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Osmoregulation in Plants: Implications for Agriculture[1]

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SYNOPSIS. Drought and salinity stress are the major causes of historic and modern agricultural productivity losses throughout the world. Both drought and salinity result in osmotic stress that may lead to inhibition of growth. Salinity causes additional ion toxicity effects mainly through perturbations in protein and membrane structure. In contrast to animals, which rely on Na^+/K^+-ATPases for the expulsion of osmotica, plants rely on plasma membrane and endosomal ATPase activities to generate proton gradients to drive ion extrusion and intracellular sequestration. Consequently, most angiosperms, including all major crop species, have a diminished capacity for Na^+ transport and tolerance to high salinity. New insights into the molecular mechanisms of Na^+/K^+ discrimination, Na^+ extrusion and compartmentation, water transport, and osmolyte biosynthesis and function have led to genetically engineered plants with improved salt, drought, and cold tolerance. A deeper understanding of the complex signal transduction and regulatory responses to osmotic stress promises novel strategies for improving salinity and drought tolerance that will be of practical benefit to agriculture.

INTRODUCTION

Drought and soil salinization in both dry land and irrigated agricultural settings are major factors in limiting agricultural productivity worldwide. More than 20% of all cultivated lands are estimated to be salt-stressed with up to one-third of agricultural lands being salt-affected in certain countries (Flowers and Yeo, 1995). Irrigation water typically contains some salts, which eventually build up, leading to salinization related losses in productivity for up to half of the areas under irrigation (Seckler *et al.*, 1998; Flowers and Yeo, 1995). Attempts to generate plant varieties with improved salinity or drought tolerance using selection-based breeding strategies have proved largely unsuccessful (Flowers and Yeo, 1995; Flowers *et al.*, 2000). This failure can be explained by the well-recognized complexity or multigenic nature of salinity and drought tolerance traits (Cushman and Bohnert, 2000; Flowers *et al.*, 2000). Although it may be feasible to improve abiotic stress tolerance using whole plant phenotypic or physiological selection strategies and pyramiding breeding schemes, such approaches, even those that employ marker-assisted selection, are slow and require massive screening efforts to identify specific quantitative trait loci. Attempts to engineer improved tolerance using single or multi-gene transfer of ''candidate'' genes offer far more rapid and promising improvements in stress tolerance. Such genetic engineering strategies, however, are limited by an incomplete understanding of stress tolerance mechanisms. Here, I highlight some of the unique characteristics of plant osmoregulation and discuss how advances in our understanding of these processes will lead to the development of crop plants with improved drought and salinity tolerance.

OSMOREGULATORY MECHANISMS IN PLANTS

The chemiosmotic regulatory systems of plant and fungal cells differ fundamentally from those found in animal cells. Animal cells rely on a primordial Na^+ chemiosmotic circuit consisting of Na^+/K^+-ATPase ''pumps'' to drive the efflux of $3Na^+$ and influx of $2K^+$ coupled to ATP hydrolysis. This active Na^+ extrusion creates an electrochemical Na^+ gradient across the plasma membrane to drive secondary symport and antiport carriers that, in turn, regulate nu-

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trient uptake and pH. In contrast, plants appear to lack plasma membrane Na^+/K^+-ATPases. Thus, plants utilize H^+-ATPases for primary extrusion or sequestration of protons to generate H^+ electrochemical gradients, which drive secondary ion and nutrient transport processes via H^+-symport/antiport systems. These H^+-ATPase pumps also modulate both intracellular and extracellular pH.

Except in the case of extreme halophytic archaebacteria, viable cellular processes in animals, fungi, and plants depend upon the maintenance of low cytoplasmic Na^+ and Cl^- concentrations and a high K^+/Na^+ ratio, because K^+ counteracts the inhibitory effects of Na^+ (and Li^+). Like animal cells, most plant cells maintain cytosolic K^+ concentrations in the range of 100–200 mM and Na^+ values in the low mM range (1–10 mM) up to a maximum of 100 mM (Maathuis and Amtmann, 1999). In contrast to K^+, an essential cation for maintaining biochemical interactions of the cytoplasm, Na^+ is not essential for, but does facilitate, volume regulation and growth in most plants. However, at high concentrations Na^+ limits growth (Blumwald, 2000). Ironically, the productivity of irrigated agricultural regions is generally many times greater than non-irrigated areas, yet irrigated crops are most susceptible to detrimental salinity effects. Therefore, genetic engineering of crop plants to improve their capacity for Na^+ transport and sequestration is an important goal for meeting the future food and fiber demands of a rapidly growing human population.

Many plants, such as extreme halophytes, display Na^+ dependence for optimal growth and development and have developed specialized structures such as salt glands and bladders to accommodate high salt concentrations in tissues (Glenn *et al.*, 1999). Others have developed whole plant strategies for avoiding stress such as accelerated completion of ontogeny. However, these specialized adaptations are lacking in most major crop species. Furthermore, the precise impact of osmotic and ionic effects on cell growth, division, phytohormone balance, and death in the context of the whole plant are complex and require further investigation (Munns, 2001). Therefore, emphasis is placed on the molecular genetic mechanisms controlling osmotic regulation at the cellular level, mainly because the action and regulation of most osmoregulatory components has not been fully explored in the context of the whole plant. The challenge for the coming decade will be to integrate information gathered at the molecular genetic and cellular levels with the complexity of whole plant physiology.

HIGH- AND LOW-AFFINITY K^+ AND NA^+ CARRIERS

Exposure of plants to high extracellular NaCl concentrations favors Na^+ uptake through a system of K^+-selective, Na^+-selective and non-selective pathways located at both the plasma and vacuolar membranes (Amtmann and Sanders, 1999; Czempinski *et al.*, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999; White, 1999; Maathuis and Amtmann, 1999; Hasegawa *et al.*, 2000; Blumwald, 2000). Multiple transport mechanisms govern K^+:Na^+ selectivity and contribute to K^+ and Na^+ uptake in higher plants (Schachtman, 2000). These systems rely upon carriers or high-affinity transporters that undergo a specific conformational change during ion transport and generally operate against a concentration gradient. Such ''up hill'' carriers are energized by coupling to an electrochemical gradient (*e.g.*, K^+-H^+ symport, K^+-Na^+ symport). The first such K^+ carrier described in higher plants, HKT1, encodes a high affinity K^+ transporter that functions as a Na^+-coupled K^+ transporter and is thought to contribute to sodium uptake in saline soil (Rubio *et al.*, 1996). Under high Na^+ concentrations, HKT1 may have more physiological relevance for Na^+ uptake than K^+ uptake (Diatloff *et al.*, 1998; Rubio *et al.*, 1999). However, its overall role in the uptake of both ions is probably minor compared with other high-affinity K^+ systems (Maathuis and Amtmann, 1999).

An HKT1 homologue recently isolated from Arabidopsis (AtHKT1) functions primarily as a selective Na^+ uptake transporter with very limited K^+ uptake activity (Uozumi *et al.*, 2000). Since AtHKT1 does not appear to couple Na^+ and K^+ or Na^+ and

H^+ uptake, it represents a likely candidate for constitutive low-affinity Na^+ uptake, a process resulting in the Na^+ toxicity following exposure to high salinity. In contrast to HKT1, a low-affinity cation carrier, LCT1, shows no homology to high-affinity carriers and exhibits non-selective, low-affinity uptake of monovalent cations (*e.g.*, K^+, Na^+, Rb^+) as well as calcium and cadmium (Schachtman *et al.*, 1997; Clemens *et al.*, 1998). Like AtHKT1, this carrier may play a significant role in Na^+ uptake under high-salinity conditions, however, details of its transport mechanism have not yet been established.

A second family of high-affinity K^+ transporters has been described in Arabidopsis (AtKUP1) (Quintero and Blatt, 1997; Kim *et al.*, 1998; Fu and Luan, 1998) and barley (HAK1) (Santa-María *et al.*, 1997). KUP/HAK transporters are extremely selective for K^+ and are blocked by mM concentrations of Na^+ (Kim *et al.*, 1998; Fu and Luan, 1998). K^+-limiting conditions induce their mRNA levels suggesting they play a primary role in high-affinity K^+ uptake. In contrast to HKT transporters, KUP/HAK transporters are thought to move K^+ by coupling to the H^+ gradient (Santa-María *et al.*, 1997). The KUP/HAK gene family consists of multiple members in plants and related transporters have been found in bacteria and fungi (Schachtman and Liu, 1999).

CATION AND ANION SELECTIVE CHANNELS

Plant genomes encode multiple classes of ion channels that mediate K^+ and Na^+ transport (see Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Maathuis and Amtmann, 1999; Zimmermann and Sentenac, 1999; Czempinski *et al.*, 1999; Blumwald, 2000; Schachtman, 2000; Blumwald *et al.*, 2000; Krol and Trebacz, 2000). Ion channels transport ions from the soil solution, secrete ions into xylem sap, and participate in signaling (Zimmermann and Sentenac, 1999; Zimmermann *et al.*, 1999). In contrast to carriers, ion channels conduct rapid, ''downhill'' dissipation of transmembrane electrochemical gradients, often under the control of membrane potentials that dictate the gating properties of the channel. Monovalent cation-selective plasma membrane channels are divided into three classes depending on their electrophysiological behavior and ion selectivity.

Inward rectifying channels

K^+ inward rectifying channels (KIRCs) conduct current in the direction of the cytoplasm and are highly selective for K^+ uptake over Na^+ under physiological (micromolar) extracellular K^+ and Na^+ concentrations. In general, most KIRCs are a major pathway for low-affinity K^+ uptake, but appear not to play a significant role in Na^+ flux into plant cells (Amtmann and Sanders, 1999). This class is exemplified by AKT1, which plays a principle role in K^+ uptake by plant roots, but is unlikely to play a major role in inadvertent sodium uptake (Hirsch *et al.*, 1998; Spalding *et al.*, 1999). AKT1 and related channels are members of the *Shaker*-like K^+ channel multi-gene family and function as homotetramers (Daram *et al.*, 1997; Czempinski *et al.*, 1999; Urbach *et al.*, 2000). Additional members of this *Shaker*-like family, AKT2/AKT3, are weakly inward rectifying and are expressed in both source and sink phloem tissues. Abscisic acid (ABA) up-regulates AKT2 expression, suggesting AKT2 participates in ion equilibrium during drought stress (Lacombe *et al.*, 2000*b*). Novel gene families encoding putative cyclic-nucleotide-gated and calmodulin-regulated K^+-uptake channels have also been described in barley (Schuurink *et al.*, 1998) and Arabidopsis (Köhler *et al.*, 1999).

Outward rectifying channels

A second class of ion channels present in many different plant species and tissues are K^+ outward rectifying channels (KORCs) (Amtmann and Sanders, 1999). The gating characteristics of KORCs are highly selective for K^+ efflux and function mainly in K^+ release. A KORC expressed in the root pericycle and xylem parenchyma of Arabidopsis, SKOR, plays a major role in the release of K^+ into xylem sap for transport to shoots (Gaymard *et al.*, 1998; Lacombe *et al.*, 2000*a*). ABA application down-regulates SKOR mRNA accumulation suggesting that this channel controls K^+ accumu-

lation in shoots as part of the adaptive response to drought stress (Gaymard *et al.*, 1998). Database searching or ''*in silico*'' cloning identified a new, so-called KCO (K^+ channel family related to the Ca^{2+} activated, outwardly rectifying) category of K^+ channels. Exemplified by KCO1 from Arabidopsis, this family member is an outwardly rectifying K^+ channel that resembles the 'two pore' K^+ channels from animals and contains four predicted transmembrane domains (Czempinski *et al.*, 1997). Additional members of this family have been cloned from potato (StKCO1, 2) and Arabidopsis (AtKCO1, 2) with a third Arabidopsis member of this family, AtKCO3, representing a novel type of plant K^+ related to animal Kir-type channels (Czempinski *et al.*, 1999). AtKCO1 and AtKCO2 differ from related ''two-pore'' K^+ channels in mammals, insects, and fungi by containing two C-terminal Ca^+-binding EF-hand domains, which impart calcium-dependent activation of the AtKCO1 channel in the physiological range of cytosolic calcium (150–500 nM) (Czempinski *et al.*, 1997).

Voltage-independent or non-selective cation channels

A third class of plasma membrane cation channels, voltage-independent monovalent cation channels (VICs) or non-selective cation (NSC) channels, has gating properties that are not significantly voltage-dependent and possess lower Na^+/K^+ selectivity compared with KIRCs or even KORCs. NSC channels have been characterized in plant roots, leaf epidermal, and guard cells (Tyerman and Skerrett, 1999; Davenport and Tester, 2000). Modeling studies and recent single NSC channel measurements demonstrate that Na^+ influx through VIC channels is much higher than other channel types under saline conditions (Amtmann and Sanders, 1999; White, 1999). These properties suggest that VIC channels may function as a major pathway for Na^+ entry into plant cells exposed to high external salinity (Amtmann and Sanders, 1999; White, 1999; Davenport and Tester, 2000). Due to their dominant role in Na^+ entry, VIC channels represent a prime target for genetic manipulation of salt tolerance either by improving their selectivity against Na^+ influx or by altering their expression or reducing their activity (White, 1999).

Anion channels

Like animal cells, plant cells contain anion channels that regulate anion efflux from the external environment, as well as participate in turgor- and osmoregulation, stomatal movements, nutrient transport, metal tolerance, and signal transduction (Schroeder, 1995; Barbier-Brygoo *et al.*, 2000). Anion channels are grouped according to whether they are activated by membrane depolarization, hyperpolarization, and stretching, or by second messenger molecules such as Ca^{2+}, nucleotides, or phosphorylation/dephosphorylation events. Anion channels play important roles in the perception and transduction of plant responses to light, phytohormones, pH, malate, CO_2, and elicitor molecules (Zimmermann *et al.*, 1999). They also participate in the regulation of intracellular ion gradients and have been described in the plasma membrane as well as in vacuolar, thylakoid, and inner mitochondrial membranes (Barbier-Brygoo *et al.*, 2000). Under saline conditions, membrane depolarization and extracellular Cl^- activated anion channels have been described in root cortical cells that regulate membrane potential and Cl^- uptake (Skerett and Tyerman, 1994; Tyerman *et al.*, 1997). Root xylem parenchyma cells contain anion efflux channels thought to function during initial phases of xylem loading which requires membrane depolarization to activate outward-rectifying channels and drive anion efflux (Wegner and Raschke, 1994). Molecular cloning studies have confirmed that plant genomes encode voltage-dependent chloride ion channels (CLC) related to the animal CLC-protein family (Maduke *et al.*, 2000). Details about the specific physiological roles of these anion channels are lacking due to difficulties encountered in obtaining functional channels in heterologous expression systems. However, ongoing efforts promise to provide this information (Barbier-Brygoo *et al.*, 2000).

SODIUM EXTRUSION AND COMPARTMENTATION

Aside from the K^+/Na^+ discriminating capacity of transport systems, a plant's ability to tightly control cytoplasmic Na^+ concentrations by extrusion and/or compartmentation is critical to avoid Na^+ toxicity, control ion fluxes, and regulate pH. The negative electrical membrane potential of the plasma membrane under high extracellular Na^+ concentrations forms a large Na^+ electrochemical potential gradient that favors the passive transport of Na^+ into the cell mediated by various KIRCs, KORCs, and NSCs. In higher plants, H^+-translocating plasma membrane ATPases (P-ATPase) and vacuolar H^+-ATPases (V-ATPase) participate in salinity stress tolerance by energizing active Na^+ extrusion from the cytosol and compartmentation within various endomembrane bound compartments (*e.g.*, endoplasmic reticulum, golgi, and vacuole), respectively (Sze *et al.*, 1999; Morsomme and Boutry, 2000; Ratajczak, 2000). A second electrogenic proton pump, the H^+-translocating pyrophosphatase (PP_iase), coexists with the V-ATPase in the vacuolar membrane and contributes to maintenance of vacuolar acidity under stress conditions (Maeshima, 2000). H^+ extrusion from the cell by P-ATPase or into the vacuolar lumen by H^+-ATPase or H^+-PP_iase creates an electrochemical gradient. This proton motive force is used by secondary transporters, such as plasma membrane and tonoplast Na^+/H^+ antiporters, to couple the downhill movement of protons to the extrusion or sequestration of Na^+ ions against their electrochemical potential gradient (Blumwald *et al.*, 2000). Sequestration of NaCl into the vacuole also provides a metabolically inexpensive and abundant osmoticum by which plants maintain an osmotic potential to drive water uptake into cells (Blumwald *et al.*, 2000).

Ionic or osmotic treatment often increases P-ATPase activity and mRNA expression with a concomitant rise in plasma membrane Na^+/H^+ antiport activity (Niu *et al.*, 1993; Barkla *et al.*, 1995). P-ATPase activation occurs rapidly presumably by covalent modification of the C-terminal domain and interaction with 14-3-3 proteins (Sze *et al.*, 1999; Roberts, 2000). NaCl treatment also generally increases V-ATPase proton pumping activity and expression of individual V-ATPase subunit genes (see Ratajczak, 2000; Ratajczak and Wilkins, 2000). Parallel increases in tonoplast Na^+/H^+ antiport activity correlate with sites of highest Na^+ accumulation within tissues suggesting that these transport processes are important determinants of salinity adaptation (Barkla *et al.*, 2001).

The contribution of the V-PP_iase activity to salinity stress adaptation has been more enigmatic. Recent experimentation in yeast, however, has demonstrated that V-PP_iase overexpression can restore salt tolerance to a salt-sensitive *ena1* mutant lacking plasma membrane Na^+-ATPase activity (Gaxiola *et al.*, 1999). Restoration of salt tolerance in this mutant was dependent upon Na^+ and Cl^- influx, presumably into the endosome, via a Na^+/H^+ antiporter (NHX1) and a chloride channel (GEF1), respectively (Gaxiola *et al.*, 1999), or extrusion of Na^+ at the plasma membrane (Blumwald, 2000; Blumwald *et al.*, 2000). These results clearly demonstrate the importance of V-PP_iase activity in energizing transport processes required for salt tolerance. A second type of K^+-insensitive, H^+-PP_iase gene (AVP2) has recently been described in plants, however, the subcellular distribution and functional roles of this class have not yet been established (Drozdowicz *et al.*, 2000; Nakanishi and Maeshima, 2000).

The recent molecular characterization of Na^+/H^+ antiporters in yeast and higher plants has provided conclusive evidence for their roles in salinity adaptation. Gaxiola *et al.* (1999) cloned the vacuolar Na^+/H^+ antiporter gene from *Arabiopsis thaliana* (*AtNHX1*) and showed that it can functionally substitute for a related endosomal antiport activity in yeast. A related salt-inducible, vacuolar Na^+/H^+ antiporter gene has also been characterized in rice (Fukuda *et al.*, 1999). Transgenic AtNHX1 overexpressing plants displayed higher vacuolar Na^+/H^+ antiporter activity, greater amounts of AtNHX1 protein, and improved growth at elevated NaCl levels compared to wild-type plants, confirming the role of this vac-

uolar Na^+/H^+ antiporter in salt tolerance (Apse *et al.*, 1999).

Recently, a putative plasma membrane Na^+/H^+ antiporter gene has been isolated from Arabidopsis by positional cloning (Shi *et al.*, 2000). This gene was originally identified as the SOS1 (salt overly sensitive) locus and plays a major role in salt tolerance (Zhu, 2000). SOS1 gene expression is induced by NaCl primarily in root cells surrounding the xylem suggesting that this Na^+/H^+ antiporter functions in loading Na^+ into the xylem for long-distance transport to the shoots (Shi *et al.*, 2000). Localization of SOS1 expression to the symplast/xylem interface is also consistent with the K^+ transport defect of the *sos1* mutant phenotype, if SOS1 function is linked to high affinity K^+ transport by K^+/H^+ or K^+/Na^+ symport processes. The ability to coordinate salt exclusion and mobilization from roots with transport to and compartmentation in shoots appears to be a fundamental determinant of salt tolerance and represents an attractive target for engineering improved salt tolerance. Despite the importance of Na^+/H^+ antiporters, their activity ultimately depends on H^+-ATPase and/or H^+-PP_iase proton pumping capacity. Therefore, future engineering strategies to improve Na^+ partitioning by enhancing Na^+/H^+ antiport activity should include parallel improvements in proton pumping activities. This has not been attempted to date, however, presumably due to the complexity of V-ATPase subunit structure and the cell- and tissue-specific expression patterns of P-ATPase gene family members (Sze *et al.*, 1999; Ratajczak, 2000; Ratajczak and Wilkins, 2000).

WATER TRANSPORT AND OSMOREGULATION

Together with ion and solute transporters, aquaporins or water channels, play essential roles in the regulation of water relations and osmotic balance within plant cells. Aquaporins are members of the major intrinsic protein (MIP) superfamily present in all organisms that facilitate the movement of water and other small molecules (*e.g.*, urea, glycerol) through membranes. Water absorption by cells from the xylem is a key determinant of plant cell growth and elongation (Nonami, 1998). MIPs are abundant proteins localized to the plasma membrane, the tonoplast, or other endomembranes of plant cells (Chrispeels *et al.*, 1999; Tyerman *et al.*, 1999; Kjellbom *et al.*, 1999; Johansson *et al.*, 2000). Of the 30 MIPs identified in Arabidopsis, 12 are proposed to be PIPs (plasma membrane intrinsic proteins), 12 are proposed to be TIPs (tonoplast intrinsic proteins), and the remaining six NLMs (NOD26-like MIPs) (Weig *et al.*, 1997; Schäffner, 1998; Johansson *et al.*, 2000). Although not all of these MIPs have been functionally tested, most show water transport activity (Kjellbom *et al.*, 1999; Johansson *et al.*, 2000).

The expression patterns of various aquaporins, which are abundant in root epidermal, exodermal, and endodermal cells, phloem-associated cells, and xylem parenchymal cells, suggest they facilitate membrane permeability to water where there are anatomical constraints to water transport or where large water fluxes are known to occur (Yamada *et al.*, 1995; Kaldenhoff *et al.*, 1995; Barrieu *et al.*, 1998; Yamada and Bohnert, 2000; Kirch *et al.*, 2000). However, the functional significance of water channels in maintaining water balance under osmotic stress remains unclear. Under drought or salt stress conditions, transcript or protein abundance of some aquaporins are upregulated (Yamada *et al.*, 1997; Uno *et al.*, 1998; Kirch *et al.*, 2000), whereas others are downregulated (Yamada *et al.*, 1995; Kirch *et al.*, 2000).

The transport activity of a putative plasma membrane aquaporin is regulated by reversible phosphorylation by a calcium-dependent protein kinase in a turgor-dependent manner (Johansson *et al.*, 1998). Simulated drought conditions lower apoplastic water potential, thereby decreasing *in vivo* aquaporin phosphorylation, lowering plasma membrane water permeability, and minimizing water loss (Johansson *et al.*, 1998). Other studies have suggested that transport of water via mercury-sensitive water channels actually increased in roots of rice plants subjected to artificial drought stress (Lu and Neumann, 1999). Differential distribution, turnover, and trafficking of aquaporins within endomembrane systems are

also likely to modulate water flux during osmotic stress (Kirch *et al.*, 2000). Antisense plants with reduced plasma membrane PIP expression compensated for reduced water permeability through the plasma membrane by increasing the size of their root system (Kaldenhoff *et al.*, 1998). The osmotic water permeability of protoplasts prepared from these antisense plants was three times lower than wild-type protoplasts (Kaldenhoff *et al.*, 1995). While these results clearly demonstrate the importance of aquaporins in water uptake, additional evidence for *in vivo* function of aquaporins is required to conclusively define their contribution to osmotic adaptation under stress conditions. Modification of the expression patterns or gating properties of selected aquaporins might represent possible targets for manipulating the control of water fluxes within plants.

OSMOLYTES AND OSMOPROTECTANTS

Most bacteria, alga, and plants accumulate various organic solutes such as sugars, cyclic and acyclic polyols, fructans, amino acids and amino acid derivatives, and quaternary amino and sulfonium compounds in response to desiccation, osmotic, or low temperature stress (Delauney and Verma, 1993; Bartels and Nelson, 1994; Bohnert and Jensen, 1996). Such organic solutes are collectively termed compatible solutes or osmolytes because they can accumulate to high concentrations within cells without impairing cellular function. As osmolytes, such compounds are thought to function, in part, by mass action to restore osmotic potential of the cytoplasm to drive water uptake to maintain cell turgor (Yancey *et al.*, 1982; Stoop *et al.*, 1996). These compounds may also counteract osmotic imbalances under conditions of low water potential or high ionic strength, protect or replace the water shell around proteins (Yancey *et al.*, 1982; Galinski, 1993), and stabilize protein complexes and membranes (Murata *et al.*, 1992; Papageorgiou and Murata, 1995). Although some osmolytes offer protection by osmotic effect, transgenic plants overexpressing biosynthetic enzymes for osmoprotectants, such as mannitol, glycine betaine, D-ononitol, or sorbitol, accumulate these substances in amounts too low to account for protective effects by osmotic mass action alone (Sheveleva *et al.*, 1997*a*, *b*; Sakamoto *et al.*, 1998; Huang *et al.*, 2000). Investigations using transgenic plants overexpressing biosynthetic enzymes for selected osmoprotectants have revealed alternative modes of stress protection. For example, mannitol protects oxidation-sensitive cellular structures by reducing hydroxyl radical formation or scavenging reactive oxygen species (Shen *et al.*, 1997*a*, *b*). Low concentrations of glycine betaine can improve salt and cold stress tolerance, possibly by protecting photosynthetic protein complexes (Holmström *et al.*, 2000) and reducing lipid peroxidation of cell membranes (Chen *et al.*, 2000). In addition, the transient accumulation of certain metabolites, such as proline, might serve as a safety valve to adjust cellular redox state during stress (Shen *et al.*, 1999; Kuznetsov and Shevyakova, 1999). Other osmoprotectants, such as ectoine, trehalose, and fructan, appear to function through membrane stabilization (Pilon-Smits *et al.*, 1995; Romero *et al.*, 1997; Nakayama *et al.*, 2000). The diverse function roles of osmoprotectants have made them favorite tools for genetic engineering improved osmotic stress tolerance (see Bohnert and Sheveleva, 1998; Hare *et al.*, 1998; Nelson *et al.*, 1998; Smirnoff, 1998; Nuccio *et al.*, 1999; Bohnert and Shen, 1999; Sakamoto and Murata, 2000; Rathinasabapathi, 2000). Defining the exact mechanisms of protection and the specific macromolecules being protected will undoubtedly lead to further improvements in osmolyte-mediated protection strategies. Identification and utilization of novel osmoprotectants derived from stress-tolerant organisms will also aid in such improvements (Rathinasabapathi *et al.*, 2000). Combinatorial engineering of more than one osmoprotectant and utilizing stress-inducible promoters to drive expression only under stress conditions should improve the efficacy of these approaches by reducing the metabolic costs of osmolyte production. More importantly, understanding how manipulating osmoprotectant biosynthesis influences metabolic flux through alterations in substrate supply, demand and

transport within subcellular compartments will be crucial to optimizing their use in engineering strategies (Nuccio *et al.*, 1998; Hare *et al.*, 1998; Stephanopoulus, 1999; Huang *et al.*, 2000; McNeil *et al.*, 2000).

OSMOREGULATORY COMPLEXITY AND FUTURE CHALLENGES

Bacterial, fungal, and plant cells share a set of common adaptive mechanisms for cellular osmotic and ionic stress tolerance. These adaptations, including ion transport, discrimination, and sequestration, the synthesis of osmoprotectants, oxidative stress protection, and metabolic ''weak links,'' have become obvious targets for engineering strategies (Bohnert and Shen, 1999; Holmberg and Bülow, 1998; Nelson *et al.*, 1998; Yeo, 1998; Smirnoff, 1998). In addition, molecular genetic and biochemical studies have now confirmed that a multitude of components participate in abiotic stress signaling pathways (Hasegawa *et al.*, 2000; Zhu, 2000). Understanding these signaling-response networks and exploiting them for engineering improved abiotic stress tolerance will remain a major goal for the coming decades. However, progress towards this goal has already begun. Overexpression of stress-regulated transcription factors in transgenic plants has been shown to improve the freezing, drought, and salinity tolerance (Jaglo-Ottosen *et al.*, 1998; Winicov and Bastola, 1999; Kasuga *et al.*, 1999; Winicov, 2000). Similarly, the ectopic expression of regulatory factors, protein kinases, and protein phosphatases can enhance tolerance to multiple abiotic stresses (Pardo *et al.*, 1998; Espinosa-Ruiz *et al.*, 1999; Gisbert *et al.*, 2000; Kovtun *et al.*, 2000; Saijo *et al.*, 2000). Generally, such ''regulon'' engineering approaches, using regulatory or signal transduction pathway intermediates, should be more efficient and effective than engineering strategies that utilize single endpoint determinants. Genomics approaches promise tremendous amounts of information about all osmotically induced genes. Combining information from genome-wide gene expression profiling with systematic mutational analysis of all candidate genes should permit a comprehensive understanding of the regulation and integration of osmoregulatory processes in plants (Cushman and Bohnert, 2000; Hasegawa *et al.*, 2000). Such information will be indispensable to our ability to engineer more effective crop protection strategies to meet the agricultural demands of the 21st century.

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