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Adaptations to Environmental Stresses

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INTRODUCTION

Environmental stresses come in many forms, yet the most prevalent stresses have in common their effect on plant water status. The availability of water for its biological roles as solvent and transport medium, as electron donor in the Hill reaction, and as evaporative coolant is often impaired by environmental conditions. Although plant species vary in their sensitivity and response to the decrease in water potential caused by drought, low temperature, or high salinity, it may be assumed that all plants have encoded capability for stress perception, signaling, and response. First, most cultivated species have wild relatives that exhibit excellent tolerance to abiotic stresses. Second, biochemical studies have revealed similarities in processes induced by stress that lead to accumulated metabolites in vascular and nonvascular plants, algae, fungi, and bacteria (Csonka, 1989; Galinski, 1993; Potts, 1994). These metabolites include nitrogen-containing compounds (proline, other amino acids, quaternary amino compounds, and polyamines) and hydroxyl compounds (sucrose, polyols, and oligosaccharides) (McCue and Hanson, 1990). Accumulation of any single metabolite is not restricted to taxonomic groupings, indicating that these are evolutionarily old traits. Third, molecular studies have revealed that a wide variety of species express a common set of genes and similar proteins (for example, Rab-related proteins and dehydrins) when stressed (Skriver and Mundy, 1990; Vilardell et al., 1994). Although functions for many of these genes have not yet been unequivocally assigned, it is likely, based on their characteristics, that these proteins play active roles in the response to stress.

Learning about the biochemical and molecular mechanisms by which plants tolerate environmental stresses is necessary for genetic engineering approaches to improving crop performance under stress. By investigating plants under stress, we can learn about the plasticity of metabolic pathways and the limits to their functioning. Also, questions of an ecological and evolutionary nature need investigation. Are the genes that confer salt tolerance on halophytes and/or drought tolerance on xerophytes evolutionarily ancient genes that have been selected against in salt- and drought-sensitive plants (glycophytes) for the sake of productivity? Or have some species obtained novel genes in their evolutionary history that have enabled them to occupy stressful environments? How will the introduction of genes conferring stress tolerance into highly productive species affect crop productivity in the field?

Regardless of the evolutionary history of extremely tolerant species, they remain a rich source of genes for introduction into other species to enhance stress tolerance. The dicot order Caryophyllales provides a good example. Families of the order Caryophyllales, such as the Cactaceae, Chenopodiaceae, and Aizoaceae, include many species that occupy extreme habitats and produce rather exotic compounds that may confer tolerance in these habitats. For example, in 10 of 12 families of the Caryophyllales, anthocyanins are replaced by betalaines. Species abound that contain tertiary or quaternary amines and sulfonium compounds or polyols (Hanson et al., 1994). In addition, species with C_4 photosynthesis, C_3/C_4 intermediate photosynthesis, and Crassulacean acid metabolism (CAM) are prevalent, likely reflecting adaptation to a low-water habitat. Similarly, the resurrection plant *Craterostigma plantagineum* (Bartels et al., 1990) is one example of more than 100 known extremely desiccation-tolerant species that are distributed among several plant orders (Gaff, 1981). *C. plantagineum* is most notable for a massive reversible fluctuation of carbohydrates during periods of either desiccation or rehydration (Bianchi et al., 1991). The unusual sugar 2-octulose accumulates in growing leaves of well-watered plants, but it is rapidly converted to sucrose when a desiccation period begins. Indeed, the accumulation of sucrose and other carbohydrates turns out to be a common feature of desiccation-tolerant seeds of many plant species. Individual genes or genes for entire pathways, such as those leading to enhancement of quaternary amine biosynthesis, carbohydrate accumulation, or CAM (see later discussion), could be introduced to plants either lacking these genes or not expressing them under stress conditions.

Pathways involving more familiar compounds may also be exploited to produce stress-tolerant plants. Some of these may not require obtaining genes from tolerant species: overexpressing or altering regulatory features of an endogenous gene or a gene from a different, but stress-intolerant, species might be sufficient. Relevant examples are the engineered overexpression of genes for enzymes that increase putative osmoprotectant compounds such as proline, polyols, or fructans (Delauney and Verma, 1993; Tarczynski et al., 1993; Pilon-Smits et al., 1995). Some of these metabolites, which

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are produced through ubiquitous pathways, have already been demonstrated to be efficient in conferring tolerance on bacteria (Dandekar and Uratsu, 1988; Potts, 1994; Nomura et al., 1995). Similarly, spinach and other chenopods, which are only moderately water stress tolerant, accumulate glycine-betaine and related compounds (Weretilnyk and Hanson, 1990; Summers and Weretilnyk, 1993; Hanson et al., 1994; Ishitani et al., 1995), and genes responsible for this accumulation could be utilized for transfer. Finally, manipulation of enzymes in the proline metabolic pathway might also be an effective approach, to judge from the positive correlation between proline accumulation and water stress tolerance (McCue and Hanson, 1990; Delauney and Verma, 1993).

Sorting out the mechanisms by which plants might adapt to abiotic stresses is still difficult because more needs to be learned about the molecular and genetic basis of stress resistance. Until now, mechanisms have been described qualitatively, using terms such as "water-use efficiency," "ion homeostasis," or "osmotic adjustment." Only recently has progress been made to substantiate these descriptions by recognizing their biochemical foundations.

APPLICATION OF NEW METHODS TO OLD PROBLEMS

In the past, many studies of abiotic stress tolerance have monitored the physiological status of a stressed plant compared with unstressed controls. Mechanisms have been deduced from such descriptions, but in general these studies have not included molecular and genetic analysis of stress tolerance principles. Most important, knowledge from physiological studies has led to only a few studies on the biochemical mechanisms underlying tolerance and sensitivity to abiotic stress factors. The challenge now is to utilize this vast store of accumulated information involving molecular and biochemical analyses and modeling of the emerging mechanisms in transgenic plants.

One promising genetic avenue is the mapping of quantitative trait loci that relate performance and yield to drought, low-temperature, or salinity tolerance. Thus, regions of chromosomes can be identified that carry genes that improve stress tolerance. A lucid example is the work on such loci in crosses of Chinese spring wheat and cultivated wheat, which differ in their degree of salinity tolerance (Dvorak and Zhang, 1992; Dubcovsky et al., 1994). The means to clone and identify mapped genes rapidly are straightforward. Certainly, for the major crop species—rice, corn, wheat, potato, and barley—and for some model species, all genetic material will be available soon in a cloned, accessible form (Höfte et al., 1993; Newman et al., 1994; Sasaki et al., 1994). In addition, our growing understanding of biochemical mechanisms involved in stress tolerance makes it possible to search for specific genes, a strategy made feasible by the development of the polymerase chain reaction. Another technique, differential display polymerase chain reaction (Liang and Pardee, 1993), may make finding stress-induced transcripts even more feasible, but the technique has potential pitfalls and is yet largely untested in plant research. Finally, many mutated lines of Arabidopsis are available in which genes are tagged by insertion (Feldmann, 1991). Having characterized a DNA or transcript sequence with a putative significance in conferring stress tolerance is, however, no longer sufficient, and in the future, emphasis must be placed on functional characterizations and biochemical integration of molecular and genetic data.

Testing anticipated contributions of particular genes to stress tolerance does not necessarily have to be done in a plant. Based on similarities in biochemical pathways and responses to stress between *Saccharomyces cerevisiae* and plants, the many known mutants in yeast metabolism can be exploited, for example, by expressing plant cDNA libraries in a yeast strain carrying a specific mutation in a stress pathway (Frommer et al., 1993; Schirmer et al., 1994). One example is the detection of potassium channel proteins, which are essential for ion homeostasis under stress conditions, by yeast complementation (Anderson et al., 1992; Sentenac et al., 1992). Analysis of cloned plant genes, especially when the function of the gene is in a membrane, can also be performed by expression in Xenopus oocytes. The functional characterization of aquaporins, proteins that lead to facilitated water permeability, has been accomplished in this way (Maurel et al., 1993; Daniels et al., 1994). The complexity of the stress syndrome, however, requires that many different techniques be utilized. Faster progress could certainly be made if groups of investigators with complementary expertise worked together.

A MODEL HALOPHYTE, *MESEMBRYANTHEMUM CRYSTALLINUM*

Mesembryanthemum crystallinum (ice plant) (Aizoaceae, Caryophyllales) is native to the Namibian Desert of southern Africa and is adapted to growth in high sodium and under drought and low-temperature conditions. Its genome is approximately twice the size of that of Arabidopsis (DeRocher et al., 1990; Meyer et al., 1990). The ice plant shows developmentally regulated cell-specific polyploidy, up to 128N, which is also influenced by stress. Its development is characterized by pronounced phase changes separating seedling establishment, juvenile and adult growth, flowering, and seed formation. Each phase exhibits easily scorable morphological, physiological, and biochemical markers (Bohnert et al., 1994; Cushman and Bohnert, 1995). Growth and phase changes are plastic and may be influenced by external conditions, light, temperature, CO_2, and stresses that limit water availability. The ice plant's development and phase changes are reflected in the expression of a number of marker genes (Meyer et al., 1990; Cushman and Bohnert, 1995; Yamada et al., 1995). Finally, the ice plant has been used in many stress physiology studies. Although our knowledge about the magnitude of changes in gene expression is still limited, it appears that transcriptional regulation

is the source of developmental changes in gene expression, whereas both transcriptional and post-transcriptional controls are important during stress (Cushman et al., 1990; DeRocher and Bohnert, 1993).

One example of the interplay between transcriptional and post-transcriptional controls is the induction of the CAM pathway, which increases water use efficiency (Bohnert et al., 1992). The CAM pathway is characterized by closed stomata during the daytime and by malate fluctuations during day–night cycles. Opening stomata only at night reduces transpiratory water loss, the essential advantage of plants with CAM over those with C_3 photosynthesis (for a review of C_3 and C_4 photosynthesis, see Furbank and Taylor, 1995, this issue). Reducing water loss is also essential for the ice plant's tolerance to prolonged salinity stress. CO_2 that is taken up at night is assimilated into oxaloacetate by the CAM characteristic enzyme, phosphoenolpyruvate carboxylase (PPC), and finally into malate. The malate is stored in the vacuole, from where it is mobilized during daytime, providing CO_2 for the action of ribulose-1,5-bisphosphate carboxylase/oxygenase. A housekeeping PPC (encoded by the *Ppc2* gene, which is constitutively expressed at a low level) provides C_4 intermediates to the citric acid cycle. The CAM-specific isoform of PPC (encoded by *Ppc1*) shows low basal expression in young plants, and little mRNA can be detected unless the plants are stressed. Water stresses lead to fivefold transcriptional activation of *Ppc1*, followed by a more than 100-fold accumulation of its mRNA and the resulting accumulation of the CAM-specific PPC. CAM induction is, however, more complex than a simple induction of *Ppc1* and other genes/enzymes of the pathway. High salinity in plants less than ~4 weeks old does not lead to full *Ppc1* induction; induction remains incomplete (Cushman et al., 1990; Vernon et al., 1993a; Bohnert et al., 1994). Productive CAM switching is correlated with a phase change that commences when plants are ~5 weeks old, depending on growth conditions. That is, the onset of CAM photosynthesis is controlled by a developmental program accelerated by stress. These results illustrate an important point, namely, that plant stress responses must be seen in a developmental context.

Even though CAM induction in young ice plants is incomplete, such plants survive severe stress, which indicates that there must be additional mechanisms that act in the short term to confer stress tolerance on the ice plant. Three such mechanisms have been identified: induced polyol biosynthesis, regulation of ion uptake and compartmentation, and facilitated water uptake.

Induced Polyol Biosynthesis

Accumulation of polyols, either straight-chain metabolites such as mannitol and sorbitol (Bieleski, 1982) or cyclic polyols such as *myo*-inositol and its methylated derivatives (Loewus and Dickinson, 1982), is correlated with tolerance to drought and/or salinity, based on polyol distribution in many species, including bacteria, yeasts, marine algae, higher plants, and animals. Polyols seem to function in two ways that are difficult to separate mechanistically: osmotic adjustment and osmoprotection. In osmotic adjustment, they act as osmolytes, facilitating the retention of water in the cytoplasm and allowing sodium sequestration to the vacuole or apoplast. Alternatively, protection of cellular structures (possibly by scavenging active oxygen) might be accomplished through interactions of such osmolytes, often termed compatible solutes (see following discussion), with membranes, protein complexes, or enzymes. Proline, quaternary ammonium, and tertiary sulfonium osmolytes are zwitterions at physiological pH. Although they are ionic, they have no net charge. Their osmoprotective function in the cytosol may be related to their unique chemistry. Those polyols that are nonreducing sugars may also store excess carbon under environmental stress conditions (Bieleski, 1982; Ford, 1984; Paul and Cockburn, 1989; Smirnoff and Cumbes, 1989; Vernon et al., 1993b).

The importance of altered metabolism under abiotic stress, for example, the diversion of carbon to polyol biosynthesis, is exemplified, as shown in Figure 1, by the metabolic reactions originating from the glucose-6-phosphate pool. Figure 1 outlines the inositol biosynthetic pathway catalyzed by inositol-1-phosphate synthase (INO1) and inositol monophosphatase as well as pathways that originate from inositol and inositol-1-phosphate. There are several interesting aspects of the inositol biosynthetic pathway. First, it is essential for membrane biosynthesis and signaling functions in all organisms, because INO1 is the sole enzyme catalyzing entry into the pathway. Connected to this pathway in the ice plant (and in other species with different evolutionary histories) is a pathway that leads to accumulation of methylated inositols, catalyzed by inositol *O*-methyltransferase (IMT1). Inositol and inositol-1-phosphate also fuel the production of other compounds that have been correlated with stress tolerance, for example, gums, cell wall–located carbohydrates, carbohydrates in glycoproteins, and mucilages. Plants use inositol to synthesize vegetative storage carbohydrates such as stachyose and verbascose, which are stress induced in some species. Yet another product of this pathway is phytate, inositol-hexa*kis*phosphate, which serves as phosphate storage for seed. The reactions outlined in Figure 1 can be considered a paradigm for what can constitute abiotic stress tolerance: the extension, possibly through the evolution of new genes, of an essential step in phospholipid biosynthesis to other pathways, in which the product of the new pathway serves an osmoprotective function.

In the ice plant, the *Imt1* gene (Figure 1) is under strict environmental control at any developmental age. The gene is transcriptionally inducible only by salt stress and low temperature (Vernon and Bohnert, 1992; Bohnert et al., 1994). Activity of the IMT1 enzyme leads to the production of D-ononitol, which is further epimerized to D-pinitol. Pinitol increases in stressed ice plants to become the major low–molecular weight carbon compound, with concentrations exceeding 700 mM in cytosol and chloroplasts (Paul and Cockburn, 1989; Adams et al., 1992). Recent data indicate that the *Ino1* gene is similarly regulated (M. Ishitani, A. Lahiri Majumder, A. Bornhouser, C.B.

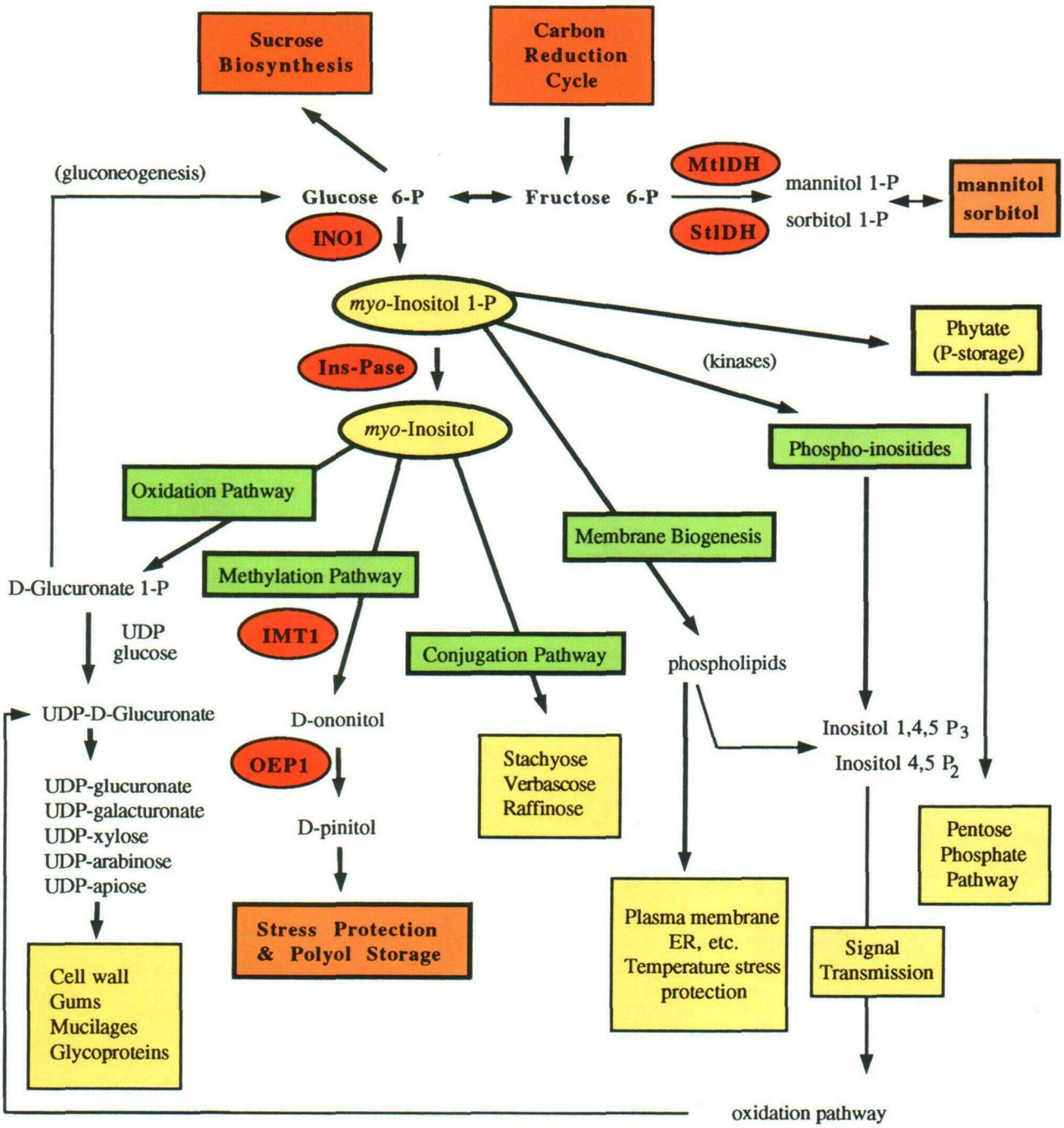

Figure 1. *Myo*-inositol, Polyols, and Their Metabolism.

Myo-inositol is synthesized from glucose-6-phosphate (glucose 6-P) by inositol 1-P synthetase (INO1) and inositol 1-P phosphatase (Ins-Pase). Mannitol and sorbitol originate from the glucose 6-P/ fructose 6-P pool by way of mannitol 1-P dehydrogenase (MtlDH) or aldose 6-P reductase (StlDH) to form mannitol 1-P or sorbitol 6-P. *Myo*-inositol 1-P can be further phosphorylated to phytate (Ins-P_6) as a storage source of phosphate in seed (Loewus and Dickinson, 1982), the carbon going to the pentose phosphate pathway upon germination (Biswas et al., 1984). Inositol 1-P can be utilized for the synthesis of the plasma membrane phosphoinositides phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP), and phosphatidylinositol biphosphate (PIP_2), which are involved in signal transduction processes as sources of the second messengers inositol 1,4,5-P_3 and inositol 4,5-P_2 (Gross and Boss, 1993). Inositol 1-P is involved in the formation of phospholipids and membrane biogenesis. Inositol also combines with galactose to form galactinol, which serves as a source of galactosyl residues to be incorporated into various members of the raffinose series of vegetative storage carbohydrates (Kandler and Hopf, 1982). Inositol is also methylated to D-ononitol by inositol *O*-methyltransferase (IMT1) and converted to D-pinitol by ononitol epimerase (OEP1). These methylated inositols serve as protective osmolytes and are only slowly turned over. In addition, inositol can be oxidized to D-glucuronate and then to UDP-D-glucuronate to form noncellulosic cell wall components such as gums, mucilages, and glycoproteins (Loewus and Loewus, 1983).

Michalowski, and H.J. Bohnert, manuscript in preparation). This ensures increased carbon flux to polyol biosynthesis under conditions of salt stress.

Accepting an osmoprotective function for accumulating compounds from the inositol biosynthetic pathway is only one part of the story. There seems to be additional meaning in the fact that methylated inositols accumulate. Methyl group transfers catalyzed by specific methyltransferases (Kagan and Clarke, 1994) are involved in several other pathways that lead to the accumulation of quaternary amines, such as glycine betaine, tertiary sulfonium compounds (dimethylsulfoniopropionate), polyamines (spermine), lignin precursors (sinapic acid; see Whetten and Sederoff, 1995, this issue), and the growth regulator ethylene (McCue and Hanson, 1990; Kawelleck et al., 1992; Hanson et al., 1994). Like the methylated inositols, quaternary amines and tertiary sulfonium species have frequently been assigned functions in osmoprotection and/or osmotic adjustment (McCue and Hanson, 1990). The various methyltransferases use *S*-adenosylmethionine (SAM) as cosubstrate, producing, after transfer of the methyl group, *S*-adenosylhomocysteine (SAH), a strong competitive inhibitor of the methylation reaction. Correlative support for transmethylations being involved in the enhancement of stress tolerance may be deduced from the accumulation of transcripts for SAM synthetase in tomato during osmotic stress (Espartero et al., 1994). This affects the SAM to SAH ratio. The importance of methylation in stress tolerance is also supported by the recent report that overexpression of the yeast gene *Hal2*, which encodes a phosphatase that recycles adenosine trapped in 3′-phosphoadenosine-5′-phosphate (after transfer of the sulfate group), increases salinity tolerance in yeast (Murguia et al., 1995). Adenosine scavenging is connected to the pathway producing sulfur-containing amino acids and to methylation reactions that utilize SAM.

Regulated Ion Uptake and Compartmentation

A second mechanism that protects the young ice plant against water stress is the regulation of ion uptake and compartmentation (Adams et al., 1992). The ice plant takes up sodium when it is available and deposits it in a gradient along its axis, with the highest amounts in the youngest tissues. This gradient parallels the increase in D-pinitol. Particularly high accumulations of sodium and pinitol have been observed in a morphological specialization of the ice plant, the epidermal bladder cells, which are developmentally preformed but increase in size dramatically when plants are salt stressed. Sodium concentrations exceeding 1 M have been measured in these cells (Adams et al., 1992). The ability of the ice plant to use sodium as an osmoticum confined to vacuoles in growing parts of the plant (compensated by D-pinitol accumulation in the cytoplasm) is in stark contrast to glycophytic plants, which attempt to limit sodium uptake or partition sodium into older tissues that serve as storage compartments that are eventually sacrificed (Cheeseman, 1988).

Facilitated Water Permeability

A third mechanism for stress protection appears to be regulation of facilitated water permeability. Transcripts of *Mip* (major intrinsic protein) genes whose abundance changes under salt stress have been isolated from root cDNA libraries of ice plants (Yamada et al., 1995). The encoded MIPs are homologous to plant and animal aquaporins, or water channels, as well as to glycerol facilitator proteins from bacteria (Chrispeels and Agre, 1994). Expression of different *Mip* mRNAs after injection into Xenopus oocytes and determination of water permeability demonstrate that the encoded proteins function as water channels. Two classes of plant aquaporins, located in the plasma membrane and tonoplast, respectively, have been identified (Johnson et al., 1990; Kammerloher et al., 1994). Transcripts of two ice plant *Mip* genes are found predominantly in cells presumably involved in water flux, that is, the root epidermis, the root tip before a functional central cylinder is formed, the endodermis, and regions surrounding strands of xylem cells in roots after secondary growth (Yamada et al., 1995).

An interesting difference exists between the ice plant and Arabidopsis in the stress regulation of transcript levels for closely related aquaporins. Whereas increases in salinity lead to higher transcript levels for one of the plasma membrane aquaporins, RD28, in Arabidopsis (Yamaguchi-Shinozaki et al., 1992; Daniels et al., 1994), the ice plant *Mip* transcripts decline transiently and recover as the leaves regain turgor in a time course that parallels the accumulation of pinitol (Vernon and Bohnert, 1992; Yamada et al., 1995). Although the changes in transcript levels in Arabidopsis are not reflected by a similar increase in aquaporin protein (Daniels et al., 1994), differences in gene activity between this glycophyte and the halophytic ice plant seem to indicate differences in how stress is perceived and/or how the signal is processed. This may be due to differences in growth regulator sensitivity, for example, different perception of abscisic acid (ABA), which is generally considered a stress hormone. Salt stress leads to ABA increases in both plants (Yamaguchi-Shinozaki et al., 1992; Thomas and Bohnert, 1993), but the ice plant seems to "interpret" changes in ABA in a way that is different from Arabidopsis.

GLYCOPHYTE MECHANISMS FOR STRESS TOLERANCE

Mechanisms very similar to those that seem to function in stress protection in the halophytes have emerged from the analysis of glycophytic species, supporting the contention that stress tolerance mechanisms are ubiquitous. Because stress tolerance is a multigenic trait, the biochemical pathways leading to products or processes that improve tolerance are likely to act additively and, possibly, synergistically. Thus, the advantages of halophytes and xerophytes over glycophytes may

result simply from the more efficient performance of a few basic biochemical tolerance mechanisms.

Induced Compatible Solute Biosynthesis

One way all organisms tolerate abiotic stress to some degree is by accumulating solutes termed compatible because they do not interfere with normal biochemical reactions. We discuss here recent reports but also refer the reader to past reviews (Yancey et al., 1982; Csonka, 1989; Delauney and Verma, 1993; Galinski, 1993; Rhodes and Hanson, 1993; Bartels and Nelson, 1994). Comprehensive biochemical studies of the various compatible solutes effective as osmoprotectants are a necessary first step for engineering of plant metabolic pathways. A recent study exemplifies this point. A thorough examination of the Plumbaginaceae found that various members of this family accumulate not only glycine-betaine but also, or alternatively, other quaternary ammonium zwitterions, including choline-*O*-sulfate, β-alanine-betaine, proline-betaine, and hydroxyproline-betaine (Hanson et al., 1994). These compounds show similar efficacy as osmoprotectants in bacterial assays. In addition, a correlation has been observed between the particular compound accumulated and the natural environment of each species. For example, species growing in sulfate-containing soil synthesize choline-*O*-sulfate, β-alanine-betaine accumulates in species grown under saline conditions, and proline-derived betaines are accumulated by species growing in dry environments. It is not yet known why different abiotic conditions should favor the accumulation of different osmolytes in members of one family. Study of biochemical pathways leading to these individual compounds may provide insight into how to engineer plants to tolerate complex environments. Conversely, systematic studies of cereal crops for their lack of compatible solute accumulation under stress (Rathinasabapathi et al., 1993) have identified targets for introducing genes and their encoded pathways both to improve crops and to measure the efficacy of a given compatible solute. Focus by different investigators on a single model system, such as transgenic tobacco, for testing the various solutes and other mechanisms would be useful.

Biochemical pathways (and corresponding genes) identified in bacteria (Galinski, 1993) are equally valuable subjects for testing concepts of stress tolerance. Introducing the bacterial *mtlD* (mannitol-1-phosphate dehydrogenase) gene into tobacco provided slightly higher salinity tolerance compared with the wild type (Tarczynski et al., 1993), indicating the possibility that even more potent osmoprotectants than mannitol may be utilized to engineer stress-tolerant plants. The most effective compatible solutes, as shown by in vitro enzyme stabilization studies, are ectoine and hydroxyectoine, which are unusual methylated cyclic amino acids, and trehalose (Lippert and Galinski, 1992). Potentially, these could be accumulated in plants and provide increased tolerance.

Regulated Ion Uptake and Compartmentation

Ion uptake and compartmentation are crucial not only for normal growth but also for growth under dry and saline conditions, because both stresses lead to a disturbance of ion homeostasis. Key components for homeostasis, especially under salinity stress, are genes encoding Na^+/H^+ antiporters. Physiological studies have observed sodium excretion in the root system and vacuolar deposition of sodium in leaves. The results argue for the existence of a Na^+/H^+ antiport activity that either removes sodium from the cell or compartmentalizes it in the vacuoles. To our knowledge, no plant Na^+/H^+ antiporter protein has been purified. Complementation of bacterial and yeast mutants may be the best strategy to accomplish the task, similar to strategies that have provided the sequences for the plant high- and low-affinity potassium uptake systems (Anderson et al., 1992; Sentenac et al., 1992; Schachtman and Schroeder, 1994). Alternatively, sequences with homologies with known Na^+/H^+ antiporter genes might be revealed as more expressed sequence tags become available. Another strategy is to screen for cesium-insensitive Arabidopsis mutants (Sheahan et al., 1993). Because cesium is probably taken up by a monovalent cation channel, mutations may reveal a Na^+/H^+ antiport or, alternatively, potassium channels or transporters. The availability of genes for these proteins will pave the way for understanding the regulation and mechanism of ion compartmentation and pH regulation controlling fundamental processes in stressed and unstressed plants.

Despite lack of progress in isolating a plant Na^+/H^+ antiporter, a putative antiporter has been isolated from yeast (Jia et al., 1992). The isolation method, which should be applicable also to plants, was based on screening for lithium sensitivity: lithium is usually transported by carriers with affinity for sodium, and, based on the high sensitivity of all cells to lithium, low concentrations can be utilized during screening, avoiding selection for osmotolerance. Lithium-resistant yeast lines obtained following transformation with a genomic DNA library contained plasmids carrying a gene, *sod2*, which encodes a protein with weak overall similarity with human and *Escherichia coli* antiporters. Overexpression of *sod2* under the control of a highly expressed promoter results in increased sodium tolerance; furthermore, the basis of resistance of mutagenized cells that were selected for lithium resistance was shown to be amplification of the *sod2* gene.

The finding that overexpression of the yeast *sod2* gene increases sodium tolerance is somewhat surprising, because one might have predicted that simple overexpression of such a gene would disturb pH regulation. The finding that pH regulation is not affected implies that endogenous H^+-ATPases can compensate for the increased amount of this antiporter in yeast. However, the supracellular nature of plants poses a problem not encountered by a unicellular organism in that plants must not only exclude sodium from cells but also transport and store it. Whereas global overexpression of a Na^+/H^+ antiporter in a whole plant (for example, using the cauliflower

mosaic virus 35S promoter) might disturb pH regulation excessively, overexpression solely in the root epidermis might make transgenic plants better sodium excluders.

Studies on salinity stress responses in yeast have provided further information about the importance of control over intracellular ion levels. The *Hal1* gene, which encodes a soluble protein of unknown function, improves salinity tolerance when overexpressed and decreases tolerance when defective. Increased expression leads to accumulation of potassium, suggesting a role for this protein in influencing potassium homeostasis (Gaxiola et al., 1992). Genes with homology with *Hal1* exist in higher plants.

Facilitated Water Flux

The discovery of carrier proteins specific for water is an important step toward understanding the mechanisms by which plants adapt to water stress (for review, see Chrispeels and Maurel, 1994). Water channels facilitate flux of water along an existing osmolarity gradient. Expression of tonoplast and plasma membrane aquaporin transcripts, some of which are water stress inducible, is correlated with cell elongation (Guerrero et al., 1990; Ludevid et al., 1992; Yamaguchi-Shinozaki et al., 1992; Daniels et al., 1994). Also, an Arabidopsis blue light–responsive transcript with homology with aquaporins is expressed primarily in expanding cells (Kaldenhoff et al., 1995). Whether water flux is the primary factor limiting cell expansion under either well-watered or stress conditions is not clear. Cell wall metabolism and relaxation are considered the major initiator and control point for cell enlargement (Cosgrove, 1993). Under stress conditions, especially in glycophytes, the inability to transport and compartmentalize inorganic solutes or to synthesize organic solutes may, however, be additional limiting factors.

Water channels can be blocked or closed by phosphorylation (Chrispeels and Agre, 1994). Could this type of regulation be involved in root-to-shoot signaling under conditions of stress? A deviation of water flow from a value expected based on actual water potential might constitute a signal.

COMPLEXITY OF STRESS TOLERANCE

Alteration of gene expression is always involved in preparing plants for an existence under stress. The question is whether the regulatory elements are stress specific and, further, whether each is unique to a specific stress-tolerant species. Even more important, would engineering of plants for stress tolerance have to take into account a myriad of changes in signaling, gene activation, and protein modification? We do not think so. Mechanisms that control stress perception itself, and gene expression after stress perception, are most likely universal in the plant kingdom, considering the distribution of stress-adapted plants in many different families, the occurrence of stress-tolerant relatives for many glycophytic species, and the genetic variability in stress tolerance of crop plants. After all, the machinery through which gene expression responses to a changing environment are mediated is present in guard cells in all plants (Assmann, 1993, 1994). Similarly, gene expression programs very much like those that operate during drought stress are also operative during seed desiccation (Bray, 1993; Delseny et al., 1994). However, differences may exist between naturally stress tolerant and sensitive plants that determine in which cell, in which tissue, or during which developmental stage a stress-mediating pathway is active. In addition, the ways in which gene expression responses to stress are inducible in a timely and spatially sensible fashion in tolerant plants may be different in stress-sensitive species.

Under severe stress, a plant adapts its metabolism and alters its development. Several of the necessary changes (Figure 2) may be ordered on a scale of increasing complexity. Low-complexity mechanisms, similar to compatible solute production, ion uptake and partitioning, and possibly facilitated water uptake, would include the synthesis of other compounds, such as membrane lipids, LEA (late embryogenesis abundant) proteins, isoforms of chaperones, or proteins recruited from other functions (for example, osmotin, which evolved primarily as a pathogenesis-related protein; LaRosa et al., 1992; Raghothama et al., 1993). We view these as low-complexity mechanisms because they appear to involve changes in a single biochemical pathway. For example, the engineering of D-pinitol biosynthesis may require that only two genes be manipulated. There may be additional changes necessary, for example, to increase flux to inositol, but such changes are still relatively simple. As is the case for polyols and quaternary ammonium compounds, expression of a single low-complexity mechanism would be marginally protective. We also include as low complexity those transcription factors (Nelson et al., 1994) or RNA binding proteins (Breiteneder et al., 1994) that are induced by stress. Additionally, protection is likely provided by adjustments in amount and/or activity of proteins that assure membrane functioning in compartmentation and in ion and pH homeostasis, that is, various proton- translocating ATPases, ion channels/transporters. In this context, comparative biochemical analyses of such proteins from glycophytes and halophytes should be done. It is important to discern whether, for example, plasma membrane ATPases from the halophyte *Atriplex nummularia* are structurally or functionally different from their homologs in Arabidopsis and/or whether their activity is differently regulated in glycophytes and halophytes (Niu et al., 1993). For protection of higher order processes, we believe that several low-complexity mechanisms must be induced coordinately.

High-complexity mechanisms would be changes that protect major processes such as photosynthesis and respiration and those that preserve structures such as the cytoskeleton, the cell wall, or plasma membrane–cell wall interactions (Botella et al., 1994). Chromosome and chromatin structure changes, for example, DNA methylation, polyploidization,

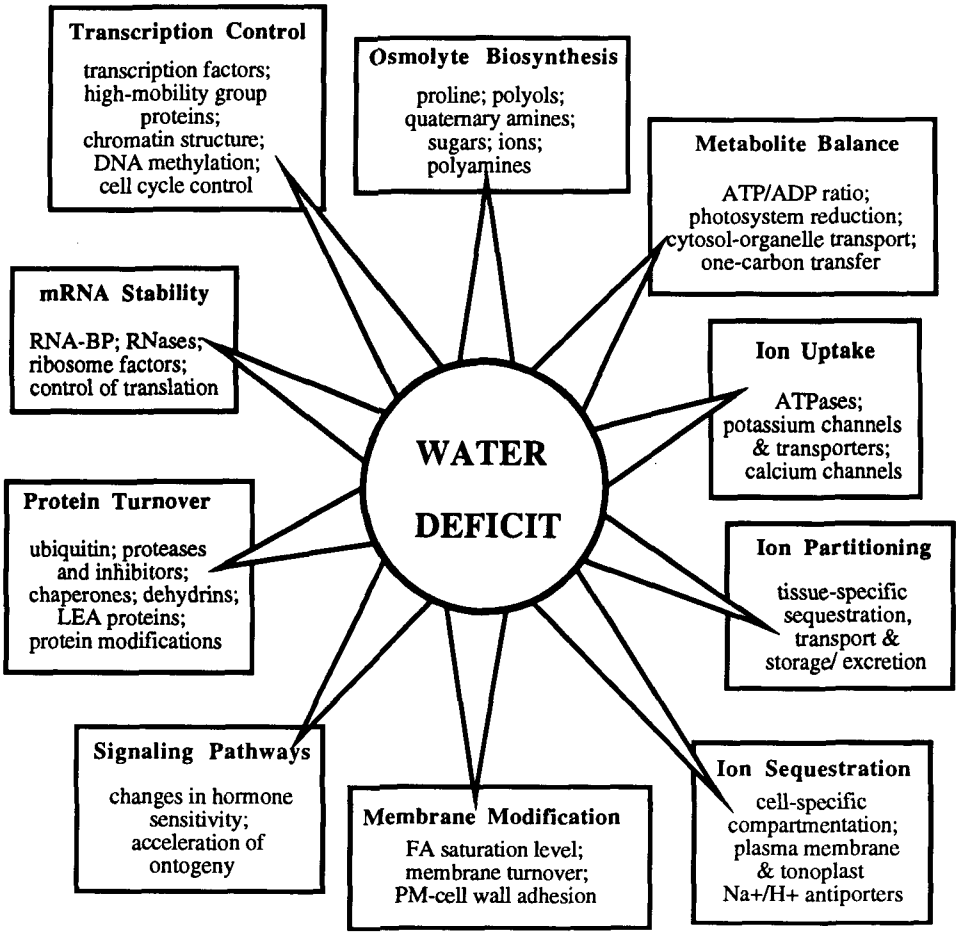

Figure 2. Organismal Responses to Water Deficit.

Under stress from water, many cellular processes change. These changes allow the plant to maintain metabolism and restore conditions that allow for continued growth under stress. Modified from Bray (1993).

amplification of specific sequences, or DNA elimination (Walbot and Cullis, 1985), would also be high complexity and result in developmental changes. Higher complexity mechanisms, such as increased water-use efficiency, would be more difficult to engineer, considering the plethora of changes necessary in different biochemical pathways to cope with altered stomatal conductance and photosynthesis.

The processes that are affected by drought, high salinity, and low temperature in all plants (Figure 2) are those that are utilized by stress-tolerant plants in coping most efficiently with water deficit. This list would likely be very similar for stresses not primarily caused by water deficit, such as nutrient excess or deficiency, heat shock, anaerobiosis, or flooding. They could be perceived as very similar disturbances in, for example, ion balance. Many components involved in the initial perception and transduction could be similar, but they could diverge at the metabolic level, where specific responses are elicited. This may be seen from the analysis of several stress-responsive promoters. Regions in promoters have been identified that distinguish different types of stress (Wilhelm and Thomashow, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993, 1994; Baker et al., 1994).

TESTING HYPOTHESES IN TRANSGENIC ORGANISMS

Mature fields in science work with a set of theories that allows predictions and serves as guidelines for applications. Rather than adding more phenomenological and anecdotal evidence to an already impressive body of observations concerning environmental stress tolerance, it seems appropriate to begin engineering traits associated with tolerance. There are several

traits whose correlative association with tolerance can be tested.

One of these traits is radical scavenging during stress. That engineering of radical scavenging may improve tolerance is based on the observation of increased oxygen radical production in the light under water stress conditions (Foyer, 1994). Superoxide dismutases (SODs) are found in all aerobic organisms to detoxify active oxygen species. Different isoforms of SOD exist in the cytosol, chloroplasts, and mitochondria. The expression of some forms is dramatically induced during stress conditions in *Nicotiana plumbaginifolia* (Bowler et al., 1991; Tsang et al., 1991). Transgenic tobacco plants that overexpress SOD are more tolerant than untransformed controls to a superoxide-generating herbicide and to ozone (Herouart et al., 1993).

Another trait that could be manipulated is acquisition or enhancement of thermotolerance (O'Connell, 1994). Heat shock, characterized by responses that are mediated at the level of transcription (Czarnecka-Verner et al., 1994), leads to the synthesis of heat shock proteins (HSPs), while synthesis of most "normal" proteins is suspended. The HSPs include chaperones targeted to different organelles with functions in protein folding and, possibly, in marking incorrectly folded proteins for turnover (Vierling, 1991). That HSPs are involved in thermotolerance acquisition may be deduced from a recent report indicating that the Arabidopsis HSP100 cognate can complement a heat shock–sensitive yeast mutant lacking HSP100 to allow survival at elevated temperature (Schirmer et al., 1994).

Low temperature and freezing, though distinct stressors, share a common factor: both may compromise membrane integrity (Guy, 1990; Thomashow, 1990). Changing membrane fluidity using plant and bacterial fatty acid desaturases has been tested in engineered plants (Murata et al., 1992; Wolter et al., 1992). By increasing the levels of desaturated lipids, decreased chilling sensitivity was observed; conversely, higher susceptibility to chilling was obtained by increasing the amounts of 16:0 fatty acids. These results have been challenged by the observation of a chilling-tolerant Arabidopsis mutant with a higher content of high–melting-point fatty acids than that found in most chilling-sensitive plants (Wu and Browse, 1995), probably indicating that there are multiple factors involved in chilling sensitivity. Freezing stress also leads to oxygen radical production, because the light-harvesting reactions continue to function, while biochemical reactions are severely restricted. Overexpression of a SOD gene in alfalfa has been shown to ameliorate oxygen radical stress and protect against injury caused by freezing (McKersie et al., 1993).

Osmolyte production is yet another potential stress-protection mechanism in transgenic plants. We have chosen to test polyol functions by introducing the bacterial *mtlD* gene into tobacco and Arabidopsis, neither of which normally synthesizes mannitol (Tarczynski et al., 1992; Thomas et al., 1995). Expression of the *mtlD* gene led to mannitol accumulation in transformed plants. Further investigation of transgenic plants indicates that mannitol provides some protection when plants are exposed to high salinity, although not at all times during development (Tarczynski et al., 1993). Whereas young plants die when stressed by half seawater, plants that have developed source leaves for carbohydrate export survive such stress treatment. The relatively low levels of polyols observed, 5 to 10 mM in total cell water of these transgenic tobacco plants, may indicate that protection is not proportional to the accumulated amount but that polyols might exert specific protective effects even at low concentrations. We have shown recently that seeds of Arabidopsis plants that contain and express the *mtlD* gene germinate more readily on media containing NaCl (ranging from 100 to 400 mM) than do wild-type seeds (Thomas et al., 1995), but that prolonged stress is not tolerated. The mechanisms by which even low levels of mannitol could provide tolerance are not understood. Mannitol and possibly polyols in general, which make up a considerable percentage of all assimilated CO_2 (Bieleski, 1982), could serve several functions: as compatible solutes, as low–molecular weight chaperones, or as scavengers of stress-induced oxygen radicals (Smirnoff and Cumbes, 1989). In these functions, they could limit injury to macromolecular complexes, membranes, or processes such as photosynthesis. Similarly, the engineered accumulation of fructans in tobacco leads to enhanced performance under drought stress (Pilon-Smits et al., 1995), but, as for polyols, the mechanisms that allow these molecules at least temporarily to sustain growth under stress are not known.

In these experiments, the underlying rationale has been to test hypotheses that were based on physiological observations through transgenic modifications of biosynthetic and metabolic pathways. The results indicate that higher stress tolerance may be achieved by engineering, that tolerance is only marginally increased by the transfer of a single trait, and that we must therefore utilize multiple mechanisms to engineer water stress tolerance.

THE PATH FORWARD

Plant stress sensitivity and tolerance have been difficult to dissect mechanistically in the past because of the many unknown genes involved, a lack of models (or the use of too many different models), and the inability to separate clearly development and stress responses. These three difficulties are, we believe, about to be solved. First, Arabidopsis and yeast, in particular, have become superior sources for genes that subsequently can be obtained from other model systems. Progress has been made in dissecting the genetic basis of salinity and drought stress tolerance in plants such as Arabidopsis and wheat (Saleki et al., 1993; Dvorak et al., 1994). *Ceratopteris richardii*, a fern with a haploid growth phase, could provide an additional, particularly effective genetic system (Hickok et al., 1991), and it has already been utilized to screen for components involved in salinity tolerance. Second, the many advantages of yeast as a genetic and expression system include physiological responses to abiotic stresses that are similar to those of plants.

Craterostigma, Atriplex, and ice plant, although they are not amenable to genetic analysis, could be xerophytic and halophytic models for biochemical analysis and could serve as a source for genes. Third, the use of Arabidopsis mutants will soon provide a rough blueprint of plant development. This blueprint can be used to delineate stress responses from developmental programs in other species.

In addition, the study of stress responses will enhance our knowledge of basic plant biochemistry. By studying how plants respond to severe stresses, we learn more about metabolism, its flexibility, its limits, and its diversity. Gene cloning and the attendant descriptions of cell-specific, tissue-specific, and developmental stage–specific expression of stress-responsive genes have built an invaluable resource. Tools are available to map, characterize, and identify genes whose functions or cellular locations are increasingly easier to identify from the analysis of mutants. As the molecular foundations of long-known physiological observations become known, we begin to understand the biochemical meaning and significance of genes that additively govern environmental stress tolerance.

A shift is imminent in the emphasis of our approaches to understand stress tolerance. What has emerged through molecular, genetic, and biochemical studies indicates that a limited number of mechanisms are involved in tolerance acquisition, although the number of important genes still exceeds the resolving power of genetic screens. Equally important, in glycophytes, halophytes, and xerophytes alike, very similar stress tolerance mechanisms have evolved that are based on a limited number of principles. The relative importance of these mechanisms, their biochemical detail, and their synergy can be tested in transgenic plants. Future work must include more emphasis on biochemical analysis of cloned genes. Also, a better design of inducible and cell-, tissue-, and stage-specific expression of transgenes must evolve. What has been learned from the recent experiments after transfer of single mechanisms indicates that multiple gene transfers, which are possible, must be a next step to exploit fully the knowledge accumulated through physiological studies.

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