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REVIEW

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Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance

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Abstract Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and the natural status of the environment. Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050. Therefore, breeding for drought and salinity stress tolerance in crop plants (for food supply) and in forest trees (a central component of the global ecosystem) should be given high research priority in plant biotechnology programs. Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific stress-related genes. These genes are involved in the whole sequence of stress responses, such as signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging. Recently, research into the molecular mechanisms of stress responses has started to bear fruit and, in parallel, genetic modification of stress tolerance has also shown promising results that may ultimately apply to agriculturally and ecologically important plants. The present review summarizes the recent advances in elucidating stress-response mechanisms and their biotechnological applications. Emphasis is placed on transgenic plants that have been engineered based on different stress-response mechanisms. The review examines the following aspects: regulatory controls, metabolite engineering, ion transport, antioxidants and detoxification, late embryogenesis abundant (LEA) and heat-shock proteins.

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Abbreviations HSF: heat-shock factor · Hsp: heat-shock protein · LEA protein: late embryogenesis abundant protein · ROS: reactive oxygen species · SP1: stable protein 1 · TF: transcription factor

Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer 1982; Bray et al. 2000). Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001a). Drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano et al. 1999; Zhu 2001a). Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins (Smirnoff 1998). As a consequence, these diverse environmental stresses often activate similar cell signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2000; Knight and Knight 2001; Zhu 2001b, 2002) and cellular responses, such as the production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes (Vierling

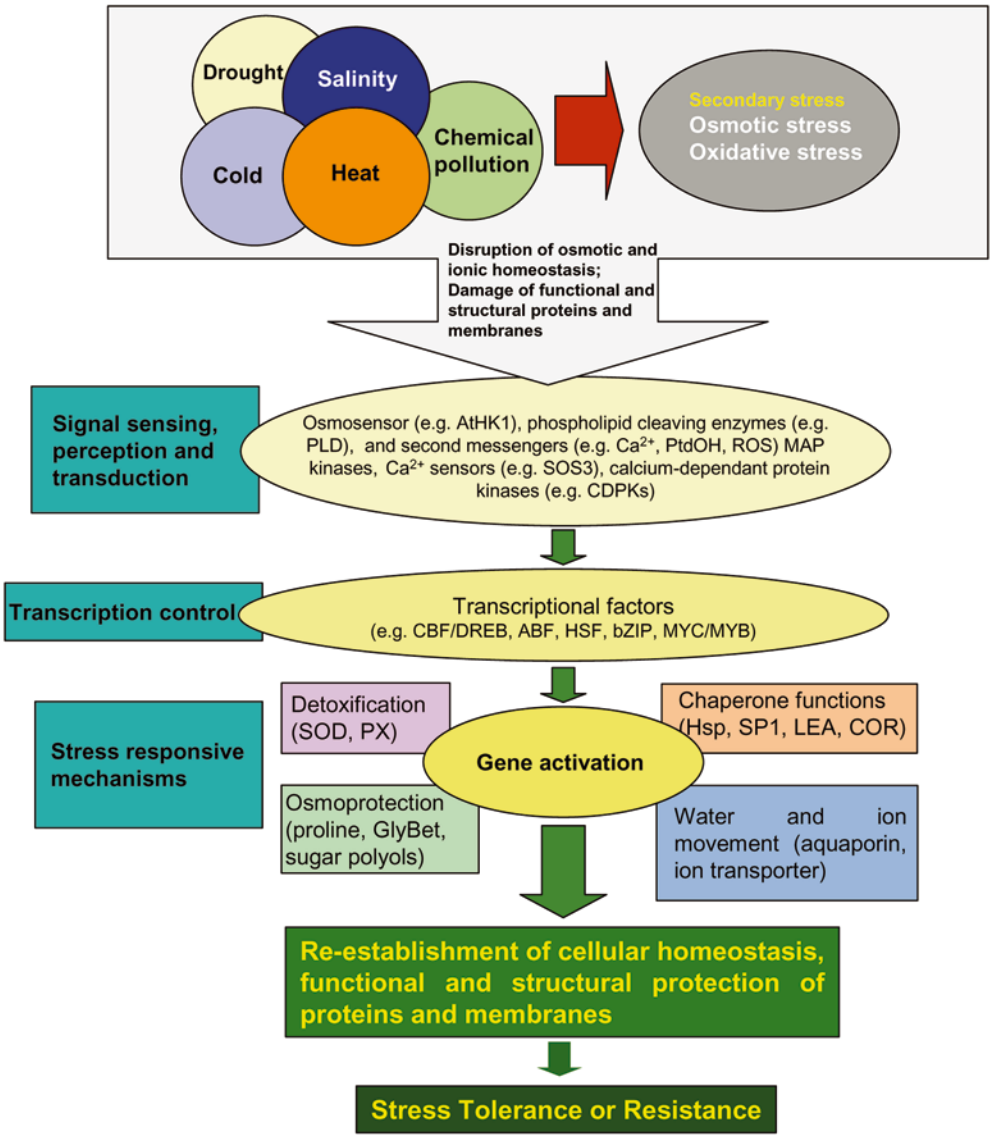

Fig. 1 The complexity of the plant response to abiotic stress. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected, and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects, or temperature, membrane fluidity changes) trigger the downstream signaling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signaling and gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death

and Kimpel 1992; Zhu et al. 1997; Cushman and Bohnert 2000).

The complex plant response to abiotic stress, which involves many genes and biochemical-molecular mechanisms, is schematically represented in Fig. 1. The ongoing elucidation of the molecular control mechanisms of abiotic stress tolerance, which may result in the use of molecular tools for engineering more tolerant plants, is based on the expression of specific stress-related genes. These genes include three major categories: (i) those that are involved in signaling cascades and in transcriptional control, such as MyC, MAP kinases and SOS kinase (Shinozaki and Yamaguchi-Shinozaki 1997; Munnik et al. 1999; Zhu 2001b), phospholipases (Chapman 1998; Frank et al. 2000), and transcriptional factors such as HSF, and the CBF/DREB and ABF/ABAE families (Stochinger et al. 1997; Schöffl et al. 1998; Choi et al. 2000; Shinozaki and Yamaguchi-Shinozaki 2000); (ii) those that function directly in the protection of membranes and proteins, such as heat-shock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA) proteins (Vierling 1991; Ingram and Bartels 1996; Tomashow 1998, 1999; Bray et al. 2000), osmoprotectants, and free-radical scavengers (Bohnert and Sheveleva 1998); (iii) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters (Maurel 1997; Serrano et al. 1999; Tyerman et al. 1999; Zimmermann and Sentenac 1999; Blumwald 2000). The readers can refer to many excellent reviews on this topic (Vierling 1991; Ingram and Bartels 1996; Bohnert and Sheveleva 1998; Smirnoff 1998; Tomashow 1998, 1999; Serrano et al. 1999; Blumwald 2000; Bray et al. 2000; Cushman and Bohnert 2000; Hasegawa et al. 2000; Shinozaki and Yamaguchi-Shinozaki 1997, 2000; Serrano and Rodriguez-Navarro 2001; Zhu 2002).

To maintain growth and productivity, plants must adapt to stress conditions and exercise specific tolerance

mechanisms. Plant modification for enhanced tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. In contrast to most monogenic traits of engineered resistance to pests and herbicides, the genetically complex responses to abiotic stress conditions are more difficult to control and engineer. Present engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. The current efforts to improve plant stress tolerance by gene transformation have resulted in important achievements; however, the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental side effects, make this task extremely difficult.

The primary objective of this review is to report recent advances in the stress-response mechanisms and their biotechnological applications in plants. Emphasis is given to transgenic plants that were engineered for stress tolerance, based on different mechanisms of stress response. The following major mechanisms and aspects are discussed: regulatory controls, osmolyte engineering, ion transport, detoxification mechanisms and the role of LEA and Hsps. The different transgenic plants that are discussed in this review are summarized in Table 1.

Transcription factors and their significance in plant stress tolerance

Plant stress responses are regulated by multiple signaling pathways that activate gene transcription and its downstream machinery. Plant genomes contain a large number of transcription factors (TFs); for example,

Table 1 Mechanisms, genes and genetically modified plant species implicated in plant responses to abiotic stress

Mechanism	Genes	Species	Reference
Transcription control	CBF1	*Arabidopsis thaliana*	Jaglo-Ottosen et al. 1998
	DREB1A	*A. thaliana*	Kasuga et al. 1999
	CBF3	*A. thaliana*	Gilmour et al. 2000
	CBFs	*Brassica napus*	Jaglo et al. 2001
	CBF1	*Lycopersicon esculentum*	Hsieh et al. 2002
	CBF4	*A. thaliana*	Haake et al. 2002
	AtMYC2 and AtMYB2	*A. thaliana*	Abe et al. 2003
	ABF3 or ABF4	*A. thaliana*	Kang et al. 2002
	HSF1 and HSF3	*A. thaliana*	JH Lee et al. 1995; Prändl et al. 1998
	HsfA1	*L. esculentum*	Mishra et al. 2002
	spl7	*Oryza sativa*	Yamanouchi et al. 2002
Compatible solute			
Proline	P5CS	*Nicotiana tabacum*	Kishor et al. 1995; Konstantinova et al. 2002; Hong et al. 2000
	ProDH	*A. thaliana*	Nanjo et al. 1999
Myo-inositol	IMT1	*N. tabacum*	Sheveleva et al. 1997
Sorbitol	*stpd1*	*N. tabacum*	Sheveleva et al. 1998
Antioxidants and detoxification	CuZn-SOD	*N. tabacum*	Gupta et al. 1993a, 1993b; Pitcher and Zilinskas 1996
	Mn-SOD or Fe-SOD	*Medicago sativa*, *N. tabacum*	McKersie et al. 1996, 1999, 2000; Van Camp et al. 1996
	GST and GPX	*N. tabacum*	Roxas et al. 1997
	*chy*B	*A. thaliana*	Davison et al. 2002
	Aldose-aldehyde reductase	*N. tabacum*	Oberschall et al. 2000
Ion transport	*AtNHX1*	*A. thaliana*	Apse et al. 1999
		B. napus	Zhang et al. 2001
		L. esculentum	Zhang and Blumwald 2001
	SOS1	*A. thaliana*	Shi et al. (2003)
	HAL1	*Cucurbita melo*	Bordas et al. 1997 Rus et al. 2001
	AVP1	*A. thaliana*	Gaxiola et al. 2001
Hsps and molecular chaperones	Hsp17.7	*Daucus carota*	Malik et al. 1999
	Hsp21	*A. thaliana*	Härndahl et al. 1999
	AtHSP17.6A	*A. thaliana*	Sun et al. 2001
	DnaK1	*N. tabacum*	Sugino et al. 1999
	SP1	*Populus tremula*	Wang et al. 2003
LEA-type proteins	COR15a	*A. thaliana*	Artus et al. 1996; Steponkus et al. 1998; Jaglo-Ottosen et al. 1998
	HVA1	*O. sativa*	Xu et al. 1996
		Triticum aestivum	Sivamani et al. 2000
	WCS19	*A. thaliana*	Ndong et al. 2002

Arabidopsis dedicates about 5.9% of its genome coding for more than 1,500 TFs (Riechmann et al. 2000). Most of these TFs belong to a few large multigene families, e.g. MYB, AP2/EREBP, bZIP and WRKY. Individual members of the same family often respond differently to various stress stimuli; on the other hand, some stress-responsive genes may share the same TFs, as indicated by the significant overlap of the gene-expression profiles that are induced in response to different stresses (Bohnert et al. 2001; Seki et al. 2001; Chen et al. 2002; Fowler and Thomashow 2002; Kreps et al. 2002). Some key examples are discussed below.

The dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) bind to DRE and CRT *cis*-acting elements that contain the same motif (CCGAC). Members of the CBF/DREB1 family, such as CBF1, CBF2, and CBF3 (or DREB1B, DREB1C, and DREB1A, respectively) are themselves stress-inducible. DREB/CBF proteins are encoded by AP2/EREBP multigene families and mediate the transcription of several genes such as *rd29A*, *rd17*, *cor6.6*, *cor15a*, *erd10*, *kin1*, *kin2* and others in response to cold and water stress (Ingram and Bartels 1996; Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998; Seki et al. 2001; Thomashow et al. 2001).

Significant improvement of stress tolerance was found upon overexpression of a single TF in engineered *Arabidopsis thaliana* plants. *Arabidopsis* cold acclimation is associated with the induction of COR (cold-regulated) genes by the CRT/DRE *cis*-regulatory elements (Thomashow 1998). Jaglo-Ottosen et al. (1998) showed that increased expression of *Arabidopsis* CBF1 induces the expression of the cold-regulated genes *cor6.6*, *cor15a*, *cor47*, and *cor78*, and increased the freezing tolerance of non-acclimated *Arabidopsis* plants. *Arabidopsis* transformation with the DREB1A gene (Kasuga et al. 1999), driven by either the strong constitutive promoter of the cauliflower mosaic virus (35SCaMV) or by a DRE-containing promoter from the dehydration-induced gene (*rd29A*), resulted in a marked increase in tolerance to freezing, water and salinity stress. Similar to the CBF1 transgene, constitutive expression of DREB1A transcription factor resulted in increased expression of its downstream targeted genes, such as *rd29A*, *rd17*, *cor6.6*, *cor15a*, *erd10*, and *kin1*. Overexpression of CBF3 in *Arabidopsis* also increased freezing tolerance and, more interestingly, resulted in multiple biochemical changes associated with cold acclimation: elevated levels of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose (Gilmour et al. 2000). Plants overexpressing CBF3 also had elevated Δ^1-pyrroline-5-carboxylate synthetase (P5CS) transcript levels, suggesting that the increase in proline levels had resulted, in part, from increased expression of the key proline biosynthetic enzyme P5CS.

Components of the *Arabidopsis* CBF/DREB cold-response pathway were also found in *Brassica napus* and other plant species (Jaglo et al. 2001). Constitutive overexpression of the *Arabidopsis* CBF genes in transgenic *B. napus* plants induced expression of orthologs of *Arabidopsis* CBF-targeted genes and increased the freezing tolerance of both non-acclimated and cold-acclimated plants. Recently, expression of *Arabidopsis* CBF1 in tomato plants has been shown to confer elevated tolerance to chilling and oxidative stress (Hsieh et al. 2002). However, the expression of *cor* genes was not induced, while reactive oxygen species (ROS) scavenger genes, e.g. *CAT1*, were activated. Recently, a close CBF/DREB1 homolog in *Arabidopsis*, CBF4, was isolated (Haake et al. 2002). The expression of CBF4 is rapidly induced during drought stress and by abscisic aid (ABA) treatment, but not by cold, thereby distinguishing it from CBF/DREB1 transcription factors. Overexpression of CBF4 under the constitutive CaMV35S promoter resulted in the expression of cold- and drought-induced genes under non-stress conditions, and the transgenic *Arabidopsis* plants showed more tolerance to freezing and drought conditions (Haake et al. 2002).

ABA signaling plays a vital role in plant stress responses as evidenced by the fact that many of the drought-inducible genes studied to date are also induced by ABA. Two TF families bZIP and MYB, are involved in ABA signaling and its gene activation. Many ABA-inducible genes share the (C/T)ACGTGGC consensus, *cis*-acting ABA-responsive element (ABRE) in their promoter regions (Guiltinan et al. 1990; Mundy et al. 1990). Several ABRE-binding proteins, including rice TRAB and *Arabidopsis* AREB/ABF and ABI5, which interact with ABRE and regulate gene expression, have been isolated (Hobo et al. 1999; Choi et al. 2000; Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000; Uno et al. 2000; Kang et al. 2002). Recently, Abe et al. (2003) showed that the *Arabidopsis* MYB transcription factor proteins AtMYC2 and AtMYB2 function as transcriptional activators in ABA-inducible gene expression, suggesting a novel regulatory system for gene expression in response to ABA, other than the ABRE-bZIP regulatory system.

Constitutive expression of ABF3 or ABF4 demonstrated enhanced drought tolerance in *Arabidopsis* plants, with altered expression of ABA/stress-responsive genes, e.g. *rd29B*, *rab18*, *ABI1* and *ABI2* (Kang et al. 2002). Several ABA-associated phenotypes, such as ABA hypersensitivity and sugar hypersensitivity, were observed in transgenic plants. Moreover, salt hypersensitivity was observed in ABF3- and ABF4-overexpressing plants at the germination and young seedling stage, indicating the possible participation of ABF3 and ABF4 in the salt response at these particular developmental stages. Improved osmotic-stress tolerance in 35S:AtMYC2/AtMYB2 transgenic plants, as judged by an electrolyte-leakage test (Abe et al. 2003), is yet another example of how plant engineering with transcriptional activators of ABA signaling can provide a means of improving plant stress tolerance.

Similar to osmotic stress, the heat-shock response is primarily regulated at the transcriptional level.

Thermo-inducibility is attributed to conserved *cis*-regulatory promoter elements (HSEs) that are the binding sites for the *trans*-active heat-shock factors (HSFs; Schöffl et al. 1998). The HSEs share a common consensus sequence "nGAAnnTTCnnGAAn". Plant HSFs, which are further categorized into three classes, A, B and C, appear to be a unique family containing a number of members: 21 from *Arabidopsis*, more than 16 from tomato, and 15 from soybean (Nover et al. 2001). Hsps are chaperones, which function during both normal cell growth and stress conditions; therefore it is not surprising that HSFs provide diverse functions that differentially control the activation of heat-shock genes (Morimoto 1998; Schöffl et al. 1998; Mishra et al. 2002). It has been shown that overexpression of HSF1 and HSF3 (class A) leads to the expression of several *hsp* genes conferring thermo-tolerance in transgenic plants (JH Lee et al. 1995; Prändl et al. 1998). In tomato plants, overexpression of HsfA1 resulted in heat-stress tolerance, while HsfA1 antisense plants and fruits were extremely sensitive to elevated temperatures (Mishra et al. 2002). Analysis of the transgenic plants disclosed that HsfA1 has a unique role as a master regulator for the synthesis of other HSFs such as HSFs A2 and B1 as well as Hsps. HSFs may also play a role in controlling cell death. The rice *spl* (spotted leaf) gene *spl7* encodes a class-A HSF, and the *spl7* transgenic rice showed no lesions (spotted leaf) or delay in development of lesions (Yamanouchi et al. 2002). The experiment suggested that *spl7* might participate in controlling cell death that is caused by environmental stresses such as high temperature.

These studies demonstrate the important role of TFs in the acquisition of stress tolerance, which may ultimately contribute to agricultural and environmental practices. Although plant transformation with stress-responsive TFs permits overexpression of downstream stress-associated multiple genes, it may also activate additional non-stress genes that adversely affect the normal agronomic characteristics of a crop. One common negative effect of TF-modified plants is the growth retardation in transgenic plants that constitutively express TFs (Kasuga et al. 1999; Hsieh et al. 2002; Kang et al. 2002; Abe et al. 2003). For example, a positive correlation was found between the levels of DREB1A expression, the level of expression of the target gene RD29A, and the degree to which plants growth is stunted (Liu et al. 1998). These negative effects can be partially prevented by the use of stress-inducible promoters that control the expression of the TF (Kasuga et al. 1999).

Applications of compatible-solute engineering

Compatible solutes, or osmolytes, accumulate in organisms in response to osmotic stress. The primary function of compatible solutes is to maintain cell turgor and thus the driving gradient for water uptake. Recent studies indicate that compatible solutes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and/or proteins (Lee et al. 1997; Hare et al. 1998; Bohnert and Shen 1999; McNeil et al. 1999; Diamant et al. 2001). Compatible solutes fall into three major groups: amino acids (e.g. proline), quaternary amines (e.g. glycine betaine, dimethylsulfoniopropionate) and polyol/sugars (e.g. mannitol, trehalose). Overexpression of compatible solutes in transgenic plants can result in improved stress tolerance.

Proline is synthesized from glutamate via glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C). P5C synthase (P5CS) catalyzes the conversion of glutamate to P5C, followed by P5C reductase (P5CR), which reduces P5C to proline. In the reverse reaction, proline is metabolized to glutamate in a feed-back manner, via P5C and GSA with the aid of proline dehydrogenase (ProDH) followed by P5C dehydrogenase (P5CDH). Transgenic tobacco (*Nicotiana tabacum*) overexpressing the *p5cs* gene that encodes P5CS produced 10- to 18-fold more proline and exhibited better performance under salt stress (Kishor et al. 1995). Freezing tolerance was achieved by transforming tobacco with the same gene (*p5cs*; Konstantinova et al. 2002). Because P5CS is the rate-limiting enzyme in proline biosynthesis in plants, and is subject to feedback inhibition by proline, removal of the feedback inhibition can result in high level of proline accumulation (Hong et al. 2000). Transgenic tobacco overexpressing the mutated form of *Vigna aconitifolia* P5CS (P5CSF129A) accumulated about 2-fold more proline than plants expressing wild-type P5CS when the feedback inhibition induced by proline was eliminated and this difference was further increased in plants under salt stress. The elevated proline also reduced free-radical levels in response to osmotic stress and significantly improved the ability of the transgenic seedlings to grow in a medium containing up to 200 mM NaCl (Hong et al. 2000).

An alternative strategy for sustaining a high level of proline during stress is to down-regulate proline catabolism. Proline dehydrogenase (ProDH) catalyzes the first step of proline degradation. Repression of *Arabidopsis* proline dehydrogenase (AtProDH) mRNA in antisense transgenic plants resulted in the constitutive accumulation of proline (Nanjo et al. 1999). AtProDH-antisense-transgenic plants showed more tolerance to freezing (−7 °C) and to NaCl (600 mM) than wild-type and vector-transformed plants.

Betaines are quaternary ammonium compounds, i.e. amino acid derivatives in which the nitrogen atom is fully methylated. In plants, glycine betaine, a representative member of this group of osmolytes, is synthesized in the chloroplast from choline by a two-step process. The first step (choline to betaine aldehyde) is mediated by choline monooxygenase (CMO), which can be induced by drought and salinity (Russell et al. 1998). The second step (betaine aldehyde to glycine betaine) is catalyzed by betaine aldehyde dehydrogenase (BADH),

an NAD-dependent dehydrogenase. Many important crops, such as rice, potato and tomato, do not accumulate glycine betaine and are therefore potential candidates for the engineering of betaine biosynthesis (McCue and Hanson 1990). Genetic engineering for glycine betaine biosynthesis in non-accumulating plants has been extensively reported (Lilius et al. 1996; Hayashi et al. 1997; Alia et al. 1998a, 1998b; Sakamoto et al. 1998, 2000; also see review of Sakamoto and Murata 2002, and references therein). Transgenic plants expressing bacterial choline-oxidizing enzymes displayed increased tolerance to various stresses such as high salt concentrations and extreme temperatures (reviewed in Sakamoto and Murata 2001). The main constraint to glycine betaine accumulation in transgenic plants appears to be the endogenous choline supply (Nuccio et al. 1998). Therefore, up-regulation of the de novo synthesis of choline to increase glycine betaine synthesis is also imported in non-accumulators, which express foreign choline-oxidizing enzymes (Nuccio et al. 1998; Huang et al. 2000). In addition, correct subcellular targeting of the inserted gene also has a significant impact on its expression and expected functions (Sakamoto et al. 1998; Konstantinova et al. 2002).

A number of "sugar alcohols" (mannitol, trehalose, *myo*-inositol and sorbitol) have been targeted for the engineering of compatible-solute overproduction. Tarczynski et al. (1993) introduced a bacterial gene that encodes mannitol 1-phosphate dehydrogenase into tobacco plants, resulting in mannitol accumulation and enhanced tolerance to salinity. In addition, transgenic tobacco plants carrying a cDNA encoding *myo*-inositol *O*-methyltransferase (IMT1) accumulated D-ononitol and, as a result, acquired enhanced photosynthesis protection and increased recovery under drought and salt stress (Sheveleva et al. 1997). When engineering these 'sugar polyols' in plants, pleiotropic effects sometimes occurred. Abnormal phenotypes associated with sorbitol accumulation were also found in transgenic tobacco transformed with *stpd1*, a cDNA encoding sorbitol-6-phosphate dehydrogenase from apple (Sheveleva et al. 1998). Plants with low levels of sorbitol (less than 2–3 μmol g^{-1} fresh weight) developed normally, but necrotic lesions and reduced shoot and root growth, as well as low fertility, were associated with an excessive sorbitol accumulation. The adverse effects observed in these sorbitol overproducers may have resulted from a disturbance in carbohydrate transport and allocation (Sheveleva et al. 1998).

Recently, Garg et al. (2002) showed rice tolerance to multiple abiotic stresses by engineering trehalose overexpression without the negative pleiotropic effects observed in some other studies. The modest increase in trehalose levels in transgenic lines, using either the tissue-specific or stress-dependent promoters, resulted in a higher capacity for photosynthesis and a concomitant decrease in the photo-oxidative damage during stress. Although the elevated trehalose levels do not seem to account for osmoprotection activity, the findings demonstrate the feasibility of engineering rice for increased tolerance and enhanced productivity through tissue-specific or stress-dependent overproduction of trehalose.

Genetic manipulations of compatible solutes do not always lead to a significant accumulation of the compound (except some cases of proline over-production; Chen and Murata 2002), suggesting that the function of compatible solutes is not restricted to osmotic adjustment, only. Accumulation of compatible solutes may also protect plants against damage by scavenging of reactive oxygen species, and by their chaperone-like activities in maintaining protein structures and functions. Engineered overproduction of these compatible solutes provides an opportunity to generate more tolerant plants. Incorrect gene expression of compatible solutes often causes pleiotropic effects (e.g. necrosis and growth retardation) due to disturbance in endogenous pathways of primary metabolisms. For agricultural practices, over-synthesis of compatible solutes should not account for the primary metabolic costs. Moreover, to minimize the pleiotropic effects, the over-production of compatible solutes should be stress-inducible and/or tissue specific (Garg et al. 2002).

Antioxidants and detoxification genes

Salt, drought, heat and oxidative stress are accompanied by the formation of ROS such as O_2, H_2O_2, and OH^- (Price et al. 1989; Moran et al. 1994, Mittler 2002), which damage membranes and macromolecules. Plants have developed several antioxidation strategies to scavenge these toxic compounds. Enhancement of antioxidant defense in plants can thus increase tolerance to different stress factors. Antioxidants (ROS scavengers) include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase, as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Additional compounds, such as osmolytes, proteins (e.g. peroxiredoxin) and amphiphilic molecules (e.g. tocopherol), can also function as ROS scavengers (Bowler et al. 1992; Noctor and Foyer 1998).

Transgenic tobacco plants overexpressing chloroplastic Cu/Zn-SOD showed increased resistance to oxidative stress caused by high light and low temperatures (Gupta et al. 1993a, 1993b). Transgenic alfalfa plants (*Medicago sativa*) expressing Mn-SOD evinced reduced injury from water-deficit stress, as determined by chlorophyll fluorescence, electrolyte leakage and re-growth (McKersie et al. 1996). In another study, transgenic alfalfa plants expressing either Mn-SOD or Fe-SOD had increased winter survival rates and yields (McKersie et al. 1999, 2000). However, this was not associated with protection against primary injury caused by freezing, and the Fe-SOD transgenic alfalfa did not show greater tolerance to oxidative stress (McKersie et al. 2000). As

proposed by the authors, Fe-SOD overexpression may reduce secondary injury symptoms and thereby enhance recovery from the stress experienced during the winter. Mn-SOD-expressing transgenic tobacco showed a 2- to 3-fold reduction in leaf injury compared to wild-type plants following exposure to ozone (Van Camp et al. 1996). In addition, overexpression of Cu/Zn-SOD in the cytosol of transgenic tobacco plants was found to confer partial resistance to ozone-induced foliar necrosis (Pitcher and Zilinskas 1996). Transgenic tobacco seedlings, overexpressing cDNA which encodes an enzyme with both glutathione *S*-transferase (GST) and glutathione peroxidase (GPX) activity, grew significantly faster than control seedlings following exposure to chilling or salt stress (Roxas et al. 1997).

In *Arabidopsis*, overexpression of the *chy*B gene that encodes β-carotene hydroxylase (an enzyme active in the zeaxanthin biosynthetic pathway) resulted in a 2-fold increase in the pool of the xanthophyll cycle (Davison et al. 2002). These transgenic plants showed greater tolerance to high light and increased temperatures, and it was suggested that the stress protection was most likely due to the action of zeaxanthin in preventing oxidative damage to membranes.

Transgenic tobacco plants expressing alfalfa aldose-aldehyde reductase, a stress-activated enzyme, showed reduced damage when exposed to oxidative stress and increased tolerance to heavy metals, salt and dehydration stress (Oberschall et al. 2000). This novel enzyme is an NADPH-dependent aldose/aldehyde reductase, which is believed to be involved in detoxification by reducing the level of reactive aldehydes.

Targeting detoxification pathways is an appropriate approach for obtaining plants with multiple stress-tolerance traits (Bartels 2001). It is expected that with our increasing understanding of those pathways, it will become possible to produce transgenic plants that can be sustained under true field conditions with multiple environmental stresses.

Engineering for ion transport

Osmotic stress, ion toxicity and high salt content in the soil and the irrigation water, especially Na^+ and Cl^-, significantly impair plant growth. Ion transporters selectively transport ions and maintain them at physiologically relevant concentrations while Na^+/H^+ antiporters also play a crucial role in maintaining cellular ion homeostasis, thus permitting plant survival and growth under saline conditions. The Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across membranes and have a variety of functions, such as regulating cytoplasmic pH, sodium levels and cell turgor (Serrano et al. 1999). Plant Na^+/H^+ antiporters have been isolated from *Arabidopsis* (AtNHX1, SOS1; Gaxiola et al. 1999; Shi et al. 2000) and rice plants (Fukuda et al. 1999) and from the halophytic plants *Atriplex gmelini* (Hamada et al. 2001) and *Mesembryanthemum crystallinum* (Chauhan et al. 2000). Overexpression of the vacuolar Na^+/H^+ antiporter *AtNHX1* in *Arabidopsis* plants (Apse et al. 1999) promoted growth and development in potting medium irrigated with up to 200 mM sodium chloride. This salinity tolerance was positively correlated with elevated levels of AtNHX1 transcript, and with protein and vacuolar Na^+/H^+ antiporter activity. Transgenic *Brassica napus* plants overexpressing AtNHX1 were able to grow, flower and produce seeds, in the presence of 200 mM sodium chloride, even though they accumulated sodium at a rate of up to 6% dry weight. Moreover, their seed yields and seed oil quality were not altered by the high soil salinity (Zhang et al. 2001). Similarly, transgenic tomato plants overexpressing this gene were able to grow, flower and produce fruit in the presence of 200 mM sodium chloride (Zhang and Blumwald 2001). Although the tomato leaves accumulated high sodium concentrations, the fruits displayed very low sodium content, demonstrating the potential to maintain fruit yield and quality at high salt levels.

The *A. thaliana* plasma membrane Na^+/H^+ antiporter, encoded by the SOS1 gene, was suggested to be essential for salt tolerance (Shi et al. 2002), and recently Shi et al. (2003) reported that overexpression of SOS1 improves salt tolerance in transgenic *Arabidopsis*. The authors also showed that the increased salt tolerance was correlated with reduced Na^+ accumulation.

Saccharomyces cerevisiae cation transport systems, such as HAL1 and HAL3, are involved in the regulation of K^+ and Na^+ transport, respectively. Shoots from transgenic melon plants expressing the HAL1 gene showed some level of salt tolerance in vitro (Bordas et al. 1997), and transgenic tomato lines expressing the HAL1 gene were found to be more salt-tolerant than the wild-type plants, as judged by both callus and plant growth in short-term experiments (Gisbert et al. 2000). Such transgenic lines also demonstrated better fruit yield under salt stress (Rus et al. 2001).

In plants, protons are used as coupling ions for ion transport systems, and the proton gradient, generated by proton pumps found in the cell membrane, is the driving force for nutrient uptake (Serrano et al. 1999). Three distinct proton pumps are responsible for the generation of the proton electrochemical gradients (Sze et al. 1999): (i) the plasma membrane H-ATPase pump (PM H-ATPase) which extrudes H^+ from the cell and thus generates a proton motive force; (ii) the vacuolar-type H-ATPase pump (V-ATPase); (iii) the vacuolar H-pumping pyrophosphatase pump (H-PPase). The latter two acidify the vacuolar lumen and other endomembrane compartments. *Arabidopsis* plants were transformed with a vacuolar H^+-PPase pump that is encoded by a single gene, *AVP1* (Gaxiola et al. 2001), which can generate an H^+ gradient across the vacuolar membrane, similar in magnitude to that of the multisubunit vacuolar H^+-ATPase pump. These transgenic plants expressed higher levels of AVP1 and were more resistant to salt and drought than wild-type plants. It

was also found that the resistant phenotypes had an increased vacuolar proton gradient, resulting in increased solute accumulation and water retention.

Maintenance and re-establishment of cellular ion homeostasis during stress and/or following stress is extremely important for plant survival and growth, especially for prevention, compartmentation or exclusion of sodium ions under salinity stress. Overexpression of the genes involved in cellular ion homeostasis have already resulted in significant improvement; however more careful utilization of specific genes, including targeting to different types of cells and organelles, should result in even greater salt-stress tolerance under true field conditions.

The role of Hsps and LEA-type proteins

To cope with environmental stress, plants activate a large set of genes leading to the accumulation of specific stress-associated proteins (Vierling 1991; Ingram and Bartels 1996; Bohnert and Sheveleva 1998; Thomashow 1999; Hoekstra et al. 2001). Heat-shock proteins (Hsps) and late embryogenesis abundant (LEA)-type proteins are two major types of stress-induced proteins that accumulate upon water, salinity, and extreme temperature stress. They have been shown to play a role in cellular protection during the stress (Vierling and Kimpel 1992; Boston et al. 1996; Close 1996; Ingram and Bartels 1996; Waters et al. 1996; Thomashow 1998).

Hsps and molecular chaperones

Dysfunction of enzymes and proteins usually accompanies abiotic stress. Therefore, maintaining proteins in their functional conformations and preventing aggregation of non-native proteins are particularly important for cell survival under stress. Many stress-responsive proteins, especially Hsps, have been shown to act as molecular chaperones, which are responsible for protein synthesis, targeting, maturation and degradation in a broad array of normal cellular processes. Furthermore, molecular chaperones function in the stabilization of proteins and membranes, and in assisting protein refolding under stress conditions (Vierling 1991; Hendrick and Hartl 1993; Boston et al. 1996; Hartl 1996; Waters et al. 1996; Török et al. 2001).

Among five conserved families of Hsps (Hsp100, Hsp90, Hsp70, Hsp60 and sHsp), the small heat-shock proteins (sHsps) are found to be most prevalent in plants. sHsps are Hsps that vary in size from 12 to 40 kDa (Vierling 1991). Various studies have shown that plant sHsps are not only expressed in response to heat shock but also under water, salt, and oxidative stress, and at low temperature (Almoguera et al. 1993; Alamillo et al. 1995; Sabehat et al. 1998; Härndahl et al. 1999; Hamilton and Heckathorn 2001). In the resurrection plant *Craterostigma plantagineum*, sHsp-like proteins are expressed constitutively in vegetative tissues (Alamillo et al. 1995). Two tomato sHsps, *tom66* and *tom111*, were induced by low temperature in pre-heated fruits (Sabehat et al. 1998). In another study, Hsp21 was found to be involved in oxidative stress (Härndahl et al. 1999). Recently, Hamilton and Heckathorn (2001) suggested that sHsps might act as antioxidants in protecting Complex-I electron transport in mitochondria during NaCl stress. Moreover, *At-HSP17.6A* expression was induced by heat and osmotic stress as well as during seed development (Sun et al. 2001). In addition, sHsps are involved in many developmental processes, such as embryo development, seed germination, somatic embryogenesis, pollen development, and fruit maturation (Waters et al. 1996).

Plant sHsps show less sequence similarity than Hsps of other organisms. The sequence similarity spans approximately 100 amino acids proximal to the carboxyl-terminal, and exhibits pronounced homology with the α-crystallin family (Waters et al. 1996). Plant sHsps, like other sHsps and α-crystallins, tend to form large oligomeric complexes (Chen et al. 1994; GJ Lee et al. 1995; Collada et al. 1997; Suzuki et al. 1998). The major chaperone activity of sHsps is to bind and hold denatured substrates in a folding-competent state for subsequent refolding by a chaperone network (Horwitz 1992; Ehrnsperger et al. 1997; Lee et al. 1997; Veinger et al. 1998; Haslbeck et al. 1999; Ding and Candido 2000; Studer and Narberhaus 2000). However, some members of the plant sHsps can also stabilize or reactivate inactivated enzymes (GJ Lee et al. 1995; Hook and Harding 1998; Muchowski and Clark 1998; Haslbeck et al. 1999; Smýkal et al. 2000; Marini et al. 2000; Sun et al. 2001).

sHsps, as well as other Hsps, are believed to play an important role in plant stress tolerance. Carrot transgenic cells and regenerated plants, which constitutively expressed the carrot Hsp17.7 gene, showed more thermotolerance than the vector controls (Malik et al. 1999). In contrast, heat-inducible Hsp17.7 antisense lines were less thermotolerant than the vector controls. Under high light conditions, transgenic *Arabidopsis* that constitutively expressed a chloroplast Hsp21 were more resistant to heat stress than the wild-type plants (Härndahl et al. 1999). The results lead us to suggest that Hsps overproduction may also protect plants from oxidative stress. Recently, Sun et al. (2001) demonstrated that the expression of *Arabidopsis AtHSP17*.6A is regulated by heat shock and osmotic stress, and is induced during seed maturation. Overexpression of *AtHSP17*.6A in *A. thaliana* conferred higher osmotolerance; however, the *AtHSP17*.6A transgene failed to increase heat tolerance.

DnaK1, a member of Hsp70 from the halotolerant *Cyanobacterium aphanothece*, was overexpressed in the cytosol of transgenic tobacco plants and was found to improve their salt tolerance (Sugino et al. 1999). Under salt stress, the CO_2 fixation rate decreased to 40% in the control plants while its activity in the transgenic plants was approximately 85%. In addition, leaf sodium

concentrations were significantly increased in control plants but those of the transgenic plants remained at levels similar to the non-stressed plants.

The LEA-type proteins

LEA-type proteins have been found in a wide range of plant species in response to water deficit resulting from desiccation, cold and osmotic stress. LEA-type proteins fall into a number of families, with diverse structures and functions (Close 1996; Ingram and Bartels 1996; Thomashow 1998). Predictions of secondary structures suggest that most LEA proteins exist as random coiled α-helices (Bray et al. 2000). It was therefore proposed that most LEA and dehydrin proteins exist as largely unfolded structures in their native state, although a few members exist as dimers or tetramers (Ceccardi et al. 1994; Kazuoka and Oeda 1994).

Hydrophilicity is a common characteristic of LEA-type and other osmotic stress-responsive proteins. LEA proteins have been grouped together with other osmotic stress-induced proteins from *Saccharomyces cerevisiae* and *Escherichia coli* into a class of proteins termed hydrophilins, based on criteria of high hydrophilicity index (>1.0) and glycine content ($>6\%$; Garay-Arroyo et al. 2000). Heat stability is another notable feature of LEA proteins, i.e. they do not coagulate upon boiling (Close et al. 1989; Ceccardi et al. 1994; Houde et al. 1995; Thomashow 1998, 1999). Another common characteristic of LEA-type proteins is that, in most cases, their related gene expression is transcriptionally regulated and responsive to ABA (Mundy and Chua 1988; Skriver and Mundy 1990; Leung and Giraudat 1998).

The functions of LEA-type proteins are largely unknown. Nevertheless, their considerable synthesis during the late stage of embryogenesis, their induction by stress and their structural characteristics (hydrophilicity, random coils and repeating motifs) permits the prediction of some of their functions. It has been suggested that LEA-type proteins act as water-binding molecules, in ion sequestration and in macromolecule and membrane stabilization (i.e. chaperone-like activity; Dure 1993a, 1993b; Close 1996; Ingram and Bartels 1996; Thomashow 1998, 1999). COR85, a group-II LEA protein, was shown to be involved in cryoprotection of freezing-sensitive enzymes (Kazuoka and Oeda 1994). Moreover, it was found that COR15am, the mature COR15a polypeptide, acts directly as a cryoprotective protein by inhibiting the formation of hexagonal II phase lipids, a major type of freeze-induced membrane lesion in non-acclimated plants (Steponkus et al. 1998).

Overexpression of COR15a, which was targeted to the chloroplasts, increased freezing tolerance of chloroplasts in vivo, and of protoplasts in vitro (Artus et al. 1996). This increase most likely resulted from the membrane-stabilizing effect of COR15a (Artus et al. 1996; Steponkus et al. 1998). However, the protective effect of COR15a was insignificant for the survival of whole plants during freezing (Jaglo-Ottosen et al. 1998). Xu et al. (1996) reported that the expression of HVA1, an LEA III family protein in barley, confers tolerance to water deficiency and salt stress in transgenic rice plants. Moreover, constitutive expression of the same protein in transgenic wheat plants improved biomass productivity and water-use efficiency under water-deficit conditions (Sivamani et al. 2000). More recently, Ndong et al. (2002) reported that constitutive expression of a wheat chloroplast LEA-like protein (WCS19) in *Arabidopsis* resulted in a significant increase in freezing tolerance.

Overexpression of a single LEA-type protein is not always sufficient to confer plant stress tolerance. Transgenic tobacco plants that had been transformed with three *Craterostigma plantagineum* cDNAs, *pcC6-19* (homologous with rice *rab16*), *pcC3-06* (homologous with *lea* D29) and *pcC27-45* (homologous with *lea* 14), expressed high levels of the encoded proteins, but this increase did not result in drought tolerance (Iturriaga et al. 1992). In contrast, multi-expression of LEA-type proteins activated by their common transcription factors was found to correlate with stress tolerance in transgenic plants (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Jaglo et al. 2001). This suggested that the LEA-type proteins might function synergistically with other members.

Perspective

Complex traits of abiotic stress phenomena in plants make genetic modification for efficient stress tolerance difficult to achieve. However, the modification of a single trait (e.g. TFs, antiporters, and others) resulted in several cases in significant improvements in stress tolerance, as discussed earlier.

In addition to TFs, the modulation of upstream signaling regulators such as rice calcium-dependent protein kinase (OsCDPK) or *Arabidopsis* glycogen synthase kinase (AtGSK1), can also be a promising method for improving stress tolerance in plants (Piao et al. 2001; Saijo et al. 2000). However, little is known about the molecular mechanisms underlying these signaling components. Moreover, alteration of further upstream molecules in the pathway often activates a much wider network of genes, other than stress-specific ones. Such 'overactivation' may have deleterious effects on total plant performance, eventually becoming useless for agricultural practices.

The discovery and use of new stress-tolerance-associated genes, as well as heterologous genes, to confer plant stress tolerance (including those unique to extreme-growth-environment organisms e.g. halophytes, thermophilic organisms), has been the subject of ongoing efforts to obtain tolerant plants. Recently, we reported on a stress-responsive gene, *sp1*, which is a representative member of a novel protein family. SP1 is a homo-oligomeric protein that possesses exceptional stability under a variety of harsh conditions, such as boiling, proteolysis, detergents and high-salt

denaturation. Biochemical analysis demonstrated that SP1 functions as a molecular chaperone in protecting and repairing different heat-labile enzymes (Wang et al. 2001b, 2002; Wang et al. unpublished data). Preliminary results showed that elevated expression of SP1 is correlated to salt-stress tolerance (Wang et al. 2003; Barak 2003). Like many newly discovered genes, SP1 may serve as a candidate for modifying plant stress tolerance. A further understanding of the biochemical and molecular mechanisms underlying stress should be achieved with the advent of functional genomics, transcriptomics and proteomics. Many heterologous genes, such as the bacterial *mtlD* gene producing mannitol, bacterial choline-oxidizing enzyme and yeast *HAL* genes (Tarczynski et al. 1993; Lilius et al. 1996; Bordas et al. 1997) have been used successfully in genetically modified plants. Recently, transgenic tobacco transformed with the animal cell death suppressors Bcl-xL and Ced-9 showed enhanced resistance to UV-B, paraquat, salt, cold and wounding stress (Mitsuhara et al. 1999; Qiao et al. 2002). The utility of foreign genes in modifying plants opens a new avenue with a wide range of gene resources. Nevertheless, the availability of many diverse metabolites in plants, the different post-transcriptional or translational modifications of selected foreign genes, as well as human health and environmental considerations, should be taken into account before foreign genes are designed for expression in plants.

While adaptation to stress under natural conditions has some ecological advantages, the metabolic and energy costs may sometimes mask and limit its benefit to agriculture and result in yield penalty. Therefore, the improvement of abiotic stress tolerance of agricultural plants can only be achieved, practically, by combining traditional and molecular breeding (Kasuga et al. 1999; Dunwell 2000; Wang et al. 2001a). Thus, a comprehensive breeding strategy for abiotic stress tolerance should include the following steps and approaches: (i) conventional breeding and germplasm selection, especially of wild relevant species; (ii) elucidation of the specific molecular control mechanisms in tolerant and sensitive genotypes; (iii) biotechnology-oriented improvement of selection and breeding procedures through functional genomics analysis, use of molecular probes and markers for selection among natural and bred populations, and transformation with specific genes; and (iv) improvement and adaptation of current agricultural practices.

An ideal genetically modified crop should possess a highly regulated stress-response capability that does not affect crop performance when stress is absent. In this respect, conventional breeding and selection techniques will continue to make a contribution (Wang et al. 2001a). While certain transgenic crops have already been moved from the laboratory, only a few stress-resistant transgenic crops have been evaluated in field trials under real stress conditions (Dunwell 2000). In addition, most of the genetically modified stress-tolerant plants generated to date are non-agronomic plants. When facing the deleterious effects of drought and salinity, it is imperative that more crops, which are genetically resistant to abiotic stress, be designed, tested, and eventually released for application as new commercial varieties.

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