that greater shoot K^+ content of the relatively salt-tolerant rice cultivar Nona Bokra cosegregated with the presence of an allelic variant of *SKC1* (shoot K^+ content) with greater activity relative to that of the salt-sensitive Koshihiraki variety [55]. SKC1 (renamed OsHKT1;5) is a plasma membrane K^+-independent, Na^+-selective transporter that is preferentially expressed in the parenchyma cells surrounding xylem vessels. The greater Na^+ concentration in the xylem sap and leaves of the salt-sensitive variety would be a consequence of a weaker *SKC1* allele and reduced Na^+ reabsorption from the xylem. Quantitative genetic analyses in wheat have also led to the identification of two loci, *Nax1* and *Nax2*, that reduced Na^+ accumulation in the leaf blade by excluding Na^+ from the xylem by two different mechanisms [50]. The process controlled by *Nax2* was confined to the roots and had the effect of reducing the transport of Na^+ from root to shoot, presumably by improved discrimination between Na^+ and K^+ at the point of xylem loading. The *Nax1* locus enhanced the retention of Na^+ in the leaf sheath, thus restricting further passage to the leaf blade [50]. High-resolution mapping and sequencing analyses of known Na^+ transporter genes have suggested that the *Nax1* and *Nax2* loci are attributable to polymorphisms in wheat *HKT* genes encoding proteins of the subfamily 1 with preferred Na^+ transport [56,57]. These results strongly indicate that Na^+ exclusion from the transpiration stream may be an important mechanism in the salt tolerance of cereals, alike many other plant species [4]. It should be pointed out however that most studies concerning QTL analysis for salt tolerance are based in Na^+ and/or K^+ content in tissues or organs, and not directly in salt tolerance. Often, higher K^+/Na^+ ratios are regarded as determinants of salt tolerance itself without considering any other agronomical or physiological traits. In fact, the *SKC* QTL of rice did not

show a significant correlation coefficient with survival to salt stress [58]. A clear difference should be made between QTLs responsible of ionic balance and QTLs for salt tolerance.

Members of subfamily 2 of HKT transporters mediate Na^+ uptake by roots, particularly when extracellular K^+ is limiting [59,60]. The transcript level of several subfamily 2 *HKT* genes has been shown to increase by K^+-starvation in cereals and downregulated by salinity (reviewed in [43••]). At low-K^+ availability, moderate levels of Na^+ actually promote plant growth by replacing K^+ for osmotic adjustment [42]. Thus, subfamily 2 HKTs may be involved in providing 'nutritional' Na^+ under K^+ starvation rather than in salinity stress [43••,60].

Comparisons of unidirectional Na^+ fluxes and rates of net accumulation of Na^+ in roots indicate that 70–95% of the Na^+ fluxed into the root symplast is extruded back to the apoplast, and that small differences in Na^+ exclusion capacity lead to major changes in the net accumulation of Na^+ [4,50]. In *Arabidopsis*, the plasma membrane Na^+/H^+ exchanger SOS1 facilitates Na^+ homeostasis by extruding the ion from root epidermal cells at the root–soil interface [61,62] (Figure 2). *SOS1* is preferentially expressed in xylem parenchyma cells and analyses of the Na^+ root/shoot partitioning in roots of *sos1* plants under different salt regimes indicate that SOS1 participates in the redistribution of Na^+ between the root and shoot, likely working in concert with AtHKT1;1 at the plasma membrane of xylem parenchyma cells [48,61,63] (Figure 2). At the parenchyma–xylem interface, the efflux of Na^+ from the parenchyma cells and loading of the xylem sap must be active (i.e. energetically costly) owing to the plasma membrane potential, negative inside, and would require a Na^+/H^+ exchanger such as SOS1 [61]. Additional evidence of the involvement of SOS1 in long-distance Na^+ transport has been produced recently in the halophytic *Arabidopsis*-relative *Thellungiella salsuginea* (a.k.a. *T. halophila*) and in tomato [64,65]. Lower net Na^+ flux was observed in the xylem sap of tomato plants with suppressed SOS1 activity [65]. Downregulation of *ThSOS1* in *Thellungiella* increased Na^+ accumulation in the root tip and in the stele. Maximal Na^+ accumulation, concomitant with a decrease in the K^+ content, was found in the root xylem parenchyma. These cells presented a Na^+–K^+ ratio more than 12 times higher than equivalent cells in wild-type plants.

Reduced or abolished activity of SOS1 interferes with K^+ nutrition and long-distance transport ([65] and references therein). Mutations in rice and *Arabidopsis* HKT Na^+ transporters also reduce K^+ accumulation in shoots during salt exposure [48,55]. Since greater Na^+ levels in the xylem sap of *athkt1;1* mutants were accompanied by reduced xylem sap K^+ levels, the coupling of Na^+ unloading by the xylem-localized HKT transporters with K^+ loading into xylem vessels via depolarization-activated K^+ channels has been hypothesized [43••]. HKT-mediated Na^+ uptake from xylem vessels would induce membrane depolarization of xylem parenchyma cells, which in turn could activate depolarization-activated outward-rectifying K^+ channels. Reduced contents of K^+ in Na^+-loaded xylem parenchyma cells in SOS1-suppressed *Thellungiella* plants are coherent with this model [64]. On the other hand, cytosolic Na^+ has been shown to inhibit the K^+ channel AKT1 involved in K^+ uptake by roots [41]. AKT1 is apparently a target of salt stress in *sos1* plants, resulting in poor growth because of impaired K^+ uptake. Mutant analyses showed that *akt1* seedlings were salt sensitive during early seedling development, but *skor* seedlings were normal. The SKOR channel mediates K^+ release into the xylem vessels from xylem parenchyma cells. Thus, the effect of Na^+ on K^+ transport is probably more important at the uptake stage than at the xylem loading stage.

The activity of the SOS1 exchanger is regulated through protein phosphorylation by the SOS2–SOS3 kinase complex in *Arabidopsis* [62,66]. SOS2/CIPK24 is a serine/threonine protein kinase of the SnRK3/CIPK family. SOS3/CBL4 is a myristoylated, membrane bound Ca^{2+} sensor belonging to the recoverin-like family of SCaBPs/CBLs. Upon Ca^{2+} binding, SOS3 binds to and enhances the protein kinase activity of SOS2 [67]. Besides activating SOS2, SOS3 was shown to recruit SOS2 to the plasma membrane to facilitate interaction with SOS1 [66]. SOS2 also interacts with SCaBP8/CBL10 to form an alternative protein kinase complex that regulates SOS1 at the plasma membrane [68]. SOS2 has recently been shown to phosphorylate SCaBP8/CBL10 at its C-terminus [69], thus adding a new layer of regulation to CBL proteins besides Ca^{2+} binding and fatty acyl modifications [70]. This phosphorylation was induced by salt stress, occurred at the membrane, stabilized the SCaBP8–SOS2 interaction, and enhanced plasma membrane Na^+/H^+ exchange activity [69]. Surprisingly, interaction of SOS2/CIPK24 with SCaBP8/CBL10 may also result in localization of the kinase complex at the vacuolar membrane where it mediates salt tolerance by regulating the accumulation of Na^+ in shoot tissues by an as yet undefined mechanism that may involve regulation of the Na^+/H^+ exchange at the tonoplast [71,72]. Regulation of the tonoplast V-ATPase by SOS2 in the absence of CBL proteins has also been reported [73]. Presumably, the post-translational modifications of SCaBP8/CBL10 or the interaction of combinatorial protein kinase complexes with specific targets in different cellular membranes may ultimately define the localization of the protein kinase *in vivo*.

The identification of additional SOS2-interacting proteins indicates a connection between SOS2 and ROS signaling [74•]. SOS2 physically interacts with the H_2O_2 signaling protein nucleoside diphosphate kinase 2 (NDPK2) and with catalases 2 and 3. A *sos2 ndpk2* double mutant did not accumulate H_2O_2 in response to salt stress, suggesting that

it is altered signaling rather than H_2O_2 toxicity alone that is responsible for the increased salt sensitivity of the *sos2 ndpk2* double mutant relative to single mutants. The effect of NDPK2 on H_2O_2 signaling and stress sensitivity may be mediated at least in part by the interaction with and stimulation of two H_2O_2-responsive mitogen-activated protein kinases, AtMPK3 and AtMPK6. The interaction of SOS2 with NDPK2 occurred at the FISL motif, the same protein domain required for SOS2 interaction with SOS3 and adjacent to the interaction domain with the protein phosphatase ABI2. Interaction with SOS2 inhibited NDPK2 histidine autophosphorylation, indicating modulation of NDPK2 activity and, in turn, of its downstream targets MPK3 and MPK6. The link between the SOS pathway for Na^+ stress and ROS signaling is further substantiated by the interaction of the C-terminal cytoplasmic tail of SOS1 with RCD1 (radical-induced cell death 1), a regulator of oxidative-stress responses [75], and by the isolation of *enh1* mutant as an enhancer of the salt sensitivity of the *sos3* mutant [76]. The *ENH1* gene encodes a chloroplast-localized rubredoxin-like protein that shows greatest sequence similarity to rubredoxin proteins that play a role in superoxide detoxification. The *enh1* mutation causes enhanced accumulation of ROS, particularly under salt stress. Since *sos2* but not *sos3* mutants show increased sensitivity to oxidative stress and the *enh1* mutation does not enhance *sos2* phenotypes, it appears that ENH1 also functions in the SOS2-dependent ROS signaling. The ion transporter SOS1 affects *ENH1* expression under salt stress by a process that may involve RCD1.

Regulatory processes involving CIPK–CBL modules have also been described for the control of K^+ channels facilitating K^+ uptake. A genetic screen for inability to grow in low external K^+ demonstrated that the protein kinase CIPK23 is required for K^+ uptake by roots under low-K^+ conditions. CIPK23 bound to one of the two alternative subunits CBL1 and CBL9 directly phosphorylates and activates the plasma membrane K^+ channel AKT1 [77••]. The interaction between CIPK23 and AKT1 involved the kinase domain of the CIPK and the ankyrin repeat domain of the channel [78•]. CIPK23 was also identified in a genetic screen for enhanced drought tolerance. In the *cipk23* mutant, reduced transpirational water loss from leaves coincided with enhanced ABA sensitivity of guard cells during opening and closing, without noticeable alterations in ABA content in the plant [79]. The *cbl1 cbl9* double mutant, but not the *cbl1* or *cbl9* single mutants, exhibited altered phenotypes for stomatal responses and low-potassium sensitivity. Thus, plasma membrane-localized CBL1–CIPK23 and CBL9–CIPK23 complexes simultaneously regulate K^+ transport processes in roots and in stomatal guard cells. For channel inactivation, the 2C-type protein phosphatase AIP1 (for AKT1-interacting PP2C 1) physically interacts and inhibits AKT1, but channel protein dephosphorylation was not shown. Interestingly, Luan's lab has recently reported that PP2CA, another member of the PP2C phosphatase family, inhibits the activity of the guard cell anion channel SLAC1 by two mechanisms: first, by dephosphorylating the SLAC1 channel protein and second, by physical interaction with the SLAC1-activating protein kinase OST1, leading to inhibition of the kinase independently of phosphatase activity [80]. OST1 (SnRK2.6) is a member of the *Arabidopsis* SnRK2-type protein kinase family that includes other members that function in the ABA response. OST1 was shown to phosphorylate and activate SLAC1 but whether PP2CA dephosphorylated the amino acid residue(s) phosphorylated by OST1 was not formally demonstrated [80]. Further work should elucidate the details of this regulatory mechanism. These results demonstrate that two opposing regulatory pathways involving SnKR2/3 kinases and 2C-type phosphatases function in guard cells. The CIPK23–AIP1 module controls cell expansion and stomatal aperture by the likely regulation of inward K^+-channels that remain to be identified (K^+-channel proteins AKT1–3 and KAT1 are expressed in guard cells), whereas the OST1–PP2CA module mediates cell shrinkage and stomatal closure by regulating ion efflux via the anion channel SLAC1. A similar CIPK–PP2C switch may operate in Na^+ efflux processes since SOS2 interacts with the 2C-type protein phosphatase ABI2 [81]. In this case also, protein–protein interactions may be as important as phosphorylation–dephosphorylation events for signal output. Structural data and protein competition assays show that the binding of ABI2 and SOS3 to SOS2 is mutually exclusive and that SOS2 may be inhibited by the binding of ABI2 [67]. Presumably, Ca^{2+}-bound SOS3 displaces ABI2 and activates SOS2 upon salt-induced Ca^{2+} signals. Together, these findings provide evidence that the SnRK2 and SnRK3/CIPK protein kinases together with 2C-type protein phosphatases form a network of molecular switches that regulate ion transporters in plants that are relevant to salinity and water stress tolerance.

Concluding remarks

Many factors related to the plant response to water and salinity stress have been identified and many of these factors have already been shown effective for engineering stress tolerance in model plants. The manipulation of signaling factors has produced the most impressive results arguably because they control a broad range of downstream events, which results in superior tolerance. They are potential candidates for multiple stress tolerance owing to the interlaced nature of stress signaling networks. The structural details of the ABA–receptor interactions that are being elucidated may pave the way for the development of agonist molecules or the molecular tailoring of proteins (e.g. modulation of the activity of key proteins guided by structural knowledge of these target proteins) that could be useful for crop plants to cope with water shortage. Controlled stress-dependent activation or deactivation of signal components might function as a

molecular switch for the biotechnological manipulation of stress responses. Effective expression systems, including cell type-specific and stress-inducible promoters will be required to fine-tune the plant response to stress according to the time and circumstances for the onset of the environmental insult. Commonly used constitutive promoters are not always effective or can have negative effects on plant growth or development. The importance of cell type-specific processes is best exemplified by AtHKT1;1. Constitutive expression of *AtHKT1;1* causes increased shoot accumulation of Na^+ and reduced salinity tolerance, whereas specific transgene expression in the stele of roots has the opposite effect, that is Na^+ exclusion from the shoot and enhanced salinity tolerance [52••].

Admittedly, the vast majority of stress genes have been tested using model plants in highly controlled experimental settings. Engineering crop plants with improved stress tolerance is still in its early stages. The number of transgenic crop plants that have undergone field trials is undoubtedly small, but some have produced encouraging results [17••,82]. Engineered mechanisms of stress tolerance might promote survival during periods of intense or prolonged stress or maintain crop's productivity under conditions of moderate environmental stress. Most of the basic research has been focused on early developmental stages. In many cases, however, the reproductive parts of crop plants are harvested and future progress in producing stress tolerant crops relies on research efforts to improve reproductive success.

Last, plants respond to specific clues of water deficit and salinity stress within seconds, yet the mechanisms by which plants perceive the presence of salts or sense turgor remain obscure. Filling this gap in our knowledge is a pressing need given the importance of these initial steps of plant responses to changes in the environment.

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