19 Fischer, K. *et al.* (1994) The 24 kDa outer envelope membrane protein from spinach chloroplasts: molecular cloning, *in vivo* expression and import pathway of a protein with unusual properties, *Plant Mol. Biol.* 25, 167–177
20 Tranel, P.J. *et al.* (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway, *EMBO J.* 14, 2436–2446
21 Hirsch, S. *et al.* (1994) A receptor component of the chloroplast protein translocation machinery, *Science* 266, 1989–1992
22 Kessler, F. *et al.* (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery, *Science* 266, 1035–1039
23 Muckel, E. and Soll, J. (1996) A protein import receptor of chloroplasts is inserted into the outer envelope membrane by a novel pathway, *J. Biol. Chem.* 271, 23846–23852
24 Waegemann, K. and Soll, J. (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts, *Plant J.* 1, 149–158
25 Kerber, B. and Soll, J. (1992) Transfer of a chloroplast-bound precursor protein into the translocation apparatus is impaired after phospholipase C treatment, *FEBS Lett.* 306, 71–74
26 Lübeck, J. *et al.* (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane, *EMBO J.* 15, 4230–4238
27 Nielsen, E. *et al.* (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone, *EMBO J.* 16, 935–946
28 Theg, S.M. *et al.* (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes, *J. Biol. Chem.* 264, 6730–6736
29 Schnell, D.J., Kessler, F. and Blobel, G. (1994) Isolation of components of the chloroplast protein import machinery, *Science* 266, 1007–1012
30 Perry, S.E. and Keegstra, K. (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins, *Plant Cell* 6, 93–105
31 Hinnah, S. *et al.* (1998) Reconstitution of a protein translocation channel of chloroplasts, *EMBO J.* 16, 7351–7360
32 Ma, Y. *et al.* (1996) Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope, *J. Cell Biol.* 134, 315–327
33 Kourtz, L. and Ko, K. (1997) The early stage of chloroplast protein import involves Com70, *J. Biol. Chem.* 272, 32264–32271
34 Wu, C., Seibert, F.S. and Ko, K. (1994) Identification of chloroplast envelope proteins in close proximity to a partially translocated chimeric precursor protein, *J. Biol. Chem.* 269, 32264–32271
35 Kessler, F. and Blobel, G. (1996) Interaction of the protein import and folding machineries in the chloroplast, *Proc. Natl. Acad. Sci. U. S. A.* 93, 7684–7689
36 Caliebe, A. *et al.* (1998) The chloroplastic protein import machinery contains a Rieske type iron–sulphur cluster and mononuclear iron binding protein, *EMBO J.* 16, 7342–7350
37 Hamman, B.D. *et al.* (1997) The aqueous pore through the translocon has a diameter of 40–60 Å during cotranslational protein translocation at the ER membrane, *Cell* 89, 535–544
38 Siegel, V. (1997) Recognition of a transmembrane domain: another role for the ribosome? *Cell* 90, 5–8
39 Walker, D. *et al.* (1996) Ricin A chain fused to a chloroplast-targeting signal is unfolded on the chloroplast surface prior to import across the envelope membranes, *J. Biol. Chem.* 271, 4082–4085
40 Clark, S.A. and Theg, S.M. (1997) A folded protein can be transported across the chloroplast envelope and thylakoid membranes, *Mol. Biol. Cell* 8, 923–934

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# Improving stress tolerance in plants by gene transfer

Niklas Holmberg and Leif Bülow

**Plant productivity is greatly influenced by environmental stresses, such as freezing, drought, salinity and flooding. One of the ways in which tolerance to these factors can be achieved is by the transfer of genes encoding protective proteins or enzymes from other organisms. Key approaches currently being examined are engineered alterations in the amounts of osmolytes and osmoprotectants, saturation levels of membrane fatty acids, and rate of scavenging of reactive oxygen intermediates.**

As the earth's population increases, new means of improving crop productivity must be found to increase the resources available. One way of doing this is to develop crops that are more tolerant to such abiotic stresses as drought, flooding, heat, radiation, salinity, chilling and freezing (Fig. 1), so that new land can be brought under cultivation. The problem with traditional plant breeding for achieving this end is that it is time consuming and laborious; it is difficult to modify single traits; and it relies on existing genetic variability. However, genetic engineering can now be used as a relatively fast and precise means of achieving improved stress tolerance. Many organisms have evolved traits that enable them to survive in extreme environments, and thus the gene(s) that confer these properties can potentially be introduced into higher plants.

For several years, microorganisms have been used as models to characterize the properties of stress-induced genes and their products from a variety of organisms. Recently, however, attention has been directed towards higher plants, and there have been successful attempts to introduce stress-tolerance-conferring genes[1]. This review focuses on recent attempts to protect higher plants from abiotic stress by gene transfer.

## Responses to a changing environment

Organisms are continually exposed to environmental stresses that influence their development, growth and productivity. Stress-relieving genes that are transcribed might encode enzymes involved in a particular metabolic pathway, regulatory proteins or

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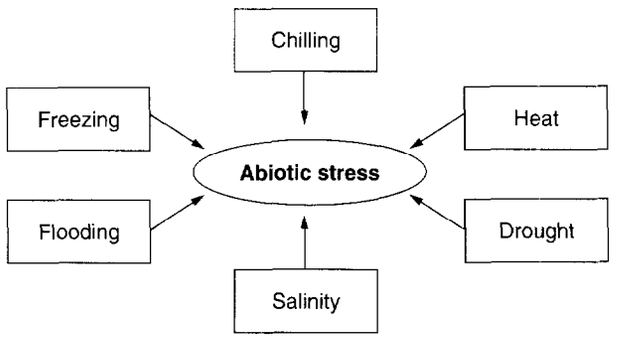


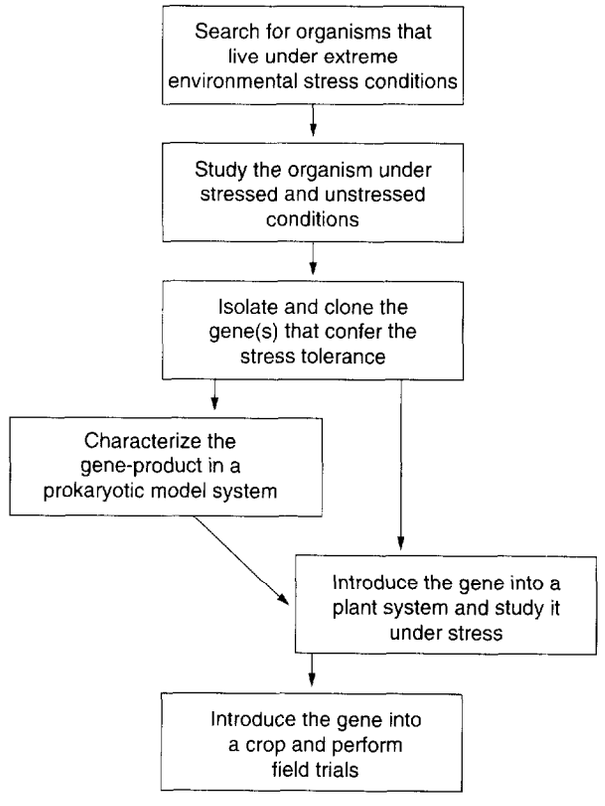

**Fig. 1.** Different environmental stress factors resulting in abiotic stress. Except flooding, they all induce osmotic stress.

proteins with specific protective properties. Stress responses enable the organism to adapt to an unfavourable situation – often by changing the metabolic flow.

With the exception of flooding, the major abiotic stresses all result in water-deficit stress. The cell membrane serves as an impermeable barrier to macromolecules and most low molecular mass substances. When the extracellular solute concentrations are altered or extracellular ice forms, there is a flux of water from the cells, causing a decrease in turgor and an increase in concentrations of intracellular solutes[2], putting a strain on membranes and macromolecules. Minor limitations in water availability cause a reduced photosynthetic rate, but further reductions lead to a complete inhibition of photosynthesis. Under conditions in which photosynthesis is impaired, but chloroplasts are exposed to excess excitation energy[3], there is photoreduction of oxygen and concomitant production of reactive oxygen intermediates, such as superoxides and peroxides, which damage membranes and enzymes[4].

There are several biochemical functions involved in the response of the plant cell to osmotic stress, such as ion exclusion, ion export, cell wall modifications, osmotic adjustments and osmoprotection[1]. Furthermore, plant cells contain antioxidant enzyme systems, such as peroxidases and superoxide dismutases, which scavenge reactive oxygen intermediates[4].

## Box 1. Strategy for creating a more stress-tolerant plant using genetic engineering[a]




[a]Note that there are few reports of transgenic crops evaluated in field trials under realistic stress conditions.

### Expressing foreign genes in plants: potential problems

The basic strategy of transferring an ability to respond to stress in a particular way into non-tolerant crops can be divided into six basic steps. These range from discovering a naturally occurring stress-tolerant organism to creating a stress-tolerant higher plant, and are described in Box 1. However, there are many obstacles to foreign gene expression in higher plants, and these are summarized in Box 2; the ability to overcome these obstacles is essential for creating stress-tolerant plants.

#### *Transformation*

Techniques for transferring foreign genes into the genomes of plants include electroporation, polyethylene glycol-mediated gene transfer, microinjection, particle bombardment and *Agrobacterium*-mediated gene transfer[5]. *A. tumefaciens*-based transformation is the most widely used system for introducing genes into dicotyledons (such as tobacco, potato and *Arabidopsis*), whereas particle bombardment is the most frequently used for monocotyledons (such as maize, wheat and rice)[6]. All the techniques carry certain problems.

#### *Expression systems*

When the concentration of an expressed protein is in direct relation to its biological activity, it is essential that the introduced

## Box 2. Potential pitfalls when expressing foreign genes in higher plants

- Transformation. Techniques for transforming the host plant are available in most cases, but there are exceptions.
- Adequate expression. Adequate expression of the gene is essential, with high expression at the right time.
- Cellular localization. Expression in specific tissues and organelles is often essential for achieving the desired results.
- Post-translational modifications. Correct processing and folding are often prerequisites for function.
- Prosthetic-group acquisition. Limitations in prosthetic group acquisition may inactivate foreign enzymes.
- Precursor availability. Precursor shortage will place limitations on product formation.
- Inhibitory environments. Suboptimal enzyme activity may be because of abnormal pH, temperature or salt concentrations.
- Side-reactions of new compounds. Endogenous enzyme activities may deplete the product pool or form toxic compounds.

gene is expressed in high amounts (although this problem may not be acute with enzymes if the catalytic turnover is sufficiently high). In order to accomplish high expression rates, a strong, constitutive promoter is needed. The CaMV 35S promoter drives high expression throughout the plant[7] and has a broad host range – for example, it has been used in tobacco, potato, alfalfa, *Arabidopsis* and rice. More-specific promoters are needed in order to direct expression to a particular tissue. For example, the promoter derived from the *Arabidopsis* Rubisco small subunit gene, *ats1A*, only drives expression in green tissues[8]. Other types are the granule-bound starch synthase *G28* promoter[9] and patatin type I promoter[10], which direct gene expression specifically to potato tubers. Different plant promoters induced by drought, cold, salinity, abscisic acid (ABA) and anoxia have been used to drive the expression of the reporter gene *GUS* in transgenic plants. For example, the osmotin promoter[11] responds to salinity, drought and ABA, and the glyceraldehyde-3-phosphate dehydrogenase 4 promoter[12] responds to anoxia. These promoters should be ideal for limiting expression to the precise moment when the stress-relieving gene is needed.

An intriguing way to achieve high expression is to use scaffold attachment regions (SARs). These regions have been shown to increase expression by >100-fold per copy in tobacco cells[13]. The SARs may act by forming loops in the chromatin structure, thereby reducing the severity or likelihood of gene silencing in cells containing a limited number of transgenes. However, high amounts of the mRNA of a particular gene do not guarantee corresponding amounts of the product: problems with codon usage, translational regulation and post-translational modification may limit yield.

*Cellular localization*

A first sign of abiotic stress is often injury to specific membranes. Chilling and freezing affect the membrane fluidity, and perturb membrane-bound processes such as the photosynthetic apparatus. The formation of reactive oxygen intermediates, a secondary effect of stress, also damages membranes. In this latter case, chloroplast membranes are especially affected because they are the source of oxygen-radical production. Damage to vital membranes and membrane-bound proteins involved in energy-producing processes subsequently influences the metabolism of the whole plant. In many cases, it may be desirable to locate membrane-modifying, osmoprotectant-producing, or radical-scavenging enzymes to defined organelles. Transit peptides are therefore often necessary to ensure correct localization of the enzymes expressed. A typical transit sequence used to direct polypeptides to chloroplasts is from the small subunit of Rubisco[13,14].

*Proteolysis, protein folding and prosthetic group acquisition*

There is a risk of proteolytic degradation when expressing short polypeptides in plants. This problem can be overcome by fusing the polypeptide to another protein, but this risks changing the biological activity of the original polypeptide. One such fusion partner is *Staphyloccocal* protein A, or domains thereof. The Spa domain of protein A has been used when expressing antifreeze proteins from fish in plants[15]. An additional advantage of using protein A as a fusion partner is the potential to detect and purify the expressed protein using IgG antibodies.

There have been no attempts to facilitate folding in transgenic plants by co-expressing chaperone proteins. However, experiments with microorganisms suggest that this might be necessary for the expression of large enzymes that contain multiple disulphide bridges.

Oxygen-radical-scavenging enzymes are usually dependent on metal-containing prosthetic groups; hence, the local availability of metals such as iron, zinc, copper or manganese may be prerequisite for functional activity. Metals are also vital parts of the porphyrin groups of the light-harvesting pigments, and thus competition for these compounds could occur if they are not present in excess.

*Precursor availability*

The amount of precursor available is critical when introducing an enzyme that catalyses the production of osmoprotectants and osmolytes such as glycine betaine, proline, mannitol or trehalose. These compounds have some effects at millimolar concentrations, but optimum protection may not be reached until molar concentrations are present. The precursor can either be extracted from the environment or be synthesized in the cell via existing metabolic pathways. Hence, it is desirable that enzyme systems that catalyse precursor formation are under feedback control, so that the pool of precursor will be replenished (even though a large part of metabolism will be shifted towards osmoprotectant synthesis). It is also important to investigate the intracellular transport of the precursor, especially if the osmoprotectant/osmolyte-producing enzyme is localized to a particular organelle.

*Inhibitory environments*

The intracellular conditions in the transgenic plants (e.g. pH, temperature, redox potential, ionic strength and presence of metals) will affect foreign proteins or enzymes that normally operate under different conditions. These problems will be minimized if the introduced gene is taken from a closely related plant.

*Side-reactions of new compounds*

It is impossible to foresee exactly all metabolic changes that might occur when a foreign gene is expressed in a plant. Unwanted side-reactions could degrade the pool of the desired product, or worse, toxic compounds may be formed from the decomposition or enzymatic degradation of the product. Careful analysis of the transgenic plants, for example using NMR, is extremely valuable for the detection of any metabolic changes.

## Improved stress tolerance in higher plants

Several different approaches to improve the stress tolerance of plants by foreign gene transfer have been attempted (Table 1). The most consistently successful approach is the introduction of genes encoding enzymes that catalyse the conversion of a naturally occurring substrate into a product with osmoprotective properties. Other important genes encode membrane-modifying enzymes; radical-scavenging enzymes; stress-induced proteins; and hypoxia/anoxia-reducing proteins.

*Production of osmoprotective compounds*

The accumulation of low molecular mass osmoprotectants and osmolytes (such as quaternary amines, amino acids and sugar alcohols) is considered to be an important strategy that plants use to overcome environmental stress. However, some species are able to accumulate such compounds more efficiently than others. For example, rice, potato and tobacco accumulate limited amounts of the potent osmoprotective compound glycine betaine. This makes them excellent targets for introducing osmoprotectant/osmolyte-producing enzyme systems. Two mechanisms are thought to lie behind the activity of these substances:

- The ability to raise the osmotic potential of the cell, thus balancing the osmotic potential of an externally increased osmotic pressure.
- The ability to stabilize membranes and/or macromolecular structures.

**Table 1. Foreign genes expressed in transgenic plants**

| Gene | Origin | Host | Stress | Refs |
|---|---|---|---|---|
| *BetA* | *E. coli* | Tobacco | Salinity | 16 |
| *BetA* | *E. coli* | Potato | Freezing | G. Lilius *et al.*, unpublished |
| *codA* | *Arthrobacter globiformis* | *Arabidopsis* | Salinity and drought | 17 |
| *p5cs* | *Vigna aconitifolia* | Tobacco | Drought | 18 |
| *Mltd* | *E. coli* | *Arabidopsis* | Salinity | 19 |
| *Mltd* | *E. coli* | Tobacco | Salinity | 20 |
| *TPS1* | *Saccharomyces cerevisiae* | Tobacco | Drought | 21 |
| *SacB* | *Bacillus subtilis* | Tobacco | Drought | 22 |
| *fad7* | *Arabidopsis* | Tobacco | Chilling | 23 |
| *Des9* | *Anacystis nidulans* | Tobacco | Chilling | 24 |
| *HVA 1* | Barley | Rice | Salinity and drought | 6 |
| *Afp* | Winter flounder | Tobacco | Freezing | 26 |
| *afa3* | Winter flounder | Tomato | Freezing | 18 |
| *Mn-Sod* | *Nicotiana plumbaginifolia* | Alfalfa | Drought and freezing | 15 |
| *Mn-Sod* | *N. plumbaginifolia* | Tobacco | Oxidative | 30 |
| *Fe-Sod* | *Arabidopsis* | Tobacco | Oxidative | 31 |
| *Gr/Cu,Zn-Sod* | *E.coli/Rice* | Tobacco | Oxidative | 33 |
| *vhb* | *Vitreoscilla stercoraria* | Tobacco | Hypoxia and anoxia | 35 |

Choline dehydrogenase from *E. coli* catalyses the conversion of choline to glycine betaine via the intermediate betaine aldehyde. When the *betA* gene, which encodes choline dehydrogenase, was introduced into tobacco[16] and potato (G. Lilius *et al.*, unpublished), salt-tolerant and freezing-tolerant phenotypes were achieved, respectively. Even though the enzyme localizes to the cytoplasm, which only occupies approximately 5% of the total cell volume, it is unclear whether the glycine betaine concentrations achieved (5 $\mu mol^{-1}\ g^{-1}$ dry weight in the potato) are high enough to facilitate osmotic adjustments. Instead, stabilization of cellular structures and macromolecules might underlie both phenotypes. Another possibility for introducing glycine betaine into transgenic plants is by using choline oxidase from *Arthrobacter globiformis* or *A. pascens*. This enzyme catalyses both steps in the conversion of choline into glycine betaine. A salt- and freezing-tolerant phenotype was also created by introducing the *codA* gene from *A. globiformis*, encoding choline oxidase, into *Arabidopsis*[17]. Using a transit peptide, the enzyme was localized to the chloroplasts, where glycine betaine concentrations of up to 50 mM were estimated; this may be high enough to facilitate osmotic adjustment.

Structurally, the amino acid proline resembles glycine betaine, and they also both have osmoprotective properties. The *p5cs* gene from moth bean (*Vigna aconitifolia*), encoding a bifunctional enzyme containing the catalytic activities of γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase, has been used to transform tobacco[18]. This enzyme, $\Delta^1$-pyrroline-5-carboxylate synthetase, catalyses the conversion of glutamate to $\Delta^1$-pyrroline-5-carboxylate, which is then reduced to proline. Transgenic tobacco expressing this enzyme produced 10- to 18-fold more proline than control plants, amounting to about 6.5 $mg\ g^{-1}$ fresh leaf under drought-stress conditions. Furthermore, the transgenic plants overproducing proline were demonstrated to have enhanced biomass production and flower development under salt stress. However, the decreased osmotic potential in the leaf sap of the transgenic tobacco during water stress raises serious questions regarding the relationship between the overproduction of proline and water-stress tolerance. The most obvious result of overproducing an osmolyte in plant cells would be an increased osmotic potential of the leaf sap, facilitating osmotic adjustment to the imposed water stress.

Mannitol, a sugar alcohol, has been overproduced in both *Arabidopsis* and tobacco by expressing the bacterial *mltd* gene, encoding mannitol 1-phosphate dehydrogenase[19,20]. Seeds of transgenic *Arabidopsis* plants were germinated in elevated salt concentrations and possessed enhanced germination rates. The maximal mannitol concentrations achieved were 100–150 mM, and thus the improved stress tolerance was attributed more to the osmoprotective properties of mannitol (e.g. the stabilization of subcellular membranes and/or macromolecular structures) than to osmotic adjustment. Similar mannitol concentrations were achieved by expressing the *mltd* gene in tobacco, which led to improved biomass production under salt-stress. Trehalose, a non-reducing disaccharide, has also been overproduced in tobacco[21]. This was accomplished by introducing the *TPS1* gene, encoding trehalose-6-phosphate synthase from yeast. The transgenic tobacco plants were assessed for drought tolerance and, although the trehalose concentration was <5 mM in the cytosol, both improved water retention and desiccation tolerance were demonstrated. Again, these results cannot be explained by osmotic adjustments facilitated by trehalose, and appear to be caused by the osmoprotective properties of trehalose itself.

In another approach to improve the drought tolerance in transgenic tobacco, a fructan-producing enzyme system was introduced[22]. The *Bacillus subtilis sacB* gene, encoding a fructosyl transferase, was fused to carboxypeptidase-Y vacuolar targeting signal from yeast, directing the expressed polypeptide to the vacuole. The plants contained about 0.35 mg fructan $g^{-1}$ fresh weight and had increased biomass under drought stress. The authors suggested that the fructan may stimulate root development and thereby increase the ability of the plant to absorb water.

### *Improved membrane flexibility*

There is a strong correlation between chilling sensitivity and the degree of unsaturation of fatty acids in plastid membranes of various higher plants[23]. The presence of centrally positioned *cis*-double bonds in the membrane lipid lowers the phase-transition temperature to approximately 0°C. Thus, by introducing enzymes capable of catalysing the formation of *cis*-double bonds in saturated or mono-saturated fatty acids, a chilling-resistant phenotype could theoretically be achieved. This hypothesis has been tested in two reports[24,25]. A chloroplast ω-3 fatty acid desaturase gene, *Fad7*, from *Arabidosis*, was introduced into transgenic tobacco[24]. The enzyme catalyses the formation of both dienoic and trienoic fatty acids. Tobacco expressing ω-3 fatty acid desaturase had increased amounts of dienoic and trienoic fatty acids, and consequently also had enhanced chilling resistance. A broad-specificity Δ9-desaturase gene (*Des9*) from the cyanobacterium *Anacystis nidulans* was also introduced into tobacco[25]. This enzyme introduces a *cis*-double bond in specific saturated fatty acids in various membrane lipids. Because the lipid biosynthetic activities in higher plants are localized to the plastid and endoplasmatic reticulum, the enzyme was fused to a transit peptide of the pea Rubisco small subunit in order to achieve correct targeting. The amounts

of the affected Δ9-monosaturated fatty acids in the transgenic plants were increased up to 17-fold. Furthermore, when tobacco plants were exposed to 1°C for 11 d, the *Des9*-expressing plants showed no signs of chlorosis, in contrast to wild-type plants.

### *Stress-induced proteins*

The transcription of genes encoding the late-embryogenesis-abundant (LEA) proteins (first characterized in seed embryos) is activated under abiotic stress. It has been hypothesized that these proteins have a protective effect, and this was tested by introducing the *HVA1* gene, encoding a group 3 LEA protein from barley, into rice[6]. The transgenic plants expressed a large amount of HVA1 – 0.5–2.5% of the total soluble protein in the leaves. The plants had improved osmotic stress tolerance, as indicated by delayed development of damage symptoms and improved recovery after drought stress.

The group 3 LEA proteins share an intriguing structural similarity with type 1 antifreeze proteins (AFPs) found in certain arctic fish species. These proteins contain 11-amino-acid motifs that form amphiphilic α-helical secondary structures. The AFPs have a unique ability to neutralize ice nucleators and inhibit ice recrystallization. Several attempts have been made to express AFPs in transgenic plants[15,26]. However, even though inhibition of ice recrystallization was detected in transgenic tobacco[15], and it has been demonstrated that the accumulation of AFPs is cold specific[26], there is still no evidence for protection from freezing damage *in vivo*.

### *Scavenging reactive intermediates*

Several reports have shown that salt, freezing and drought stress are also accompanied by the formation of reactive oxygen intermediates. These toxic molecules damage membranes, membrane-bound structures and macromolecules, especially in the mitochondria and chloroplast[25], resulting in oxidative stress[4]. Evidence for this is that freezing- and salinity-tolerant plants also have well-developed antioxidant defences, and by pretreating plants with one form of stress it is often possible to increase the tolerance to a different stress factor. Transgenic plants have been used more recently to study the relationship between abiotic stress tolerance and a functional antioxidant-defence system[27]. Antioxidant systems in plants consist of enzymes that can scavenge oxygen radicals, such as superoxide dismutases (SODs), peroxidases, catalases and glutathione reductases[4].

The SODs are essential components in almost all plant antioxidant defences, catalysing the dismutation of two superoxide radicals into oxygen and hydrogen peroxide. The SOD isoenzymes can be divided into three different classes according to their metal cofactor: copper/zinc (Cu,Zn); manganese (Mn); and iron (Fe). Plants generally contain Fe-SOD and/or Cu,Zn-SOD in the chloroplasts; Mn-SOD in the mitochondria; and Cu,Zn-SOD in the cytosol[28]. When removing superoxide radicals, SOD and peroxidases/catalases often work in concert, with SOD mainly removing superoxide radicals and concomitantly produced peroxide removed by peroxidases and catalases.

The Mn-SOD cDNA from *Nicotiana plumbaginifolia* has been introduced into alfalfa[29]. Through the use of two different transit peptides, the Mn-SOD was expressed either in the chloroplasts or the mitochondria. A three-year field trial indicated that yield and survival of the transgenic plants were significantly improved. However, it may be too simplistic to think that the observed effects of the transgene are solely caused by the introduced Mn-SOD activity. A more plausible explanation is that the overall stress-defence system is enhanced by the peroxide produced, as it has been shown to elicit several stress-tolerance-conferring genes[30]. Mn-SOD has also been expressed in both chloroplasts and mitochondria of transgenic tobacco[31]. Ozone was used to evaluate the ability of the transgene to protect plants from ambient oxidative stress. Transgenic plants in which the Mn-SOD was targeted to the chloroplasts demonstrated a threefold and twofold reduction in leaf injury as compared with wild-type and transgenic, Mn-SOD-expressing (mitochondria) tobacco, respectively. This can be explained by the ability of ozone to increase the formation of 8-hydroguanine in the chloroplast DNA and to destroy chlorophylls and carotenoids.

Another of the SOD isoenzymes, Fe-SOD from *Arabidopsis*, has also been expressed in transgenic tobacco[32]. When targeted to the chloroplast, this enzyme protected both the plasmalemma and photosystem II against superoxide generated during illumination of leaf discs impregnated with methyl viologen by scavenging radicals. However, overproduction of Fe-SOD did not improve the tolerance to chilling-induced photoinhibition in leaf-disc assays or to salt stress at the whole-plant level.

An interesting approach for increasing oxidative stress tolerance relies on the expression of glutathione reductase (GR) and Cu,Zn-SOD, both separately and together in the cytosol of transgenic tobacco[33]. The genes encoding these enzymes were derived from *E. coli* and rice, respectively. Leaf discs of transgenic and control plants were subjected to paraquat, a superoxide-generating herbicide that mimics photooxidative stress; the tolerance was assessed by measuring electrolyte leakage. The GR/Cu,Zn-SOD-expressing plants exhibited less damage than wild-type, GR and Cu,Zn-SOD controls. Furthermore, a GR-antisense construct was also introduced into tobacco, rendering a paraquat-sensitive phenotype. Collectively, these results show that both GR and Cu/Zn-SOD play crucial roles in protecting plants from oxidative stress, and thus probably also from abiotic stress.

### *Hypoxia- and anoxia-reducing proteins*

Oxygen stress is often caused by flooding. Oxygen is involved in respiration and several crucial biosynthetic pathways such as the synthesis of chlorophylls. Plants have evolved different strategies to cope with hypoxia: altered root architecture; increased internal oxygen transport by a larger aerenchyma; radial loss of soluble substances by altered cortex structure; induction of enzymes, export mechanisms and enzyme systems to avoid toxication by fermentation end-products; and production of oxygen-binding proteins.

Plant hemoglobins were first discovered in the nitrogen-fixing root nodules, occupied by *Rhizobium* or *Frankia*-type bacteria, of leguminous plants. It was later suggested that a hemoglobin gene may be a component of all plants, and that the role for non-legume hemoglobin was not to facilitate oxygen diffusion, but rather to function as an oxygen-sensing device. However, characterization of the non-symbiotic hemoglobin genes of *Casuarine glauca* has increased speculation that the hemoglobin may facilitate oxygen diffusion[34]. This hypothesis was tested by expressing a bacterial gene, encoding *Vitreoscilla* hemoglobin (VHb) in transgenic tobacco[35]. The VHb-producing plants exhibited intriguing characteristics: compared with non-transgenic control lines, their germination time was halved and the dry weight was doubled after 35 d growth. Moreover, the enhanced growth of the plants was associated with a 30–40% increase in total chlorophyll content. As seed germination occurred under anaerobic or micro-aerobic conditions, a plausible explanation for the shorter germination times may be a shorter fermentation period during germination. This may lead to less toxic waste products from the fermentation process. The increased chlorophyll concentration in the transgenic plant may be explained by the strong oxygen dependence of the chlorophyll biosynthesis, which at the onset of seedling development occurs at the expense of respiration.

## Future prospects

All the necessary techniques exist for isolating stress-response genes and introducing them into crops. Moreover, many advances have recently been achieved in understanding the metabolic network in plants – essential for predicting the effects of introducing a foreign gene. Now it needs to be known how plant roots sense environmental stress and how stress signals are transduced into altered gene expression. Plant hormones, such as ABA and 1-aminocyclopropane-1-carboxylic acid (ACC), play important roles, but their actions are still not fully understood. It is also important to mimic nature and only activate the necessary genes during stress, in order to minimize effects on unstressed growth. One way to achieve this is to use osmotically inducible promoters from naturally occurring stress-response systems.

One approach for engineering extreme stress tolerance may be to introduce genes from different stress responses into a single plant. If, for example, osmoprotectant-producing, membrane-modifying, and superoxide-scavenging enzymatic activities are all present in a single species, there is a strong possibility that they could work in concert to overcome abiotic stress. This could be achieved either by transformation with multiple genes or by cross-breeding plants containing different stress-tolerance genes. These are theoretically straightforward operations, but there may be severe perturbances to the metabolic network of plants containing several foreign enzymatic activities. Thus, it is of paramount importance to target the location, control the level and time of expression, and ensure precursor availability for each enzyme in order to avoid negative effects.

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## References

1 Bray, E.A. (1997) Plant responses to water deficit, *Trends Plant Sci.* 2, 48–54
2 Lichtentaler, H.K. (1995) Vegetation stress: an introduction to the stress concept in plants, *J. Plant. Physiol.* 148, 4–14
3 Smirnoff, N. (1993) The role of active oxygen in the response of plants to water deficit and desiccation, *New Phytol.* 125, 27–58
4 Allen, R.D. (1995) Dissection of oxidative stress tolerance using transgenic plants, *Plant Physiol.* 107, 1049–1054
5 Walden, R. and Schell, J. (1990) Techniques in plant molecular biology – progress and problems, *Eur. J. Biochem.* 192, 563–576
6 Xu, D. *et al.* (1996) Expression of a late embryogenesis abundant protein gene, *HVA 1*, from barley confers tolerance to water deficits and salt stress in rice, *Plant Physiol.* 110, 249–257
7 Benfey, P.N. and Chua, N. (1990) The cauliflower mosaic virus 35 S promoter: combinatorial regulation of transcription in plants, *Science* 250, 959–966
8 Krebbers, E. *et al.* (1988) Four genes in two diverged subfamilies encode the ribulose-1,5-bisphosphate carboxylase small subunit polypeptides of *Arabidopsis thaliana*, *Plant Mol. Biol.* 11, 745–759
9 Rohde, W. *et al.* (1990) Structural and functional analysis of two *waxy* gene promoters from potato, *J. Genet. Breed.* 44, 311–315
10 Rocha-Sosa, M. *et al.* (1989) Both developmental and metabolic signals activate the promoter of a class I patatin gene, *EMBO J.* 8, 23–29
11 Raghothama, K.G. *et al.* (1993) Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter, *Plant Mol. Biol.* 23, 1117–1128
12 Köhler, U. *et al.* (1996) A promoter for strong and ubiquitous anaerobic gene expression in tobacco, *Plant J.* 10, 175–183
13 Allen, G.C. *et al.* (1996) High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco, *Plant Cell* 8, 899–913
14 Schreier, P.H. *et al.* (1985) The use of nuclear encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts, *EMBO J.* 4, 25–32
15 Hightower, R. *et al.* (1991) Expression of antifreeze proteins in transgenic plants, *Plant Mol. Biol.* 17, 1013–1021
16 Lilius, G., Holmberg, N. and Bülow, L. (1996) Enhanced NaCl stress tolerance in transgenic tobacco expressing bacterial choline dehydrogenase, *Biotechnology* 14, 177–180
17 Hayashi, H. *et al.* (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress, *Plant J.* 12, 133–142
18 Kavi Kishor, P.B. *et al.* (1995) Overexpression of $\Delta^1$-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants, *Plant Physiol.* 108, 1387–1394
19 Thomas, J.C. *et al.* (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*, *Plant Cell Environ.* 18, 801–806
20 Tarczynski, M. and Bohnert, H. (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol, *Science* 259, 508–510
21 Holmström, K-O. *et al.* (1996) Drought tolerance in tobacco, *Science* 379, 683–684
22 Pilon-Smits, E.A.H. *et al.* (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress, *Plant Physiol.* 107, 125–130
23 Murata, N. *et al.* (1982) Composition and positional distribution of fatty acids in phospholipids from leaves of chilling sensitive and chilling resistant plants, *Plant Cell Physiol.* 23, 1071–1079
24 Kodama, H. *et al.* (1994) Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco, *Plant Physiol.* 105, 601–605
25 Ishizaki-Nishizawa, O. *et al.* (1996) Low-temperature resistance of higher plants is significantly enhanced by a nonspecific cyanobacterial desaturase, *Nat. Biotechnol.* 14, 1003–1006
26 Kenward, K.D. *et al.* (1993) Accumulation of type I fish antifreeze protein in transgenic tobacco is cold specific, *Plant Mol. Biol.* 23, 377–385
27 Rennenberg, H. and Polle, A. (1994) Protection from oxidative stress in transgenic plants, *Biochem. Soc. Trans.* 22, 936–939
28 Slooten, L. *et al.* (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in chloroplasts, *Plant Physiol.* 107, 737–750
29 McKersie, B.D. *et al.* (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase, *Plant Physiol.* 111, 1177–1181
30 Prasad, T.K. *et al.* (1994) Evidence for chilling-induced oxidative stress in maize seedlings and the regulatory role for hydrogen peroxide, *Plant Cell* 6, 65–74
31 Van Camp, W. *et al.* (1994) Elevated levels of superoxide dismutase protect transgenic plants against ozone damage, *Biotechnology* 12, 165–168
32 Van Camp, W. *et al.* (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts, *Plant Physiol.* 112, 1703–1714
33 Aono, M. *et al.* (1995) Paraquat tolerance of transgenic *Nicotiana tabacum* with enhanced activities of glutathione reductase and superoxide dismutase, *Plant Cell Physiol.* 36, 1687–1691
34 Jacobsen-Lyon, K. *et al.* (1995) Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca*, *Plant Cell* 7, 213–223
35 Holmberg, N. *et al.* (1997) Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production, *Nat. Biotechnol.* 15, 244–247

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