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REVIEW

Transgenic plants with improved dehydration-stress tolerance: progress and future prospects

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Abstract

This review summarizes the recent progress made towards the development of transgenic plants with improved tolerance to water stress and salinity. Of the various strategies employed, emphasis has been given to the genes engineered for the biosynthesis of osmoprotectants and osmolytes. This review also briefly discusses the importance of the use of specific stress inducible promoters and the future prospects of transgenic plants with improved agronomic traits.

Additional key words: compatible solutes, environmental stress, overexpression, rd29A promoter, regulatory-proteins.

Introduction

The growth and productivity of crop plants depend largely on their vulnerability to environmental stresses. High salinity, water deficit, and temperature stresses are the major constraints that limit agricultural production (Boyer 1982). Plants respond to these conditions with an array of biochemical and physiological adaptations, which involve, the function of many stress-related genes. Hence any attempt to improve the stress tolerance requires a better understanding of the underlying physiological, biochemical and molecular events (Cherian and Reddy 2003). To date, various approaches have been tested to produce stress tolerant plants by the use of classical genetic methods as well as improved plant breeding techniques. However, the strategy of gene transfer to crop plants from their more tolerant wild relatives using classical genetic methods has been of limited success (Flowers and Yeo 1995). Genetic engineering has allowed the introduction of new pathways for the biosynthesis of various compatible solutes into plants, resulting in the production of transgenic plants with improved stress tolerance (Chen and Murata 2002).

The response of plants to different stress conditions varies, and different kinds of stress often lead to identical or similar responses. At the cellular level, abiotic stresses, especially water deficit (drought and salinity) cause a decrease in pressure potential. Cell solutes concentrate due to water loss and they are actively accumulated to keep the cytoplasm osmotically balanced. Osmotic adjustment is therefore a major component of abiotic stress tolerance in plants and contributes to pressure potential maintenance. The common solutes employed in osmotic adjustment include various quarternary amines, aminoacids, or sugar alcohols. Enhanced accumulation of these osmolytes facilitates the retention of water in the cytoplasm and the protection of membranes, protein complexes, and cellular structures. Furthermore, plant cells contain antioxidant enzyme systems, such as peroxidases and superoxide dismutases, which scavenge reactive oxygen intermediates and provide protection against oxidative stress.

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Abbreviations: ABA - abscisic acid; ABRE - ABA-responsive element; BADH - betainealdehyde dehydrogenase; CBF - C-repeat binding protein; CDH - choline dehydrogenase; CMO - choline monoxygenase; COD - choline oxidase; COR - cold regulated; CuCOR19 - *Citrus unshiu* cold-regulated gene encoding a 19-kDa protein; DREB - dehydration responsive element binding protein; GB - glycinebetaine; GSA - glutamic-γ-semialdehyde; LEA - late-embryogenesis abundant; P5C - Δ^1-pyrroline-5-carboxylate; P5CR - P5C reductase; P5CS - P5C synthetase; WUE - water use efficiency.

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Biochemical pathways that lead to the production of some of these osmoprotectants are mostly known (Kavi Kishor *et al*. 1995, Hayashi *et al*. 1997). Genes encoding several of the related enzymes have been cloned, and expression of specific genes indicated that tolerance is conferred by genetically encoded mechanisms. There are many proposed transgenic routes for stress improvement in plants. One example is the transference of genes involved in osmolyte synthesis (Kavi Kishor *et al*. 1995, Hayashi *et al*. 1997). Another approach is the isolation and transference of genes directly implicated in stress tolerance such as halotolerance genes (Serrano and Gaxiola 1994, Cervera *et al*. 2000). Similarly, the transfer of barley late embryogenesis (LEA) gene (Wu and Ho 1997), introduction of protein kinase or a calcium/ calmodulin-dependent gene (Pardo *et al*. 1998, Sheen 1998) was reported to be beneficial.

In this review, we limit our discussion to transgenic plants engineered towards dehydration-stress tolerance, particularly due to salinity and drought with emphasis on osmolyte biosynthesis genes. The possibilities for increasing tolerance to other abiotic stresses such as waterlogging, temperature, metals (*e.g.* Al) were reviewed elsewhere (Dennis *et al*. 2000, Grover *et al*. 2000, 2001, 2003, Datta 2002, Samac and Tesfaye 2003). Some of the important genes involved in the manipulation of dehydration-stress tolerance and the performance of transgenic plants are presented below.

Osmolyte biosynthesis genes

Glycinebetaine: Glycinebetaine (GB) is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals and microorganisms (Rhodes and Hanson 1993, Jagendorf and Takabe 2001). At high concentrations, GB does not interfere with cytoplasmic functions and it stabilizes the structure and function of many macromolecules. So, it belongs to a group of compounds that are collectively known as compatible solutes. GB appears to be a critical determinant of stress tolerance in plants. Physiological studies on transgenic plants have suggested that GB might accelerate *de novo* protein synthesis during stress adaptation and recovery (Alia *et al*. 1999).

Plant transformation with the aim of manipulation in glycinebetaine biosynthesis pathway was studied (Nakamura *et al*. 1997, Hayashi and Murata 1998, Sakamoto and Murata 2001; Table 1). Alteration of GB synthesis is possible by changing the *1*) choline monoxygenase/betaine aldehyde dehydrogenase pathway (CMO/BADH), *2*) choline dehydrogenase/betaine aldehyde dehydrogenase pathway (CDH/BADH), and *3*) direct choline oxidase pathway (COD). Among these the COD pathway clearly has an advantage over the CDH/BADH and CMO/BADH pathways because of the single step conversion of choline to glycinebetaine and also COD does not require any cofactors for this catalysis. For example, transgenic cyanobateria and other plants engineered to synthesize glycinebetaine involving the COD pathway acquired resistance to cold, drought and salt stress by GB accumulation (Deshnium *et al.* 1995, Nomura *et al*. 1995, Huang *et al*. 2000). Similarly, Lilius *et al*. (1996) introduced the *E. coli betA* gene encoding choline dehydrogenase into tobacco and the transgenic plants demonstrated enhanced salt tolerance.

Gao *et al*. (2000) reported the successful transformation and regeneration of the woody plant, Japanese persimmon, with the *codA* gene. The successful integration of *codA* in *Brassica juncea* enhanced tolerance to salt stress (Prasad *et al.* 2000). Plants overexpressing *codA* gene with increased tolerance to salt, cold, high temperature and freezing tolerance were reported (Hayashi *et al*. 1997, Alia *et al*. 1998a,b, Sakamoto *et al*. 1998, 2000). The possible mechanism for the glycinebetaine action could be in some cases the stabilization of structure and function of proteins since the GB concentrations obtained were considered insufficient for osmotic adjustment (Takabe *et al*. 1998, Sakamoto and Murata 2001). A major role of GB might be to protect membranes and macromolecules from the damaging effects of stress.

Crops such as rice and potato are generally non-accumulators of GB. External application of GB has been shown to increase the tolerance to freezing, cold and salt stress (Nakamura *et al*. 1997, Hayashi *et al*. 1998). Transgenic rice plants overproducing glycinebetaine have been reported (Sakamoto *et al*. 1998, Kishitani *et al*. 2000). Holmstrom *et al*. (2000) reported accumulation of betaine in transgenic tobacco expressing the two *E. coli* genes *betA* and *betB*. It was shown that transgenic lines expressing only *betA* accumulate betaine, although accumulation is increased 2 - 3 fold in transgenic plants producing both enzymes. These transgenic lines exhibit increased tolerance to salt stress and also enhanced tolerance to photoinhibition at low temperatures. Under various stress conditions GB protects the photosystem 2 (PS 2) in transgenic cells of *Synechococcus* and in higher plants (Deshnium *et al*. 1997, Alia *et al*. 1999, Holmstrom *et al*. 2000). A role of GB in protecting membrane integrity was also inferred from the enhanced tolerance to temperature stress that was observed during imbibition to seeds (Alia *et al*. 1998a,b).

There is also some evidence for the involvement of GB in the protection of the transcriptional and translational machinery under stress conditions (Rajendrakumar *et al*. 1997, Allard *et al*. 1998). Bourot *et al*. (2000) have suggested that GB can assist *in vivo* folding in a chaperon-like manner. Transgenic tobacco plants overexpressing phosphoethanolamine *N*-methyl-transferase show enhanced synthesis of choline and glycinebetaine (McNeil *et al*. 2001). More recently it was found that the co-expression of *N*-methyltransferase genes in *Synechococcus* caused accumulation of a

Table 1. Stress responses of transgenic plants overexpressing genes for the biosynthesis of glycinebetaine and proline.

Gene	Gene product and function	Transgenic plant	Performance of transgenic plants	Reference
CodA	choline oxidase (glycinebetaine synthesis)	*Synechococcus*	tolerance to 0 - 4 M NaCl	Deshnium *et al*. 1995, 1997
CodA	choline oxidase (glycinebetaine synthesis)	*Arabidopsis*	plants tolerated 0 - 4 M NaCl for 48 h and 0.1 M NaCl for 20 d	Hayashi *et al*. 1997, 1998
CodA	choline oxidase (glycinebetaine synthesis)	*Arabidopsis*	tolerant to cold and heat at 0 and 55 °C, respectively	Alia *et al*. 1998a,b
CodA	choline oxidase (glycinebetaine synthesis)	rice	salt tolerance at 0.15, 0.1 M NaCl and cold tolerance at 5 °C	Sakamoto *et al*. 1998
CodA	choline oxidase (glycinebetaine synthesis)	*Arabidopsis*	increased freezing tolerance at -5 to -10 °C	Sakamoto *et al.* 2000
CodA	choline oxidase (glycinebetaine synthesis)	*Arabidopsis*	enhanced tolerance to salt stress at the reproductive stage	Sulpice *et al*. 2003
CodA	choline oxidase (glycinebetaine synthesis)	Japanese persimmon	successful regeneration and salt tolerance at 0.1 M NaCl	Gao *et al.* 2000
CodA	choline oxidase (glycinebetaine synthesis)	*Brassica*	better growth and seed germination under salt stress	Prasad *et al*. 2000
CodA/*cox*	choline oxidase (glycinebetaine synthesis)	*Arabidopsis*	increased salt, drought and freezing tolerance	Huang *et al.* 2000
CodA/cox	choline oxidase (glycinebetaine synthesis)	tobacco	enhanced salt tolerance at 0.15 M NaCl	Huang *et al.* 2000
CodA/cox	choline oxidase (glycinebetaine synthesis)	*Brassica*	increased salt and drought tolerance	Huang *et al.* 2000
bet A, bet B	choline dehydrogenase and betaine aldehyde dehydrogenase	*Synechococcus*	increased salt tolerance at 0.2 and 0.4 M NaCl for 4 d	Nomura *et al*. 1995
modified *bet A*	choline dehydrogenase (glycine betaine synthesis)	rice	salt and drought tolerant at 0.15 M NaCl and low relative humidity	Takabe *et al*. 1998
modified *bet A*	choline dehydrogenase (glycine betaine synthesis)	tobacco	increased salt/cold tolerance at 0.2 M NaCl and at 4 °C	Holmstrom *et al*. 2000
bet A	choline dehydrogenase (glycine betaine synthesis)	tobacco	better growth at 0.3 M NaCl	Lilius *et al.* 1996
badh	betaine aldehyde dehydrogenase (glycinebetaine synthesis)	carrot	growth at 400 mM NaCl	Kumar *et al*. 2004
ApDMT	dimethylglycine methyltransferase (glycinebetaine synthesis)	*Synechococcus*	salt tolerance sufficient for it to grow in sea water	Waditee *et al.* 2005
ApGSMT	glycinesarcosine methyltransferease (glycinebetine synthesis)	*Arabidopsis*	high betaine accumulation and improved seed yield under stress	Waditee *et al.* 2005
PEAMT cDNA	phosphoethanolamine *N*-methyltransferase (choline biosynthesis)	tobacco	enhanced synthesis of choline and glycinebetaine	McNeil *et al.* 2001
p5cs	Δ^1-pyrroline-5-carboxylate synthetase (proline synthesis)	tobacco	enhanced biomass and flower development under salt stress and water stress	Kavi Kishor *et al.* 1995
p5cs	Δ^1-pyrroline-5-carboxylate synthetase (proline synthesis)	rice	increased biomass when subjected to salt and water stress	Zhu *et al.* 1998
p5cs	Δ^1-pyrroline-5-carboxylate synthetase (proline synthesis)	wheat	increased tolerance to salt stress	Sawahel and Hassan 2002
p5cs	Δ^1-pyrroline-5-carboxylate synthetase (proline synthesis)	*Chlamydomonas*	higher accumulation of proline and better growth under Cd stress	Siripornadulsil *et al.* 2002
NtHAL3	HAL3 proteins (involved in coenzyme A biosynthetic pathway)	tobacco	improved tolerance to salt, osmotic and lithium stress	Yonamine *et al*. 2004

significant amount of GB and conferred salt tolerance to a freshwater cyanobacterium sufficient for it to become capable of growth in seawater (Waditee *et al*. 2005). *Arabidopsis* plants expressing *N*-methyltransferase genes also accumulated GB in roots, stems, leaves, and flowers and improved seed yield under stress conditions (Waditee

et al. 2005). These results show the usefulness of glycine *N*-methyltransferase genes for the improvement of abiotic stress tolerance in crop plants.

Overexpression of GB by manipulation of BADH *via* chloroplast genetic engineering may prove to be an important strategy in order to confer salt tolerance on desired crops. One such recent study shows that transgenic carrot plants expressing BADH grew in the presence of high concentrations of NaCl (up to 400 mM), the highest level of salt tolerance reported so far among genetically modified crop plants (Kumar *et al*. 2004).

Proline: Proline also does not interfere with the normal biochemical reactions and act as osmoprotectant during osmotic stress. Proline accumulation has not only been observed in plants but also in protozoa, invertebrates and certain eubacteria (Mc Cue and Hanson 1990, Delauney and Verma 1993). The accumulation of proline in response to environmental stress indicated that its synthesis is a nonspecific response to decreased water potential (Reddy and Iyengar 1999). The role of proline during stress is a subject of controversy and interesting because it accumulates to very high levels under adverse conditions. It appears that proline affect the enzymes stabilizing their active conformation and in this way protect enzymes against conformational perturbances caused by ions (McCue and Hanson 1990). In plants, proline is synthesized either from glutamate (Glu) or from ornithine. The pathway from Glu is the primary route for the synthesis of proline under conditions of osmotic stress and nitrogen limitation, while a pathway from ornithine predominates at high levels of available nitrogen (Delauney *et al*. 1993). The proline biosynthesis pathway from Glu is thought to involve conversion of Glu to proline via the intermediates γ-glutamylphosphate, glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C); catalysed by two enzymes, the Δ^1-pyrroline-5-carboxylate synthetase (P5CS), and Δ^1-pyrroline-5-carboxylate reductase (P5CR).

Genetic engineering of proline biosynthesis pathway led to increased osmotolerance and salinity stress tolerance in transgenic plants (Table 1). For example, transgenic tobacco plants overexpressing the mothbean P5CS accumulates 10 fold more proline than did control plants (Kavi Kishor *et al*. 1995). Overproduction of proline in rice with P5CS gene increased its biomass under water stress (Zhu *et al*. 1998). *Arabidopsis* plants overexpressing a CBF3 transcriptional activator had elevated P5CS transcript and proline contents (Gilmour *et al*. 2000). Transgenic wheat plants with high content of proline and increased tolerance to salt stress were reported (Sawahel and Hassan 2002). Overexpression of *NtHAL3* genes also conferred increased proline biosynthesis and the enhancement of salt, osmotic, and lithium tolerance in cultured tobacco cells (Yonamine *et al*. 2004). Recently, tobacco plants tansformed to accumulate different osmolytes (proline, fructans and glycinebetaine) successfully survived freezing stress (Parvanova *et al*. 2004).

Polyamines: Polyamines are a group of nitrogenous cellular compounds that accumulate under a variety of abiotic stress conditions including salt and drought (Krishnamurthy and Bhagwat 1989). The involvement of polyamines in abiotic stress responses was first documented through putrescine accumulation in response to suboptimal potassium levels in barley (Richards and Coleman 1952). Since then, a connection has been suggested between increased putrescine content and abiotic stress (Bouchereau *et al*. 1999). The physiological role of putrescine in abiotic stress responses is a subject of controversy, as it is difficult to establish a direct cause-and-effect relationship.

Genetic manipulation of polyamine metabolism has become a valuable tool for studying their physiological roles in plants. Plant polyamine content has been modulated by the overexpression/down-regulation of arginine decarboxylase (*adc*), ornithine decarboxylase (*odc*), and *S*-adenosylmethionine decarboxylase (*samdc*) (Minocha and Sun 1997, Capell *et al*. 1998, Roy and Wu 2001, Kakkar and Sawhney 2002, Thu-Hang *et al*. 2002, Capell *et al*. 2004). Overexpression of spermidine synthase in *Arabidopsis* enhances tolerance to multiple stresses. When compared with wild type plants, the T_2 and T_3 transgenic plants exhibited a significant increase in spermidine synthase activity and spermidine content in leaves together with enhanced tolerance to various stresses such as chilling, freezing, salinity, hyperosmosis, drought, and paraquat toxicity (Kasukabe *et al*. 2004). Similarly, overexpression of *Datura stramonium* samdc cDNA in transgenic rice is found sufficient for accumulation of spermidine in leaves and spermidine and spermine in seeds (Thu-Hang *et al*. 2002). Transgenic carrot cell lines overproducing mouse ornithine decarboxylase, which converts ornithine to the diamine putrescine, were able to withstand salt and osmotic stress over a short period (Minocha and Sun 1997). Rice plants overexpressing oat arginine decarboxylase gene were reported (Capell *et al*. 1998, Roy and Wu 2001). The overexpression of *adc* and *samdc* genes increased polyamine content in transgenic rice and conferred tolerance to drought stress (Capell *et al*. 2004).

Mannitol: In addition to above discussed osmolytes, there are also reports on the accumulation of compounds related to sugar metabolism during plant response to water stress and osmotic adjustment. Among them, some of the most extensively studied are the sugar alcohol mannitol, trehalose, fructans and D-ononitol. In the following section we discuss about these osmolytes and the stress responses of transgenic plants engineered by modifying the responsible enzymes in their biosynthesis pathway (Table 2).

The osmolyte mannitol is normally synthesized in

Table 2. Stress responses of transgenic plants overexpressing genes for the biosynthesis of osmolytes other than glycinebetaine and proline.

Gene	Gene product and function	Transgenic plant	Performance of transgenic plants	Reference
TPS1	trehalose-6-phosphate synthetase (trehalose synthesis)	tobacco	increased drought tolerance, better survival of leaves after 15 d water stress	Romero *et al.* 1997
TPS1	trehalose-6-phosphate synthetase (trehalose synthesis)	potato, tobacco	increased drought tolerance	Yeo *et al.* 2000, Zhao *et al.* 2000
otsA	trehalose-6-phosphate synthetase (trehalose synthesis)	tobacco	increased dry mass and more efficient photosynthesis under drought stress	Pilon-Smits *et al.* 1998
otsB	trehalose-6-phosphate synthetase (trehalose synthesis)	rice	sustained plant growth, less photooxidative damage, better mineral balance	Garg *et al.* 2002
IMT1	D-ononitol (myo-inositol *O*-methyltransferase)	tobacco	improved performance under drought and salt, higher photosynthetic rate	Sheveleva *et al.* 1997
mt1D	mannitol-1-phosphate dehydrogenase(mannitol synthesis)	tobacco	better growth under high salinity	Tarczynski *et al.* 1993
mt1D	mannitol-1-phosphate dehydrogenase(mannitol synthesis)	*Arabidopsis*	enhanced seed germination under high salinity	Thomas *et al.* 1995
mt1D	mannitol-1-phosphate dehydrogenase(mannitol synthesis)	tobacco	increased tolerance to oxidative stress, increased retention of chlorophyll under stress	Shen *et al.* 1997
mt1D	mannitol-1-phosphate dehydrogenase(mannitol synthesis)	tobacco	marginally increased dry mass under salt stress	Karakas *et al.* 1997
mt1D	mannitol-1-phosphate dehydrogenase(mannitol synthesis)	wheat	improved growth performance under water stress and salinity	Abebe *et al.* 2003
SacB	fructosyltransferase (fructan synthesis)	tobacco	better growth under PEG-induced osmotic stress	Pilon-Smits *et al.* 1995
Adc	arginic decarboxylase (putrescine synthesis)	rice	minimized chlorophyll loss under drought stress	Capell *et al.* 1998
Datura adc	arginic decarboxylase (putrescine synthesis)	rice	higher content of putrescin and tolerant to drought stress	Roy and Wu 2001, Capell *et al.* 2004
Odc	(ornithine decarboxylase (putrescine synthesis)	carrot	cell lines could withstand high salt content over a short period	Minocha and Sun 1997
SPDS cDNA	spermidine synthase (spermidine synthesis)	*Arabidopsis*	enhanced tolerance to chilling, freezing, salinity and drought	Kasukabe *et al.* 2004
Samdc cDNA	*S*-adenosylmethionine decarboxylase (spermidine synthesis)	rice	significant accumulation of spermidine in leaves and spermidine and spermine in seeds	Thu-Hang *et al.* 2002

numerous plant species, but not in tobacco and *Arabidopsis*. Mannitol accumulation increases when plants are exposed to low water potential (Patonnier *et al.* 1999). Mannitol has been proposed to enhance tolerance to water deficit stress primarily through osmotic adjustment (Loescher *et al.* 1992). Besides its function in osmotic adjustment, mannitol improves tolerance to stress through scavenging of hydroxyl radicals ($OH^{\bullet}$) and stabilization of macromolecular structures (Abebe *et al.* 2003).

Eventhough, tobacco and *Arabidopsis* plants are non-accumulators of mannitol, the overexpression of *E. coli* gene for mannitol-1-phosphate dehydrogenase (*mt1D*) resulted in the biosynthesis of mannitol and increased salinity tolerance in these species (Tarczynski *et al.* 1993, Thomas *et al.* 1995). In the seeds of this *Arabidopsis* plants, the concentration of mannitol reached 10 μmol g^{-1}(d.m.) and they were able to germinate in medium with 400 mM NaCl, whereas control seeds ceased to germinate at 100 mM NaCl. However, Karakas *et al.* (1997) could see only a marginal increase in growth and dry mass at salt stress in transgenic tobacco with the same gene. Shen *et al.* (1997) reported increased resistance to oxidative stress in transgenic tobacco plants by targeting mannitol biosynthesis to chloroplasts. The ectopic expression of the *mt1D* gene for the biosynthesis of mannitol in transgenic wheat improves tolerance to water stress and salinity both at the callus and whole-plant level (Abebe *et al.* 2003).

Trehalose: Trehalose is a rare sugar with unique abilities to protect biomolecules from environmental stresses and is present in many bacteria, fungi and some desiccation-tolerant higher plants. Increasing trehalose accumulation in crop plants could improve drought and salinity tolerance (Penna 2003). In bacteria, this sugar is produced by action of the two enzymes trehalose phosphate synthase, which produces trehalose phosphate

(T-6-P), and trehalose phosphate phosphatase, which degrade T-6-P into trehalose. When these two enzymes are expressed in plants (Goddijn *et al.* 1997, Pilon-Smits *et al.* 1998) the transgenic plants produced larger leaves, altered stem growth and improved stress tolerance. When grown under drought stress, two selected transgenic tobacco plants showed 28 and 39 % higher total dry mass than the controls. It is proposed that beneficial results can be achieved by modifying T-6-P *via* the inhibition of endogenous trehalase, an enzyme that hydrolyses trehalose into two glucose moieties (Goddijn *et al.* 1998). Expression of yeast trehalose-6-phosphate synthetase gene (*TPS1*) in transgenic tobacco plants exhibited multiple phenotypic alterations and improved drought tolerance (Romero *et al.* 1997). Two bacterial genes, trehalose-6-phosphate synthetase (*otsA*) and trehalose-6-phosphate phosphatase (*otsB*), were introduced into tobacco and the transgenic plants were found to be larger, exhibiting better growth under drought stress (Pilon-Smits *et al.* 1998). Transgenic potato and tobacco plants exhibited significantly enhanced tolerance to drought, when the yeast gene for TPS1, driven by 35S promoter of cauliflower mosaic virus (CaMV) in potato, and by drought-inducible promoter of RD29 in tobacco (Zhao *et al.* 2000, Yeo *et al.* 2000). Trehalose overproducing transgenic rice plants showed high tolerance to different abiotic stresses (Garg *et al.* 2002). These transgenic plants maintained optimal K^+/Na^+ ratios necessary for cellular functions.

Fructans: Fructans are polyfructose molecules and many plants use them as storage sugars. In contrast to starch, fructans are soluble. For this reason it is assumed that fructans may play a role in the osmotic adjustment. The effects of fructans on the abiotic stress tolerance was assessed by transforming tobacco plants with a construct that contained the *sacB* gene for levansucrase from *Bacillus subtilis* fused to the vacuole-sorting signal of carboxypeptidase Y from yeast and placed downstream of the constitutive 35S promoter (Pilon-Smits *et al.* 1995). The transgenic plants performed significantly better than controls under drought conditions, having 55 % more rapid growth rate, 33 % greater fresh mass and 59 % greater dry mass over wild type plants. When sugar beet plants were transformed using the same construct, fructan accumulated to 0.5 % of their dry mass in roots and shoots and transgenic plants showed significantly better growth under drought stress than wild type plants (Pilon-Smits *et al.* 1999). Even though, several assumptions about the function of fructans exist, the known data available are not sufficient to substantiate any hypothesis (Pilon-Smits *et al.* 1995). However, it is quite clear that fructans promote the process of root branching, and thus increasing root surface and water uptake (Datta 2002).

D-ononitol: Myo-inositol and its derivatives are commonly studied with respect to cell signalling and membrane biogenesis, but they also participate in stress responses in plants and animals, particularly to salt stress (Nelson *et al.* 1998). In some plants, inositol provides substrate for the production and accumulation of the compatible solutes ononitol and pinitol (sugar alcohols) and help to lower the cytoplasmic osmotic potential and to balance sodium accumulation in vacuoles. Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance was studied (Nelson *et al.* 1998). The results indicate that inositol metabolism provides more than an alteration in the allocation of carbon destined to become a stable osmolyte and can also act as a facilitator of sodium sequestration and protecting photosynthesis.

Only few results are available on the genetic transformation of myo-inositol *O*-methyltransferase (IMT1) in plants. For example, expression of a cDNA encoding myo-inositol *O*-methyltransferase (IMT1) in tobacco during salt and drought stress resulted in the accumulation of methylated inositol (D-ononitol) (Sheveleva *et al.* 1997, 2000). When these transgenic plants were exposed to salt or drought stress, D-ononitol accumulated to concentrations that exceeded 35 μmol g^{-1} (f.m.) in the cytosol. Also, the CO_2 photosynthetic fixation was inhibited to a lesser extent during salt or drought stress in the transgenic plants that accumulated D-ononitol than in wild type plants.

Above discussed osmolytes are important and provide sufficient protection against water stress and salinity in transgenic plants. The observed tolerance was more attributed to the osmoprotectant effect of compatible osmolytes rather than to their contribution to osmotic adjustment (Chinnusamy *et al.* 2005).

Protection factors of macromolecules

LEA and LEA related: Osmotic stress induces late-embryogenesis-abundant (LEA) proteins, conferring dehydration tolerance to vegetative tissues in plants. LEA proteins and chaperones (heat-stress induced proteins) have shown to be involved in protecting macromolecules like enzymes, lipids and mRNAs from dehydration (Yamaguchi-Shinozaki *et al.* 2002; Table 3). LEA proteins were first characterized in cotton. During seed desiccation and in response to water stress, LEA proteins accumulate mainly in the embryo. LEA gene expression or LEA protein accumulation in plants under stress conditions indicate their role in plant stress tolerance. However, there is no clear experimental evidence supporting the exact functions of LEA proteins and the physiological roles of LEA proteins remain largely unknown (Xu *et al.* 1996).

Table 3. Stress responses of transgenic plants overexpressing LEA and LEA related genes, transport proteins and transcription factors.

Gene	Gene product and function	Transgenic plant	Performance of transgenic plants	Reference
Osm1, Osm4	24-kD protein	tobacco	retarded leaf senescence and improved seed germination at 200 mM NaCl	Barthakur *et al*. 2001
TLP-D34	thumatin-like protein (PR-5 member related to osmotin)	rice	enhanced osmotic adjustment and fungal protection	Datta *et al*. 2000
HVA1	group 3 LEA protein	rice	increased tolerance to water deficit and salt stress	Xu *et al*. 1996, Wu and Ho 1997
HVA1	group 3 LEA protein	wheat	increased biomass and WUE under stress	Sivamani *et al*. 2000
COR15a	15 kDa protein	*Arabidopsis*	increased freezing tolerance of chloroplasts and protoplasts	Steponkus *et al*. 1998
CuCOR19	19 kDa protein	tobacco	enhanced cold tolerance and inhibited lipid peroxidation	Hara *et al*. 2003
Yeast CAN/CNB	regulatory proteins (signal transduction)	tobacco	enhanced capacity to survive NaCl shock (250 mM)	Pardo *et al.* 1998
AtNHX1	Na^+/H^+ antiporter (transport proteins)	*Arabidopsis*	sustained growth and development up to 200 mM NaCl	Apse *et al*. 1999
AtNHX1	Na^+/H^+ antiporter (transport proteins)	*Brassica*	plants able to grow, flower and produce seeds in presence of 200 mM NaCl	Zhang *et al.* 2001
AtNHX1	Na^+/H^+ antiporter (transport proteins)	tomato	plants grow and fruit at very high salinity stress	Zhang and Blumwald 2001
HAL1	ion homeostasis	melon, *Arabidopsis*	plants showed increased salt tolerance	Bordas *et al.* 1997, Yang *et al.* 2001
HAL1	ion homeostasis	tomato	improved salt tolerance, able to retain more K^+ under salt stress	Gisbert *et al.* 2000, Rus *et al.* 2001b
HAL2	ion homeostasis	citrus	tolerance not recorded	Cervera *et al.* 2000
AtHal3	ion homeostasis	*Arabidopsis*	improved salt tolerance	Albert *et al.* 2000
SOS1	Na^+/H^+ antiporter (transport proteins)	*Arabidopsis*	plants could grow and remain green under 200 mM NaCl	Shi *et al.* 2000, 2002
ApNhaP	Na^+/H^+ antiporter (transport proteins)	*Synechococcus*	able to grow in sea water	Waditee *et al.* 2002
AVP1	transport protein (H^+- pump)	*Arabidopsis*	enhanced tolerance to salinity and drought stress	Gaxiola *et al.* 2001
Alfin1	zinc finger transcription factor	alfalfa	improved salt tolerance	Winicov and Bastola 1999
OSISAP1	zinc finger transcription factor	tobacco	improved tolerance to cold, dehydration and salt stress	Mukhopadyay *et al*. 2004
ABF3, ABF4	ABRE binding factors	*Arabidopsis*	reduced transpiration and enhanced drought tolerance	Kang *et al.* 2002
CBF1	transcription factor	tomato	plants more resistant to water deficit	Hsieh *et al.* 2002a,b
CBF1, CBF3	transcription factor	*Arabidopsis, Brassica*	increased freezing tolerance	Jaglo-Ottosen *et al*. 1998, Gilmour *et al.* 2000, Jagglo *et al*. 2001
CBF4	transcription factor	*Arabidopsis*	plants more tolerant to freezing and drought stress	Haake *et al.* 2002
DREB1A	transcription factor	*Arabidopsis*	increased salt, drought, and cold tolerance	Kasuga *et al*. 1999
DREB1A	transcription factor	tobacco	improved drought and low temperatures stress tolerance	Kasuga *et al*. 2004
DREB1A	transcription factor	wheat	improved resistance to water stress	Pellegrineschi *et al.* 2004
OsDREB	transcription factor	*Arabidopsis*	higher tolerance to drought, salt and freezing stress	Dubouzet *et al.* 2003
AtMYC2, AtMYB2	transcription factor	*Arabidopsis*	higher osmotic tolerance, hyper-sensitivity to ABA	Abe *et al.* 2003
CpMYB10	transcription factor	*Arabidopsis*	fast germination under NaCl and sorbitol	Villalobos *et al.* 2004
PDH45	DNA helicase 45	tobacco	grow to maturity and set viable seeds under continuous salinity	Mishra *et al.* 2005

Genes encoding LEA-type proteins are of different kinds such as RD (responsive to dehydration), ERD (early response to dehydration), KIN (cold inducible), COR (cold regulated), and RAB (responsive to ABA)

(Shinozaki and Yamaguchi-Shinozaki 2000, Zhu 2002). Overexpression of a barley group 3 LEA protein gene, *HAV1* in transgenic rice, showed better stress tolerance under salt and drought stress than wild-type plants (Xu *et al*. 1996, Wu and Ho 1997, Zhu *et al*. 1998). It has been found that certain specific LEA proteins may not be responsible for desiccation tolerance or their presence alone is not sufficient to prevent injury under desiccation conditions and probably LEA proteins together with soluble sugars contribute to the tolerance (Xu *et al*. 1996).

Expression of LEA-type genes under osmotic stress is regulated by both ABA-dependent and independent signaling pathways (Chinnusamy *et al*. 2005). Constitutive overexpression of *ABF3* and *ABREB2* in *Arabidopsis* enhances expression levels of target LEA-type genes (*RAB18* and *RD29*). These transgenic plants are hypersensitive to ABA, sugar and salt stress at the germination stage, but drought tolerant during seedling stage (Kang *et al*. 2002). Transgenic *Arabidopsis* plants overexpresing *AtMYC2* and *AtMYB2* show higher osmotic stress tolerance, although their salt tolerance is not known (Abe *et al*. 2003).

Dehydrins: Dehydrin, a group-2 LEA protein, is one of the several ubiquitous water-stress-responsive proteins in plants (Ingram and Bartels 1996; Table 3). Cryo-protective activities for freeze-sensitive enzymes were indicated in dehydrins from citrus (Hara *et al*. 2001). Recent genetic or transgenic approaches have also shown the relationship between dehydrin accumulation and cold acclimation (Steponkus *et. al*. 1998, Seki *et al*. 2002, Hara *et al*. 2003).

Osmotin was originally identified from salt adapted tobacco cells (Singh *et al*. 1985). The accumulation of this protein was correlated with osmotic adjustment (LaRosa *et al*. 1987). mRNAs encoding proteins with homology to osmotin have been isolated from many plant species (Kononowicz *et al*. 1993). In addition to their role as osmoprotectants, these genes can function in defense against pathogens (Liu *et al*. 1994). Transgenic potato overexpressing tobacco osmotin was reported (Liu *et al*. 1994). The osmotin gene is induced by fungal and viral protein in addition to osmotic stress as a result of dehydration, high salt and cold stress (LaRosa *et al*. 1992). Transgenic tobacco plants overexpressing osmotin gene showed retarded leaf senescence and improved seed germination under salt stress (Barthakur *et al*. 2001, 2002). Transgenic rice containing the coding region of a thaumatin-like protein (TLP-D34), a PR-5 protein conferred enhanced fungal protection and osmotic adjustment in rice (Datta *et al*. 2000).

Membrane proteins

Water channel proteins and transporters are proteins that function in the transport of water, sugars and proline (amino acid) through plasma membrane to adjust osmotic pressure under stress conditions (Yamaguchi-Shinozaki *et al*. 2002). Studies on sodium transport proteins such as AtHKT1 and AtNHX1 indicate that they can protect plant cells from the drying effect of salt by transporting sodium ions from the cell cytoplasm to vacuole. Genetic manipulation of salt uptake transport proteins in *Arabidopsis thaliana* showed increased salt tolerance (Moffat 2002). For example, overexpression of Na^+/H^+ antiporters *in Arabidopsis* plants promotes substantial growth and development under NaCl stress (Apse *et al*. 1999, Shi *et al*. 2000, 2002). Similar results have been reported for transgenic tomato and canola overexpressing *AtNHX1* (Zhang and Blumwald 2001, Zhang *et al*. 2001). Also overexpression of *AVP1* encoding the vacuolar H^+-pump in *Arabidopsis* led to increased water retention and drought tolerance (Gaxiola *et al*. 2001). Recently, Rus *et al*. (2001a) show that AtHKT1 transcript is a salt tolerant determinant that confers salt tolerance.

An alternate methodology to generate salt tolerant plants is the introduction of halotolerant genes involved in the regulation of ion homeostasis such as *HAL1*, *HAL2* and *HAL3* from yeast (Cervera *et al*. 2000, Albert *et al*. 2000, Yang *et al*. 2001). Overexpression of *HAL1* in tomato improved salt tolerance by maintaining a high internal K^+ concentration and decreasing intracellular Na^+ during salt stress (Gisbert *et al*. 2000, Rus *et al*. 2001b). Similar results were also reported in other plants (Bordas 1997, Albert *et al*. 2000). Modification of calcium related protein is another method claimed to improve plant stress tolerance. For example the introduction of a calcium/ calmodulin-dependent gene and the functional calcineurin activity led to increased salt tolerance in transgenic plants (Yang *et al*. 1997, Pardo *et al*. 1998, 1999). Other stress induced genes such as *OSISAP1* (encoding a zinc-finger protein) from rice, *PDH45* (encoding pea DNA helicase 45) are recently being characterized and their over-expression led to increased tolerance to cold, dehydration and salt stress in transgenic plants (Mukhopadhyay *et al*. 2004, Mishra *et al*. 2005).

Regulatory proteins

Transcription factors are small molecules that attach to specific sites on a DNA molecule in order to activate or deactivate expression of certain genes. Transcription factors could regulate the production of special proteins such as chaperons (heat shock proteins) under temperature and CBFs (C-repeat binding proteins) under

dehydration stresses. These regulatory proteins help to protect plant cells from the detrimental effects of heat or water-deficit stress.

Plant transformation with genes for regulatory proteins is an important approach towards the molecular studies on abiotic stress (Thomashow 2001). Analyses of the expression of dehydration-induced genes have shown that at least four independent signal pathways function in response to dehydration. Two are abscisic acid (ABA) dependent (Abe *et al*. 1997), and two are ABA-independent. ABA-independent regulation of LEA-type genes is mediated by transcription factors that activate DRE/CRT *cis*-elements of LEA-type protein encoding genes. Overexpression of *CBF* genes (*CBF1-4*) has resulted in activation of *DRE/CRT cis* elements and led to the expression of *LEA*-type genes (Jaglo *et al*. 2001, Haake *et al*. 2002), enhancing freezing and osmotic stress tolerance of transgenic *Arabidopsis* (Jaglo-Ottosen *et al*. 1998, Gilmour *et al*. 2000) and *Brassica napus* (Jaglo *et al*. 2001), and chilling and drought tolerance of tomato (Hsieh *et al*. 2002a,b).

Introduction of dehydration responsive element binding proteins (DREB) family genes under the control of different promoters were reported (Dubouzet *et al*. 2003). Transgenic *Arabidopsis* with cDNA encoding DREB1A (with dehydration response element B1A – a homologue of CBF1) using 35S CaMV promoter or stress inducible rd29A promoter was reported (Kasuga *et al*. 1999, Yamaguchi-Shinozaki *et al*. 2002). Transgenic wheat plants expressing *DREB1A* gene under the control of rd29A promoter showed substantial resistance to water stress in comparison with controls (Pellegrineschi *et al*. 2004). The stress inducible expression of this gene has minimal effects on plant growth and provided greater tolerance to stress conditions than genes driven by the 35S promoter. Recent studies on *Arabidopsis*, transformed with a *CpMYB10* promoter fused to *GUS* gene show reporter expression under ABA and stress conditions in several organs. Overexpression of *CpMYB10* cDNA in *Arabidopsis* led to desiccation and salt tolerance of transgenic lines, exhibiting glucose insensitive phenotypes indicating the involvement of this gene in stress tolerance and altering the ABA and glucose signaling responses (Villalobos *et al*. 2004).

The multiple abiotic stress tolerance, in addition to enhanced expression, of LEA-type genes shown by CBF-overexpressing transgenic plants, were attributed in part to the accumulation of compatible osmolytes (Gilmour *et al*. 2000), and enhanced oxidative stress tolerance (Hsieh *et al*. 2002a,b). However, it is not clear how CBF overexpressing plants activate the osmolyte biosynthesis or antioxidant defense pathways. Studies on genome expression analysis showed that CBF overexpression could trigger AP2 domain proteins such as RAP2.1 and RAP2.6, a putative zinc finger protein and R2R3-MYB73, that may regulate osmolyte biosynthesis and antioxidant defense genes (Fowler and Thomashow 2002). It was also reported that overexpression of a zinc finger transcription factor ALFIN1 activates MsPRP (NaCl responsive gene) expression and increases salt tolerance of alfalfa (Winicov and Bastola 1999). Therefore, transfer of CBFs and other transcription factors to crop plants under the control of stress specific promoters is a valid approach for engineering tolerance to multiple stresses.

Progress in transgenic plants for dehydration-stress tolerance

Transgenic technologies have widened the scope for crop improvement. During the past decade several crop plants were generated using gene transfer technologies for improved survival, better growth and yield traits. Abiotic stress reactions, especially to water deficit and high salinity are complex and involve many genes, with each individual gene playing a unique role in determining the overall stress tolerance and acclimation of plants (Chen and Murata 2002). This makes the possibilities of abiotic stress improvement rather difficult and slow when compared to quality traits or biotic stress. However, considerable progress in producing transgenic plants with improved tolerance to various stress conditions were achieved, albeit with varying degrees of success.

Among them, some of the research results of the recent years claimed to have considerable increment in abiotic stress tolerance are the development of transgenic rice with trehalose gene, expression of the *mt1D* gene for the biosynthesis of mannitol in transgenic wheat, the overexpression of polyamine biosynthesis genes *adc* and *samdc* in rice, and the plastid expressed *badh* gene in carrot plants (Garg *et al*. 2002, Abebe *et al*. 2003, Capell *et al*. 2004, Kumar *et al*. 2004). The recent claim of transgenic rice overexpressing trehalose gene is a remarkable achievement in transgenic research for abiotic stress tolerance, as they grow better under three types of abiotic stresses namely, drought, salinity, and cold temperatures.

Over the past several years other studies were also conducted on detoxification enzymes and transgenic plants were produced for improved oxidative stress tolerance including environmental stresses (Moffat 2002, Datta 2002, Sunkar *et al*. 2003). In the coming years, it will be likely that the knowledge gained from stress tolerant transgenic model plants such as *Arabidopsis* will be incorporated into additional crop plants and their potential field testing may allow their feasibility to be used in various crop-improvement programs.

Use of stress inducible promoters

With the identification of several stress genes, there is an urgent need to express them with the help of suitable promoters. With constitutive promoters a gene is expressed in the majority of tissues throughout plant development and hence has a number of drawbacks for the efficient use in genetically improved crops of the future (Gittins *et al*. 2000). For example, constitutive overproduction of trehalose (Romero *et al*. 1997) or polyamines (Capell *et al*. 1998) causes abnormalities in plants grown under normal conditions. The use of stress inducible specific promoters may protect transgenic plants from such growth abnormalities.

This has been demonstrated by the stable transformation of ABA-inducible expression system as well as the expression of gene encoding a key proline synthesizing enzyme in rice (Su *et al*. 1998, Su and Wu 2004). The use of stress inducible promoters in these studies successfully removed inhibition of growth under low-stress conditions. Most of the stress promoters contain an array of stress-specific *cis*-acting elements that are recognized by the requisite transcription factors. Identification and characterization of stress-induced promoters, particularly those induced by anaerobic conditions, low or high temperature and salt stresses, have been reported (Busk and Pages 1998, Grover *et al*. 2001). Recently, Kasuga *et al*. (2004) overexpressed the *DREB1A* cDNA in *A. thaliana* driven by either the constitutive CaMV 35S or the stress inducible rd29A promoter (Kasuga *et al*. 1999). Plants in which *DREB1A* was expressed constitutively showed morphological abnormalities under unstressed conditions, whereas plants expressing the *DREB1A* under the control of rd29A were healthy and highly tolerant to abiotic stress. More recently, the use of a combination of the *Arabidopsis DREB1A* gene and stress-inducible rd29A promoter, improved drought and low-temperature stress tolerance in transgenic tobacco (Kasuga *et al*. 2004) and their result indicates that a combination of the rd29A promoter and *DREB1A* is useful for improvement of various kinds of transgenic plants that are tolerant to environmental stress.

Also, in biotechnological applications it will be useful to limit transgene expression to particular tissues by the use of tissue specific promoters, a strategy drawing increasing attention recently (Gittins *et al*. 2001). Another strategy is to target the products to specific organelles such as the accumulation of glycinebetaine or mannitol in chloroplasts and their high level expression under abiotic stress (Shen *et al*. 1997, Sakamoto *et al*. 1998, Kumar *et al*. 2004).

Future prospects

The feasibility of transgenic approaches for improved abiotic stress tolerance has been widely accepted. However, many of the transgenic plants produced in the past several years with single-gene transfer could only bring marginal stress tolerance. Therefore, the use of multigene approach with simultaneous transfer of several genes offers substantial promise because of the possible inheritance of all the transgenes in the same locus (Bajaj *et al*. 1999). At present, some of the proposed strategies for producing highly stress tolerant transgenic plants were the use of stress inducible specific promoters, testing of transgenic plants up to several generations to confirm the stable high level expression and the possible use of homozygous lines for field testing. Methodologies such as RNAi and transposon insertional knockouts for the candidate genes and signaling pathways are expected to produce more stress-tolerant crop plants in the near future (Xiong and Zhu 2002). Also the possible future targets for transformation includes integration of data gained from genomic and post genomic projects. The knowledge gained from the genomic and post genomic projects together with proteomics research promises a great deal for the transgenic research and for the agriculture of tomorrow.

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