Engineering crops for tolerance against abiotic stresses through gene manipulation

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Plant genetic engineering took birth in the mid-eighties when, for the first time, plants were successfully engineered for improved virus, herbicide and insect resistance. This sphere has been ever-increasing since then. Abiotic stresses (such as high salt levels, low water availability leading to drought, excess water leading to flooding, high and low temperature regimes, etc.) adversely affect crop plants. The genetic responses of plants to these stresses are complex involving simultaneous expression of a number of genes. Till the early-nineties it was inconceivable that there would be any success in attaining the goal of improving resistance of crop plants to abiotic stresses. Continuing efforts of the stress biologists have resulted in engineering of plants resistant to low temperature, high temperature and excess salinity. A satisfactory progress has also been achieved in the area of generating plants resistant to water stress and flooding. While what has been achieved is impressive, it is still a challenging task to pyramid useful genes for high-level resistance to such stresses. The limiting factor in extension of biotechnology to abiotic stresses is the lack of information on what are the 'useful genes'—genes which would lead to better stress tolerance. We have reviewed how these genes are being searched to enable further development of strategies for stress management in crop plants. This is important because the strategies for coping with the abiotic stresses (and also for several other applications in plant biotechnology) have also come through the research work of scientists working on as diverse organisms as bacteria and fish.

By the turn of the century, the burgeoning population will exert considerable pressure on agriculture. To meet the increased demands, it is imperative to look for tools not only to increase the crop productivity but also to ensure protection against loss of potential productivity due to environmental vagaries. Plant genetic engineering techniques could be effectively utilized to exploit some of the untapped potentials to increase the harvestable crop yield. These techniques are fast developing and it is expected that agriculture in the 21st century will include a significant proportion of transgenic plants.

Plant genetic engineering methods involve specific gene manipulations either through over-expression or silencing of alien/native genes. If a gene (from any source; prokaryotic or eukaryotic, plants or animals) can be identified which can lead to betterment of the crops, it is possible to transfer that gene into a desired host, using these tools and techniques. Thus plant genetic engineering has an advantage over the conventional breeding methods: (i) Genes from any biological species

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(ranging from bacteria to man) can be transferred to crop species by genetic engineering methods. (ii) Introduction of molecular changes by genetic engineering takes lesser time compared to plant breeding methods. (iii) Often transfer of specific single gene(s) by plant breeding approach is associated with the simultaneous transfer of undesired genes and this problem can be avoided with plant genetic engineering.

In plant genetic engineering there are three main steps. (i) To identify, isolate, clone and characterize the desired gene. (ii) To transfer and incorporate the gene into the desired host system. (iii) To regulate the expression patterns of transgene (the transferred gene) in the desired tissues so that the gene is expressed in appropriate levels at the desired time with respect to desired developmental stage of the plant[1,2]. Research is being carried out for optimizing all these requirements to suit the various plant systems, and several of the important plant species (including important cereals, pulses and oil crops) have become amenable to transformation. Mainly transformation has been through *Agrobacterium tumefaciens*, a soil-bacterium, the 'gene ferry' in the hands of plant genetic engineers. In the recent years, methods such as use of particle gun have further added to the arsenal of the available methods. Further, a host of DNA sequences (the so-called promoter sequences including CaMV 35S promoter, actin1 promoter, ubiquitin1 promoter, etc.) are available for the regulation of transgenes. It is possible now to obtain the desired level of gene expression in either the constitutive (without any internal or external inducer) or the inducible (in response to a given inducer) modes[3]. The main limiting factor in extension of plant genetic engineering methods is now the availability of the 'MAG' – 'Most Appropriate Gene'.

Abiotic stresses and crop yield

It is commonly observed that field crops experience a multitude of stress conditions which are broadly classified into two categories, viz. biotic (viral, insect and fungal pathogens etc.) and abiotic stresses (such as those imposed by presence of excess salts, less or excess water, low and high temperatures, etc. see Table 1).

A study in USA showed that only 10–20% of the potential yield of plants like maize, wheat, rice and peanuts is harvested under stress-prone ecosystems[4]. This situation is more pertinent in Indian ecosystems where farming is mostly at the mercy of weather conditions. Therefore, it is important that we attempt to reduce the losses in biomass caused by various stresses. The severity of environmental stresses often varies with location and crop. Table 2 lists some of the important abiotic stresses affecting the major crop plants in different locales of India.

Table 1. Selective abiotic stresses which affect crop plants

Drought
Flooding
Heavy metals
High temperature
Low temperature
Ozone
Salinity/alkalinity
UV irradiation

Genetic engineering for tolerance to abiotic stresses – basic tenets

How far can genetic engineering of plants help in raising stress-tolerant crop plants? The limiting factor in extending plant genetic engineering methods to any application appears to be the availability of the desired gene(s). Therefore, identification/isolation of the genes, which would provide tolerance to abiotic stresses, is the most critical input in this endeavour (for the sake of simplicity, the terms 'resistance' and 'tolerance' have been interchangeably used in the present discussion).

Since every living system 'fights back' the adverse conditions in its best possible way, plants are no exceptions. A logical approach to identify genes related to stress tolerance is to understand the natural defence mechanisms of plants and improve upon them by suitable gene manipulations. Plant physiologists/biochemists have shown that the onset of stress conditions elicits several metabolic/physiological changes associated with optimal metabolism of carbohydrates, lipids, nucleic acids and proteins[5–7]. In the light of these changes being either 'deteriorative' (without any direct role in stress tolerance) or adaptive in nature (with a definite role in imparting stress tolerance), it is important to delineate the stress-associated metabolic events. One approach in this direction is the use of sublethal stress regimes, as sublethal stress conditions generally provide protection against the ensuing lethal stress levels[1,8–10]. Thus metabolic alterations (at least some) during sublethal stress conditions are more likely to play a role in inducing stress tolerance.

In principle, stress-responsive genes can be analysed using targeted or non-targeted strategy. The former relies upon the availability of relevant biochemical information (in terms of a defined enzyme, protein, a biochemical

Table 2. Major abiotic stresses affecting some of the important crops

Crop	Stress agents
Brassica	Drought, low and high temperatures, salinity
Chickpea	Drought, low and high temperatures, salinity
Cotton	Drought, low temperature, salinity
Pigeonpea	Drought, low temperature
Rice	Drought, low temperature, salinity, submergence
Wheat	Drought, high temperature, salinity/alkalinity, submergence

reaction or a physiological phenomenon). This approach is more precise, methodical, and its chances of success are high. The nontargeted or the shotgun strategy to obtain a desired gene is indirect. This strategy banks on random analysis of stress-related alterations in cell processes/gene expression. For instance, it includes examination of protein profiles before and after stress treatment to select proteins which are specifically altered, or use differential hybridization to hybridize cDNA library with mRNA from both the control and experimental (stressed) plants. As this strategy can work even though there is no prior information about the gene or gene product, it appears to be the best choice due to a dearth of sufficient information on precise biochemical changes elicited in order to acquire stress tolerance. We shall get back to these approaches later in this review.

Once the desired gene is identified, the molecular biologist can design expression cassettes by subcloning a suitable promoter at the 5′ end of the gene (to allow regulated expression), terminator sequences at the 3′ end (to prevent read-through), and then together with a selectable marker gene (such as those associated with resistance to herbicides, antibiotics, etc.) transfer the gene to the cells of the target species. After the transformed cells (usually callus cells) are selected, tissue culture technique is used in regenerating plants from the transformed cells. This is carried out in controlled conditions in an aseptic milieu. The plants raised from the transformed cells are referred to as T_0 plants. The gene of interest is identified by polymerase chain reaction (PCR), and copies of the transgenes and copies of loci to which it has been incorporated in the host system are identified by Southern blot analysis. This is a critical step because if multiple insertions are obtained, often the transgene is unstable and its expression is suppressed[11]. The expression level of the transgene is quantified by Northern blotting and Western blotting. The seeds from the selfed T_0 plants, T_1 seeds, are used to raise T_1 plants, and the T_2 seeds are used for producing homozygous state, to prevent further segregation of the transgene. This work is labour-intensive, takes three–four growth seasons, and needs to be carried out in controlled-conditions to prevent gene flow from the transgenics to untransformed plants. The latter point is critical because a given gene may potentially have disastrous consequences in natural ecosystems. Once the homozygous lines are obtained, trials are carried out to find out how the transgene has enabled the plant to cope with a particular stress condition.

In the present review, we have emphasized the isolation and characterization of various stress-responsive proteins and the genes that can potentially be manipulated towards engineering crops for improved stress tolerance (for want of space the list of references are kept to minimum in this review as they can be easily accessed from the recently published books/chapters/research papers).

Targeted approach for engineering tolerance to abiotic stresses – how to go for it?

As stated above, the prerequisite for the targeted approach is the availability of some background information. This can be illustrated by considering osmotic stress conditions (brought about by drought, salt and low temperature stresses) in relation to maintenance of turgor by means of osmotic adjustment. Osmotic adjustment enhances the capacity of plant tissues to maintain turgor and protoplast volume despite reduction in tissue water content. Thus it allows turgor- and volume-mediated processes to continue to a more negative water potential values. On this basis it is inferred that accumulation of osmotically-active compounds in cells in response to osmotic stress is an important component for the induction of stress tolerance[12]. One further needs to know how these osmotically-active substances are synthesized in the cells as plant genetic engineering methods work at the level of genes, not at the level of processes/reactions. What are the enzymes needed for the synthesis of osmotically-active compounds? Is there any rate-limiting step in the synthesis of such compounds? Once the rate-limiting step is known, the next aim would be to identify, isolate and clone the gene for the enzyme which limits the rate of synthesis. A great deal of success has been achieved in raising transgenic plants with over-expression of osmotically-active compounds such as proline, glycine-betaine and certain sugars. Specific examples are discussed in the later section of this article.

However, several reactions/processes are thought to act in concert for providing effective tolerance against abiotic stresses. Unfortunately, our understanding of the biochemical/physiological processes contributing to stress tolerance is far from complete. Therefore, a shotgun approach has been exploited to examine various cellular processes/gene expression alterations which take place concomitant to stress effects.

Shotgun approach for engineering tolerance to abiotic stresses – what are stress proteins?

A major point which has emerged from the studies on plant–environment interactions is that the response of crop plants to abiotic stress involves several gene alterations at the same time, hence is multi- or polygenic. Molecular studies further support this notion. In response to salinity stress, it has been shown that more than 100 transcripts are affected at the same time[13]. With respect to protein analyses, a large number of proteins show up/down regulation due to stresses as revealed by one/two-dimensional protein gel analysis[10,14]. Protein and RNA analyses show that the existing physiological/

biochemical knowledge of plant responses is scanty and several mechanisms are yet to be understood. One approach is to analyse all the proteins/RNA species induced as a response to stress. The proteins which are up-regulated by stress conditions are referred by the general term 'stress proteins'. For improving tolerance to abiotic stresses, a large number of investigators are examining stress proteins. Such proteins have been noted in response to high temperature, low temperature, salinity, drought and several other stress factors[1,9,10,14–16]. In rice, analysis of stress-associated changes in genes/proteins has been made in response to salt[17–22], drought[17,22–24], cold[22,25,26], and high temperature[19,22,27–32].

High temperature stress-responsive proteins/genes

Among the best characterized (genetically and biochemically) stress proteins are the heat shock proteins (HSPs). Nearly all organisms from bacteria to man respond to heat shock (HS, 5 to 10°C more than the ambient temperature) by synthesizing specific HSPs. Further, the genes encoding HSPs, referred to as hsp genes, have been isolated, characterized and sequenced in a range of biological systems. A list of selected plant heat shock genes/proteins is presented in Table 3. As far as possible, we are listing the names of different HSPs with capital letters (such as HSP 70 for the 70 kDa family of HSPs) and names of gene(s) encoding for HSPs with small alphabets (such as hsp70 for genes encoding HSP 70 proteins). The same pattern of nomenclature is followed with respect to other stress proteins in Tables 4–6. Detailed biochemical/molecular information on HSPs has been reviewed in several recent chapters/papers[9,10,33,34]. The salient features of HSPs are presented in Table 7.

Table 3. A selective list of heat shock genes/proteins in a range of plant species

Plant species	hsp genes/HSPs
Arabidopsis thaliana	HSP 70-1, *hsp17.4*, *hsp18.2*, *hsp 81.1*, *hsp81.2*, *hsp101*, *hsp17.6*, *hsp22*
Brassica oleracea	HSPs of 90, 88, 86, 74, 69, 66, 47, 43, 42, 27, 23, 21, 19 and 18 kDa
Catharanthus roseus	*hsp90*
Daucus carota	*hsp70*
Glycine max	*hsp7.6-L*, *hsp17.5-M*, *hsp17.5-E*, *hsp22*, *hsp101*, LMW class I HSPs
Gossypium hirsutum	HSPs of 100, 94, 89, 75, 60, 58, 37, 21 kDa
Helianthus annus	HSPs of 17.6 and 17.9 kDa, ubiS
Hordeum vulgare	HSPs of 94, 85, 76, 71, 39, 32, 24 kDa and *hsp90* gene
Lycopersicon peruvianum	Seven LMW HSPs (15-20 kDa), polyubiquitin
Oryza sativa	HSPs of 30, 33, 90, 104 and 110 kDa, *hsp16.9 A*, *hsp16.9B*, *hsp82B*, ubiquitin
Phaseolus vulgaris	*hsp70*
Pharbitis nil	*hsp83A*, *hsp83B*
Phaseolus aureus	HSP 70
Pisum sativum	HSPs of 21, 18.1 and 62
Solanum tuberosum	HSP 70 and ubiquitin
Sorghum bicolor	HSPs of 94, 73, 60, 34, 30 and 24 kDa
Triticum aestivum	HSPs of 60, 58, 46, 40, 14, 118, 90, 70 and 18 kDa
Vigna radiata	HSPs of 114, 79, 73, 70, 60, 56, 51, 46 and 18 kDa
Zea mays	HSPs of 108, 89, 84, 76, 73, 60, 30, 23 and 18 kDa, *hsp70*

Low temperature, salinity and water stress-responsive proteins/genes

Apart from high temperature, stress genes/proteins responsive to low temperature, drought, and salt stresses have also been reported. Selected examples of these genes/proteins are presented in Tables 4–6.

Cellular functions of stress proteins

The rate of protein biosynthesis shows a general decline during stress conditions[31,34–36]. Despite an overall reduction in protein synthesis activity, it is noteworthy that cells preferentially synthesize stress proteins. Although stress protein is an 'umbrella term', it is unequivocally shown, at least in certain instances, that stress proteins

Table 4. Selected examples of low temperature responsive genes/proteins in plants

Plant species	Genes/proteins
Arabidopsis thaliana	*rab18*, *kin1*, *2*, *lti30*, *40*, *45*, *65*, *78*, *cor6.6*, *15a*, *b*, *47*, *corpHH7.2 28*, *29*, *67* clones
Brassica napus	Proteins of 22–23 kDa
B. oleracea	7 kDa protein
Bromus inermis	Proteins of 22 , 25, 165, 190 and 200 kDa and corresponding cDNA clones
Cucumis sativus	Proteins of 25, 38, 50, 70 and 80 kDa
Glycine max	HSP 70-related protein
Hordeum vulgare	Proteins of 45 and 75 kDa, *pAF93*, *pT59*, *pAO29*, *86*, *hva1*, *blt14*, *101*
Lycopersicon esculentum	Proteins of 27 and 35 kDa
Medicago sativa	15 kDa protein, *cas18*
Oryza sativa	Proteins of 95, 75, 50, 25, 21 and 14 kDa, *psaB*, *psbB*, *rbcL*, *atpE*, *rbcS*, *cab*, *rab16A*, *21*-like gene, *lip 5*, *9*, *19*
Solanum tuberosum	*CI7*, *13*, *19*, *21* clones
Spinacea oleracea	*cor85*, *140*, Proteins of 85 and 160 kDa, CAP 79, 160
Triticum aestivum	*wcs19* and *120*
Zea mays	*adh1*

play a crucial role in assisting the cells to carry out their metabolic activities during adverse conditions[10,14,36]. The role of a stress protein becomes clear by showing homology (if there) of its amino acid sequence or the nucleotide sequence with any established protein/enzyme molecule, or by unravelling the functional attributes of a defined stress protein and thus work out its identity. This point can be understood by studying alcohol dehydrogenase (ADH). Application of hypoxic/anoxic conditions (caused by submergence or flooding stress because diffusion of oxygen in water is extremely low) leads to up-regulation of several proteins, called anaerobic proteins or ANPs[37], one of which has been identified to be the ADH protein[38]. As ADH protein has a role in ethanolic fermentation of the glucose, anoxic conditions induce ethanolic fermentation through participation of ADH (as well as pyruvate decarboxylase or PDC protein), and this up-regulation of ethanolic fermentation has a role in generation of low levels of ATP under conditions when normal ATPs levels are not formed[39]. Most of the other ANPs have also turned out to be the enzyme linked with carbohydrate utilization by glycolysis and ethanolic fermentation pathways. These include PDC, glyceraldehyde 3-phosphate dehydrogenase, sucrose synthase, enolase, aldolase, etc.[40]

HSPs represent novel stress proteins, lacking any homology with the established enzymes. Accumulation of HSPs has been related with acquisition of induced thermotolerance in both animal and plant systems[10]. This contention has basically emerged from the fact that the course of HSP synthesis and the development of the acquired thermotolerance are temporally correlated[8,10]. Further support for this hypothesis has come through experiments showing that when the HSPs are not allowed to be synthesized by selective mutagenesis of the hsp genes[41], or when the HSPs are inactivated by antibody binding, the cells fail to develop thermotolerance[42]. For a long time, little was known about the cellular roles of HSPs. In 1988, two groups of workers showed that the members of the *hsp70* gene family are involved in the transport of proteins synthesized in the cytoplasm into the cell organelles such as ER[43,44]. These observations indicated that the HSP 70 proteins perform the role of molecular chaperones. The HSP 60 and HSP 90 family of proteins are also shown to function as molecular

Table 5. *Selected examples of water stress responsive genes/proteins in plants*

Plant species	Genes/proteins
Arabidopsis thaliana	*lti64, 78, rd17, 22, 29A, B, AT HB-7* (homeobox gene), *Atmyb2, erd5*
Brassica napus	BnD 22
Daucus carota	DC 8 protein
Gossypium hirsutum	Several families of LEA proteins
Glycine max	P5CR clone
Helianthus annus	mRNAs corresponding to hsp 17.6 and hsp 17.9
Hordeum vulgare	Several families of LEA proteins
Lycopersicon esculentum	*le16*, mRNA corresponding to 18–19 and 64 kDa polypeptides, lipid transfer proteins, proteases, ubiquitin, TAS 12, 14
Oryza sativa	Protein of 23 kDa, *rab16A, rab16B, rab16C, rab16D, rab21, rab25*
Pisum sativum	pPsB12 clone
Sorghum bicolor	BADH 1, 15 clone
Triticum aestivum	Em 1a, 1b, WSP 23
Vigna radiata	Several cDNA clones
Zea mays	*rab17, 28*, several families of LEA proteins

Table 6. *Salt stress responsive genes/proteins in selected crop species*

Plant species	Genes/proteins
Arabidopsis thaliana	*At myb2, sal1*
Brassica napus	BnD 22
Citrus sinensis	Cit-SAP
Hordeum vulgare	*hva1*, 26 and 27 kDa proteins
Lycopersicon esculentum	Osmotin, TSW 12, le 16
L. pimpinellifolium	14.5 kDa protein
Medicago sativa	*psm1409, pa9*
Nicotiana tabacum	Proteins of 30 and 43 kDa, osmotin
Oryza sativa	*rab21, salT*, Em gene, SAP 90, 104

Table 7. Salient properties of plant heat shock proteins

- HSPs are either high (80–100 kDa, HMW-HSPs), intermediate (68–73 kDa, IMW-HSPs) or low molecular weight (15–20 kDa, LMW-HSPs) proteins.
- Selected HSPs are synthesized and accumulated in response to water stress, abscisic acid, excess NaCl, chilling and anoxia apart from high temperature stress .
- Majority of the HSPs are encoded by nuclear genes and localized in the cytoplasm. Selected HSPs are localized in endoplasmic reticulum. Some HSPs are transported from the cytoplasm towards the interior of nuclei, mitochondria, and chloroplasts.
- HSPs are mostly transiently synthesized in cells. Further, the synthesis and accumulation of HSPs exhibit both time- and temperature-dependent patterns.
- Accumulation of selected HSPs has been reported in field-grown plants in response to natural diurnal changes in temperature. It is quite possible that all the plant species synthesize HSPs under field conditions.
- HSPs are highly conserved proteins as related proteins from diverse species show a marked homology in their structure and function.
- Selected HSPs are noted to be constitutively synthesized in flowers, pollen, embryos, pods and seeds of several plant species, indicating that HSPs are also developmentally-regulated proteins.
- Some of the hsp genes are not expressed in uninduced tissues and the expression is only observed when the cells are subjected to stress conditions. On the other hand, selected hsp genes are expressed even in uninduced conditions and the expression levels are noted to be up-regulated in response to stress conditions.
- The transcriptional regulation of hs genes is mediated by a core DNA sequence called the heat shock element (HSE), located in promoter region of the hs genes towards the 5′ side of the TATA box. This sequence has a 5 bp module (-nGAAn-) arranged as contiguous inverted repeat (nGAAnnTTCnnGAAn).
- For the regulation of hs promoter, *trans*-acting transcription factors termed as the heat shock factors (HSFs) have been identified that bind specifically to the HSEs. In limited cases, HSF-encoding genes have also been cloned.

chaperones[45]. The HSP 104 in yeast mediates the process of resolubilization of heat-inactivated proteins from insoluble aggregates[46]. Finally, ubiquitin has been shown to possess protease activity, and this HSP aids in removal of denatured and nonfunctional proteins, thus preventing proteotoxicity[47].

While significant progress has been made in isolation and partial characterization of new stress proteins responsive to low temperature, salinity and water stress, little is known about the precise biochemical reactions in which these stress proteins are implicated. By using various approaches it should be possible to analyse the functional significance of such diverse array of stress proteins. This can be undertaken by working out the amino acid sequence (partial or complete) of these proteins (or the nucleotide sequence of the corresponding genes) to find out the extent to which these sequences are homologous to known enzymes/proteins. Another way to achieve this is to genetically alter the level of desired stress proteins in transgenic plants and study the consequent changes in physiology/biochemistry. With modern molecular biology techniques, it is possible to either over-express any gene or under-express it (anti-sense technology). Production of plants with sense or antisense gene constructs may enable the scientists to work out the significance of these genes and for a large number of stress genes, this is an active research area of plant molecular biology today.

Transgenic crop plants for improved tolerance to abiotic stresses

We have stated above that several proteins/genes/ mechanisms are correlated with stress responses. However, the number of genes with defined role in stress tolerance are just a few. Most of the physiological/biochemical studies have not proved to be of direct importance with the viewpoint of plant genetic engineering. Stress-associated reduced photosynthetic rates, increased stomatal resistance, decreased rate of the light reaction of photosynthesis, decreased rate of ribulose bis phosphate carboxylase-oxygenase enzyme activity, decreased uptake of water and nutrients, decreased rates of enzyme activities which utilize nitrate such as nitrate reductase and nitrite reductase, decreased protein biosynthesis activity, etc. have been well-documented[5–7]. But, nearly all reactions in the cell are expected to be affected once the conditions are noncongenial. The need is to dissect the complexity of the physiological/biochemical responses in 'component-reactions' and work out the rate-limiting step and then isolate and characterize such genes, as well as to separate the 'primary responses' from the 'secondary responses'. This is because if the primary responses are genetically manipulated, it is likely that secondary responses would automatically be taken care of. The biochemical/physiological studies in most cases have not been carried out to present a clear picture.

We now highlight the reports in which response of crop plants to abiotic stresses has been manipulated by changing the genetic make-up in the past 4–5 years.

- Osmotically-shocked cells synthesize and accumulate massive amounts of osmoprotectory compounds. Such compounds possibly help the cells to lower their osmotic potential, to draw water from the outside medium[1]. Examples of osmoprotectory compounds include glycine, glycinebetaine, choline, proline, sugars and sugar alcohols[1]. Mannitol as an osmoprotectory compound is primarily found in microbes. By introducing mannitol-1-phosphate dehydrogenase gene (*mtlID*) isolated from *E. coli*, Tarczynski *et al.*[48] showed over-expression of mannitol in tobacco plants. These transgenic plants showed tolerance to high NaCl levels (250 mM). Seeds of transgenic *Arabidopsis* transformed with *mtlID* gene under control of CaMV 35S promoter over-produced mannitol and germinated in a medium supplemented with high amounts of NaCl[49].
- Proline is accumulated in significant amounts in a variety of plants subjected to salt/drought stresses[50]. Exogenous application of proline protects plant tissues from stress conditions[51]. Increase in proline levels is considered to help the cells in osmoprotection as well as in regulating their redox potential, scavenging hydroxy radicals, and in protection against denaturation of various macromolecules[51]. Transgenic tobacco plants over-expressing pyrroline-5-carboxylate synthetase gene have been recently produced[52]. These plants over-produce proline in the tissues and show enhanced level of protection when challenged with excess NaCl.
- Synthesis of HSPs is controlled at the level of transcription through interactions involving *cis*-acting promoter sequences and *trans*-acting HSFs. Lee *et al.*[53] over-expressed *hsf* gene in *Arabidopsis* plants and showed that transgenic plants over-expressing this gene have increased thermotolerance.
- Pilon-Smits *et al.*[54] have shown that over-expression of *sacB* gene from *Bacillus subtilis* leads to high level of fructans in tobacco cells, and this is associated with increased drought tolerance.
- Antifreeze proteins or AFPs are the proteins found in polar arctic fishes. It is suggested that these proteins help these fishes to survive under freezing conditions. Hightower *et al.*[55] have shown that over-expression of chemically-synthesized antifreeze protein gene *ala3* leads to improved freezing tolerance of tobacco plants.
- Murata *et al.*[56] have shown that desaturation of fatty acids is a critical factor in protection of membranes against cold stress conditions. Glycerol 3-phosphate acetyl transferase gene from squash when over-

expressed in tobacco, leads to increased low temperature tolerance.

- LEA proteins represent a category of characteristic proteins which are abundant during late embryogenesis[57]. Distribution of LEA proteins is widespread amongst diverse plant genera[14,57,58]. It has recently been shown that *hva1* gene (which encodes for a specific class of LEA proteins) when over-expressed in rice leads to increased salt tolerance[59].
- Increased water and salt stress tolerance has recently been achieved in rice plants through overexpression of *cor47* gene[60]. This gene was originally shown to be a low temperature-responsive gene.
- Glycine-betaine is an extremely efficient osmoprotectant. Lilius *et al.*[61] transformed tobacco plants with *betA* gene (which encodes for choline dehydrogenase protein responsible for conversion of choline to betaine aldehyde) from *E. coli*. The *betA* gene introduction rendered the transgenic tobacco plants tolerant to high concentrations of salt. Betaine aldehyde dehydrogenase (BADH) is the second enzyme in this pathway responsible for conversion of betaine aldehyde to glycine betaine. Holmstrom *et al.*[62] transformed *betB* gene from *E. coli* to tobacco and showed that increased BADH leads to improved resistance to betaine aldehyde and osmotic stress. In soil-bacterium *Arthrobacter globiformis*, *codA* gene (encoding for choline oxidase) is responsible for choline to glycine betaine conversion. In a recent study, transformation of *Arabidopsis* with the *codA* gene from this bacterium rendered the transgenic plants tolerant to both salt and cold stress[63].
- *sal1* gene (induced by salt stress) participates in sulphur assimilation and phosphoinositide signalling pathways. Quintero *et al.*[64] introduced *sal1* gene from *Arabidopsis* to yeast cells and showed that this gene confers high salt tolerance in transformed yeast cells.

Concluding remarks

While techniques for genetic transformation of crop plants are being widely practiced for varied applications, there is still a lot to be achieved for identifying the mechanisms/reactions/genes responsible for imparting tolerance to abiotic stresses. There appears to be a big gap in what physiologists/biochemists have shown with respect to identifying the mechanisms for stress tolerance and what molecular biologists can achieve with respect to tempering the genes for altering their expression levels. This is perhaps because molecular biology has developed at a faster pace than the physiological/biochemical sciences (for the reason that plant molecular biologists have freely 'borrowed' tools and techniques from molecular biologists working with microbes/animals because these are same, however the physiologists/biochemists cannot often do this because the systems are quite different). In essence, what we are saying is that plant genetic engineering does not have the requisite support for the identification of the candidate genes to be transferred for improving stress tolerance.

Still, genetic engineering for improved tolerance to abiotic stresses is the need of the hour as the existing cultivars in most cases are capable of giving much higher biomass than what we harvest. While it is true that the response of plants to these stresses is multi-genic, the recent success achieved in genetic engineering against excess salt, high and low temperatures as well as less or excess water by altering individual single genes as highlighted in this review is therefore noteworthy. It is to be appreciated that when number of genes are independently contributing for stress tolerance trait and if manipulation of one or two genes can improve the tolerance say by 5–10%, the effort is still not a waste. Since it has now been possible to pyramid different genes, in future it should be possible to isolate and manipulate a large number of genes in one organism. The present need is to isolate and test more number of 'candidate' genes which would improve stress tolerance. The past successes in identifying the relevant genes has come through: (i) application of targeted approach wherein genes for specific adaptive biochemical reactions were employed (such as those for proline synthesis, mannitol synthesis, fructan synthesis, unsaturation of fatty acids, etc.), and (ii) shot-gun approach wherein genes which are expressed under stress conditions and for which no specific role has as yet been proven (such as LEA proteins, antifreeze proteins, etc.) were employed. While the former case represents the success of physiologists and biochemists, the latter case of employing stress proteins represents success of molecular biologists. The pyramiding of different genes by plant genetic engineering is going to be the hallmark in future studies in this arena.

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