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Genetic Engineering for Abiotic Stress Tolerance in Plants

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Abstract

Development of plant varieties with a high level of tolerance to abiotic stresses is crucial for establishing full yield potential and to stabilize production. Due to the multitude of abiotic stresses and their complex genetic control the progress of breeding for tolerance to abiotic stresses using conventional approaches has not been very rewarding. Recent advances in cellular and molecular biology have made it possible to clone important genes and mobilize them in any organism across barriers of sexual hybridization for stable expression and transmission. All living organisms have evolved mechanisms for avoidance and/or tolerance to one or more of the abiotic stresses. Plants producing crucial enzymes or proteins from various organisms involved in abiotic stress tolerance mechanisms have shown significant advantage over their wild type controls under stressed environment. The enhanced level of compatible osmolytes, radical scavengers and other transgene products correlated with the degree of tolerance. Further understanding of the molecular mechanisms of stress perception, signal transduction and response by plants and other organisms may help to engineer plants with high levels of tolerance to multiple stresses. Perspectives and additional approaches for further improving the tolerance to abiotic stresses through genetic engineering are discussed.

1. Introduction

Optimum expression of a plant phenotype which is engaged in productivity is highly dependent on favorable interaction of the genotype and environment during various stages of plant development. A number of abnormal environment parameters such as drought, salinity, cold, freezing, high temperature, anoxia, high light intensity and nutrient imbalances *etc.* are collectively termed as abiotic stresses. The abiotic stresses often adversely affect the plant productivity in the best plant growing environments and restrict expansion of cultivation to marginal areas thus jeopardizing the food security to the ever increasing global population. Unfavorable abiotic factors accounted for about 70% average yield losses in eight major U. S. crops as estimated on the basis of differences in their record yield (potential yield) and average yield [1].

Abiotic stresses lead to dehydration or osmotic stress through reduced availability of water for vital cellular functions and maintenance of turgor pressure. Stomata closure, reduced supply of CO_2 and slower rate of biochemical reactions during prolonged periods of dehydration, high light intensity, high and low temperatures lead to high production of Reactive Oxygen Intermediates (ROI) in the chloroplasts causing irreversible cellular damage and photo inhibition. Excessive contents of heavy metals (*e.g.* Cu, Zn, Pb, and Cd) and other inorganic ions (Na, Al, B, As and Mn) in problem soils are restricting normal cultivation of plants on one-fourth of the world soils. Ultra violet radiation from 280-320 nm wavelength (UV-B) may pose a potential threat to plant health and productivity with the depletion of ozone stratospheric layer due to certain undesirable but avoidable human activities.

Plants have evolved mechanisms to respond to various abiotic stresses at morphological, anatomical, cellular and molecular levels. Some responses to tolerance or adjustment are highly species specific whereas others are fairly common even among plants belonging to different families and orders, microorganisms and animals. In response to dehydration or osmotic stress a series of compatible osmolytes are accumulated for osmotic adjustment, water retention and free radical scavenging [2]. Similarly, overexpression of certain enzymes such as superoxide dismutase, ascorbate peroxidase and glutathione reductase has been implicated in free radical detoxification and scavenging of free radicals under oxidative stress [3].

Attempts have been made to understand the molecular basis of tolerance to certain abiotic stresses. Enzymes responsible for the production of compatible

Table 1. Genetic engineering of plants for tolerance to abiotic stresses.

Stres	Gene/Enzyme	Source	Transgenic Plant	Reference
Osmotic	Delta-pyrroline-5-carboxylate synthetase (P5CS)	Mothbean (*V. aconitifolia*)	Tobacco *Arabidopsis*	[9] [8]
Drought and Salinity	Mannitol-1-phosphate dehydrogenase (*mt1D*)	*E. coli*	Tobacco	[10,11,49]
Cold and Salt	Choline oxidase (*cod*A)	*Arthrobactor globiformis*	*Arabidopsis*	[13]
Salt	Choline dehydrogenase (*bet*A)	*E. coli*	Tobacco	[14]
Cold	Omega-3-fatty acid desaturase (*fad7*)	*Arabidopsis*	Tobacco	[39]
Drought	Trehalose-6-phosphate synthase	Yeast	Tobacco	[15,16]
Drought	Levan sucrase (Sac B)	*Bacillus subtilis*	Tobacco	[17]
Salt	HAL1	Yeast	*Cucumis melo*	[8]
Salt and Drought	Lea protein (HVA1)	Barley	Rice	[50]
Oxidative and Chilling	Cu/Zn super oxide dismutase (Cu/Zn SOD)	Pea	Tobacco	[48]
Heat	Heat Shock Protein soybean 101kD and	Yeast	Soybean	[34]
	AtHSP101	Yeast	*Arabidopsis*	[35]
Cadmium	Metallothionein-I (MT-I)	Mouse	Tobacco	[32]
Copper	Metallothionein-like (PsMTA)	Pea	*Arabidopsis*	[31]
Aluminum	Citrate synthase (CSb)	*P. aeruginosa*	Tobacco Papaya	[33]

osmolytes and radical scavenging have been cloned and characterized and used for genetic transformation of stress susceptible genotypes or mutants to confirm their unequivocal role in stress protection and relief. ABA a phytohormone produced in response to several abiotic stresses has been implicated in signal transduction for inducing the plant defense response to abiotic stresses. ABA, responsive elements of several stress inducible promoters have been identified and the search for transcription factor binding to these elements is in progress. There appears to be a great parallelism between ABA and salicylic acid as the signal molecules for activation of plant defense response to multiple abiotic and biotic stresses.

In the absence of well documented and reliable sources of genetic resistance to abiotic stresses in the germplasm of crop plants and their related wild species for sound breeding programs using conventional approaches, improvement of tolerance to the abiotic stresses through genetic engineering may be the second best available alternative.

Recent advances in cellular and molecular biotechnology have made it possible to clone genes of economic importance and mobilize them among microorganisms, plants and animals across genetic barriers of gene transfer. The development of efficient procedures for plant regeneration from a large number of plant species and improved vector systems based on Ti plasmids of *Agrobacterium*, direct DNA transfer methods, transposable elements, series of promoters, marker genes and a large number of cloned genes, have made genetic manipulations more precise and directed [4]. As a result of these developments, a series of genes has been transferred through various transformation techniques including genes for several agronomically important traits such as herbicide resistance, disease and insect resistance and quality characteristics [5-7]. These developments have also resulted in precise understanding of the genome organization and regulation of gene expression in higher plants.

In this review the efforts and status of enhancing tolerance to some of the predominant abiotic stresses through genetic engineering are summarized (**Table 1**).

2. Complexed stresses by osmoticum, dehydration and salinity

Proline has been recognized as a potent and compatible osmoprotectant which is accumulated in high concentrations in glycophytes and halophytes in response to osmotic stress such as drought and high salinity. Two important enzymes for the biosynthesis of proline *i.e.* Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR) have been cloned from several

plants and their expression studied under various abiotic stresses and ABA application. Yoshiba *et al.* [8] found that the gene P5CS of *Arabidopsis thaliana* was induced by dehydration, high salt and ABA treatments but not by heat or cold treatments whereas P5CR was not induced to a significant extent with any of the treatments suggesting that the P5CS may only play a major role in the biosynthesis of proline under osmotic stress.

Kishore *et al.* [9] used the P5CS gene cloned from *Vigna aconitifolia* under CaMV35S promoter for genetic transformation of *Nicotiana tabacum* cv. Xanthi. Some transgenic plants expressing a high level of P5CS mRNA also accumulated high level of P5CS protein. The transgenic plants produced 10 to 18-fold more proline than the control plants. Under drought stress the proline content increased from about 80 μg/g fresh leaf (before stress) to about 3000 μg/g (after stress) in control and from 1000 μg/g to an average of 6500 μg/g in transgenic lines. Wilting in the transgenic plants was less severe and delayed by 2-3 days in transgenic plants as compared with the wild type (WT) control plants. The osmotic potentials of the leaf sap from transgenic plants were decreased less under water-stress conditions compared to WT plants. Under salt stress conditions roots of transgenic plants were 40% longer and had 2-fold greater biomass compared to WT. The transgenic plants also had twice the number of capsules and number of seeds per capsule as compared to the untransformed plants. Their results demonstrated that proline acts as an osmotic protectant and its overproduction in the transgenic plants increased tolerance to drought and salt stress.

Tarczynski *et al.* [10] reported that transgenic tobacco plants with the bacterial gene encoding for mannitol-1-phosphate dehydrogenase (*mtlD*) accumulated sugar alcohol mannitol to a maximum concentration of 100 mM whereas no mannitol was detected from the control lines. They also tested the response of mannitol accumulating transgenic tobacco plants under salinity stress. No differences were detected between control plants and mannitol producing plants in the absence of NaCl. The transgenic plants showed increased tolerance at high salinity (250 mM NaCl) after 30 days of exposure as indicated by their greater height and decreased weight loss relative to that of the WT control. The transgenic plants produced new roots and leaves and flowered. The *in vivo* presence of mannitol protected the tobacco plants against high salinity.

Salt stress reduced dry weight in WT plants by 44% but did not reduce the dry weight in transgenic plants possessing the *mtlD* gene [11]. Based on its concentration, in relation to other carbohydrates, mannitol could have contributed directly very little to the differences in full turgor osmotic potential between salt stressed transgenic and WT plants. Inositol, a naturally occurring polyol in tobacco, accumulated in response to salt stress equally well in both transgenic and wild type plants. No significant differences were found between transgenic and WT plants for any of the parameters evaluated under drought stress. It was concluded that the mannitol, a relatively minor osmolyte in the transgenic plants, might have indirectly enhanced osmotic adjustment and salt tolerance. Relatively slower growth of the transgenic plants and not the mannitol *per se* may have been the cause of salt tolerance.

Glycinebetaine, a quaternary amine, is another important compatible solute, which is widely distributed among plants and protects plants on exposure to salt and cold stress. In plants like spinach and barley betaine is synthesized from choline by oxidation of choline to betaine aldehyde and then to betaine. The first step is catalyzed by choline mono-oxygenase while the second by nuclear coded gene for betaine aldehyde dehydrogenase. The soil bacterium *Arthrobacter globiformis* accumulates a high level of betaine which contains choline oxidase (*cod*A) which catalyses the conversion of choline to betaine. Deshnium *et al.* [12] cloned *cod*A cDNA from *Arthrobacter* and transformed cyanobacterium *Synechococcus* which accumulated 60-80 mM betaine providing it tolerance to salt stress and low temperature. Hayashi *et al.* [13] transformed *Arabidopsis thaliana* with the cloned *cod*A gene under CaMV35S promoter and sequences encoding transit peptide of the small subunit of Rubisco. At 300 mM NaCl the seeds of the wild type plants did not germinate at all whereas all seeds of the transformed lines germinated indicating that the codA transformed plants had enhanced tolerance for salt stress. The transformed plants grew slowly at 200 mM NaCl whereas none of the wild type plants grew. After incubation at 400 mM NaCl for two days the photo system II activity of control plants disappeared totally whereas the transformed plants had maintained more than 50% activity. When exposed to low temperature the transformed plants did not show any chlorosis. These observations demonstrate that the accumulation of glycine betaine through genetic engineering in *Arabidopsis* enhanced its ability to tolerate salt and cold stress. Lilius *et al.* [14] also reported an 80% increase in salt tolerance between the transgenic tobacco plants expressing *E. coli bet*A gene encoding choline dehydrogenase over the wild type plants. The accumulation of betaine in the transgenic plants, however, was not reported.

A high concentration of osmoprotectant trehalose, a non reducing disaccharide, which effectively stabilizes

dehydrated enzymes and lipid membranes *in vitro* can be found in some resurrection plants. Holmström *et al.* [15] transformed tobacco with a construct containing the gene encoding the trehalose-6-phosphate synthase subunit (TPS1) of yeast trehalose synthase is driven by the promoter of the Rubisco small subunit gene. Trehalose was present in the leaves, flower buds and roots of the TPSI positive transformants. Leaves of seven transgenic lines grown in a green house had 0.8 to 3.2 mg trehalose per g dry weight as compared to 0.06 mg per g in non-transformed or vector transformed plants. Trehalose accumulation decreased the growth rate of TPS1 positive plants by 30-50% under optimum growing conditions without any obvious morphological changes. Three week old TPS1 positive seedlings of line 8 were subjected to air drying along with non-transformed and vector transformed controls. The control seedlings wilted after 2h of drying and totally collapsed after 7h whereas the TPS1 positive seedlings were only marginally affected. After rehydration, the trehalose producing plants recovered and recommenced growth while the controls died. Similar, results were obtained with detached leaves and 4-month old *in vitro* propagated plants. The small amount of trehalose ($\leq$5 mM in cytosol) may not be enough for the needed osmotic adjustment and hence the trehalose might have improved water retention and enhanced desiccation tolerance through stabilization of cellular structures and macromolecules.

Romero *et al.* [16] also transformed tobacco with the yeast trehalose-6-phosphate synthase gene driven by CaMV35S promoter. Trehalose was present in the leaves of transgenic plants up to 0.17 mg per g fresh weight while only negligible in control plants. Sucrose and glucose content in the leaves of transgenic plants was reduced. A high proportion of trehalose accumulating plants showed certain morphological changes such as loss of apical dominance, stunted growth, lanceolate leaves and sterility. Drought stress was provided by withholding water for 15 days. Drought stress dramatically affected the control plants while the alleviation of wilting symptoms in transgenic plants was found to be correlated with the morphological changes. Holmström *et al.* [15] however, did not observe any morphological changes in the TPS1 transgenic plants. Although all the transgenic plants expressing trehalose had better desiccation tolerance than the control, the morphologically altered plants, however, had the highest tolerance. A low amount of trehalose in the leaves, changes in morphology and alteration in sugar metabolism suggests that the drought tolerance of the trehalose accumulating plants might not be attributed to osmo adjustment by trehalose *per se* but be due to its pleiotropic effect through other pathways.

Fructans, the polyfructose molecules with various degrees of polymerization, are produced by many plants experiencing seasonal drought and are stored in soluble form in vacuoles. Pilon-Smits *et al.* [17] transformed *N. tabacum* cv. Petit Havana with a *Sac* B gene from *Bacillus subtilis* encoding for levan sucrase and vacuolar sorting signal from yeast placed under CaMV35S promoter. Homozygous fructan accumulating plants and the non-transformed plants were tested for drought tolerance under hydroponic conditions with the addition of PEG 10,000 up to 5% and 10% (w/v). Under unstressed conditions no significant differences were observed between the transformed and WT plants. The drought stressed transgenic plants performed significantly better and gave higher fresh weight (+35%) and dry weight (+59%) than the wild type plants. The differences were especially pronounced for the roots. The transgenic plant roots increased 73% in weight under stress than the WT. The fructan level in transgenic plants was increased by 7-fold under induced drought stress. Under all conditions the total non structural carbohydrate content was higher in the transgenic plants. The increase in non-structural carbohydrates and enhanced root development might have led to better growth of transgenic plants under drought stress.

A salt tolerance gene *HAL1* from yeast was used to transform commercial cultivars of melon (*Cucumis melo* L.) by Bordas *et al.* [18]. *In vitro* cultured shoots of transgenic plants were evaluated for salt tolerance on shoot growth medium containing 1% NaCl. Although roots and vegetative growth was reduced, transgenic plants showed a higher level of tolerance than the WT plants.

3. Anaerobiosis/anoxia

Most plants are highly sensitive to anoxia during submergence. An important aspect of the adaptation to oxygen limitation include metabolic changes such as avoidance of self poisoning and cytoplasmic acidosis, maintenance of adequate supplies of energy and sugar. During anoxia, ATP and NAD^+ are generated not in the Krebs cycle and the respiratory chain but via glycolysis and fermentation. A number of enzymes of the anaerobic pathways such as alcohol dehydrogenase and pyruvate decarboxylase induced during anoxia have been cloned and characterized [19-21]. Using rice seedlings, Umeda and Uchimiya [20] observed the coordinated expression of genes whose products are involved in glycolysis and alcohol fermentation under submergence stress. They analyzed the mRNA level in submergence-tolerant rice FR13A and submergence-sensitive IR42 and showed these

genes including glucosephosphate isomerase, phosphofructokinase, glyceraldehydephosphate dehydrogenase and enolase may change in FR13A. Furthermore, Kawai *et al.* [22] showed adenylate kinase, which is known to supply ADP in energy-producing systems, also induced faster in FR13A.

In many organisms lactic acid is a prominent end product of anaerobic metabolism. An unfavorable consequence of lactate accumulation is cytoplasmic acidosis, a decrease in cellular pH which may finally lead to cell death [23-25]. Bucher *et al.* [26] developed tobacco with the constitutive capacity of ethanolic fermentation by expressing a pyruvate decarboxylase gene derived from the obligate anaerobe *Zymomonas mobilis*. As a result, during the first 2-4 h of anoxia, acetaldehyde accumulated to 10- to 35-fold and ethanol to 8- to 20-fold higher levels than in non-transgenic plants.

Several anaerobic stress inducing promoters have also been analyzed. Kyozuka *et al.* [27] demonstrated that maize *Adh*1 promoter was strongly induced (up to 81-fold) in roots of seedling after 24 h of anaerobic treatment. For gene expression to obtain anaerobiosis tolerant plants, a desirable promoter should not be active under aerobic condition.

4. Heavy metal

Optimum growth and productivity and even cultivation of most of the plants is severely restricted in soils with elevated levels of one or more inorganic ions such as sodium in saline soils; Al, and Mn in acidic soils and heavy metals Cu, Zn Pb, Ni, Cd *etc.* due to mining, industrial affluents and other human activities. Some of the ecotypes and wild relatives of the cultivated plants possess high levels of tolerance to the toxic concentrations of the inorganic ions. Major genes for tolerance to some of the ions such as Al, As, B, Cd and Cu have been identified [28] in certain crop plants and the breeding programs for developing tolerant varieties have some success. The molecular basis of tolerance is, however, not known in plants. In animals, however, metal detoxification is known to be mediated by low molecular weight metal binding proteins called metallothioneins [29]. In higher plants small metal binding ligands called phytochelatins synthesised from glutathione by phytochelatin synthase [30] on application of heavy metals are functionally analogous to metallothioneins. The specificity of phytochelatins and correlation with metal tolerance, however, has not been established. Evans *et al.* [31] also examined the phenotypic effect of constitutive expression of *PsMTA* in transgenic *E. coli* and *Arabidopsis*. Copper accumulation by *PsMTA* positive *E. coli* was approximately 8-fold greater than in control cells. Similarly, the *PsMTA* positive *Arabidopsis* plants accumulated several fold more Cu than the untransformed plants indicating that *PsMTA* could be involved in Cu detoxification. Pan *et al.* [32] transformed tobacco with a mouse metallothionein-I (MT-I) gene and found that the growth of the transgenic plants was unaffected up to 200 μM cadmium whereas the control plants developed leaf chlorosis at only 10 μM cadmium concentration.

In plants with genetic resistance to Al toxicity, the Al exclusion and uptake from root tips have been found to be correlated to their increased capacity to release organic acids such as citric acid which chelates Al^{3+} outside the plasma membrane. de la Fuente *et al.* [33] developed transgenic tobacco and papaya that overexpressed a citrate synthase gene (*CSb*) from *Pseudomonas aeruginosa* in their cytoplasm. Tobacco lines expressing *CSb* had up to 10-fold higher level of citrate in their root tissues and one of the lines released 4-fold citrate extracellularly whereas in papaya there was only 2 to 3-fold increase of citric acid production. Over production of citric acid was shown to result in Al tolerance in both the species.

5. Heat and Cold

Temperate and subtropical plants are highly susceptible to high temperature during early tillering, flower initiation, anthesis and grain filling stages leading to substantial reduction in their productivity. In response to high temperature all organisms, including plants, synthesize a set of proteins called as heat shock proteins (HSPs) which have been classified into several families according to their molecular masses. Many HSPs are constitutively expressed (HSC) and are essential for growth and metabolism at normal growth temperature during various stages of development. The induction of HSPs at permissive temperatures have been associated with the acquisition of thermotolerance to withstand short periods of an otherwise lethal temperature. A number of HSPs have been reported to act as molecular chaperones to assist the folding, assembly and transport of other proteins, prevent protein aggregation at high temperatures and promote degradation of heat damaged proteins for restoration of cellular functions after stress.

The genes for a number of HSPs and heat shock cognates have been cloned, but their role for tolerance during heat stress has not been unequivocally established through genetic engineering of higher plants. The HSP104 of the yeast *Saccharomyces cerevisiae* belonging to HSP100 gene family promotes survival at high temperature. Lee *et al.* [34] and Schirmer *et al.* [35] isolated high temperature inducible 101 kD

HSP from cDNA libraries of soybean (*HSP101*) and *Arabidopsis* (*At HSP101*), respectively. On genetic transformation of yeast both the genes complemented the thermotolerance defect of yeast caused by the deletion of the *Hsp104* gene suggesting their possible role in heat tolerance in higher plants.

Constitutive HSP70 and heat inducible HSP70 genes of proteins of the HSP70 family are present in all organisms including higher plants. Lee and Schoffl [36] investigated the expression of HSC70/HSP70 in *Arabidopsis* transformed with heat inducible antisense HSP70 gene. In the transgenic lines HSP70 gene was not induced by heat shock and also the level of Hsp70 mRNA was greatly reduced. The antisense RNA down regulation was only specific for HSP70 family which did not affect the induction of mRNA and the level of small HSP18. The acquisition of thermotolerance was negatively affected in the antisense transgenic plants. The time required to turn off the heat shock transcription factor during recovery from heat stress was considerably prolonged in antisense plants compared to wild type line implying a dual role of HSP70 in plants, protective role in thermotolerance and auto regulation of heat shock response.

As discussed by Murata *et al.* [37], the chilling sensitivity of plants is closely correlated with the degree of unsaturation of fatty acids in the phosphatidylglycerol of chloroplast membranes. Plants with a high proportion of *cis*-unsaturated fatty acids, such as spinach and *Arabidopsis*, are resistant to chilling, whereas species like squash with only a small proportion are not. The chloroplast enzyme glycerol-3-phosphate acyltransferase seems to be important for determining the level of phosphatidylglycerol fatty acid unsaturation. Thus they demonstrated for the first time that the level of fatty acid unsaturation of phosphatidylglycerol and the degree of chilling sensitivity of tobacco can be manipulated by transformation with cDNAs for glycerol-3-phosphate acyltransferases from squash and *Arabidopsis*.

High levels of trienoic unsaturated fatty acids (16: 3 and 18: 3) in membrane lipids of plants have been associated with chilling tolerance and cold acclimation. A number of *fad* mutants of *Arabidopsis* defective in fatty acid desaturase are more susceptible to chilling [38]. A gene for omega-3-fatty acid desaturase of chloroplasts has been cloned which successfully complements the *fad7* mutant of *Arabidopsis* for accumulation of trienoic fatty acids. Kodama *et al.* [39] developed transgenic tobacco plants for the chloroplast omega-3-fatty acid desaturase gene (*fad7*) isolated from *Arabidopsis*. The transgenic plants containing a high level of 16: 3 and 18: 3 fatty acids had corresponding decreased level of their 16: 2 and 18: 2 precursors. The suppression of the leaf growth and the low temperature induced chlorosis were significantly reduced in the transgenic plants with the *fad7* gene indicating that the increased levels of trienoic fatty acids enhanced cold tolerance.

6. Shading

Optimum supply of nutrients and efficient photosynthesis are conducive to biomass production but the allocation of assimilates within the developing plant determines the harvest index and economic yield. In pure stand canopy as well as in mixed cropping, competition for light energy invokes shade avoidance syndrome manifested by rapid growth and extension of stem and petiole at the expense of leaves, storage and reproductive organs thus predisposing plants to lodging, susceptibility to diseases and insect pests and a lower harvest index [40]. Although the development of semi-dwarf varieties of wheat and rice in the 60s has led to their higher harvest index and grain yield by overcoming some of the defects of the tall genotypes yet the competition among plants for light energy continues to operate in canopies under intensive cultivation practices. The photosynthetic pigments in plants absorb the visible radiation (400-700 nm) and reflect and transmit far red (FR) radiation beyond 700 nm. The FR wave band between 700-800 nm predominating in the dense plant stands have been implicated in proximity perception for initiating shade avoidance syndrome. The FR reflection signals are perceived by the photoreceptors called phytochromes which possess distinct photo sensory functions [41]. Phytochrome (*phy*A) mediating the inhibition of stem growth on etiolated plants in response to FR wave length 710-720 nm is rapidly degraded and down regulated in light grown plants. Robson *et al.* [42] obtained transgenic tobacco lines expressing a high level of heterologous oat *phy*A apoprotein. In growth chamber experiments the growth of the *phy*A expressing plants was unaffected under fluorescent white light but was inhibited under FR supplementation. Internode growth of WT plants was low under white light but was markedly increased under FR supplementation which simulated the canopy environments for induction of shade avoidance response. The level of growth inhibition of transgenic plants correlated with the level of *phy*A production. Under field trials at various planting densities from 20 to 100 cm, the transgenic plants were indistinguishable from the WT at the lowest plant density but became progressively shorter as the plant density increased. This phenomenon termed as "proximity conditional dwarfing" led to a 15 to 20% increase in harvest index (expressed as leaf biomass as a proportion of total biomass) in transgenic plants under high plant density

thus demonstrating the suppression of shade avoidance response under high level of *phy*A expression. Further understanding of the molecular basis of interaction of various phytochromes among themselves and with R: FR ratios in natural light environment may help to change crop plant architecture to avoid shade stress and obtain maximum production under high plant density, mixed cropping and agroforestry.

7. UV-B

The high influxes and absorption of UV-B radiation affects terrestrial plants through damage to DNA directly or indirectly through formation of free radicals, membranes by peroxidation of unsaturated fatty acids, photosystemII, phytohormones and even symbiotic relationship of plants with microorganisms. Before the evolution of terrestrial plants the earth received very heavy doses of UV-radiation. There was a high level of CO_2, a low level of oxygen and little or no ozone shield. The gradual build up of stratospheric O_3 (parallel to atmospheric O_2) which absorbs solar UV-C (<280 nm) completely and most of the UV-B accompanied and facilitated the evolution of land plants [43]. The concomitant evolution of UV-B absorbing phenolic compounds flavonoids, anthocyanins, tannins and lignins with increasing degrees of polymerization and complexity from algae to higher plants. Other defense mechanisms also played an important role in the evolution of terrestrial plants. There are large temporal and spatial differences in UV-B fluxes on the globe to which plants are exposed. The depletion of the stratospheric ozone layer may further aggravate the UV-B stress scenario. The laboratory and field studies on the effect of UV-B radiation on damage to plants under simulated conditions have not been very com prehensive or conclusive [44]. A number of secondary metabolites such as flavonoids, tannins and lignins are increased at elevated levels of UV-B radiation which screen UV-B and protect the cellular components against the UV-B damage. Mutants of *Arabidopsis* deficient in flavonoids were found to be hypersensitive to UV-B radiation [45]. Several enzymes of the pathways of UV-B absorbing phenolic compounds have been cloned and characterized which can be appropriately engineered and used for genetic transformation of UV-B sensitive plants for enhancing their tolerance against the invisible stress.

8. Oxidative stress

A number of abiotic stresses such as extreme temperatures, high light intensity, osmotic stresses, heavy metals and a number of herbicides and toxins lead to over production of reactive oxygen intermediates (ROI) including H_2O_2 causing extensive cellular damage and inhibition of photosynthesis [3]. Due to excessive reduction of PSI under high light intensity and limited supply of CO_2, due to stomatal closure, O_2 competes for electrons from PSI leading to the generation of ROI through Mehler reaction thus making the chloroplasts as the rich source of ROI. Chloroplasts have developed efficient enzymatic and non-enzymatic systems for dismutation and scavenging of superoxide radicals. Superoxide dismutase and ascorbate peroxidase are the important antioxidant enzymes whose activities are enhanced under stress conditions and are associated with stress tolerance. Cytosolic and chloroplast bound super oxide dismutase and ascorbate peroxidase have been cloned in many plants and used for genetic transformation to confirm their role for providing tolerance during oxidative stress. In one of the earlier attempts, Tepperman and Dunsmuir [46] developed transgenic tobacco overproducing petunia chloroplastic Cu/Zn SOD but did not find any tolerance to photo oxidative stress from methyl viologen herbicide and ozone fumigation in the transgenic plants. The Cu/Zn SOD are known to be susceptible to inactivation by H_2O_2 whereas the MnSOD is not. Bowler *et al.* [47] used chimeric MnSOD from *Nicotiana plumbaginifolia* with chloroplast transit peptide for genetic transformation and demonstrated that the elevated level of SOD protected the transgenic plants against cellular damage by oxygen radicals and ozone. Gupta *et al.* [48] used a chimeric gene encoding pea chloroplast Cu/Zn SOD for tobacco transformation. During exposure to moderate chilling stress (10°C and low light intensity) transgenic SOD plants retained 20% higher photosynthetic rate than the untransformed plants. After exposure to severe stress (3°C and high light intensity) for 4h the leaf discs of SOD transgenic plants regained 90% photosynthetic capacity as that before stress whereas the WT plant discs had only 30% activity. The SOD transgenic plants also showed reduced damage from the super oxide generation herbicide methyl viologen (MV) up to 1.2 μM MV. Ascorbate peroxidase (APX) is the key enzyme for scavenging H_2O_2 in cytosol and chloroplasts.

Mannitol has been recognized as the hydroxyl radical scavenger *in vitro*. To investigate the role of mannitol in radical scavenging, Shen *et al.* [49] targeted a bacterial mannitol-1-phosphate dehydrogenase gene to chloroplasts in transgenic tobacco by the addition of amino terminal transit peptide. One of the mt1D transgenic line, BS1-31 accumulated about 100 mM mannitol in its chloroplasts and had similar phenotype and photosynthetic activity as that

of the WT. The presence of mannitol in the chloroplasts of transgenic plants led to the increased tolerance to oxidative stress induced by MV as indicated by increased chlorophyll retention. Isolated mesophyll cells of BS1-31 also exhibited higher CO_2 fixation than the wild type. The cells of BS1-31 also showed higher hydroxyl radical scavenging capacity to DMSO as the probe. The production of mannitol in the chloroplast has several distinct advantages over its production in cytosol for scavenging of the most reactive hydroxyl radical at the very site of their production.

9. Perspectives and strategies for improving tolerance

The work on genetic engineering of tolerance to abiotic stresses began piece meals within a decade of the molecular understanding of pathways induced in response to one or more of the abiotic stresses. A number of the key microbial and plant genes of defense mechanisms and pathways have been cloned, engineered for high level of constitutive expression in plants and monitored for their effectiveness in model plants after genetic transformation. In most of the cases the transgenes expressed faithfully but only a limited level of tolerance was provided under stress conditions as compared to the no-transformed wild type plants. In many cases the tansgenic plants had morphological abnormalities and slower growth under nonstressed environment. The level of many compatible osmolytes responsible for osmotic adjustment was too low to be effective *per se* in providing the required water retention and osmotic adjustment. Some of the approaches and strategies for further improving the effectiveness and level of tolerance to abiotic stresses are outlined below.

The use of multiple tolerance mechanisms for one or more of the abiotic stresses through stepwise or co -transformation may help to achieve high levels of tolerance for commercial exploitation.

Only a few of the genes induced in response to abiotic stresses have been cloned and used for transformation. Additional stress response and stress tolerance genes should be cloned through complementation of abiotic stress sensitive mutants in yeast and *Arabidopsis*. The entire genome of yeast has been sequenced and the *Arabidopsis* is likely to be sequenced soon. The QTL mapping of stress tolerance in certain species, comparative mapping and map based cloning in plants may be used to screen genes which function under stress as well as those induced and expressed in response to stress.

The low level of expression of the transgenes inspite of the use of constitutive promoters can be further enhanced by organellar transformation. The bacterial genes used in many transformation attempts can be coordinately expressed in organelles to high levels without changing promoters, codon usage modification and gene silencing. Organellar transformation is likely to become a matter of routine soon.

The constitutive expression of a transgene may be desirable for continuous expression but is not always without cost and yield penalty in non-stressed environments as has been found in many transformants. For high level production of osmoprotectants it will be desirable if such genes are driven by stress inducible promoters rather than the constitutive promoters.

Molecular understanding of the stress perception, signal transduction and transcriptional regulation of abiotic stress responsive genes may help to engineer tolerance for multiple stresses. The identification of multiple stress and ABA responsive elements in the 5′ promoter sequences can help to isolate transcription factors which may coordinately express multiple stress responsive genes. Exposure of plants to several abiotic stresses especially those causing osmotic stress results in increased biosynthesis of ABA which in turn induces expression of stress related genes.

Genes responsible for adaptation of certain plants to extreme habitats such as the desiccation tolerant resurrection plant *Craterostigma plantagineum* and halophyte *Mesembryanthemum crystallinum* should be cloned and used for genetic transformation of stress sensitive plants. *C. plantagineum* accumulates unusual sugar, 2-octulose in leaves in well-watered plants which is rapidly converted to sucrose on desiccation. The ice plant responds to drought or salt stress by switching from C_3 mode of photosynthesis to Crassulacean acid metabolism (CAM) which allows the plant to fix CO_2 at night reducing transpiratory water loss. The key enzyme of CAM pathway phosphoenol pyruvate (PEP) carboxylase along with other enzymes is regulated by osmotic stress at the transcriptional and translational levels. Inositol-1-phosphate synthase (INO1) is the key enzyme for diversion of carbon from glucose-6-phosphate pool to series of polyol biosynthesis through myo-inositol-1-phosphate. Several polyols and their derivatives are the integral parts of stress tolerance mechanisms. Transgenic plants with high level expression of INO1 during stress and thus with sufficient myo-inositol pool will be capable of deploying multiple defense mechanisms. Late embryo abundant (LEA) proteins usually expressed in seeds during maturation are also induced by osmotic stress and respond to ABA. LEA have been speculated to function in water retention, ion sequestration and molecular chaperones for providing cellular protection. A number of LEA genes have been cloned and characterized some, which are referred to as dehydrins or RABs, should be tested

through genetic transformation of osmotic stress sensitive plants [50]. Some isoforms of osmotin proteins belonging to pathogen related 5PR antifungal proteins are also induced by many abiotic stresses. These may be involved in providing protection against osmotic stress imposed by many pathogens.

Understanding the molecular mechanism for providing protection against biotic and abiotic stresses may lead to a generalized master mechanism for stress tolerance. Optimum homeostasis is always a key to living organisms for adjusted environments. Thus, abiotic stress accompanying a number of biological phenomena must be precisely investigated by consideration of plant homeostasis.

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