PLANT CELLULAR AND MOLECULAR RESPONSES TO HIGH SALINITY

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Abstract  Plant responses to salinity stress are reviewed with emphasis on molecular mechanisms of signal transduction and on the physiological consequences of altered gene expression that affect biochemical reactions downstream of stress sensing. We make extensive use of comparisons with model organisms, halophytic plants, and yeast, which provide a paradigm for many responses to salinity exhibited by stress-sensitive plants. Among biochemical responses, we emphasize osmolyte biosynthesis and function, water flux control, and membrane transport of ions for maintenance and re-establishment of homeostasis. The advances in understanding the effectiveness of stress responses, and distinctions between pathology and adaptive advantage, are increasingly based on transgenic plant and mutant analyses, in particular the analysis of Arabidopsis mutants defective in elements of stress signal transduction pathways. We summarize evidence for plant stress signaling systems, some of which have components analogous to those that regulate osmotic stress responses of yeast. There is evidence also of signaling cascades that are not known to exist in the unicellular eukaryote, some that presumably function in intercellular coordination or regulation of effector genes in a cell-/tissue-specific context required for tolerance of plants. A complex set of stress-responsive transcription factors is emerging. The imminent availability of genomic DNA sequences and global and cell-specific transcript expression data, combined with determinant identification based on gain- and loss-of-function molecular genetics, will provide the infrastructure for functional physiological dissection of salt tolerance determinants in an organismal context. Furthermore, protein interaction analysis and evaluation of allelism, additivity, and epistasis allow determination of ordered relationships between stress signaling components. Finally, genetic activation and suppression screens will lead inevitably to an understanding of the interrelationships of the multiple signaling systems that control stress-adaptive responses in plants.
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INTRODUCTION

It has been two decades since salinity stress biology and plant responses to high salinity have been discussed in this series (65, 80) and one decade since salinity tolerance in marine algae has been covered (129). Together with a monograph, still unsurpassed in its comprehensive discussion of the biophysical aspects of plant abiotic stresses (141), these reviews covered organismal, physiological, and the then-known biochemical hallmarks of stress and the bewildering complexity of plant stress responses. Much research information has subsequently been gathered about cellular, metabolic, molecular, and genetic processes associated with the response to salt stress, some of which presumably function to mediate tolerance (28–30, 36, 67, 78, 96, 105, 154, 189, 229, 240, 241, 285, 249). Our knowledge of how plants re-establish osmotic and ionic homeostasis after salt stress imposition, and then maintain physiological and biochemical steady states necessary for growth and completion of the life cycle in the new environment is fundamental to our understanding of tolerance (29, 78, 160, 182, 189, 285).

During the past decade, concerted experimental focus targeted the identification of cell-based mechanisms of ion and osmotic homeostasis as essential determinants of tolerance (37, 96, 255). Plant scientists now recognize that underlying cellular mechanisms of salt tolerance are evolutionarily more deeply rooted than previously
perceived. Consequently, research that has exploited the molecular genetic advantages of model unicellular organisms (e.g. bacteria, yeast, and algae) has been highly applicable to, and will continue to provide, greater understanding of higher plant salt tolerance (36, 48, 87, 181, 203, 230). However, it is obvious that plant cells become specialized during ontogeny. Cell differentiation and spatial location within plant tissues affect what adaptive mechanisms are most important to proper functioning of specific cells within the organism. In fact, integrative hierarchical functioning among cells, tissues, and organs, in a developmental context, certainly is an essential requisite for salt tolerance of the organism (29, 78, 96, 173, 230, 285). It is acknowledged that whole plant studies are as necessary as always, but will be more insightful, because the prevailing reductionist approaches of the past have provided mechanistic understanding that can be appreciated as the basis for integration of cellular level tolerance to the whole plant.

Algal genera such as *Dunaliella*, which includes many extreme halophytes (flora of saline environments), and higher plants in the halophyte category, for example, species of *Atriplex* and *Mesembryanthemum crystallinum* (2, 18, 19, 59, 89, 184, 263), have provided substantial insight into the response of halophytes to stress, which by comparison with that of non-halophytes (glycophytes) has led to greater comprehension of adaptive mechanisms. More recently, plant molecular genetic models, in particular *Arabidopsis thaliana*, have provided inroads through the investigative power and causal demonstration of gain- and loss-of-function molecular genetic experimentation to elucidate both cellular and organismal mechanisms of salt tolerance (145, 146, 279, 293, 295). We feel that the preceding 20 years of research, coupled with powerful new genetic tools, have brought the field to the brink of understanding the genetic mechanisms underlying the physiological and ecological complexity of salt tolerance. We summarize in this review salient research advances in the topic area since the previous reports in this series (65, 80, 129). We discuss briefly basic physiological stress responses and then outline how new molecular and genetic tools have begun to link and integrate the stress-responsive signaling cascades with the effectors (identified as physiological, biochemical and eventually genetic components) that mediate adaptive processes, indicating that signaling controls and mediates salt adaptation.

**SALINE STRESS AND PLANT RESPONSE**

**Salt Stress**

High salinity causes both hyperionic and hyperosmotic stress effects, and the consequence of these can be plant demise (78, 182, 285). Most commonly, the stress is caused by high Na\(^+\) and Cl\(^-\) concentrations in the soil solution. Altered water status most likely brings about initial growth reduction; however, the precise contribution of subsequent processes to inhibition of cell division and expansion and acceleration of cell death has not been well elucidated (174, 285). Membrane
disorganization, reactive oxygen species, metabolic toxicity, inhibition of photosynthesis, and attenuated nutrient acquisition are factors that initiate more catastrophic events (65, 80, 173, 285).

Salt Movement through Plants

Movement of salt into roots and to shoots is a product of the transpirational flux required to maintain the water status of the plant (66, 285). Unregulated, transpiration can result in toxic levels of ion accumulation in the aerial parts of the plant. An immediate response to salinity, which mitigates ion flux to the shoot, is stomatal closure. However, because of the water potential difference between the atmosphere and leaf cells, and the need for carbon fixation, this is an untenable long-term strategy of tolerance (173, 285).

To protect actively growing and metabolizing cells, plants regulate ion movement into tissues (66, 174). One mode by which plants control salt flux to the shoot is the entry of ions into the xylem stream. Still debated is the extent to which symplastic ion transport through the epidermal and cortical cells contributes to a reduction in Na\(^+\) that is delivered to the xylem (44, 66). However, at the endodermis, radial movement of solutes must be via a symplastic pathway, as the Casparian strip constitutes a physical barrier to apoplastic transport (44, 66).

The accumulation of large quantities of ions in mature and old leaves, which then dehisce, has often been observed under salt stress (66, 174). In a function as ion sinks, old leaves may restrict ion deposition into meristematic and actively growing and photosynthesizing cells. An alternative possibility is that cellular ion discrimination is a natural consequence of transpirational and expansive growth fluxes, cell morphology, and degree of intercellular connection. Meristematic cells, which are not directly connected to the vasculature, are less exposed to ions delivered through the transpiration stream, and their small vacuolar space is not conducive to ion storage. De facto, the solute content of tissues containing cells with little vacuolation (e.g. meristematic regions) is predominated by organic osmolytes and in tissues with highly vacuolated cells by ions (22, 280).

Lessons from Halophyte and Glycophyte Comparisons

Halophytes require for optimal growth electrolyte (typically Na\(^+\) and Cl\(^-\)) concentrations higher or much higher than those found in nonsaline soils. How and within which range of NaCl (roughly defined from 20 to 500 mM NaCl) these plants respond best is complex, and has led to a number of classification attempts (65, 78, 80). Halophytes seem to lack unique metabolic machinery that is insensitive to or activated by high Na\(^+\) and Cl\(^-\) (65, 181, 182, 214). Instead, plants ultimately survive and grow in saline environments because of osmotic adjustment through intracellular compartmentation that partitions toxic ions away from the cytoplasm through energy-dependent transport into the vacuole (10, 22, 78, 90, 182, 249, 285). Some halophytes exclude Na\(^+\) and Cl\(^-\) through glands and bladders, which are specialized structures that seem to be evolutionarily late inventions by which halophytes gain an edge over glycophytes. Osmotic adjustment of both
halophytes and glycophytes is also achieved through the accumulation of organic solutes in the cytosol, and the lumen, matrix, or stroma of organelles (Figures 1, 2; see color plates) (182, 213, 285).

A principal difference between halophytes and glycophytes is the capacity of the former to survive salt shock. This greater capacity allows haplophytes to more readily establish metabolic steady state for growth in a saline environment (33, 40, 49, 97, 184). Responsiveness to salinity and at least some ability to establish an adapted new steady state is not unique, however, to halophytes inasmuch as both glycophyte cells and plants exhibit substantial capacity for salt tolerance provided that stress imposition is gradual (8, 37, 96). The question then remains as to whether particular biochemical mechanisms of halophytes are better activated or are preactivated, allowing a more rapid and successful overall response to saline stress. Because of the diversity of halophyte species, there is no simple answer to this question, but unique adaptive responses to NaCl stress are not apparent in the overwhelming majority of halophytes (65, 78, 285).

Whereas glycophytes restrict ion movement to the shoot by attempting control of ion influx into root xylem, halophytes tend more readily to take up Na\(^+\) such that the roots typically have much lower NaCl concentrations than the rest of the plant (3, 37). It seems that a major advantage that halophytes have over glycophytes is not only more responsive Na\(^+\) partitioning but more effective capacity to coordinate this partitioning with processes controlling growth, and ion flux across the plasma membrane, in both cellular and organismal contexts. This may explain why halophytes can use, and perhaps rely on, Na\(^+\) and Cl\(^-\) for osmotic adjustment that then supports cell expansion in growing tissues and turgor in differentiated organs (3, 78, 80, 285). In a saline environment, the ability to take up and confine Na\(^+\) to leaves lowers the osmotic potential of aerial plant parts; this then facilitates water uptake and transport and lowers the metabolic cost for the production of osmolytes. Contrarily, the necessity for efficient vacuolar deposition of Na\(^+\) exacts a higher cost for H\(^+\) pumping, and possibly requires additional mechanisms for the acquisition of ion nutrients (principally K\(^+\)). Also, the osmotic benefits of storing Na\(^+\) and Cl\(^-\) as abundant, cheap osmolytes are limited by the available vacuolar space. Therefore, continued growth, i.e. the production of new vacuoles, may be a factor limiting tolerance (139).

**SALT STRESS TOLERANCE DETERMINANTS:**

**Effectors and Signaling Components**

We define determinants of salt stress tolerance as effector molecules (metabolites, proteins, or components of biochemical pathways) that lead to adaptation and as regulatory molecules (signal transduction pathway components) that control the amount and timing of these effector molecules. Stress adaptation effectors are categorized as those that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport, and transducers of long-distance response coordination (12, 131, 140, 170, 180, 182, 213, 265). Listed in Table 1 are
Figure 1  Cellular homeostasis established after salt (NaCl) adaptation. Indicated are the osmolytes and ions compartmentalized in the cytoplasm and vacuole, transport proteins responsible for Na and Cl homeostasis, water channels, and electrochemical potentials across the plasma membrane and tonoplast. Included are organelles (chloroplast (cp), mitochondrion (mt), and peroxisome (perox)) for which the importance of ROS scavenging is implicated.
Figure 2 Osmolytes/Osmoprotectants. Listed are common osmolytes involved in either osmotic adjustment or in the protection of structure. In all cases, protection has been shown to be associated with accumulation of these metabolites, either in naturally evolved systems or in transgenic plants (after 28; 189).
<table>
<thead>
<tr>
<th>Components</th>
<th>Suggested mechanisms and/or metabolic functions</th>
<th>Gene/Protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
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</tr>
<tr>
<td>LEA/dehydrins</td>
<td>Function unknown (protein stability); control of desiccation; preventing the generation of or detoxifying ROS;</td>
<td>HVA1, various</td>
<td>34, 253, 281</td>
</tr>
<tr>
<td>ROS scavenging</td>
<td>inhibiting OH⁻-production from Fenton-reaction; increases in ROS scavenging enzymes</td>
<td>classes Fe-SOD, Mn-SOD</td>
<td>31, 268, 276, 83, 217, 252</td>
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<td></td>
<td></td>
<td>GP, PHGPX, ASX, Catalase</td>
<td>12, 187, 239, 5, 122, 244</td>
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<td></td>
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<td>Gst/Gpx</td>
<td>81, 264</td>
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<tr>
<td>Chaperones</td>
<td>Heat-/cold-/salt-shock proteins; protein folding</td>
<td>Hsp, Csp, Ssp</td>
<td>88, 186</td>
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<td></td>
<td></td>
<td>DnaJ</td>
<td>296</td>
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<tr>
<td><strong>Carbohydrates</strong></td>
<td><strong>Osmolytes and/or compatible solute</strong></td>
<td></td>
<td></td>
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<tr>
<td>Polyols</td>
<td>Inhibiting OH⁻-production from Fenton-reaction; osmotic adjustment; redox control</td>
<td>Mtl, Int1, Stldh</td>
<td>257, 258, 272, 232, 233, 234, 236, 237, 238</td>
</tr>
<tr>
<td>Fructan (levansucrase)</td>
<td>Osmoprotection</td>
<td>SacB</td>
<td>200</td>
</tr>
<tr>
<td>Trehalose</td>
<td>Osmoprotection; (signaling?)</td>
<td>Tps; Tpp, trehalase</td>
<td>79, 106</td>
</tr>
<tr>
<td><strong>Quaternary N-compounds</strong></td>
<td><strong>Osmoprotection</strong></td>
<td></td>
<td></td>
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<tr>
<td>Glycine betaine</td>
<td>Protein protection, one-carbon sink</td>
<td>codA</td>
<td>98, 161</td>
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<tr>
<td>Dimethyl sulfonium compounds</td>
<td></td>
<td>BADH</td>
<td>194, 209</td>
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<td></td>
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<td>CMO</td>
<td>4, 109, 219</td>
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<td>73, 212, 216</td>
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<td></td>
<td></td>
<td>189</td>
</tr>
<tr>
<td>Amino acid/derivatives</td>
<td>Osmotic adjustment (&amp; possibly other functions)</td>
<td>(\text{Proline} )</td>
<td></td>
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<tr>
<td></td>
<td>Substrate for mitochondrial respiration; redox control; nitrogen balance/storage, transport</td>
<td>(\text{P5CS/P5CR} ) (125, 220)</td>
<td></td>
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<tr>
<td>Ectoine</td>
<td>Osmoprotectant, (signaling?)</td>
<td>(\text{EctA,B,C (operon)} ) (149, 190)</td>
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<tr>
<th>Inadvertent (\text{Na}^+) uptake</th>
<th>Control over potassium uptake</th>
<th>(\text{Hkt1, Hak1} ) (182, 218)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{K}^+)-transporters</td>
<td>High affinity (\text{K}^+) uptake; possibly significant contribution to sodium uptake</td>
<td>(\text{182, 218, 222})</td>
</tr>
<tr>
<td>(\text{K}^+)-channels</td>
<td>Low affinity or dual affinity (\text{K}^+) uptake; minimal contribution to sodium uptake</td>
<td>(\text{9, 228, 247})</td>
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<td></td>
<td>Sodium stimulation of potassium uptake</td>
<td>(\text{182, 218, 222})</td>
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<tr>
<th>Sodium partitioning</th>
<th>Tissue/cell-specific deposition of sodium</th>
<th>(\text{Na}^+/\text{H}^+) antiporters</th>
<th>(\text{3, 76, 10, 72, 148, 184, 274, 18, 263})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Na}^+/\text{H}^+) transport</td>
<td>Vaccum storage of (\text{Na}^+) as an osmoticum and/or plasma membrane (\text{Na}^+) exclusion/export</td>
<td>(\text{Hkt1, Hak1} ) (182, 218)</td>
<td></td>
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<tr>
<td>or antiport</td>
<td>Establishing proton gradients</td>
<td>(\text{Hkt1, Hak1} ) (182, 218)</td>
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</table>

| \(\text{Na}^+/\text{myo-inositol} \) transport | Long-distance phloem & xylem transport | \(\text{ITR-family} \) \(\text{Chauhan et al, unpublished, 180}\) |

<table>
<thead>
<tr>
<th>Water relations</th>
<th>Control over water flux into and out of cells</th>
<th>(\gamma)-TIP</th>
<th>(\text{116, 158, 169, 282})</th>
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<tbody>
<tr>
<td>Water channel proteins</td>
<td>Membrane cycling controlling presence &amp; amount; posttranslational modifications</td>
<td>(\gamma)-TIP</td>
<td>(\text{116, 158, 169, 282})</td>
</tr>
<tr>
<td>(\text{AQP, MIP)})</td>
<td>Controlling transcript amounts; expected functions in tolerance for homologues that transported other small metabolites (glycerol, urea, polyols)</td>
<td>(\text{RD28} ) (\text{159, 265, 283})</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>(\text{SITIP, SIMIP} ) (\text{131, 199})</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\text{Fps1p (yeast)} ) (\text{256})</td>
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stress-regulated genes that encode stress-tolerance effectors. Regulatory molecules are cellular signal pathway components (Figure 3, see color plates), including transcription factors, that regulate salt tolerance effectors (241).

FUNCTIONAL CATEGORIES OF SALT TOLERANCE EFFECTOR DETERMINANTS

Ion Homeostasis

A hypersaline environment, most commonly mediated by high NaCl, results in perturbation of ionic steady state not only for Na\(^+\) and Cl\(^-\) but also for K\(^+\) and Ca\(^{2+}\) (182). External Na\(^+\) negatively impacts intracellular K\(^+\) influx, attenuating acquisition of this essential nutrient by cells. High NaCl causes cytosolic accumulation of Ca\(^{2+}\) and this, apparently, signals stress responses that are either adaptive or pathological. Ion homeostasis in saline environments is dependent on transmembrane transport proteins (Figure 1) that mediate ion fluxes, including H\(^+\) translocating ATPases and pyrophosphatases, Ca\(^{2+}\)-ATPases, secondary active transporters, and channels (23, 26, 182, 254). A role for ATP-binding cassette (ABC) transporters (210) in plant salt tolerance has not been elucidated, but ABC transporters regulate cation homeostasis in yeast (166).

The molecular identity of many transport proteins that putatively mediate Na\(^+\), K\(^+\), Ca\(^{2+}\), and Cl\(^-\) transport has been determined. Most of these transport proteins were identified from structure/function information or by functional complementation of transport-deficient yeast mutants (55). It is now clear that many of the transport determinants that mediate ion homeostasis in yeast and plants are very similar (229). Furthermore, it is likely that the rudiments of salt-responsive signal regulatory pathways controlling ion homeostasis in both organisms are analogous (36, 229). The evolutionary conservation of essential ion transport function and the complete compilation of plant genome databases that will be available shortly make it likely that the as-yet unidentified transport proteins involved in Na\(^+\), K\(^+\), Ca\(^{2+}\), and Cl\(^-\) homeostasis will emerge shortly. Less well understood is the physiological function of these transport systems during stress adaptation and after steady state is re-established, but again comparison with the yeast model provides a reasonable paradigm. The physiological function of some transport proteins has been confirmed recently by experimentation with plant mutants (77, 102, 288).

Plasma Membrane and Ionoplast H\(^+\) Electrochemical Gradients

Secondary active transport and electrophoretic flux across the plasma membrane and tonoplast are driven by the H\(^+\)-electrochemical potential gradients established by H\(^+\) pumps (150, 193, 254, 291). The thermodynamic steady-state conditions facilitated by the plasma membrane (H\(^+\)-ATPase) and tonoplast (H\(^+\)-ATPase and H\(^+\)-pyrophosphatase) H\(^+\) pumps in nonsaline environments are assumed to be established after plants adapt to salinity and re-establish ion homeostasis in a saline environment (Figure 1) (25, 182). The H\(^+\) electrochemical potential gradients
Figure 3 Osmotic stress regulatory molecules in plants compared to categories of yeast signal components. Vertical columns indicate type of evidence for participation of gene in plant stress signaling. Horizontal rows indicate category of signal component organized according to the yeast model hierarchy. Superscripts indicate component subtypes and appropriate references. * denotes yeast gene. For all yeast signal genes see Reference (87).
established could facilitate between $10^2$- to $10^3$-fold lower concentrations of $\text{Na}^+$ and $\text{Cl}^-$ in the cytosol relative to the apoplast or the vacuole, assuming transport proteins are present in nonlimiting amounts. However, cytosolic $\text{Na}^+$ and $\text{Cl}^-$ concentrations of cells growing in salt levels near that of seawater have been measured to be about 80 to 100 mM (22, 182). Consequently, even in seawater concentrations of $\text{Na}^+$ and $\text{Cl}^-$, energy-dependent transport of these ions into the apoplast and vacuole (so that toxic levels do not accumulate in the cytosol) can be facilitated by the capacity of the $H^+$ electrochemical potential gradients established across the plasma membrane and tonoplast. In high NaCl environments, $K^+$ remains at about 80 mM in the cytosol (22).

The plasma membrane $H^+$-ATPase is encoded by a multigene family and expression of isogenes is regulated specifically in spatial and temporal contexts as well as by chemical and environmental inducers, including salt (183, 184, 254). Furthermore, increased ATPase-mediated $H^+$ translocation across the plasma membrane is a component of the plant cell response to salt imposition (33, 273). Both an ATPase (V-type) and a pyrophosphatase are responsible for $H^+$-translocation into the vacuole and generation of $H^+$ motive force across the tonoplast (150, 254, 291). Salt treatment induces ATPase activity and $H^+$ transport of V-type pumps (23, 207, 269). This increased activity has been attributed to greater protein abundance (206), changes in kinetic properties (211), differential subunit composition (23), and transcriptional regulation (23, 25, 178). The pyrophosphatase can contribute substantially to $H^+$ transport into the vacuole. However, the relative contribution of the ATPase and pyrophosphatase to the $H^+$ electrochemical gradient across the tonoplast during salt stress or growth in saline environments is not clear (291). The pyrophosphatase has physiological roles in maintaining cytosolic pH status and turnover of pyrophosphate (291). Evidence indicates that salt treatment both up-regulates and down-regulates pyrophosphatase activity (23).

$\text{Na}^+$ and $\text{Cl}^-$ Transport Across the Plasma Membrane  
$\text{Na}^+$ and $\text{Cl}^-$ transport across the plasma membrane in a hypersaline environment must be considered in two cellular contexts, after salt stress shock and after re-establishment of ionic homeostasis. Immediately after salt stress, the $H^+$ electrochemical gradient is altered. Influx of $\text{Na}^+$ dissipates the membrane potential, thereby facilitating uptake of $\text{Cl}^-$ down the chemical gradient. An anion channel has been implicated in this passive flux (50, 100, 242). However, after steady-state conditions are re-established, including establishment of an inside negative plasma membrane potential of $-120$ to $-200$ mV, $\text{Cl}^-$ influx likely requires coupling to downhill $H^+$ translocation, presumably via a $\text{Cl}^--H^+$ symporter of unknown stoichiometry (201).

The precise transport system responsible for $\text{Na}^+$ uptake into the cell is still unknown. Physiological data indicate that $\text{Na}^+$ competes with $K^+$ for intracellular influx because these cations are transported by common proteins (Figure 1) (7, 26, 182). Whereas $K^+$ is an essential co-factor for many enzymes, $\text{Na}^+$ is not. The need for $\text{Na}^+$ as a vacuolar osmolyte in saline environments may be the
reason why plants have not evolved transport systems that completely exclude Na\(^+\) relative to K\(^+\). K\(^+\) and Na\(^+\) influx can be differentiated physiologically into two principal categories, one with high affinity for K\(^+\) over Na\(^+\) and the other for which there is lower K\(^+\)/Na\(^+\) selectivity. Many K\(^+\) transport systems have some affinity for Na\(^+\) (26, 43, 223). These include inward rectifying K\(^+\) channels (9, 288); Na\(^+\)-K\(^+\) symporter (224); K\(^+\) transporters (71, 128, 222); voltage-dependent, nonselective, outward-rectifying cation channel that mediates Na\(^+\) influx upon plasma membrane depolarization (26, 225); and voltage-independent cation channels (7, 26, 275). The size of the Na\(^+\) electrochemical potential gradient across the plasma membrane, resulting from the large inside negative membrane potential and the Na\(^+\) chemical potential, implicates electrophoretic flux as the principal mode of intracellular Na\(^+\) influx. Low-affinity K\(^+\) transport has been ascribed to inward K\(^+\) channels (152). However, at least two inward K\(^+\) channels mediate high-affinity uptake of K\(^+\) (9, 102). The Na\(^+\)-K\(^+\) transporter and K\(^+\) transporters, with dual high and low affinity, may contribute substantially to Na\(^+\) influx. Regardless of the transport systems involved in K\(^+\) and Na\(^+\) acquisition, it is clear that a single genetic locus (SOS3), which encodes a signal transduction intermediate, modulates high- and low-affinity K\(^+\) uptake (145, 146). Ca\(^{2+}\) can facilitate higher K\(^+\)/Na\(^+\) selectivity, and interestingly, external Ca\(^{2+}\) can suppress the K\(^+\) acquisition deficiency of sos3. The transport systems involved in K\(^+\) acquisition by roots and loading to the xylem have been summarized recently (152, 153).

Na\(^+\) efflux across the plasma membrane and compartmentalization into vacuoles or pre-vacuoles is mediated presumably by Na\(^+\)/H\(^+\) antiporters, regardless of whether or not the membrane potential is inside positive or negative. Although Na\(^+\)-ATPase activity has been described for some algae, there is no evidence that such a pump exists in cells of higher plants (26, 182). To date, a plasma membrane antiporter is implicated from physiological data obtained with isolated membrane vesicles (26, 57, 97). However, since so many plant transport systems are analogous to yeast, it is likely that NHA1/SOD2 orthologs will be identified that are responsible for Na\(^+\) efflux across the plasma membrane (115, 204).

**Na\(^+\) and Cl\(^-\) Vacuolar Compartmentation**  Compartmentation analyses indicate that in seawater concentrations of NaCl, both Na\(^+\) and Cl\(^-\) are sequestered in the vacuole of plant cells and represent the primary solutes affecting osmotic adjustment in this compartment (22, 90, 249). Na\(^+\) compartmentation in the vacuole requires energy-dependent transport, and an immediate effect of NaCl treatment is vacuolar alkalization (27, 82, 156). Na\(^+\)/H\(^+\) antiporter activity has been associated with tonoplast vesicles (27, 57), and this is presumed to be at least partially responsible for the alkalization. Recently, plant cDNAs encoding NHE-like proteins were isolated that can functionally complement a yeast mutant deficient for the endomembrane Na\(^+\)/H\(^+\) transporter, NHX1 (10, 72, 76; FJ Quintero, MR Blatt & JM Pardo, unpublished). Overexpression of an NHE-like antiporter substantially enhanced salt tolerance of Arabidopsis, confirming the function of the transporter in Na\(^+\) compartmentation (10). The tonoplast Cl\(^-\) transport
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Determinants are predicted to be a channel or a carrier that couples Cl\(^-\) influx to the H\(^+\) gradient. A +50 mV (inside positive) tonoplast membrane potential would be sufficient to facilitate an almost tenfold concentration of Cl\(^-\) in the vacuole based on electrophoretic flux through an anion permeable channel (16, 50, 99). Secondary active transport (H\(^+\)/anion antiporter) has been proposed also (254).

**Ca\(^{2+}\) Homeostasis** Experimental evidence implicates Ca\(^{2+}\) function in salt adaptation. Externally supplied Ca\(^{2+}\) reduces the toxic effects of NaCl, presumably by facilitating higher K\(^+\)/Na\(^+\) selectivity (46, 137, 145). High salinity also results in increased cytosolic Ca\(^{2+}\) that is transported from the apoplast and intracellular compartments (132, 151). The resultant transient Ca\(^{2+}\) increase potentiates stress signal transduction and leads to salt adaptation (132, 163, 197, 221, 277). A prolonged elevated Ca\(^{2+}\) level may, however, also pose a stress; if so, re-establishment of Ca\(^{2+}\) homeostasis is a requisite. Ca\(^{2+}\) transport systems have been summarized in recent reviews (192, 218).

**Endocytosis and Prevacuolar Trafficking** The extent to which endocytotic or prevacuolar compartments contribute to intracellular ion compartmentation in plants is yet to be assessed (157). The prevacuole compartments may be sorted for exocytosis or fusion with the tonoplast, or retained in the cells without fusion to the central vacuole (70, 142). In fact, a dramatic characteristic of NaCl-adapted tobacco cells is the presence of numerous small vacuoles (prevacuoles), and transvacuolar strands (22, 24, 41). This and other cytological changes of adapted cells are characteristic of meristematic cells (60), and indicative that a form of developmental arrest, perhaps as a result of altered cell cycle progression or cell division to expansion transition, occurs during salt adaptation (24, 41). Note also that cytological characteristics of plasmolyzed root cells of the halophyte *Atriplex nummularia* include plasma membrane invaginations, vesiculation, Hechtian strands, and numerous small vacuoles (prevacuoles) (183). Frommer et al (70) have suggested that *ArNHX1* (encodes a tonoplast Na\(^+\)/H\(^+\) antiporter) overexpression in transgenic plants may affect salt tolerance (10) by disrupting the trafficking of the Na\(^+\)/H\(^+\) antiporter such that it is localized not only to prevacuoles but also to the tonoplast or plasma membrane, thereby increasing the capacity for compartmentation of Na\(^+\) away from the cytosol.

**Osmolyte Biosynthesis**

One response, which is probably universal, to changes in the external osmotic potential is the accumulation of metabolites that act as “compatible” solutes, i.e. they do not inhibit normal metabolic reactions (38, 68, 284). With accumulation proportional to the change of external osmolarity within species-specific limits, protection of structures and osmotic balance supporting continued water influx (or reduced efflux) are accepted functions of osmolytes. Frequently observed metabolites with an osmolyte function are sugars (mainly sucrose and fructose),
sugar alcohols (glycerol, methylated inositols), and complex sugars (trehalose, raffinose, fructans). In addition, ions (K⁺) or charged metabolites [glycine betaine, dimethyl sulfonium propionate (DMSP), proline and ectoine (1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrimidine)] are encountered (Figure 2). The accumulation of these osmolytes is believed to facilitate “osmotic adjustment,” by which the internal osmotic potential is lowered and may then contribute to tolerance (52, 149, 160). Compatible solutes are typically hydrophilic, which suggests they could replace water at the surface of proteins, protein complexes, or membranes, thus acting as osmoprotectants and nonenzymatically as low-molecular-weight chaperones.

This biophysical view of the function(s) of such solutes is supported by many studies [for reviews see (30, 181, 285)]. Compatible solutes at high concentrations can reduce inhibitory effects of ions on enzyme activity (39, 245) to increase thermal stability of enzymes (74), and to prevent dissociation of enzyme complexes, for example, the oxygen-evolving complex of photosystem II (194). One argument raised against such studies is the high effective concentration necessary for protection in vitro, which is usually not matched in vivo. Considering the cellular concentration of proteins, protection may be achieved at lower solute concentrations found in vivo. It may not be the concentration in solution that is important. Glycine betaine, for example, protects thylakoids and plasma membranes against freezing damage or heat destabilization even at low concentration (118, 290). This indicates that the local concentration at a surface may be more important than the absolute amount.

**Metabolic Pathways** Typically, pathways leading to osmolyte synthesis are connected to pathways in basic metabolism that show high flux rates (28, 160, 189). Examples are the biosynthetic pathways leading to proline (52), glycine betaine (160, 208), D-pinitol (108, 272), or ectoine (75). The pathways from which these osmolytes originate are situated in amino acid biosynthesis from glutamic acid (proline) or aspartate (ectoine), choline metabolism (glycine betaine), and myo-inositol synthesis (pinitol). The enzymes required for pathway extensions that lead to these osmolytes are often induced following stress. In higher plants this has been documented for glycine betaine (93), D-pinitol (108, 180, 272), proline (177, 287, 289), and the operon, encoding three enzymes, required for ectoine accumulation is also stress induced (149, 190). Ectoine may be restricted to bacteria; it has not yet been detected in plants, but transgenic expression of the three genes from *Halomonas elongata* and *Marinococcus halophilus* in tobacco, leading to ectoine amounts in the micromolar range, provided some protection (176; M Rai, G Zhu, N Jacobsen, A Somogyi & HJ Bohnert, unpublished). DMSP synthesis, found in Gramineae and Compositae and in many algae, is increased under conditions of salinity (262). The metabolic requirements for the synthesis of DMSP and the related glycine betaine are providing a paradigm to engineer metabolic pathways for osmotic stress tolerance (189).

**Osmolyte Functions** Irrespective of the seemingly clear-cut importance of osmolytes, what the various accumulators might signify remains a matter of debate.
The terms osmoprotection and osmotic adjustment focus on biophysical and physiological characters. Following this view, the multiplicity of accumulating metabolites may be interpreted to reflect the relative enzymatic capacities of various biochemical pathways in different species. In reviewing physiological studies, Greenway & Munns (80) come down on the side of pathology, or favor a view of osmolytes as sinks of reducing power following metabolic disturbance that might be mobilized as a source of carbon and nitrogen once stress is relieved. More recently, new arguments interpreting biochemical and molecular analyses, based on mutant analysis and transgenic approaches, have been introduced into the discussion (29, 30, 95, 189, 294). Plants engineered to synthesize and moderately accumulate a number of osmolytes showed marginally improved performance under abiotic stress conditions. The effects seen with modest increases in mannitol, fructans, trehalose, ononitol, glycine betaine, or ectoine, and with strong increases in proline amount indicate that the purely osmotic contribution of these metabolites to stress tolerance may not describe their function completely, i.e. that the pathway leading to a particular osmolyte may be more important than accumulation per se (30, 95, 112, 181).

Proline accumulation provides an example. Under stress, the imbalance between photosynthetic light capture and NADPH utilization in carbon fixation may alter the redox state and lead to photoinhibition. Proline synthesis, following transcriptional activation of the NADPH-dependent P5C-synthetase (P5CS), could provide a protective valve whereby the regeneration of NADP$^+$ could provide the observed protective effect (52, 95, 125, 271, 286). An argument against accumulation per se can be seen from the analysis of the sos1 mutant of Arabidopsis, deficient in K$^+$ uptake in which proline accumulated to levels twofold higher than in wild type under moderate salt stress but the plants were not tolerant (53, 145). The regulated synthesis of both proline synthesis and degradation enzymes indicate that cycling between precursor and product may be important (130, 175, 270). Supportive evidence for a nonosmotic function of at least some osmolytes comes from transgenic overexertion of osmolyte-producing enzymes (233, 234) with moderate accumulation of osmolytes leading to the protection of the carbon reduction cycle by reducing the production of hydroxyl radicals generated by a Fenton reaction. There may be more than one function for a particular osmolyte and, based on results from in vitro experiments (92, 192), different compatible solutes could have different functions. Among these functions a role in prevention of oxygen radical production or in the scavenging of reactive oxygen species (ROS) may be paramount (12, 185, 187).

Transgenic plants have been generated to probe the effect of ROS scavenging on salinity stress tolerance, based on observations of gene expression changes in stressed plants. A putative phospholipid hydroperoxide glutathione peroxidase, PHGPX, transcript increased during salt stress in Arabidopsis and citrus (83, 252) and, also in citrus, transcripts and enzyme activities of Cu/Zn-SOD, glutathione peroxidase, and a cytosolic APX (83, 104). Catalase-deficient (antisense) tobacco showed enhanced sensitivity to oxidative stress under conditions of high light and salinity (276).
ROS scavenging as an important component of abiotic stress responses is documented by mutant analysis. The ascorbic acid-deficient Arabidopsis semidominant, soz1 accumulates only 30% of ascorbate compared with wild type, and plants show significantly higher sensitivity to oxidative stress conditions (45). Further support comes from the study of transgenic models, which have been generated to study antioxidant defenses (5, 47, 187, 191, 244). Overexpression of genes leading to increased amounts and activities of mitochondrial Mn-SOD, Fe-SOD, chloroplastic Cu/Zn-SOD, bacterial catalase, and glutathione S-transferase/glutathione peroxidase can increase the performance of plants under stress (31, 84, 85, 217, 239, 268).

Water Uptake and Transport

A few studies are pertinent to water movement under salinity stress conditions. For maize roots (but not for tobacco) high salinity caused a considerable reduction in water permeability in the cortex (13, 266), reducing the osmotic water permeability by as much as fivefold. Changes in the osmotic water permeability were reflected in changes in root hydraulic conductivity due to the fact that most of the water was flowing around cells (14). Conceivably, such changes may be caused by reducing the probability of opening of water channels or by a change in their number. Species- and stress-specific changes in water permeability may be caused by AQP phosphorylation, as demonstrated by Johansson et al (116, 117). Low water potential reduces phosphorylation of the plasma membrane PM28A in spinach. Phosphorylation is carried out by a Ca\(^{2+}\)-dependent membrane-bound protein kinase (116). PM28A, expressed in Xenopus oocytes, is phosphorylated at the site phosphorylated in vivo, and decreased phosphorylation reduced water permeability in oocytes expressing PM28A (117), which suggests that PM28A could be involved in water flow through leaf tissue. Phosphorylation has also been reported for a seed-specific TIP (158).

The control over plant AQP amount or activity would be particularly important during stress, and evidence for mobility under stress conditions is mounting (17, 269). The amount of MIP transcripts (282) and proteins (Kirch et al, unpublished) varies during salt stress in M. crystallinum, as do protein locations (269). Drought and salt stresses regulate protein amounts for the location of AQPs in the tonoplast, internal vesicles, and plasma membrane differently, indicating the existence of signaling pathways that exert control over water flux. It is intuitively clear that water channels (or water/solute channels, “aquaglyceroporins”) should have significance for water relations in stressed plants, but the dataset available on the control of water uptake in salt-stressed roots is small. Given the wealth of information from the study of AQPs in the last few years, more surprises can be expected (226).

Long-Distance Response Coordination

Although intracellular phenomena of the salt stress response are well described, much less is known about the organismal response coordination in different organs
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during development in a changing environment and even less is known about sensing mechanisms at membranes. Communication along the plant body has been revealed in the induction of flowering in the systemic signaling of pathogen attack (6, 20) or upon the disturbance, due to osmolyte changes, of source-sink relationships (215). In this context, the interaction and crosstalk between carbohydrate status, involving glucose sensing by hexokinases, and ethylene-based signal transduction (135, 292) could be a paradigm for understanding plant abiotic stress responses. Long-distance signaling under stress has been associated with root to shoot ABA transport (140, 259), possibly also in cytokinin/auxin transport, or through ethylene (58, 94, 188, 260), and by the coordinated interplay of different growth regulators (127, 170). However, more studies are needed. Although the signals appear to be predominantly chemical (132), action potentials derived from local ion movements could also be involved (32).

The evidence for salt stress-specific ABA transport stems from physiology (171, 172, 243, 259) and, more recently, from mutant analysis in Arabidopsis (64). Auxin transport, cytokinin conjugates to inositols, glucose, or amino acids, have mainly been viewed under aspects of development and tropisms (61, 120). The analysis of mutants with altered ABA responses indicated regulatory roles for the loci ABA1 (ABA biosynthetic pathway), ABI1 (calcium-modulated protein phosphatase 2C), and AXR2 (resistance to auxin and ethylene) in the differential expression of P5CS genes responsible for proline accumulation under stress, suggesting signals arriving from outside the responding cells (251). A similar conclusion was reached in the study of a nonpermeable ABA-BSA conjugate, which elicited intracellular gene expression and activated K+ currents (114). Likewise, evidence has been presented for the movement of glutathione and its conjugates and other redox carriers or oxidants into the apoplast (21, 56, 69). It seems that within the signaling cascades described by the classical plant growth regulators, a variety of other compounds will have to be included, for example, sugars and maybe other osmolytes and extracellular enzymes such as invertases, oxalate oxidases, glutathione S-transferases, and oxidoreductases. These generate a variety of chemical signals that may converge on the production of radical oxygen species, and transmembrane signal mediators, sensitive to redox states, could transfer excitation to the cell’s interior eliciting stress responses.

Recently, evidence for a metabolic connection between leaf photosynthetic capacity and root Na+ uptake has been obtained. Salt stress-dependent increases in the transport of myo-inositol through the phloem from leaves to the root system could be correlated with increased Na+ transport in the xylem (179, 180). A hypothetical connection between myo-inositol as an indicator of photosynthetic activity in leaves and increased Na+ transport is provided by the detection of Na+/myo-inositol symporter proteins, ITRs. Salt stress-inducible ITR mRNAs are located to the vascular system of the root and stem (S Chauhan, F Quigley, DE Nelson, Y Ran, HJ Bohnert, unpublished). Increased amounts of inositols may, however, have another function. IAA promotes rapid changes in phosphatidylinositol (PI) metabolites (62), and increased concentrations of inositols could enhanced PI metabolism. In yeast, the pathway leading to PI-based secondary messengers is activated by osmotic
stress with a crucial role for phosphatidylinositol-3P 5-hydroxyl kinase leading to PI-(3,5)-diphosphate (54). Altered PI signaling is indicated by the ABA- and osmotic stress-mediated induction of a PI-4-phosphate 5-kinase in Arabidopsis (165).

REGULATORY MOLECULES

We have conceptually divided genetic determinants of salt tolerance into two classes: encoding effectors responsible for remodeling the plant during adaptation, and regulatory genes that control the expression and activity of the effectors. Rapid technical developments and insightful use of yeast as a model organism have greatly facilitated our discovery of these regulator genes (155, 230, 246). Plant growth regulator/hormone physiological function(s) in plant stress responses is not surveyed in this report, as relevant and recent treatises are available (51, 127, 169, 170). Instead, plant growth regulator function is described in the context of stress regulatory determinants that have been linked to osmotic tolerance. We now summarize and provide a framework for the organization of these plant regulatory genes, comparing them to the yeast model (Figure 3).

Plant Signaling Genes as Determinants of Salt Tolerance
Based on Functional Complementation of Yeast Mutants

Determinants of plant stress tolerance have been identified, in recent years, by functional complementation of osmotic, sensitive yeast mutants. A putative MAP (mitogen-activated protein) kinase (MAPK) has been identified from Pisum sativum (PsMAPK), with 47% primary sequence identity to Hog1p, which is the MAP kinase in the yeast osmoregulatory pathway that controls glycerol accumulation. PsMAPK functionally suppressed salt-induced cell growth inhibition of hog1 (202). Combinations of Arabidopsis proteins ATMEKK1 (MAPKKK) and MEK1 (MAPKK), or ATMEKK1 and ATMKK2 (MAPKK) suppressed growth defects of pbs2Δ (wild-type allele encodes the MAPKK of the HOG pathway), implicating these as functional components of an osmotic stress MAP kinase cascade (107).

AtDBF2 kinase of Arabidopsis mediated functional sufficiency of yeast cells for LiCl tolerance (138). Overexpression of this serine/threonine kinase enhanced tolerance of plant cells to numerous stresses, including salt and drought. By analogy with yeast, it is presumed that At-DBF2 is a cell cycle–regulated protein kinase that is a component of a general transcriptional regulatory complex, like CCR4 (144), that modulates expression of genes involved in osmotic adaptation (138). AtGSK1 was isolated as a suppressor of the NaCl-sensitive phenotype of a calcineurin-deficient mutant (198). Calcineurin is a protein phosphatase type 2B that is a pivotal intermediate in a signal pathway that regulates ion homeostasis and salt tolerance of yeast (162, 163). AtGSK1 has sequence similarity to the glycogen synthase kinase 3 (GSK3) of mammalian cells and SHAGGY of Drosophila melanogaster. AtGSK1 expression induced transcription of yeast ENA1, which encodes a P-type ATPase responsible for Na⁺/Li⁺ efflux across the plasma membrane. Furthermore,
AtGSK1 suppressed the NaCl-sensitive phenotype of a mutant (mck1) deficient for a yeast GSK3 ortholog, implicating that functional complementation is based on kinase activity.

*Arabidopsis* *SAL1* was isolated by complementation of a mutant defective for ENA ATPase activity (205). A family of four genes (*ENA1–4*) that are tandemly arranged in the genome, encodes ENA. *SAL1* complemented the Li⁺-sensitive phenotype of a *ena1-4Δ* strain. *SAL1* is the plant ortholog of yeast HAL2 and encodes a protein with (2′), 5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. The nucleotidase activity converts adenosine 3′-phosphate 5′-phosphosulfate to 3′(2′)-phosphoadenosine 5′-phosphate and this catalysis functions as part of a sulfur cycle to reduce SO₄²⁻ levels in high Na⁺ environments (196). The polyphosphate phosphatase activity is presumed to have a significant function in phosphoinositide signaling (205).

Two *Arabidopsis* transcription factor-encoding cDNAs *STO* (salt tolerance) and *STZ* (salt tolerance zinc finger) suppressed the Na⁺/Li⁺-sensitive phenotype of a calcineurin-deficient (*cnb1-2Δ* or *cnbΔ*) mutant (143). *STO* is similar to *Arabidopsis* *CONSTANS* and is predicted to be a member of a multigene family. *STZ*-mediated salt tolerance is at least partially dependent on *ENA1*, implying a function that involves regulation of the Na⁺-ATPase, whereas *STO* function is independent of *ENA1*.

*NtSLT1* is a tobacco protein that suppresses the Na⁺/Li⁺-sensitive phenotype of a calcineurin-deficient (*cnbΔ*) mutant (TK Matsumoto, JM Pardo, S Takeda, I Amaya, K Cheah, et al, unpublished). Functional complementation of *cnbΔ* by *NtSLT1*, and by the *Arabidopsis* ortholog AtSLT1, can be mediated only by a peptide that is truncated at the N terminus, indicating the presence of an autoinhibitory domain on the native protein. Complementation involves transcriptional activation of *ENA1* as well as a function(s) independent of the ATPase.

**Plant Signaling Factors that Enhance Salt Tolerance of Plants**

Numerous signal or signal-like molecules have now been identified and presumed, through some evidence, to function in plants as mediators of osmotic adaptation (Figure 3). Some of these affect osmotic tolerance in genetically modified plants (Figure 3). Transcriptional modulation has always been predicted to play a major role in the control of plant responses to salt stress (240, 241, 294). Transcription factors have been identified based on interaction with promoters of osmotic/salt stress–responsive genes. These factors participate in the activation of stress-inducible genes, and presumably lead to osmotic adaptation (1, 147, 231, 240, 241, 278). Since the promoters that are controlled by these transcription factors are responsive to several environmental signals, it is not clear which transcription factors, if any, function only in salt stress responses, or if salt-specific transcriptional regulation alone is a requisite component of salt tolerance in plants. ABA-deficient and -insensitive mutants have been used to delineate transcription factors as components of osmotic stress signal transduction pathways that either involve or are independent of the growth regulator (241). Promoters of ABA-dependent osmotic
stress-responsive genes include regulatory elements that interact with basic leucine-zipper motif (bZIP), MYB, or MYC domains in DNA binding proteins (1, 235, 241). The bZIP transcription factors interact with the ABA-responsive element (ABRE) (241). The promoter of the osmotic/salt stress-responsive rd22 gene contains signature recognition motifs for MYC and MYB transcription factors. DNA binding proteins, rd22BP1 (MYC) and Atmyb2 (MYB), interact with their corresponding cis-elements and trans-activate rd22. However, the rd22 promoter does not contain ABRE motifs, which indicates that ABA dependency is mediated through another process. At least some ABA-dependent transcriptional activation appears to involve Ca\(^{2+}\)-dependent protein kinases (CDPKs) (231). Transcription factors thought to function in osmotic/salt stress gene induction, independent of ABA, include dehydration response element (DRE) binding proteins (147, 248). Two gene families have been characterized, DREB1 (which includes the previously identified CBF1), and DREB2 (which trans-activates the osmotic/salt-responsive rd29A). Both DREB1 and DREB2 family members also have domains that bind ethylene-responsive elements (147, 248).

DREB1A overexpressing transgenic plants exhibited constitutive activation of stress-responsive genes and enhanced freezing, dehydration, and salt tolerance (123, 147). Driving DREB1A with the stress-inducible rd29A promoter substantially increases salt tolerance, with minimal adverse growth effects in the absence of stress (123). Ectopic overexpression of CBF1/DREB1B in transgenic plants induces cold-responsive genes and enhances freezing tolerance (111, 261). Overexpression of the zinc finger transcription factor ALFIN1 activates MsPRP2 (NaCl-responsive gene) expression and increases salt tolerance of alfalfa (278).

In addition to transcription factors, some other signaling components affect a salt-tolerance phenotype in plants (Figure 3). Notable among these is the SOS3 gene that encodes a Ca\(^{2+}\) binding CNB-like protein. Mutation of SOS3 produces salt-sensitive plants with altered K\(^+\) transport characteristics (145, 146). Another CNB-like protein AtCLB1 is able to suppress salt sensitivity in CNB yeast mutants, but only in the presence of a mammalian CNA subunit (134). The yeast CNA/CNB genes are also able to confer salinity tolerance when overexpressed in transgenic plants (195), further implicating these regulatory molecules in plant stress signaling. It seems clear that a Ca\(^{2+}\) binding, CNB-like molecule plays an important role in osmotic stress responses in plants. Mutation of the ABI1 gene that renders plants insensitive to ABA also causes an osmotic stress-sensitive phenotype (11, 63, 133), confirming the important role of ABA in stress signaling.

Genomic Bioinformatics Will Allow Rapid Increase in Signal Gene Identification

So far, few genes encoding plant signaling proteins controlling responses to osmotic stress have been found strictly by mining gene sequence databases (Figure 3). Divergence between species may sometimes make this approach difficult. For example, considerable effort to locate the plant version of CNB was expended,
and although CNB-like proteins were eventually identified, none can interact with yeast CNA1/2 (134), which indicates that their function is apparently not the same as in yeast. The use of sequence comparisons to identify potential signal genes will undoubtedly increase rapidly and dramatically. As gene knockout technology in *Arabidopsis* becomes increasingly facile, a quick and easy test of fidelity will also be available for bioinformatic mining (15, 126, 246). Eventually this will become the dominant approach to gene discovery. Phenotypic changes such as increases in salt tolerance caused by ectopic overexpression of regulatory genes in plants are also important for crop improvement, but in many cases may not be able to address the question of necessity, i.e. whether a regulatory protein is necessary for salt tolerance in plants. In one scenario, a gene of a multigene family may be sufficient to confer a stress-tolerance phenotype when overexpressed in plants but may not be necessary because other family members can substitute for its function owing to functional redundancies. In another scenario, a gene that normally does not function in salt stress responses may confer a stress-tolerance phenotype when ectopically expressed because signaling specificity can be lost during ectopic overexpression. It is therefore important to isolate or construct loss-of-function mutations even for genes that are known to confer tolerance phenotypes when overexpressed in order to understand precisely their interrelationships.

**Organization of Plant Signaling Genes into Pathways**

Although we have identified many genes that encode signal transduction proteins that are potentially important to osmotic stress adaptation in plants, we still know little of how these gene products function in a signal pathway. It is evident from Figure 3 that, compared to yeast, we are virtually unable to draw any functional relationship directly between these many genes. Why are we able to place the yeast genes in an ordered functional signal pathway but cannot do so for the plant genes? Even though some plant genes have been shown to affect stress tolerance of yeast or even of plants, their relationships to each other remains mostly a mystery. It is known that the MP2C gene product somehow reduces the activity of SAMK (164). The plant transcription factors listed in Figure 3 all interact with particular *cis*-elements and control expression of target genes, some of which are known. For instance, ABI1 negatively controls downstream target genes, whereas AtCDPK1 positively controls the same target promoter (250). The tyrosine, dual-function phosphatase AtDsDTP1 dephosphorylates and inhibits AtMPK4 (86). *VP14* is a stress-induced gene encoding a carotenoid cleavage enzyme that is a rate-limiting step in ABA synthesis (277), thus exerting control over numerous ABA-controlled genes. NaCl downregulates in tobacco the expression of a member of the 14-3-3 protein family that has interactive functions with many signal components (42).

One of the best ways to gather information regarding interactions between plant signal components has been to determine their ability to activate downstream promoters fused to marker genes such as was shown for AtCDPK1 and ABI1 (231).
This kind of information is needed for many other signal components. In a comprehensive genetic screen, Ishitani et al (110) isolated a large number of Arabidopsis mutations that either reduce or enhance salt stress induced gene expression. The authors used the salt-, drought-, cold-, and ABA-responsive rd29A promoter fused to the firefly luciferase gene to report plant responses to salt treatments. The firefly luciferase reporter allows large-scale, nondestructive screening of mutants by real-time, high throughout low light imaging technology (110). The recessive los (low expression of osmotic stress-responsive genes) mutants define positive regulators of salt stress gene expression, while the recessive hos (high expression of osmotic stress-responsive genes) and cos (constitutive expression of osmotic stress-responsive genes) mutations are presumed to be in genes that negatively regulate stress responses. Some los, hos, and cos mutations also alter plant responses to ABA or low temperature, thus revealing points of crosstalk between salt, cold, and ABA signaling pathways. Molecular cloning of these mutations as well as their interactors, enhancers, and suppressors (Figure 4, see color plates) will eventually allow plant regulatory pathways to be built, like the ones in yeast (Figure 3).

Direct physical interaction information by yeast two-hybrid or by in vitro assays also is lacking for most plant gene products so far. Yeast two-hybrid assays have been used to demonstrate that the AtMEKK1 protein interacts with AtMEKK2/MEK and AtMPK4 (167, 168). Both genetic and biochemical evidence for interactions between many more of these plant genes and their products is now needed. On the genetic side, allelism, additivity and epistasis data must be obtained now for the plant genes where function is evident (see Figure 4). Conducting biochemical interaction studies in both yeast two-hybrid and in vitro assays as well as ability to modify through phosphorylation, dephosphorylation, etc, are logical next steps for these genes. Additional signal pathway participants can also be identified by activation or suppression screens with known mutants. Such information is now needed to establish the exact roles of signal components in stress-signaling to allow ordered signaling pathways to be revealed.

One potential pathway that is increasingly meeting these criteria involves the genes mutated at the SOS1, 2, and 3 loci. Genetic experiments determined that these loci are not additive (same pathway) and that the sos1 phenotype is epistatic to the other two, placing it furthest downstream. Recent cloning and interaction studies of SOS2 and 3 indicate that SOS3 is a CNB-like Ca^{2+} binding protein that physically interacts with and activates the SOS2 protein, which is similar to the SNF1/AMPK family of kinases (91).

As new genes enter the camp of potential mediators of stress-adaptation through bioinformatic or expression studies, evidence for their function in mediating adaptation through overexpression and gene knock-out studies must follow (Figure 4). Armed with this evidence for function, further genetic studies and interaction determinations will finally allow us to draw complete signaling pathways for plant osmotic stress responses.
Figure 4 Algorithm for discovering stress tolerance determinants.
KNOWING ALL OSMOTIC STRESS TOLERANCE DETERMINANTS: A Tall Order About to be Filled?

Although genes that participate in signal transduction ought to be constitutively expressed to allow for rapid sensing of a change in the osmotic environment, they are often also induced by osmotic change, as are some of the genes listed in Figure 3. As with other signal transduction elements and by analogy to yeast responses, this is most likely a mechanism that functions to amplify the signal response (87). It is now obvious that besides genes involved in signaling, a very large number of plant genes are transcriptionally controlled by osmotic stress, and several previous reviews have summarized these genes (35, 294). These transcripts represent “downstream controlled effector genes,” as listed in Table 1, and embody the accumulated knowledge from studies of individual genes and mechanisms. With the advent of microarray technology, this period of research is now coming to an end.

Illustrated in Figure 4 is the prevalent strategy for finding the most important genes that control salt adaptation. The focus on individual components of plant stress-tolerance responses is now rapidly being replaced by global analyses. Technological advances, summarized under the “genomics” label, make it possible to monitor the expression of many or even all genes in an organism simultaneously during the entire life cycle of the organism or in response to different stimuli. Genomics-based approaches are, however, more than a collection of novel instruments, techniques, and data production tools. Although at present a learning curve is needed to use these tools effectively, the opportunities provided by genomics are already revolutionizing experimental approaches and are beginning to produce a staggering amount of data and unexpected results. Knowledge of all osmotically induced genes is within reach because of the rapid development of gene microarray technologies (http://stress-genomics.org; http://www.zmdb.iastate.edu; 15, 103, 126). Also, the imminent completion of the *Arabidopsis* genome sequence will provide a roadmap for plant genes at least in type, if not in actual complexity and function. Equally important will be the genome sequence-based isolation of *Arabidopsis* mutants from the rapidly increasing collections of tagged plants (155, 246). By combining microarray analyses with signal transduction mutants, the subarrays of osmotically responsive genes that are under the control of specific signal components can be determined. For example, a mutation of *SOS3*, or activation of CDPK1 must affect the expression of downstream genes and pathways. With microarray technology it is expected that we can determine these and other subsets of activated genes to ascertain which combinations of signal genes control the broadest array of downstream effectors of salt adaptation. This will allow a systematic and logical approach to decide what combinations of gene modifications should be used for engineering signaling and plant metabolism to maximize salinity tolerance through genetic engineering.
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