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REVIEW

Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects

Pooja Bhatnagar-Mathur · V. Vadez · Kiran K. Sharma

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Abstract Abiotic stresses including drought are serious threats to the sustainability of crop yields accounting for more crop productivity losses than any other factor in rainfed agriculture. Success in breeding for better adapted varieties to abiotic stresses depend upon the concerted efforts by various research domains including plant and cell physiology, molecular biology, genetics, and breeding. Use of modern molecular biology tools for elucidating the control mechanisms of abiotic stress tolerance, and for engineering stress tolerant crops is based on the expression of specific stress-related genes. Hence, genetic engineering for developing stress tolerant plants, based on the introgression of genes that are known to be involved in stress response and putative tolerance, might prove to be a faster track towards improving crop varieties. Far beyond the initial attempts to insert "single-action" genes, engineering of the regulatory machinery involving transcription factors has emerged as a new tool now for controlling the expression of many stress-responsive genes. Nevertheless, the task of generating transgenic cultivars is not only limited to the success in the transformation process, but also proper incorporation of the stress tolerance. Evaluation of the transgenic plants under stress conditions, and understanding the physiological effect of the inserted genes at the whole plant level remain as major challenges to overcome. This review focuses on the recent progress in using transgenic technology for the improvement of abiotic stress tolerance in plants. This includes discussion on the evaluation of abiotic stress response and the protocols for testing the transgenic plants for their tolerance under close-to-field conditions.

Keywords Abiotic stress · Drought tolerance · Genetic engineering · Transcription factors · Transpiration efficiency

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P. Bhatnagar-Mathur · V. Vadez · K. K. Sharma (✉)
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India
e-mail: k.sharma@cgiar.org

Introduction

Abiotic stresses adversely affect growth and productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Drought, temperature extremes, and saline soils are the most common abiotic stresses that plants encounter. Globally, approximately 22% of the agricultural land is saline (FAO 2004), and areas under drought are already expanding and this is expected to increase further (Burke et al. 2006). Often crops are exposed to multiple stresses, and the manner in which a plant senses and responds to different environmental factors appears to be overlapping. Gene expression profiles of either drought- or salt-stressed barley plants indicated that although, various genes were differentially regulated in response to different stresses, they possibly induce a similar defense response (Ozturk et al. 2002).

When a plant is subjected to abiotic stress, a number of genes are turned on, resulting in increased levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection to these stresses. A key to progress towards breeding better crops under stress has been to understand the changes in cellular, biochemical and molecular machinery that occur in response to stress. Modern molecular techniques involve

the identification and use of molecular markers that can enhance breeding programs. However, the introgression of genomic portions (QTLs) involved in stress tolerance often brings along undesirable agronomic characteristics from the donor parents. This is because of the lack of a precise knowledge of the key genes underlying the QTLs. Therefore, the development of genetically engineered plants by the introduction and/or overexpression of selected genes seems to be a viable option to hasten the breeding of "improved" plants. Intuitively, genetic engineering would be a faster way to insert beneficial genes than through conventional or molecular breeding. Also, it would be the only option when genes of interest originate from cross barrier species, distant relatives, or from non-plant sources. Indeed, there are several traits whose correlative association with resistance has been tested in transgenic plants. Following these logical steps, various transgenic technologies have been used to improve stress tolerance in plants (Allen 1995).

Stress-induced gene expression can be broadly categorized into three groups: (1) genes encoding proteins with known enzymatic or structural functions, (2) proteins with as yet unknown functions, and (3) regulatory proteins. Initial attempts to develop transgenics (mainly tobacco) for abiotic stress tolerance involved "single action genes" i.e., genes responsible for modification of a single metabolite that would confer increased tolerance to salt or drought stress Stress-induced proteins with known functions such as water channel proteins, key enzymes for osmolyte (proline, betaine, sugars such as trehalose, and polyamines) biosynthesis, detoxification enzymes, and transport proteins were the initial targets of plant transformation. In fact, metabolic traits, especially pathways with relatively few enzymes, have been characterized genetically and appear more amenable to manipulations than structural and developmental traits. However, that approach has overlooked the fact that abiotic stress tolerance is likely to involve many genes at a time, and that single-gene tolerance is unlikely to be sustainable. Therefore, a second "wave" of transformation attempts to transform plants with the third category of stress-induced genes, namely, regulatory proteins has emerged. Through these proteins, many genes involved in stress response can be simultaneously regulated by a single gene encoding stress inducible transcription factor (Kasuga et al. 1999), thus offering possibility of enhancing tolerance towards multiple stresses including drought, salinity, and freezing. It is interesting to note that this "second wave" has also coincided with a better integration of genetic engineering and plant physiology.

Further, genetic engineering allows controlling the timing, tissue-specificity, and expression level of the introduced genes for their optimal function. This is an important consideration if the action of a given gene or transcription factor is desired only at a specific time, in a specific organ, or under specific conditions of stress. The basic findings on stress promoters have led to a major shift in the paradigm for genetically engineering stress-tolerant crops in recent years (Katiyar et al. 1999). The most widely used promoters in generating transgenic plants are constitutively expressed, i.e., they are turned on all the time and throughout the plant life cycle. However, in cases where the gene expression needs to be tailored to a specific organ or a specific time, such constitutive promoters may not be a suitable choice, especially for the stress-induced genes. This is because the constitutive expression of some stress-induced genes may have serious deleterious effects on the plant. Accordingly, the more recent efforts to generate transgenic plants make use of gene cassettes driven by stress-induced promoters. With an increasing number of stress genes becoming available and genetic transformation becoming more or less a routine procedure, characterization of stress-induced promoters (particularly those induced by anaerobic, low or high temperature and salt stresses) has taken a firm footing (Katiyar et al. 1999).

It is important to examine how transgenic plants are evaluated, and how the proof-of-concept of gene effect in model plants can be adapted to crop species. Unfortunately, a substantial amount of published work involving the assessment of transgenic plants under abiotic stresses has shown effect of the transgene under growth environments that are unlikely to occur in the natural conditions. So, there is a need to set basic guidelines on the protocols to be used to carry out a rigorous evaluation of the response of transgenic plants to abiotic stresses. Since most of the work carried out so far has focused on a few model plants, there is also a need to document and summarize the major achievements in crop plants.

This review summarizes the recent progress in using transgenic plant technology for the improvement of abiotic stress tolerance using examples from research targeted at drought, salinity and temperature stresses, with particular attention to how transgenic plants are evaluated.

Single action genes

Osmoprotectants

Severe osmotic stress causes detrimental changes in cellular components. In stress-tolerant transgenic plants, many genes involved in the synthesis of osmoprotectants—organic compounds such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines) and a variety of sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol) that accumulate during osmotic

adjustment—have been used to date (Vincour and Altman 2005). Many crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress-tolerant organisms. It is believed that osmoregulation would be the best strategy for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity and high temperature. Therefore, a widely adopted strategy has been to engineer certain osmolytes or by over expressing such osmolytes in plants, as a potential route to breed stress-tolerant crops.

Various strategies are being pursued to genetically engineer osmoprotection in plants. The first step involved in obtaining stress tolerant transgenic plants has been to engineer genes that encode enzymes for the synthesis of selected osmolytes (Bray 1993). This has resulted in a profusion of reports involving osmoprotectants such as glycine-betaine (Ishitani et al. 1997; Lilius et al. 1996; Hayashi et al. 1997, 1998; Alia et al. 1998, 1999; Sakamoto et al. 1998, 2000; Holmstrom 2000; McNeil et al. 2000) and proline (Delauney and Verma 1993; Nanjo et al. 1999a; Zhu et al. 1998; Yamada et al. 2005). Also, a number of "sugar alcohols" (mannitol, trehalose, myo-inositol and sorbitol) have been targeted for the engineering of compatible-solute overproduction, thereby protecting the membrane and protein complexes during stress (Tarczynski et al. 1993; Yang et al. 1996; Shen et al. 1997; Abebe et al. 2003; Holmstrom et al. 1996; Zhao et al. 2000; Pilon-Smits et al. 1995, 1998, 1999; Garg et al. 2002; Cortina and Culiáñez 2005; Gao et al. 2000). Similarly, transgenics engineered for the overexpression of polyamines have also been developed (Roy and Wu 2001; 2002; Kumria and Rajam 2002; Waie and Rajam 2003; Anderson et al. 1998; Capell et al. 2004). Studies on the identification/isolation/cloning of genes that are associated with improved flooding stress tolerance have also focused on enzymes of the glycolytic and alcohol fermentation pathways indicating that respiratory pathway is affected in a major way in response to anaerobic stress. Research on genetically altering the levels of *pdc* and *adh* in tobacco and rice has been extensively carried out to elucidate their role in submergence tolerance. Transgenic rice over- and under-expressing pyruvate decarboxylase 1 (*pdc1*) gene has also been developed, which showed a positive correlation of higher PDC activities with survival after submergence (Quimlo et al. 2000).

The results of transgenic modifications of biosynthetic and metabolic pathways in most of the above-mentioned cases indicate that higher stress tolerance and the accumulation of compatible solutes may also protect plants against damage by scavenging of reactive oxygen species (ROS), and by their chaperone-like activities in maintaining protein structures and functions (Hare et al. 1998; Bohnert and Shen 1999; McNeil et al. 1999; Diamant et al. 2001). However, pleiotropic effects (e.g. necrosis and growth retardation) have been observed due to disturbance in endogenous pathways of primary metabolisms. Also, there are also some reports showing a negative effect of osmotic stress on yield potential (Fukai and Cooper 1995). Genetic manipulations of compatible solutes do not always lead to a significant accumulation of the compound (except in some cases of proline over-production; Chen and Murata 2002), thereby, suggesting that the function of compatible solutes is not restricted to osmotic adjustment, and that osmoprotection may not always confer drought tolerance. A recent review (Serraj and Sinclair 2002) shows that virtually none of the studies that tested the effect of osmotic adjustment on yield under water stress showed any benefit at all, since some benefit of osmotic adjustment might be in the ability of plants to maintain root growth under severe stress (Voetberg and Sharp 1991). Another recent study with chickpea has also shown that osmotic adjustment provided no beneficial effect on yield under drought stress (Turner et al. 2007). Besides, the results of simulation modeling also suggest that changes in a given metabolic process, (Passioura 1977, 2007), may end up with little benefit for actual yield under stress (Sinclair et al. 2004). For agricultural practices, over-synthesis of compatible solutes should not account for the primary metabolic costs and hence to minimize the pleiotropic effects, over-production of compatible solutes should be stress-inducible and/or tissue specific (Garg et al. 2002).

Detoxifying genes

In most of the aerobic organisms, there is a need to effectively eliminate reactive oxygen species (ROS) generated as a result of environmental stresses. Depending on the nature of the ROS, some are highly toxic and need to be rapidly detoxified. In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system to scavenge the ROS. These antioxidant systems include various enzymes and non-enzymatic metabolites that may also play a significant role in ROS signaling in plants (Vranova et al. 2002). A number of transgenic improvements for abiotic stress tolerance have been achieved through detoxification strategy. These include transgenic plants over expressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases (Zhu et al. 1999; Roxas et al. 1997). Transgenic tobacco over expressing *SOD* in the chloroplast, mitochondria and cytosol have been generated (Bowler et al.

1991; Van Camp et al. 1996) and these have been shown to enhance tolerance to oxidative stress induced by methyl viologen (MV) in leaf disc assays. Overexpression of chloroplast *Cu/Zn* SOD showed a dramatic improvement in the photosynthetic performance under chilling stress conditions in transgenic tobacco (Sen Gupta et al. 1993) and potato plants (Perl et al. 1993). Tobacco transgenic plants overexpressing *MnSOD* rendered enhanced tolerance to oxidative stress only in the presence of other antioxidant enzymes and substrates (Slooten et al. 1995), thereby, showing that the genotype and the isozyme composition also have a profound effect on the relative tolerance of the transgenic plants to abiotic stress (Rubio et al. 2002). While transgenic alfalfa (*Medicago sativa*) plants cv. RA3 overexpressing *MnSOD* in chloroplasts showed lower membrane injury (McKersie et al. 1996), the tobacco transgenic plants overproducing alfalfa aldose reductase gene (*MsALR)* showed lower concentrations of reactive aldehydes and increased tolerance against oxidative agents and drought stress (Oberschall et al. 2000).

Late embryogenesis abundant (LEA) proteins

LEA proteins represent another category of high molecular weight proteins that are abundant during late embryogenesis and accumulate during seed desiccation and in response to water stress (Galau et al. 1987). Amongst the several groups of LEA proteins, those belonging to group 3 are predicted to play a role in sequestering ions that are concentrated during cellular dehydration. These proteins have 11-mer amino acid motifs with the consensus sequence TAQAAKEKAGE repeated as many as 13 times (Dure 1993). The group 1 LEA proteins are predicted to have enhanced water-binding capacity, while the group 5 LEA proteins are thought to sequester ions during water loss. Constitutive overexpression of the *HVA1*, a group 3 LEA protein from barley conferred tolerance to soil water deficit and salt stress in transgenic rice plants (Xu et al. 1996). Constitutive or stress induced expression of the *HVA1* gene resulted in the improvement of growth characteristics and stress tolerance in terms of cell integrity in wheat and rice under salt- and water-stress conditions (Sivamani et al. 2000; Rohilla et al. 2002). Although, the reported water use efficiency (WUE) was extremely low when compared to other data reported in wheat cultigens, transgenic rice (TNG67) plants expressing a wheat LEA group 2 protein (PMA80) gene or the wheat LEA group 1 protein (PMA1959) gene resulted in increased tolerance to dehydration and salt stresses (Cheng et al. 2002). Besides, protective chaperone like function of LEA proteins acting against cellular damage has been proposed (Vincour and Altman 2005), indicating the role of LEA proteins in anti-aggregation of enzymes under desiccation and freezing stresses (Goyal et al. 2005).

Transporter genes

An important strategy for achieving greater tolerance to abiotic stress is to help plants to re-establish homeostasis under stressful environments, restoring both ionic and osmotic homeostasis. This has been and continues to be a major approach to improve salt tolerance in plants through genetic engineering, where the target is to achieve Na^+ excretion out of the root, or their storage in the vacuole. A number of abiotic stress tolerant transgenic plants have been produced by increasing the cellular levels of proteins (such as vacuolar antiporter proteins) that control the transport functions. For example, transgenic melon (Bordás et al. 1997) and tomato (Gisbert et al. 2000) plants expressing the *HAL1* gene showed a certain level of salt tolerance as a result of retaining more K^+ than the control plants under salinity stress.

A vacuolar chloride channel, *AtCLCd* gene, which is involved in cation detoxification, and *AtNHXI* gene which is homologous to *NhxI* gene of yeast have been cloned and overexpressed in *Arabidopsis* to confer salt tolerance by compartmentalizing Na^+ ions in the vacuoles. Transgenic *Arabidopsis* and tomato plants that overexpress *AtNHX1* accumulated abundant quantities of the transporter in the tonoplast and exhibited substantially enhanced salt tolerance (Apse et al. 1999; Quintero et al. 2000; Zhang and Blumwald 2001). Salt Overly Sensitive 1 (SOS1) locus in *A. thaliana*, which is similar to plasma membrane Na^+/H^+ antiporter from bacteria and fungi, was cloned and overexpressed using CaMV 35S promoter. The up-regulation of *SOSI* gene was found to be consistent with its role in Na^+ tolerance, providing a greater proton motive force that is necessary for elevated Na^+/H^+ antiporter activities (Shi et al. 2000).

Multifunctional genes for lipid biosynthesis

Transgenic approaches also aim to improve photosynthesis under abiotic stress conditions through changes in the lipid biochemistry of the membranes (Grover and Minhas 2000). Adaptation of living cells to chilling temperatures is a function of alteration in the membrane lipid composition by increased fatty acid unsaturation. Genetically engineered tobacco plants over-expressing chloroplast glycerol-3-phosphate acyltransferase (*GPAT*) gene (involved in phosphatidyl glycerol fatty acid desaturation) from squash (*Cucurbita maxima*) and *A. thaliana* (Murata et al. 1992) showed an increase in the number of unsaturated fatty acids and a corresponding decrease in the chilling sensitivity. Besides,

transgenic tobacco plants with silenced expression of chloroplast ω3-fatty acid desaturase (Fad7, which synthesizes trienoic fatty acids) were able to acclimate to high temperature as compared to the wild type (Murakami et al. 2000).

Heat shock protein genes

The heat shock response, the increased transcription of a set of genes in response to heat or other toxic agent exposure is a highly conserved biological response, occurring in all organisms (Waters et al. 1996). The response is mediated by heat shock transcription factor (HSF) which is present in a monomeric, non-DNA binding form in unstressed cells and is activated by stress to a trimeric form which can bind to promoters of heat shock genes. The induction of genes encoding heat shock proteins (Hsps) is one of the most prominent responses observed at the molecular level of organisms exposed to high temperature (Kimpel and Key 1985; Lindquist 1986; Vierling 1991).

Genetic engineering for increased thermo-tolerance by enhancing heat shock protein synthesis in plants has been achieved in a number of plant species (Malik et al. 1999; Li et al. 2003; Katiyar-Agarwal et al. 2003). There have been a few reports on positive correlations between the levels of heat shock proteins and stress tolerance (Sun et al. 2001; Wang et al. 2005). Although the precise mechanism by which these heat shock proteins confer stress tolerance is not known, a recent study demonstrated that in vivo function of thermoprotection of small heat shock proteins is achieved via their assembly into functional stress granules (HSGs; Miroshnichenko et al. 2005).

Regulatory genes

Many genes that respond to multiple stresses like dehydration and low temperature at the transcriptional level are also induced by ABA (Mundy and Chua 1988), which protects the cell from dehydration (Dure et al. 1989; Skriver and Mundy 1990). In order to restore the cellular function and make plants more tolerant to stress, transferring a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels (Bohnert et al. 1995). To overcome such constraints, enhancing tolerance towards multiple stresses by a gene encoding a stress inducible transcription factor that regulates a number of other genes is a promising approach (Yamaguchi-Shinozaki et al. 1994; Chinnusamy et al. 2005). Therefore, a second category of genes of recent preference for crop genetic engineering are those that switch on transcription factors regulating the expression of several genes related to abiotic stresses.

Transcription factors

An attractive target category for manipulation and gene regulation is the small group of transcription factors that have been identified to bind to promoter regulatory elements in genes that are regulated by abiotic stresses (Shinozaki and Yamaguchi-Shinozaki 1997; Winicov and Bastola 1997). The transcription factors activate cascades of genes that act together in enhancing tolerance towards multiple stresses. Dozens of transcription factors are involved in the plant response to drought stress (Vincour and Altman 2005; Bartels and Sunkar 2005). Most of these fall into several large transcription factor families, such as AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger 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 transcription factors, as indicated by the significant overlap of the gene-expression profiles that are induced in response to different stresses (Seki et al. 2001; Chen and Murata 2002). Transcriptional activation of stress-induced genes has been possible in transgenic plants over expressing one or more transcription factors that recognize promoter regulatory elements of these genes. Two 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). Introduction of transcription factors in the ABA signaling pathway can also be a mechanism of genetic improvement of plant stress tolerance. Constitutive expression of ABF3 or ABF4 demonstrated enhanced drought tolerance in *Arabidopsis*, with altered expression of ABA/stress-responsive genes, e.g. *rd29B*, *rab18*, *ABI1* and *ABI2* (Kagaya et al. 2002). Several ABA-associated phenotypes, such as ABA hypersensitivity and sugar hypersensitivity, were observed in such plants. Moreover, salt hypersensitivity was observed in ABF3- and ABF4-overexpressing plants at the germination and young seedling stages indicating the possible participation of ABF3 and ABF4 in response to salinity at these particular developmental stages. Improved osmotic-stress tolerance in 35S:At-MYC2/AtMYB2 transgenic plants as judged by an electrolyte-leakage test was reported by (Abebe et al. 2003). Transgenic A*rabidopsis* plants constitutively over-expressing a cold inducible transcription factor (*CBF1*; CRT/DRE binding protein) showed tolerance to freezing without any negative effect on the development and growth characteristics (Jaglo-Ottosen et al. 1998). Over-expression of *Arabidopsis CBF1* (CRT/DRE binding protein) has been shown to activate *cor* homologous genes at non-acclimating temperatures (Jaglo et al. 2001). The *CBF1* cDNA when introduced into tomato (*Lycopersicon esculentum*) under the control of a CaMV 35S promoter improved

tolerance to chilling, drought and salt stress but exhibited dwarf phenotype and reduction in fruit set and seed number (Hsieh et al. 2002). Another transcriptional regulator, *Alfin1*, when overexpressed in transgenic alfalfa (*Medicago sativa* L.) plants regulated endogenous *MsPRP2* (NaCl-inducible gene) mRNA levels, resulting in salinity tolerance, comparable, to a few available salt tolerant plants (Winicov and Bastola 1999). Lee et al. (1995) produced thermo-tolerant *Arabidopsis* plants by de-repressing the activity of *ATHSF1*, a heat shock transcription factor leading to the constitutive expression of heat shock proteins at normal temperature. Several stress induced *cor* genes such as *rd29A, cor15A*, *kin1* and *cor6.6* are triggered in response to cold treatment, ABA and water deficit stress (Thomashow 1998). There have been numerous efforts in enhancing tolerance towards multiple stresses such as cold, drought and salt stress in crops other than the model plants like *Arabidopsis*, tobacco and alfalfa. An increased tolerance to freezing and drought in *Arabidopsis* was achieved by overexpressing CBF4, a close CBF/DREB1 homolog whose expression is rapidly induced during drought stress and by ABA treatment, but not by cold (Haake et al. 2002). Similarly, a *cis*-acting element, dehydration responsive element (DRE) identified in *A. thaliana*, is also involved in ABA-independent gene expression under drought, low temperature and high salt stress conditions in many dehydration responsive genes like *rd29A* that are responsible for dehydration and cold-induced gene expression (Yamaguchi-Shinozaki and Shinozaki 1993; Iwasaki et al. 1997; Nordin et al. 1991). Several cDNAs encoding the DRE binding proteins, DREB1A and DREB2A have been isolated from *A. thaliana* and shown to specifically bind and activate the transcription of genes containing DRE sequences (Liu et al. 1998). DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in drought-responsive gene expression. The transcriptional activation of stress-induced genes has been possible in transgenic plants over-expressing one or more transcription factors that recognize regulatory elements of these genes. In *Arabidopsis*, the transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes (Shinozaki and Yamaguchi-Shinozaki 1997). DREB1A cDNA under the control of CaMV 35S promoter in transgenic plants elicits strong constitutive expression of the stress inducible genes and brings about increased tolerance to freezing, salt and drought stresses (Liu et al. 1998). Strong tolerance to freezing stress was observed in transgenic *Arabidopsis* plants that overexpress *CBF1* (DREB1B) cDNA under the control of the CaMV 35S promoter (Jaglo-Ottosen et al. 1998). Subsequently, the overexpression of *DREB1A* has been shown to improve the drought- and low-temperature stress tolerance in tobacco, wheat and groundnut (Kasuga et al. 2004; Pellegrineschi et al. 2004; Behnam et al. 2006; Bhatnagar-Mathur et al. 2004, 2006). The use of stress-inducible *rd29A* promoter minimized the negative effects on plant growth in these crop species. However, overexpression of DREB2 in transgenic plants did not improve stress tolerance, suggesting involvement of post-translational activation of DREB2 proteins (Liu et al. 1998). Recently, an active form of DREB2 was shown to transactivate target stress-inducible genes and improve drought tolerance in transgenic *Arabidopsis* (Sakuma et al. 2006). The DREB2 protein is expressed under normal growth conditions and activated by osmotic stress through post-translational modification in the early stages of the osmotic stress response.

Another ABA-independent, stress-responsive and senescence-activated gene expression involves *ERD* gene, the promoter analysis of which further identified two different novel cis acting elements involved with dehydration stress induction and in dark-induced senescence (Simpson et al. 2003). Similarly, transgenic plants developed by expressing a drought-responsive AP2-type TF, SHN1-3 or WXP1, induced several wax-related genes resulting in enhanced cuticular wax accumulation and increased drought tolerance (Aharoni et al. 2004; Zhang et al. 2005). Thus, clearly, the overexpression of some drought-responsive transcription factors can lead to the expression of downstream genes and the enhancement of abiotic stress tolerance in plants (see review, Zhang et al. 2004). The regulatory genes/factors reported so far not only play a significant role in drought and salinity stresses, but also in submergence tolerance. More recently, an ethylene-response-factor-like gene *Sub1A*, one of the cluster of three genes at the *Sub1* locus have been identified in rice and the overexpression of *Sub1A-1* in a submergence-intolerant *variety* conferred enhanced submergence tolerance to the plants (Xu et al. 2006), thus confirming the role of this gene in submergence tolerance in rice.

Signal transduction genes

Genes involved in stress signal sensing and a cascade of stress-signaling in *A. thaliana* has been of recent research interest (Winicov and Bastola 1997; Shinozaki and Yamaguchi-Shinozaki 1999). Components of the same signal transduction pathway may also be shared by various stress factors such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki 1999). Although there are multiple pathways of signal-transduction systems operating at the cellular level for gene regulation, ABA is a known component acting in one of the signal transduction pathways, while others act independently of ABA. The early response genes have been known to encode transcription factors that activate downstream delayed response genes (Zhu 2002). Although, specific branches and components exist (Lee et al. 2001), the signaling pathways for salt, drought, and

cold stresses all interact with ABA, and even converge at multiple steps (Xiong et al. 1999). Abiotic stress signaling in plants involves receptor-coupled phospho-relay, phosphoionositol-induced Ca^{2+} changes, mitogen activated protein kinase (MAPK) cascade, and transcriptional activation of stress responsive genes (Xiong and Zhu 2001). A number of signaling components are associated with the plant response to high temperature, freezing, drought and anaerobic stresses (Grover et al. 2001).

One of the merits for the manipulation of signaling factors is that they can control a broad range of downstream events that can result in superior tolerance for multiple aspects (Umezawa et al. 2006). Alteration of these signal transduction components is an approach to reduce the sensitivity of cells to stress conditions, or such that a low level of constitutive expression of stress genes is induced (Grover et al. 1999). Overexpression of functionally conserved At-DBF2 (homolog of yeast DBf2 kinase) showed striking multiple stress tolerance in *Arabidopsis* plants (Lee et al. 1999). Pardo et al. (1998) also achieved salt stress-tolerant transgenic plants by overexpressing calcineurin (a Ca^{2+}/Calmodulin dependent protein phosphatase), a protein phosphatase known to be involved in salt-stress signal transduction in yeast. Transgenic tobacco plants produced by altering stress signaling through functional reconstitution of activated yeast calcineurin not only opened-up new routes for study of stress signaling, but also for engineering transgenic crops with enhanced stress tolerance (Grover et al. 1999). Overexpression of an osmotic-stress-activated protein kinase, SRK2C resulted in a higher drought tolerance in *A. thaliana*, which coincided with the upregulation of stress-responsive genes (Umezawa et al. 2004). Similarly, a truncated tobacco mitogen-activated protein kinase kinase kinase (MAPKKK), NPK1, activated an oxidative signal cascade resulting in cold, heat, salinity and drought tolerance in transgenic plants (Kovtun et al. 2000; Shou et al. 2004). However, suppression of signaling factors could also effectively enhance tolerance to abiotic stress (Wang et al. 2005). This hypothesis was based on previous reports indicating that a and b subunits of farnesyltransferase ERA1 functions as a negative regulator of ABA signaling (Cutler et al. 1996; Pei et al. 1998). Conditional antisense downregulation of a or b subunits of protein farnesyl transferase, resulted in enhanced drought tolerance of *Arabidopsis* and canola plants.

Choice of promoters

An important aspect of transgenic technology is the regulated expression of transgenes. Tissue specificity of transgene expression is also an important consideration while deciding on the choice of the promoter so as to increase the level of expression of the gene. Thus, the strength of the promoter and the possibility of using stress-inducible, developmental-stage-, or tissue-specific promoters have also proved to be critical for tailoring plant response to these stresses (Bajaj et al. 1999). Some gene products are needed in large amounts, such as LEA3, thereby necessitating the need for a very strong promoter. With other gene products, such as enzymes for polyamine biosynthesis, it may be better to use an inducible promoter of moderate strength. The promoters that have been most commonly used in the production of abiotic stress tolerant plants so far, include the CaMV 35S, ubiquitin 1 and actin promoters. These promoters being constitutive in nature, by and large express the downstream transgenes in all organs and at all the stages. However, constitutive overproduction of molecules, such as trehalose (Romero et al. 1997) or polyamines (Capell et al. 1998) causes abnormalities in plants grown under normal conditions. Also, the production of the above-described molecules can be metabolically expensive. In these cases, the use of a stress inducible promoter may be more desirable. In plants, various types of abiotic stresses induce a large number of well-characterized and useful promoters. An ideal inducible promoter should not only be devoid of any basal level of gene expression in the absence of inducing agents, but the expression should be reversible and dose-dependent. The transcriptional regulatory regions of the drought-induced and cold-induced genes have been analyzed to identify several *cis*-acting and *trans*-acting elements involved in the gene expression that is induced by abiotic stress (Shinwari 1999). Most of the stress promoters contain an array of stress-specific *cis*-acting elements that are recognized by the requisite transcription factors; for example, the transcriptional regulation of *hsp* genes is mediated by the core "heat shock element" (HSE) located in the promoter region of these genes, 5' of the TATA box. All the plant *hsp* genes sequenced so far have been shown to contain partly overlapping multiple HSEs proximal to TATA motif. Apart from these *hsp* promoters, *rd29* and *adh* gene promoters induced by osmotic stress and anaerobic stress, respectively, have also been studied. The *Arabidopsis rd29A* and *rd29B* are stress responsive genes, but are differentially induced under abiotic stress conditions. The *rd29A* promoter includes both DRE and ABRE elements, where dehydration, high salinity and low temperatures induce the gene, while the *rd29B* promoter includes only ABREs and the induction is ABA-dependent. Overexpression of DREB1A transcription factors under the control of stress inducible promoter from *rd29A* showed a better phenotypic growth of the transgenic plants than the ones obtained using the constitutive CaMV 35S promoter (Kasuga et al. 1999). A stress inducible expression of

Arabidopsis CBF1 in transgenic tomato was achieved using the ABRC1 promoter from barley *HAV22* (Lee et al. 2003). Gene expression is induced by the binding of DREB1A, which in itself is induced by cold and water stress, to a *cis*-acting DRE element in the promoters of genes such as *rd29A*, *rd17*, *cor6.6*, *cor15A*, *erd10*, and *kin1*, thereby, initiating synthesis of gene products imparting tolerance to low temperatures and water stress in plants. The regions of respiratory alcohol dehydrogenase *adh*1 gene promoter in maize and rice that are required for anaerobic induction include a string of bases called anoxia response element (ARE) with the consensus sequence of its core element as TGGTTT. Besides, other stress-responsive *cis*-acting promoter sequences like low temperature responsive elements (LTRD) with a consensus sequence of A/GCCGAC have been identified in genes such as *Cor 6.6*, *Cor 15* and *Cor 78* These basic findings on stress promoters have led to a major shift in the paradigm for genetically engineering stress tolerant crops (Katiyar-Aggarwal et al. 1999).

Physiological evaluation of stress effect

A large number of studies have evaluated different transgenic constructs in different plant species, and to different stresses such as drought, salinity and cold. The expression of the genes inserted as well as altered levels of metabolites have been reported in great detail. However, less detail is given with regard to the methods used to evaluate the stress response. Although, the transgenic construct is usually reported to have increased the tolerance to drought in most of the instances, it is then referred to as such in other papers. This lack of details applies mostly to drought stress, the protocols used for salt stress are usually better described (Tarczynsky et al. 1993; Holstrom et al. 2000), although the levels of salt stress used in some studies are far beyond what is found in a natural environment. It is understood that most of these studies are intended to assess the gene expression, often in model plants, under a particular stress, and extreme situation of stress are often used to ensure the gene expression. However, these studies may bring about some misleading conclusions from an agronomic or physiology perspective, where the assessment of stress tolerance of transgenics needs to be done with respect to its cross-talk with other stress-related genes/mechanisms and where the effects of stress need to be observed over longer periods/conditions. This is particularly important, in order to closely mimic the life span of most crops under cycles of stress, rather than short exposure to very severe stresses, although we agree that short exposures to stress are certainly adequate if the purpose is to assess gene expression only. Therefore, in the following discussion, we focus on the agronomic/physiological perspective and don't mean to challenge the quality of the work done to assess gene expression. Our intention is to try to reconcile both approaches (molecular and agronomic) toward a common focus: breeding.

Two major issues that typically need to be addressed in stress response evaluation of transgenics include: (1) Means of stress imposition, details about the stress, and growth conditions (including the intensity, timing, and quickness of imposition, etc.), and (2) "Hard" data on the response of tested materials to support conclusions (comparison within the same species). Besides, precise details about the protocols used to evaluate the performance of plants to any given stress are very essential to assess the performance of materials.

Means of stress impositions, growth conditions, and evaluations

Stress conditions used to evaluate the transgenic material in most of the reports so far, are usually too severe (Nanjo et al. 1999a; Shinwari et al. 1998; Garg et al. 2002) as plants are very unlikely to undergo such stresses under field conditions. Also, the means of evaluation are often significantly different from natural conditions. For example, Pellegrineschi et al. (2004) compared the performance of initial events of DREB1A transgenic wheat to the wild parent by withholding water to 2-week-old seedlings grown in 5 cm × 5 cm pots, and then re-watering until maturity when they were evaluated. Untransformed plants were nearly dead within 10–15 days of stress imposition, likely because of a different pattern of water use, whereas transgenic plants survived in these small pots and "passed" the evaluation successfully; such conditions would obviously not occur in the field. Besides, the type of systems used to assess plant performance, one would expect the evaluation to be made, at least, on the basis of biomass accumulated during the stress.

While the use of PEG (polyethylene glycol) in hydroponics can be useful to test certain response of plants under a given osmotic potential as reported by Pilon-Smits et al. (1996, 1999), it offers relatively different conditions than in the soil where the water reservoir is by definition finite. Here, the observation on improved growth was explained by an increased water uptake under the water potential applied, due to osmolyte production by the transgenic plant. This is quite possible in such a system because the water reservoir is unlimited in hydroponics, and because the water potential is constant. Under soil conditions, however, the volume of soil surrounding the root where water can be extracted is limited, and the water potential of that soil quickly declines upon water uptake by roots, reaching soil water potential where even the enhanced

osmolyte production of the transgenics would be unable to extract any significant additional amount of water. A more realistic test of the ability to take up water using osmotic potential-enhanced transgenics would be to compare their capacity to extract water from a soil system rather than a hydroponic system. A recent study by Sivamani et al. (2000) reported an increased WUE in the transgenic wheat. Unfortunately there was no control over the soil evaporation that probably accounts for most of the water loss and explained the very low values of WUE observed. Besides, investigating drought responses by using fresh weight (Sun et al. 2001) and other indirect estimates of performance like growth rate, stem elongation (Pilon-Smits et al. 1995; Lee et al. 2003), or survival (Pardo et al. 1998) are likely to give inconsistent results. While applying a drought stress, it is important to know the stages of drought stress that the plants are exposed to, for which, a detailed description of growth conditions, plant size, container size, water availability, and transpiration is needed. It is also crucial to report the dry weight of tested plants, possibly before and after the stress period.

Similarly, often the stress imposed has been modified from 2 days, to 2 weeks, and even 4 weeks using the same experimental conditions (Lee et al. 2003), without indicating the water holding capacity of the potting mixture used as well as the plant density. This obviously leads to different types of stresses, where the plants exposed for 2 days of water stress may well have remained in stage I when water is abundant (see below), while plants exposed to 4 weeks stress may have spent most of the time under stage III where roots may have exhausted all the available water. Also there are cases where a given quantity of water is applied to the plants on alternate days from 2 to 10 weeks (Sivamani et al. 2000), thereby, disregarding the fact that the water requirements increase dramatically during the period, and probably exposing their plants to an initial flooding before a severe stress.

Adequate protocols to apply drought and salinity stress

Unlike what seems to be a common practice in transgenic evaluation, applying drought does not consist simply in withholding water. Indeed, we cannot investigate drought responses of plants without understanding the different phases that a plant undergoes under drought in natural conditions. These steps have been described earlier (Ritchie 1982; Sinclair and Ludlow 1986). In phase I, water is abundant and plant can take up all the water required by transpiration and stomata are fully open. During that stage, the water loss is mostly determined by the environmental conditions to which the leaves are exposed. During stage II, the roots are no longer able to supply sufficient water to the shoot and stomata progressively close to adjust the water loss to the water supply, so that leaf turgor is maintained. In stage III, roots have exhausted all the water available for transpiration. Stomata are closed and virtually all the physiological processes contributing to growth, including photosynthesis are inhibited. This has been used to design dry-down experiments where the response of plants to drought is taken as a function of the fraction of soil moisture available to plant (fraction of transpirable soil water, FTSW), and not as a function of number of days after which the stress has been imposed. The former allows a precise comparison of stress imposed across experiments and environmental conditions, whereas referring to stress intensity on the number of days of exposure to stress, without referring to pot size, evaporative demand, etc., can lead to erratic and irreproducible data. Based on transpiration values, it is possible to partially compensate the water loss to apply a milder stress condition, which allows plants of different sizes to be exposed to a similar drought stress. For instance, plants exposed to water stress are allowed to lose a maximum of 70 g per day. Any water loss in excess of this value is added back on a plant basis. This allows maintaining the volumetric soil moisture content, a proxy for water stress, similar in all pots. Amount of daily water loss can be adapted to increase/decrease the level of stress. This protocol has the advantage of mimicking the situation a plant would face in the field, i.e. a progressive soil drying. This method has been successfully used at ICRISAT to assess the response of 14 transgenic events of groundnut (Fig. 1) with rd29A promoter-driven *DREB1A*

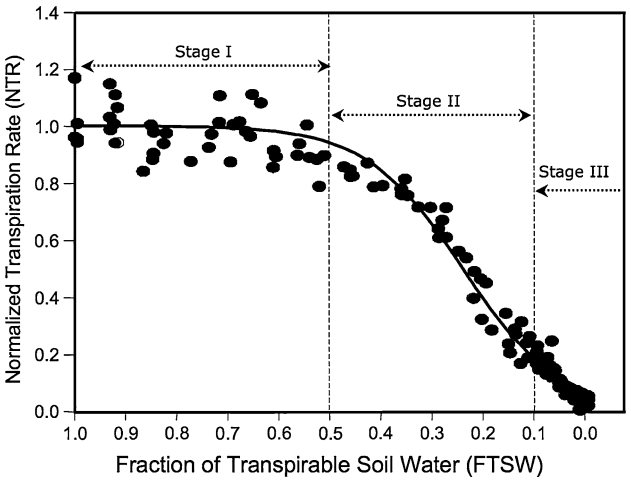

Fig. 1 A typical response curve of groundnut cultivar JL 24 to soil-drying condition. This is used to design dry-down experiments where the response of plants to drought is taken as a function of the fraction of soil moisture available to plant (fraction of transpirable soil water, FTSW), and not as a function of the number of days after which the stress has been imposed

under contained greenhouse conditions (Bhatnagar-Mathur et al. 2004, 2006).

Regarding salinity, most of the evaluations reported so far have been carried out at the seedling stage (Maliro et al. 2004), although this type of evaluation has been reported to have little correspondence, if any, with how plants will later perform under salt stress (Munns et al. 2002; Vadez et al. 2006). Besides, evaluations are made on a short-term basis by using high concentrations of salt; way above those found even in highly saline natural environment that obviously magnifies the effect of transgenics engineered to excrete salt. Therefore, protocols that use too severe concentrations of salt should be avoided. A few other subjects of contention include the treatments that are used as salt stress, and also the hypothesis about the major determinants of salt stress tolerance.

It is often assumed that the avoidance of Na^+ accumulation and toxicity confers salt tolerance in plants. Therefore, most of the transgenic work has dealt with genes involved in Na^+ extrusion from the root or Na compartmentation in the vacuoles. However, severe stresses (over 200–300 mM) in hydroponics (Behnam et al. 2006; Holmstrom et al. 2000; Lee et al. 2003) that are unlikely to occur in the natural environment will necessarily highlight those transgenics that are able to excrete Na^+ and able to maintain homeostasis, even though it may be for a short while. Whether such a strategy is adequate is still an open question. Vadez et al. (2007) reported that salinity tolerance was not related to differences in the accumulation of Na^+ in chickpea, thereby, a strategy of Na^+ excretion in chickpea would appear inadequate and similar converging data has been observed in sorghum and millet (unpublished data).

Procedures for the salinity evaluation of crops are being optimized to be carried out in soil conditions in an outdoor facility under natural conditions at ICRISAT. Here, salt stress is applied to the soil during the early stages of germination and plant development using a staggered salt application (total amount split in three applications) to avoid an osmotic shock. Besides, plants are maintained close to 80% field capacity until maturity to avoid a possible increase in salt concentration if water is not replenished regularly. The plant tolerance to stress is evaluated based on the seed yield since no correlation between the shoot biomass and seed yield under salinity has been observed (Vadez et al. 2007). It is likely that reproduction is the key physiological process affected by salinity. Therefore, transgenic research intended to improve salt tolerance should probably be focused on those processes that appear to be sensitive. A thorough investigation of these processes can only help in devising a suitable and focused transgenic approach.

Conclusions

This review summarizes the recent efforts to improve abiotic stress tolerance in crop plants by employing some of the stress-related genes and transcription factors that have been cloned and characterized. The following general conclusions emerge from this review:

1. The use of transgenes to improve the tolerance of crops to abiotic stresses remains an attractive option.
2. Options targeting multiple gene regulation appear better than targeting single genes.
3. An important issue to address is how the tolerance to specific abiotic stress is assessed, and whether the achieved tolerance compares to existing tolerance. The biological cost of production of different metabolites to cope with stress and their effect on yield should be properly evaluated.
4. A well focused approach combining the molecular, physiological and metabolic aspects of abiotic stress tolerance is required for bridging the knowledge gaps between short- and long-term effects of the genes and their products, and between the molecular or cellular expression of the genes and the whole plant phenotype under stress.
5. Thorough understanding of the underlying physiological processes in response to different abiotic stresses can efficiently/successfully drive the choice of a given promoter or transcription factor to be used for transformation.

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