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Understanding regulatory networks and engineering for enhanced drought tolerance in plants

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Drought stress is one of the major limitations to crop productivity. To develop crop plants with enhanced tolerance of drought stress, a basic understanding of physiological, biochemical and gene regulatory networks is essential. Various functional genomics tools have helped to advance our understanding of stress signal perception and transduction, and of the associated molecular regulatory network. These tools have revealed several stress-inducible genes and various transcription factors that regulate the drought-stress-inducible systems. Translational genomics of these candidate genes using model plants provided encouraging results, but the field testing of transgenic crop plants for better performance and yield is still minimal. Better understanding of the specific roles of various metabolites in crop stress tolerance will give rise to a strategy for the metabolic engineering of crop tolerance of drought.

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Introduction

When plants experience environmental stresses such as drought, salinity, high and low temperature, intense light, and excessive ozone or CO₂ they activate a diverse set of physiological, metabolic and defense systems to survive and to sustain growth. Tolerance and susceptibility to abiotic stresses are very complex. Abiotic stress is the primary cause of crop loss worldwide, causing average yield losses of more than 50% for major crops [1,2]. Plant traits that are associated with resistance mechanisms are multigenic, and thus difficult to control and engineer. Transcriptomics, proteomics and gene expression studies have identified the activation and regulation of several stress-related transcripts and proteins, which are generally classified into two major groups. One group is involved in signaling cascades and in transcriptional control, whereas

members of the other group function in membrane protection, as osmoprotectants, as antioxidants and as reactive oxygen species (ROS) scavengers. Manipulation of genes that protect and maintain cellular functions or that maintain the structure of cellular components has been the major target of attempts to produce plants that have enhanced stress tolerance.

Among the various abiotic stresses, drought is the major factor that limits crop productivity worldwide. Exposure of plants to a water-limiting environment during various developmental stages appears to activate various physiological and developmental changes. Understanding of the basic biochemical and molecular mechanisms for drought stress perception, transduction and tolerance is still a major challenge in biology. Plant modification for enhanced drought tolerance is mostly based on the manipulation of either transcription and/or signaling factors or genes that directly protect plant cells against water deficit.

Reviews on the molecular mechanisms of abiotic stress responses and the genetic regulatory networks of drought stress tolerance have been published recently [3,4,5, 6,7**]. This review focuses on the recent progress in understanding the mechanisms of gene regulation and the roles of protective metabolites in drought stress tolerance, and the progress in genetic or metabolic engineering for enhanced drought tolerance in crop plants. We have categorized these engineering efforts into two major groups: first, engineering cell signaling and gene regulation, and second, engineering for osmoprotectant accumulation.

Physiological and biochemical responses

Physiological and biochemical changes at the cellular level that are associated with drought stress include turgor loss, changes in membrane fluidity and composition, changes in solute concentration, and protein–protein and protein–lipid interactions [8]. Plant tissues can maintain turgor during drought by avoiding dehydration, tolerating dehydration or both [9]. These forms of stress resistance are controlled by developmental and morphological traits such as root thickness, the ability of roots to penetrate compacted soil layers, and root depth and mass [10]. Constitutive phenotypic traits (e.g. root thickness) are present even in the absence of stress conditions. By contrast, adaptive traits, such as osmotic adjustment and dehydration tolerance, arise in response to water deficit [11]. Reduction of photosynthetic activity, accumulation of organic acids and osmolytes, and changes in

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carbohydrate metabolism, are typical physiological and biochemical responses to stress. The reduction in photosynthetic activity is due to several coordinated events, such as stomatal closure and the reduced activity of photosynthetic enzymes. Synthesis of osmoprotectants, osmolytes or compatible solutes is one of the mechanisms that plants have evolved for adaptation to water deficit. These molecules, which act as osmotic balancing agents, are accumulated in plant cells in response to drought stress and are subsequently degraded after stress relief [12]. Osmoprotectants include amino acids, polyols, and quaternary ammonium and tertiary sulfonium compounds [13]. Early studies on changes in the carbohydrate metabolism of plants that are exposed to drought stress suggested that, under dry conditions, the hydroxyl group of polyhydroxy compounds can form a hydrogen bond with the polar heads of membrane phospholipids, and that these hydrophobic interactions are important for membrane stability [8,14–16].

Engineering cell signaling and gene regulation

Gene expression profiling using cDNA or oligo microarray technology has advanced our basic understanding of gene regulatory networks that are active during the exposure of plants to various stresses [6,17,18*]. In *Arabidopsis*, numerous genes that respond to dehydration stress have been identified and categorized as *responsive to dehydration* (*rd*) and *early response to dehydration* (*erd*) genes [19]. There are at least four independent regulatory systems for gene expression in response to water stress. Two are abscisic acid (ABA)-dependent and two are ABA-independent [20]. A *cis*-acting element, such as the Dehydration-responsive element/C-repeat (DRE/CRT), is involved in the ABA-independent regulatory systems. DRE/CRT also functions in cold- and high-salt-responsive gene expression. When the DRE/CRT-binding protein DREB1/CBF was overexpressed in transgenic *Arabidopsis* plants, changes in the expression of more than 40 stress-inducible genes were identified, and these changes led to increased freezing, salt, and drought tolerance [21–23].

Other important transcriptional regulators, such as the MYC and MYB proteins, are known to function as activators in one of the ABA-dependent regulatory systems [24]. The ABA-responsive element (ABRE) functions as a *cis*-acting element in the other ABA-dependent regulatory system. ABA-responsive element binding (AREB) basic leucine-zipper-type proteins (also known as auxin binding factors [ABFs]) have been identified as transcriptional activators in the ABA-dependent regulatory systems [25,26]. ABF3 or ABF4 overexpression conferred several ABA-associated phenotypes, such as ABA hypersensitivity, sugar hypersensitivity, and enhanced drought tolerance, along with altered expression of ABA- or stress-responsive genes such as *rd29B*, *rab18*, *ABA-INSENSITIVE1* (*ABI1*) and *ABI2* [27]. The identification of these

transcriptional activators of ABA signaling has promise for genetic engineering for enhanced drought tolerance.

In *Arabidopsis*, expression levels of several ABA synthesis genes, including the zeaxanthin epoxidase gene (*ZEP*; also known as *LOS6* [for low expression of osmotic stress-responsive genes 6]/*ABA1*), a 9-*cis*-epoxycarotenoid dioxygenase gene (*NCED3*), the aldehyde oxidase gene (*AAO3*), and the molybdenum cofactor sulfuryase gene (*MCSU*; also known as *LOS5/ABA3*), are upregulated by drought and salt stress but not obviously induced by cold [28,29]. Three cDNA clones that encode proteins (belonging to NAC transcription factor family) that bind to the 63-bp promoter region of *erd1*, which contains the CATGTG motif, have been isolated [30*]. This motif is known to play an important role in the dehydration-inducible expression of the *Arabidopsis* *ERD1* gene, which encodes a protein that is homologous to ClpA, the ATP-binding subunit of the caseinolytic ATP-dependent protease. The expression of these three cDNA clones (i.e. ANAC019, ANAC055, and ANAC072) was induced by drought, high salinity, and abscisic acid, as confirmed subsequently using the β -glucuronidase (GUS) reporter gene. Microarray analysis of transgenic plants that overexpressed NAC transcription factor proteins revealed that several stress-inducible genes were upregulated, and that these plants showed significantly increased drought tolerance. A novel AP2-domain-containing putative transcription factor gene from the model legume *Medicago truncatula* has been characterized, and the overexpression of this gene (*WAX PRODUCTION1* [*WXP1*]), is able to activate wax production and to confer drought tolerance in alfalfa (*Medicago sativa*). This transcription factor gene is distinctly different from the most-studied genes in the AP2/ERF transcription factor family, such as the AP2 genes, CBF/DREB1s, DREB2s, WIN1/SHN1 and GL15 [31].

Two transcription factors that are involved in the regulation of stomatal movements, AtMYB60 and AtMYB61, which are members of the *Arabidopsis* R2R3-MYB gene family, were characterized recently [32,33]. *AtMYB60* is specifically expressed in guard cells, and its expression is negatively modulated during drought. A null mutation in *AtMYB60* results in the constitutive reduction of stomatal opening and in decreased wilting under water stress conditions. Likewise the overexpression of *AtMYB61* also decreases the stomatal aperture. In the ABA-mediated stomatal control processes, a plant syntaxin protein plays an important role [34]. The *Arabidopsis* *osm1/syp61* (osmotic stress sensitive/syntaxin) mutant plants not only showed impaired ABA-induced stomatal closure but ABA treatment of *osm1/syp61* mutants was unable to inhibit their stomatal opening. In addition, the effects of *osm1/syp61* mutation on stomatal behavior are reflected in significant changes in water-loss characteristics and in increased sensitivities to salinity and water-deficit stress

[34]. These approaches will facilitate the engineering of stomatal activity to help plants survive water deficit conditions. In addition, the overexpression of rice *Myb4* (*OsMyb4*) in *Arabidopsis* has improved drought tolerance by increasing the accumulation of compatible solutes. Solutes such as glucose, fructose, sucrose, proline, glycine betaine and sinapoyl malate accumulate in greater concentrations in *Osm4*-overexpressing plants than in non-transgenic plants, under both non-stress and stress conditions [35].

To compare the stress responses of *Arabidopsis* and rice, transgenic rice plants were developed that constitutively express CBF3/DREB1A (CBF3) and ABF3, *Arabidopsis* genes that function in ABA-independent and ABA-dependent stress-response pathways, respectively [36]. The expression of CBF3 in transgenic rice improved tolerance of drought and high salinity, and slightly improved tolerance of low temperatures. By using the 60K rice whole-genome microarray and RNA gel-blot analyses, this group has been able to identify 12 and 7 target genes that are activated in transgenic rice plants that overexpress CBF3 and ABF3, respectively. These genes appear to acclimate the plants that express them to stress conditions. An additional 13 and 27 genes, respectively, were induced by drought stress in CBF3 and ABF3 overexpressing plants. The results of a field trial of transgenic plants that have been engineered for drought stress tolerance have been reported by Monsanto (J Heard *et al.* Abstract L 8.02, Interdrought II, Rome, September 2005); transgenic maize plants that expressed an *Arabidopsis* transcription factor (NF-YB class CCAAT-binding transcription factor) demonstrated improved drought tolerance in the field. The Monsanto data also suggest that the functions of selected transcription factors in drought stress tolerance are conserved across the dicot and monocot lineages because they have similar impacts on specific phenotypes. Even though several genes that are associated with drought tolerance were identified and characterized, only a few stress-resistant transgenic crop plants were evaluated in the field trials.

Several late embryogenesis abundant (LEA) proteins that resulted in cellular dehydration were isolated under stress conditions, and some of them (dehydrins) have been found to act as chaperones that stabilize vesicles, proteins and membrane structure in stressed plants [37,38]. Overexpression of the barley *HVA1* gene improved the performance of transgenic rice plants by protecting their cell membranes from injury under drought stress [39]. Transgenic rice plants that overexpressed the wheat LEA gene *PMA80* and transgenic wheat plants that overexpressed the barley LEA gene *HVA1* showed enhanced tolerance of water-deficit conditions [40,41]. The barley *HVA1* gene, which encodes a member of the group 3 LEA proteins, has been introduced into wheat and the transgenic plants showed improved biomass productivity and

water-use efficiency under water-deficit conditions [41]. Recently, field trials confirmed the potential of the *HVA1* gene to confer drought protection upon transgenic spring wheat [42]. The rice dehydrin gene *OsDhn1* was highly upregulated in CBF1/DREB1b transgenic rice plants, indicating that *OsDhn1* is a target of CBF/DREB1 signaling and is located downstream of CBF1/DREB1b in the stress signaling pathway [43].

Engineering for osmoprotectant accumulation

Osmoprotectants are small neutral molecules that are non toxic to the cell at molar concentration and that stabilize proteins and cell membranes against the denaturing effect of stress conditions on cellular functions [44]. Many major crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress-tolerant organisms. It has been hypothesized, therefore, that engineering the introduction of osmoprotectant synthesis pathways is a potential strategy for improving the stress tolerance of crop plants [45].

The genetic engineering of metabolic pathways for the production of osmolytes such as mannitol, fructans, trehalose, ononitol, proline, or glycinebetaine, among others, might increase resistance to drought, but the mechanism by which these osmolytes provide protection is not completely understood [46]. Usually, the osmolytes are localized in the cytoplasm of plants. The active accumulation of osmolytes decreases the cell's osmotic potential and maintains cell turgor [10]. Genetic modulation for osmolyte accumulation, however, does not always lead to osmotic adjustment in plants in response to stress. Other responses, such as the production of scavenging ROS, the induction of chaperone-like activities that protect protein structure and metabolic detoxification, are also being reported during drought stress [11].

Mannitol

Mannitol is a major photosynthetic product in many algae and higher plants and enhances tolerance to water-deficit stress primarily through osmotic adjustment [47]. The introduction of a mannitol dehydrogenase (*mtlD*) gene into wheat [48] produced a considerable increase in water stress tolerance. There was, however, no significant difference in osmotic adjustment between the *mtlD* transgenic wheat and control plants, at either the callus or whole-plant level, suggesting that the beneficial effect of mannitol resulted from protective mechanisms other than osmotic adjustment. These mechanisms are likely to involve the scavenging of hydroxyl radicals (OH^-) and/or the stabilization of macromolecules. In tobacco, mannitol protects thioredoxin, ferredoxin, and glutathione and the thiol-regulated enzyme phosphoribulokinase from the effects of OH^- [49].

Another example of the protection of sensitive enzymes and membranes from ROS is provided by D-ononitol and

myo-inositol in the cytoplasm. The tolerance of drought and salt stress in transgenic tobacco plants that overexpress the inositol methyl transferase gene (*IMT1*) from the ice plant (*Mesembryanthemum crystallinum*) is increased by the accumulation of the methylated form of inositol, D-ononitol [50].

Raffinose, galactinol and fructan

Water deficit alters the synthesis and partitioning of metabolically important carbohydrates in plants. Some of these effects on carbohydrate metabolism might be required for the photosynthetic assimilation of carbon and/or its conversion to metabolically usable forms. Other stress-induced changes in carbon metabolism might reflect adaptations for stress tolerance [51]. For example, raffinose-family oligosaccharides, such as raffinose, stachyose and galactinol, play important roles in the desiccation tolerance of plants.

Seven galactinol synthase (*GolS*)-related genes have been identified in the *Arabidopsis* genome, but little is known about their roles in the accumulation of galactinol and raffinose in plants under water-deficit conditions [52]. *Arabidopsis* plants that overexpress the *AtGolS1* and/or *AtGolS2* genes show enhanced tolerance to drought stress owing to their accumulation of galactinol and raffinose. The endogenous production of these sugar compounds in transgenic plants provides membrane protection and a reduced rate of transpiration from the leaves, which result in drought tolerance. Hence, these galactinol and raffinose act as osmoprotectants rather than by conferring an osmotic adjustment that provides an adaptation to water-stress conditions [52].

Fructans are polyfructose molecules that are soluble carbohydrates and are located in the vacuoles of many plants. Fructan metabolism plays a significant role in drought- and cold-stress tolerance in plants [53]. Because these compounds are soluble, they might play a role in the osmotic adjustment of natural fructan accumulators to different abiotic stresses by varying the degree of polymerization of the fructan pool. Tobacco and sugar beet plants that were engineered with the bacterial fructan gene showed enhanced tolerance to drought stress conditions [54,55].

Trehalose

Trehalose (α -D-glucopyranosyl-1,1- α -D-glucopyranoside) is a non-reducing disaccharide that is present in many different organisms and that functions as reserve carbohydrate and stress protectant, stabilizing proteins and membranes and protecting them from denaturation [56]. In yeast, trehalose is synthesized from UDP-glucose and glucose-6-phosphate in two reactions, which are catalyzed by trehalose phosphate synthase (TPS) and trehalose-6-phosphate-phosphatase (TPP) [57]. A family of 11 TPS genes has been identified in *Arabidopsis*, including TPPs and a subfamily of TPPs [58]. Metabolic

engineering for the accumulation of trehalose in transgenic plants that expressed the trehalose biosynthesis genes resulted in an elevated level of drought-stress tolerance in plants, even though phenotypic abnormalities were noticed in some cases [59–61,62*].

The *AtTPS1* enzyme plays a significant role in sugar and ABA signaling during vegetative development, and the overexpression of *AtTPS1* produces increased drought tolerance through these signaling processes [62*]. The level of trehalose accumulation in transgenic plants that overexpressed *AtTPS1* was slightly changed, and in contrast to the work described above, the trehalose-6-phosphate level was increased without affecting plant morphology. Controlled trehalose overproduction in rice owing to the stress-inducible or tissue-specific expression of bifunctional TPS fusion gene resulted in drought tolerance without any detrimental effect on plant growth or grain yield. In addition, the expression of this gene resulted in elevated soluble carbohydrate levels, including subtle changes in the levels of glucose, fructose, and sucrose. These results confirm the role of trehalose in sugar sensing and carbon metabolism [60].

Proline

Proline accumulation plays a highly protective role in plants that are exposed to abiotic stresses, conferring osmotic adjustment together with an increase in the levels of other osmolytes. Other suggested functions of proline are the detoxification of ROS and interaction with the hydrophobic residue of proteins. The proline biosynthetic pathway has been well characterized [63,64].

The involvement of proline in the response to water deficits has been demonstrated in transgenic tobacco that overexpressed proline biosynthesis enzymes [65,66]. The suppression of proline synthesis in transgenic plants that contain the pyrroline-5-carboxylate reductase (P5CS) gene in the antisense direction resulted in increased sensitivity to water deficit [67]. Recently, it was reported that transgenic petunia plants that overexpressed the *AtP5CS* gene from *Arabidopsis* and the *OsP5CS* gene from rice can withstand drought conditions for longer durations than wildtype plants [68]. The expression of P5CS also resulted in drought tolerance in soybean. The sense transformants, which demonstrated the earliest proline accumulation, experienced the least water loss when compared to the antisense transformants, which possessed the slowest proline accumulation [69].

Conclusions and future perspectives

Transcriptomic, proteomic and metabolic analyses have identified and characterized several genes that are induced by drought stress and the associated signaling and regulatory pathways in plants. Recent efforts on dissecting the cross-talk between drought stress and other major abiotic stress signaling pathways also provide

potential candidates for multiple stress tolerance. Most of these studies were conducted using model plants, and engineering for drought tolerance in crop plants is still in its early stages. The number of transgenic crop plants that have undergone field trials or been tested under natural water-deficit conditions is very small. Drought tolerance is a complex trait, making the transgenic production of tolerant crops a challenging prospect. Nevertheless, advances in our understanding of stress signal perception and transduction and of the associated molecular regulatory networks, together with high-throughput transformation technology, have improved the possibility of achieving this goal. This progress has made it possible to employ gene pyramiding or co-transformation for resistance to one or more stresses. The identification and characterization of tissue-specific and drought-inducible promoters will enhance these efforts.

Recent genetic engineering of regulatory elements resulted not only in enhanced plant survival in drought conditions *per se* but also in improved crop productivity under water deficit conditions. More work on crop plants is needed, however, to link physiology, systems biology and field performance. Understanding traits in crop plants that are associated with root architecture and plasticity under water-deficit conditions (e.g. osmotic adjustments in roots) and their manipulation might help to advance our knowledge of crop drought tolerance. Another major physiological constraint to sustaining and improving plant production under drought stress is plant reproductive failure under stress. Most of the research on our basic understanding of drought tolerance and its applications is focused on plant developmental stages other than just before flowering and after flowering. In most cases, however, the reproductive parts of crop plants are the harvestable yields and future success in producing drought tolerant crops relies on intensive research efforts to improve reproductive success. A comprehensive screening of metabolites during drought stress will advance our fundamental understanding of major metabolic pathways and provide direction for future metabolic engineering for drought-stress tolerance in crop plants. In the end, the value of any genes or pathways for drought tolerance in crop plants can only be judged by evidence of solid field performance.

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