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BOTANICAL BRIEFING

Metabolic Engineering for Stress Tolerance: Installing Osmoprotectant Synthesis Pathways

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Abiotic environmental stresses such as drought, salinity and low temperature are major limitations for plant growth and crop productivity. Certain plants, marine algae and bacteria have evolved a number of adaptations to such abiotic stresses: some of these adaptations are metabolic and others structural. Accumulation of certain organic solutes (known as osmoprotectants) is a common metabolic adaptation found in diverse taxa. These solutes protect proteins and membranes against damage by high concentrations of inorganic ions. Some osmoprotectants also protect the metabolic machinery against oxidative damage. Many major crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress-tolerant organisms. Therefore, it was hypothesized that installing osmoprotectant synthesis pathways is a potential route to breed stress-tolerant crops. Proving this, recent engineering efforts in model species led to modest but significant improvements in stress tolerance of transgenic plants. Synthetic pathways to two kinds of osmoprotectants—polyols and quaternary ammonium compounds—are discussed here. Results from the metabolic engineering experiments emphasize the need for a greater understanding of primary metabolic pathways from which osmoprotectant synthesis pathways branch. Future research avenues include the identification and exploitation of diverse osmoprotectants in naturally stress-tolerant organisms, and the use of multiple genes and reiterative engineering to increase osmoprotectant flux in response to stress. High-throughput genomic technologies offer a number of tools to refine this by rapidly identifying genes, pathways, and regulatory controls.
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Key words: Review, abiotic stress, osmoprotectant, compatible solute, genetic engineering.

INTRODUCTION

Abiotic stress factors such as drought, salinity and extremes of temperature have long been known as major limitations to crop productivity (Boyer, 1982). Organisms that currently live in habitats where these factors predominate have evolved various adaptations to these stresses. One approach to improve stress tolerance in crops would be to transfer the genes for these adaptive traits from the tolerant organism to the crop. However, this process has not been successful using conventional means (see Yeo and Flowers, 1989), partly because the traits are poorly described genetically and partly because of the transfer of unwanted genes during conventional crossing. Genetic transformation technology enables us to achieve gene transfer in a precise and, to some extent, predictable manner. Metabolic traits, especially pathways with few enzymes, are better characterized genetically and more amenable to such manipulations than structural and developmental traits. This Botanical Briefing reviews the potential for the metabolic engineering of pathways that result in the synthesis of osmoprotectants in plants.

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OSMOPROTECTANTS

Certain plants, marine algae, bacteria and other organisms accumulate organic solutes such as sugar alcohols, the amino acid proline, quaternary ammonium and or tertiary sulphonium compounds in response to osmotic stress (Yancey *et al.*, 1982). These compounds are termed compatible solutes (Johnson *et al.*, 1968) because even in high concentrations they do not inhibit the activity of enzymes. They also protect enzymes and membranes against deleterious effects of destabilizing ions such as Na^+ and Cl^- (Yancey *et al.*, 1982). Accumulation of compatible solutes in response to stress is a metabolic adaptation found in a number of stress-tolerant, often unrelated taxa, suggesting convergent evolution for this trait (Wyn Jones and Storey, 1981; Yancey *et al.*, 1982; Rhodes and Hanson, 1993). Although many osmoprotectant compounds confer stress protection in bacteria, marine algae, animal cells and plants, their synthetic pathways often differ in terms of enzymes and steps. Structures of representative osmoprotectants accumulated by stress-tolerant plants are shown in Fig. 1. The osmoprotectant is synthesized in response to the stress and is localized in the cytoplasm; inorganic ions such as Na^+ and Cl^- are preferentially sequestered into the vacuole (Flowers *et al.*, 1977, 1986; Bohnert *et al.*, 1995; Glenn *et al.*, 1999). Thus, this leads to turgor maintenance for the cell under osmotic stress.

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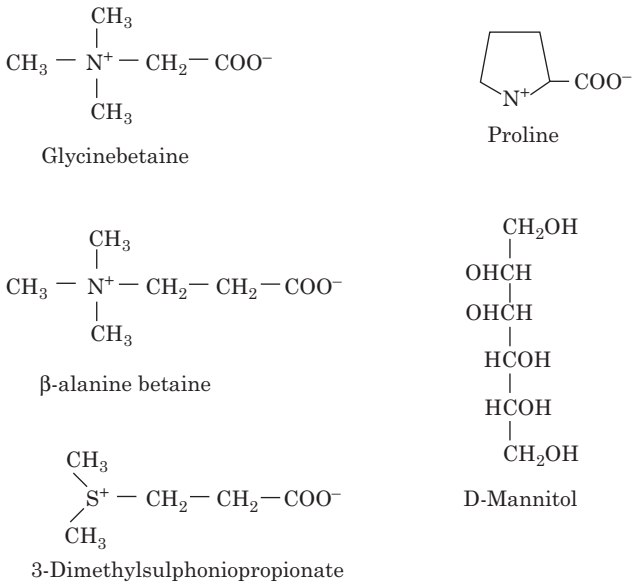

FIG. 1. Structures of representative osmoprotectants accumulated by stress-tolerant plants.

METABOLIC ENGINEERING

Metabolic engineering is the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, with the use of recombinant DNA technology (Stephanopoulos, 1999). Some of the metabolic adaptations to stress have been manipulated in model plant species using metabolic engineering. Table 1 lists examples where the expression of a transgene resulted in changes in the synthesis of an osmoprotectant and claims for a stress-tolerant phenotype. However, only a few of these studies looked thoroughly at the consequences of these manipulations on the phenotype. Nevertheless, further increases in levels of stress tolerance in both model and crop species can be expected in the future following reiterative manipulations of multiple transgenes, guided by a thorough analysis of the transgenic plants obtained so far.

INSTALLING OSMOPROTECTANT SYNTHESIS PATHWAYS

Work relating to two osmoprotectant classes—the polyols and the quaternary ammonium compounds—is reviewed here. Some generalizations can be made: firstly, the availability of the precursor to synthesize the osmoprotectant could limit the amount of osmoprotectant made in a transgenic host. Secondly, negative physiological consequences of diverting the precursor to the osmoprotectant away from primary metabolism should be considered. Thirdly, despite the availability of physiological data and techniques for assessing stress tolerance in plants, transgenic plants have only rarely been subjected to rigorous assessments of their stress-tolerant phenotype following osmoprotectant engineering.

POLYOL BIOSYNTHESIS

Polyols such as glycerol, mannitol, sorbitol and sucrose are osmoprotectants in algae, certain halophytic plants and insects exposed to freezing (Yancey *et al*., 1982). Figure 2 illustrates polyol synthesis. *Myo*-inositol derived from glucose-6-phosphate serves as a precursor to a number of metabolites, pools of which turn over slowly in the cell and which are related to membrane biogenesis, cell signalling and stress protection (Loewus and Murthy, 1999). Mannitol is synthesized by the action of NADPH-dependent mannitol 1-phosphate dehydrogenase from fructose 6-phosphate (Fig. 2). When expressed in transgenic tobacco and *Arabidopsis*, a gene encoding mannitol 1-phosphate dehydrogenase (*mtlD*) from *Escherichia coli* resulted in mannitol production and a salinity-tolerant phenotype (Tarczynski *et al*., 1993; Thomas *et al*., 1995). Further

TABLE 1. *Stress-tolerant plants obtained following expression of a transgene*

Abiotic stress factors	Engineered species	Reference
Chilling	*Nicotiana tabacum*	Murata *et al*., 1992
Salinity	*Nicotiana tabacum*	Tarczynski *et al*., 1993
Cold	*Nicotiana tabacum*	Kodama *et al*., 1994
Salinity	*Nicotiana tabacum*	Kishor *et al*., 1995
Drought	*Nicotiana tabacum*	Pilon-Smits *et al*., 1995
Drought and salinity	*Oryza sativa*	Xu *et al*., 1996
Salinity	*Nicotiana tabacum*	Lilius *et al*., 1996
Oxidative stress	*Nicotiana tabacum*	Shen *et al*., 1997*a*
Salinity and cold	*Arabidopsis thaliana*	Hayashi *et al*., 1997
Salinity and drought	*Nicotiana tabacum*	Sheveleva *et al*., 1997
Chilling and salinity	*Nicotiana tabacum*	Roxas *et al*., 1997
Freezing	*Arabidopsis thaliana*	Jaglo-Ottosen *et al*., 1998
Salinity and cold	*Oryza sativa*	Sakamoto *et al*., 1998
High temperature	*Arabidopsis thaliana*	Alia *et al*., 1998*b*
Drought, salt and freezing	*Arabidopsis thaliana*	Kasuga *et al*., 1999
Heavy metal stress	*Brassica juncea*	Zhu *et al*., 1999
Salinity	*Arabidopsis thaliana*	Apse *et al*., 1999
Salinity	*Medicago sativa*	Winicov and Bastola, 1999
Salinity, drought, low temperature	*B. napus*, *A. thaliana*, *N. tabacum*	Huang *et al*., 2000

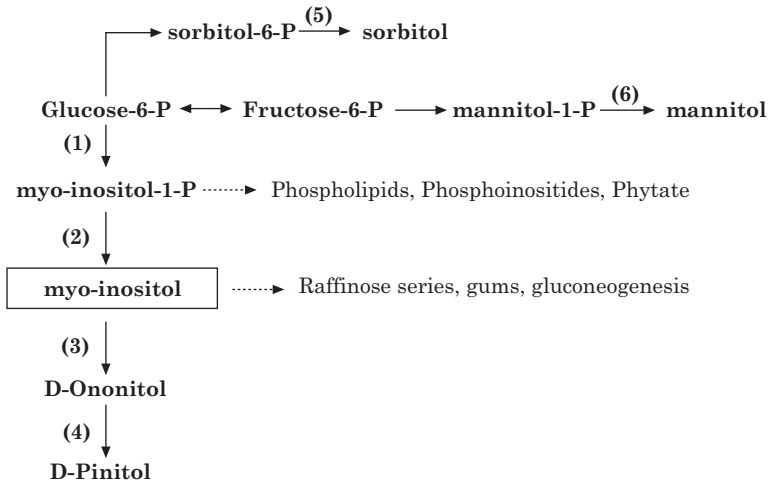

FIG. 2. *myo*-Inositol and polyol biosynthesis. Solid arrows indicate enzyme-catalysed steps. Enzymes discussed in the text are numbered (1) to (6). (1) *myo*-inositol-1-phosphate synthase; (2) *myo*-inositol-1-phosphate phosphatase; (3) *myo*-inositol *O*-methyltransferase; (4) *D*-ononitol epimerase; (5) sorbitol-6-phosphate dehydrogenase; and (6) mannitol-1-phosphate dehydrogenase. (3) and (4) are unique to the iceplant.

work suggested that mannitol only contributed to 30–40 % of the change in osmotic potential in transgenic plants (Karakas *et al*., 1997). Rather, the stress-tolerant phenotype is due to protection by mannitol against oxidation by hydroxyl radicals (Shen *et al*., 1997*a*, *b*). A recent investigation in yeast concluded that polyols may have a dual function in stress protection, both by facilitating osmotic adjustment and by supporting redox control (Shen *et al*., 1999). By over-expressing apple cDNA for sorbitol 6-phosphate dehydrogenase, sorbitol-accumulating transgenic tobacco plants have been obtained (Sheveleva *et al*., 1998). Those transgenic plants accumulating high levels of sorbitol (>15 μmol g^{-1} f. wt) exhibited growth defects and necrotic lesions, presumably due to depletion of the *myo*-inositol pool (Sheveleva *et al*., 1998).

Certain stress-tolerant plants accumulate specialized polyols (cyclitols) which may provide better stress protection than that provided by mannitol or sorbitol accumulation. The genes for the synthesis of these cyclitols and their regulation are therefore interesting for engineering crops for stress tolerance. An elegant series of studies on *myo*-inositol metabolism in a halophyte, common iceplant (*Mesembryanthemum crystallinum*), showed that *myo*-inositol is converted to the osmoprotectants *D*-ononitol and *D*-pinitol (Fig. 2) by a two-step pathway and this pathway is regulated by stress (Vernon and Bohnert, 1992; Adams *et al*., 1992; Rammesmayer *et al*., 1995; Nelson *et al*., 1998). The committing step to *myo*-inositol production, catalysed by *myo*-inositol 1-phosphate synthase, is induced by salinity (Ishitani *et al*., 1996). This enzyme is expressed in leaves of the iceplant but repressed in its roots (Nelson *et al*., 1998). Therefore, metabolic engineering of a glycophyte for the production of cyclitols could best be achieved by tissue-specific stress-inducible expression of at least three enzymes from the common iceplant: *myo*-inositol 1-phosphate synthase, *myo*-inositol *O*-methyltransferase and *D*-ononitol epimerase. However, stress-inducible synthesis of *D*-ononitol in transgenic tobacco was achieved by expressing only *myo*-inositol *O*-methyltransferase from the iceplant since *myo*-inositol synthesis in tobacco was stress-inducible. These transgenic plants exhibited salt and drought tolerance (Sheveleva *et al*., 1997).

GLYCINEBETAINE SYNTHESIS

Quaternary ammonium compounds such as glycine betaine, proline betaine, β-alanine betaine, choline *O*-sulphate and the tertiary sulphonium compound dimethylsulphoniopropionate are effective osmoprotectants widely distributed in bacteria, marine algae and many plant families (Rhodes and Hanson, 1993; Gorham, 1995; Gage and Rathinasabapathi, 1999). Glycine betaine is synthesized by a two-step oxidation of choline via betaine aldehyde. Choline has a vital role as the precursor for phosphatidylcholine, a dominant constituent of membrane phospholipids in eukaryotes. Despite this, a large proportion of free choline is diverted to glycine betaine in plants that naturally accumulate glycine betaine in response to stress. In the enteric bacterium, *Escherichia coli*, choline is oxidized by a membrane-bound choline dehydrogenase to betaine aldehyde, which in turn is oxidized to glycine betaine by a soluble betaine aldehyde dehydrogenase (Andersen *et al*., 1988). In certain soil bacteria, choline is oxidized by choline oxidase, a soluble flavo-enzyme that generates hydrogen peroxide during the reaction (Ohta-Fukuyama *et al*., 1980). The plant pathway for choline oxidation in plants is also via betaine aldehyde but involves different enzymes. In spinach and sugarbeet, choline oxidation to betaine aldehyde is catalysed by choline monooxygenase, an iron-sulphur enzyme (Burnet *et al*., 1995; Rathinasabapathi *et al*., 1997). Betaine aldehyde oxidation to glycine betaine is catalysed by betaine aldehyde dehydrogenase, a non-specific aldehyde dehydrogenase (Trossat *et al*., 1997; Vojtechova *et al*., 1997). Both these enzymes are stress-inducible stromal enzymes (McCue and Hanson, 1992; Russell *et al*., 1998). The synthetic routes to choline and glycine betaine as known in spinach are shown in Fig. 3.

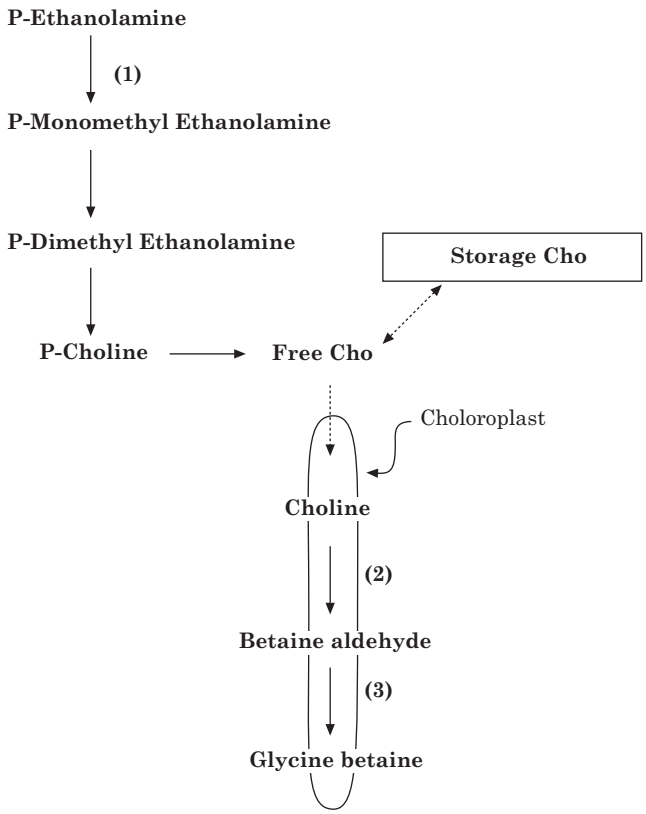

FIG. 3. Inter-relationships and compartmentation of choline and glycine betaine synthesis in spinach. Only phospho-base (P) route is shown for choline synthesis. The major fate of P-choline in all plants is to phosphatidyl choline (not shown) which also leads to free choline (see McNeil *et al.*, 2000). Solid arrows represent enzyme catalysed steps. Enzymes discussed in the text are numbered (1) to (3). (1) *S*-Adenosyl L-methionine: Phosphoethanolamine *N*–methyltransferase; (2) Choline monooxygenase; and (3) Betaine aldehyde dehydrogenase.

When genes for choline-oxidizing enzymes from *E. coli* and *Arthrobacter* sp. are expressed in microbial models lacking glycine betaine synthetic ability, the transgenic organisms synthesized glycine betaine from exogenous choline and this conferred osmotolerance (Andersen *et al.*, 1988; Rozwadowski *et al.*, 1991; Deshinium *et al.*, 1995, 1997; Nomura *et al.*, 1995). Microbial choline-oxidizing enzymes have also been expressed in transgenic tobacco and *Arabidopsis thaliana*—two species that do not naturally accumulate glycine betaine. Lilius *et al.* (1996) expressed choline dehydrogenase from *E. coli* in transgenic tobacco. Despite an apparent stress-tolerant phenotype, glycine betaine synthesis was not confirmed (Lilius *et al.*, 1996). A transgenic potato line expressing bacterial choline dehydrogenase produced small amounts of glycine betaine—about 100 nmol g^{-1} f. wt—when choline was supplied in the medium (Holmberg, 1996). This level is an order of magnitude less than that observed in natural accumulators and is osmotically insignificant. Low levels of glycine betaine could be due to poor expression of the transgene in this case or poor availability of the substrate. These alternatives were not clarified in this study (Holmberg, 1996). When choline oxidase from *Arthrobacter* was expressed in transgenic *A. thaliana*, targeting the enzyme into the stroma, about 1 μmol g^{-1} f. wt glycine betaine was measured (Hayashi *et al.*, 1997, 1998). The transgenic plants exhibited salinity and temperature stress tolerance (Alia *et al.*, 1998*a*, *b*). Similar results were also obtained in rice (Hayashi *et al.*, 1997). Although choline oxidase action is also expected to produce hydrogen peroxide, its levels were similar in wild-type and transformed plants (Hayashi *et al.*, 1997).

Expression of spinach choline monooxygenase in transgenic tobacco resulted in transgenic plants with high levels of this enzyme (Nuccio *et al.*, 1998). Since tobacco has some endogenous betaine aldehyde dehydrogenase activity, addition of choline monooxygenase alone can be expected to result in glycine betaine synthesis. Transgenic tobacco expressing spinach choline monoxygenase in the chloroplasts synthesized only very low levels (0·02 to 0·05 μmol g^{-1} f. wt) of glycine betaine in both unstressed and stressed conditions (Nuccio *et al.*, 1998). Exogenous choline and choline precursors mono- and dimethylethanolomine enhanced glycine betaine levels but ethanolomine did not, suggesting that choline synthesis in tobacco is limiting at the first methylation of ethanolomine (Fig. 3). Accordingly, extractable activity for the enzyme methylating phospho-ethanolomine was much less in tobacco than that found in spinach. This suggests that glycine betaine accumulators and non-accumulators differ in choline synthesis and its regulation. Hence increased synthesis of both choline and glycine betaine needs to be engineered, preferably under stress-inducible expression elements, to achieve a transgenic plant accumulating

glycine betaine. Similar conclusions were reached by Huang *et al.* (2000) who expressed a microbial choline oxidase in tobacco, *A. thaliana* and *Brassica napus* and achieved 1–2 μmol g^{-1} f. wt glycine betaine. Upon choline supplementation these transgenic plants made substantially more glycine betaine. In this regard *A. thaliana* and *B. napus* accumulated much more glycine betaine than tobacco, suggesting that the rigidity in choline availability is variable among different species. Recent work points out that the activity and regulation of phosphoethanolamine methylation is the limiting factor in choline synthesis in tobacco (McNeil *et al.*, 2000; Nuccio *et al.*, 2000). It is also possible that choline transport into the chloroplast differs between species (Nuccio *et al.*, 1998; Huang *et al.*, 2000).

Rigidity in choline utilization or availability is not uncommon in plant metabolism. In the evolution of the stress-tolerant plant family Plumbaginaceae, most members replaced glycine betaine with other equally effective quaternary ammonium compounds such as β-alanine betaine, proline betaine and hydroxy proline betaine (Hanson *et al.*, 1994). Part of the reason for this 'invention' appears to be metabolic, since all members of this family synthesize choline *O*-sulphate, an osmoprotectant important for sulphate detoxification (Rivoal and Hanson, 1994). Competition for choline could therefore be relieved by replacing glycine betaine by β-alanine betaine, proline betaine and hydroxy proline betaine, osmoprotectants originating from precursors β-alanine and proline (Hanson *et al.*, 1994). Other osmoprotectant synthetic pathways manipulated to obtain transgenic plants include those of proline (Kishor *et al.*, 1995; Nanjo *et al.*, 1999), fructan (Pilon-Smits *et al.*, 1995) and trehalose (Holmstrom *et al.*, 1996; Goddijn *et al.*, 1997; Romero *et al.*, 1997). Identification of enzymes and genes involved in the synthesis of novel osmoprotectants found in stress-tolerant organisms can be expected to provide more such opportunities for stress tolerance engineering (Rivoal and Hanson, 1994; Rathinasabapathi *et al.*, 2000).

ENGINEERING STRESS TOLERANCE

Osmoprotectant accumulation is only one facet of a myriad of stress-tolerant traits found in nature. Since oxidative stress is a component of drought and salinity, manipulations aimed at improving oxidative stress tolerance have also resulted in salinity tolerance (Roxas *et al.*, 1997). Some of the traits, when engineered together with osmoprotectant synthesis, can be expected to enhance whole plant stress tolerance. This could be done either via reiterative engineering or by crossing and selecting transgenic plants engineered for different traits. For example, manipulation of genes involved in ion transport together with osmoprotectant synthesis can be expected to increase a cell's ability to withstand salinity stress. The gene products involved in ion homeostasis have been identified by the use of yeast model systems (Serrano *et al.*, 1999) and by analysing mutants altered for salt sensitivity (Wu *et al.*, 1996; Liu *et al.*, 2000). This has led to identification of plant genes involved in ion transport and compartmentation, inspiring effective strategies for engineering salinity tolerant plants. Halophytic plants are capable of salt accumulation at the vacuolar compartment (Flowers and Yeo, 1988). In response to salinity, halophytes accumulate Na^+ into vacuoles, through the operation of a tonoplast Na^+/H^+ antiport. This avoids deleterious effects of Na^+ in the cytosol and maintains osmotic balance by using the ions accumulated in the vacuole (Glenn *et al.*, 1999). Chloride transport into vacuoles is via a chloride channel (Hechenberger *et al.*, 1996). Over-expression of a tonoplast Na^+/H^+ antiport protein in transgenic *Arabidopsis thaliana* resulted in increases in vacuolar sodium concentrations (Apse *et al.*, 1999). These transgenic plants were sodium chloride tolerant (Apse *et al.*, 1999). Understanding the function and regulation of other genes involved in water and ion transport (Chrispeels *et al.*, 1999) can be expected to provide important tools for engineering salinity and osmotic stress tolerance in plants.

REGULATING STRESS TOLERANCE

Osmoprotectant synthesis in naturally stress-tolerant species is highly regulated by stress. In addition to the use of stress inducible promoters for engineering osmoprotectant synthesis pathways, genes involved in stress signal sensing are additionally useful for engineering stress tolerant plants. Genes involved in stress signal sensing and a stress-signalling cascade in *A. thaliana* have therefore been of recent research interest (Winicov, 1998; Shinozaki and Yamaguchi-Shinozaki, 1999 for reviews). Expression of some of the genes in the stress signal transduction cascade is mediated by the plant growth regulator abscisic acid (ABA); others act independently of ABA. Components of the same signal transduction pathway are also shared by various stress factors such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki, 1999). By expressing a regulatory gene that could induce a number of other genes involved in stress-tolerance, transgenic plants with a stress-tolerant phenotype were achieved. For example, stress-inducible expression of the transcription factor DREB1A in transgenic *A. thaliana* resulted in improved drought, salt and freezing tolerance (Kasuga *et al.*, 1999). Based on homology in stress-signalling between yeast and plants, Pardo *et al.* (1998) achieved stress-tolerant transgenic plants by over-expressing calcineurin, a protein phosphatase known to be involved in salt-stress signal transduction in yeast. Transcription factors involved in regulating the expression of genes involved in osmoprotectant synthesis will be useful for metabolic engineering.

METABOLIC ENGINEER'S TOOL BOX

In naturally stress-tolerant plants there is a wide variety of adaptations to stress, many of which have not yet been identified at the molecular level. Understanding the function of such genes determining these factors will improve our understanding of the complexity of plant metabolism and may provide unique opportunities for the metabolic engineer. Progress made in understanding the

function of genes involved in stress tolerance in *A. thaliana*, not a naturally tolerant species, does, however, provide an important basis on which to analyse other plant genomes: the tools of 'functional genomics' (Bouchez and Hofte, 1998) will expedite this progress. However, since osmoprotectant synthetic pathways and other metabolic adaptations to stress are found in diverse taxa, a number of different model species need to be employed along with *Arabidopsis*. Microbial genes have often been used to engineer traits implicated in stress tolerance, but genes that have evolved in stress-tolerant plants and their regulation will be of special interest in the long term. Continued efforts in the identification and description of stress-tolerant taxa and physiological and molecular studies to understand their tolerance mechanisms are therefore justified.

Identification of regulatory genes and transcription factors involved in stress-inducible expression of osmoprotectant biosynthetic pathways will also be of great interest. Tools such as vectors for multiple gene transfer (e.g. Von Bodman *et al.*, 1995), stress-inducible promoters and efficient selectable markers will also need to be developed and evaluated. Following manipulations of enzyme-catalysed steps, it is imperative to verify whether the manipulation resulted in a change in the pathway flux, so that further rounds of useful manipulations could be predicted. Metabolic control analysis will be of use to determine flux control coefficients for manipulated enzymes and could provide strategies as to which steps/enzymes are the most interesting for manipulation (Stephanopoulus, 1999). Such applications depend upon developments in analytical tools for sensitive and accurate determination of metabolite pool sizes such as nuclear magnetic resonance, mass spectrometry and radiotracer technology combined with computer modelling.

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