

MINIREVIEW

Beyond osmolytes and transporters: novel plant salt-stress tolerance-related genes from transcriptional profiling dataChandan Sahi^a, Amanjot Singh^a, Eduardo Blumwald^b and Anil Grover^{a,*}^aDepartment of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, Dhaula Kuan, New Delhi 110021, India^bDepartment of Plant Sciences, Mail Stop 5, University of California, One Shields Avenue, Davis, CA 95616, U.S.A.**Correspondence***Corresponding author,
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With recent advancements in DNA-chip technology, requisite software development and support and progress in related aspects of plant molecular biology, it is now possible to comprehensively analyze the expression of complete genomes. Global transcript profiling shows that in plants, salt-stress response involves simultaneous up and downregulation of a large number of genes. This analysis further suggests that apart from the transcripts that govern synthesis of osmolytes and ion transporters, two candidate systems that have attracted much of the attention thus far, transcripts encoding for proteins related to the regulation of transcriptional and translational machineries have a distinct role in salt-stress response. In particular, induction of transcripts of specific transcription factors, RNA-binding proteins, ribosomal genes, and translation initiation and elongation factors has recently been noted to be important during salt stress. There is an urgent need to examine cellular functionality of the above putative salt-tolerance-related genes emerging from the transcriptome analysis.

High soil salinity, contributed largely by NaCl, is one of the important environmental factors that limits distribution and productivity of major crops. Salinity affects approximately 20% of world's arable land and approximately 40% of irrigated land to various degrees. Transgenic research provides much-needed flexibility in manipulation of crops by altering the expression levels of native genes or by incorporating alien genes for a desired trait, in a relatively shorter time-frame. In the past one decade of research, production of salt-stress tolerant transgenic plants by genetic engineering has been claimed in over 100 research publications (Grover et al. 2003).

Salt-stress response is shown to encompass large number of genes, including genes that show pleiotropic effects (Yang and Yen 2002). These genes are linked to different pathways and processes such as stress perception and signaling, leading to molecular, biochemical, cellular, physiological, and morphological adaptations to finally the whole-plant response (Flowers 2004, Bartels and Sunkar 2005, Chinnusamy et al. 2005, Vinocur and Altman 2005). Different stress-regulated genes may have cumulative or exclusive roles in salt tolerance. Osmotic stress and Na⁺ stress are considered to be the two major components of the plant salt-stress

Abbreviations – ABI3, abscisic acid-insensitive3; ADPRF, ADP ribosylation factor; CBF, C-repeat binding factor; DREB, dehydration-responsive element binding protein; EREBP, ethylene-responsive element binding protein; ESTs, expressed sequence tags; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCN, general control nonrepressed; HSP, heat shock protein; LEA, late-embryogenesis-abundant protein; MADS, MCM1, AGAMOUS, DEFICIENS, SRF; MAP, mitogen activated protein; MYB, myeloblastosis; MYC, myelocytomatosis; NHX1, Na⁺/H⁺ antiporter; PAL, phenylalanine ammonia lyase; PDIase, protein disulphide isomerase; PPIase, peptidyl prolyl *cis-trans* isomerase; PR, pathogenesis related; RING, really interesting new gene; ROS, reactive oxygen species; SOD, superoxide dismutase; TPR, tetratricopeptide repeat; TPS, trehalose phosphate synthase.

response. Salinity reduces the ability of plants to take up water thus leading to reduction in growth rate, due to hormonal signal generated by the roots (Munns 2002). Osmolytes like proline, glycine-betaine, trehalose, and sugar alcohols such as mannitol and sorbitol that are abundantly produced and accumulated in salt-treated cells represent a critical component of salt-stress responses. These compounds are proposed to work through lowering the osmotic potential of cells or by protecting various cellular structures and proteins during stress. Na⁺-specific biochemical perturbations further hamper the growth processes. Ion transport (influx and efflux) and maintenance of ionic homeostasis employing transporters responsible for salt uptake, exclusion, long-distance transport, and compartmentalization have also emerged as a crucial input in plant salt-stress response (Apse et al. 1999, Blumwald 2000, Blumwald et al. 2000, Qiu et al. 2002, Qiu et al. 2003, Rus et al. 2004, Serrano and Rodriguez 2002, Yamaguchi et al. 2003, Zhang and Blumwald 2001, Zhang et al. 2001). Other transport proteins implicated in salt-tolerance acquisition include aquaporins and amino acid transporters. Essentially, our current understanding of the response of plants to salt stress encompasses firstly the relatively quicker osmotic function and then the somewhat delayed increase of Na⁺ function. On the basis of this understanding, enzymes that catalyse rate-limiting steps in the biosynthesis of compatible osmolytes, proteins that protect membrane integrity and control osmotic and/or ion homeostasis and reactive oxygen species (ROS) are considered to be the examples of salt-stress-tolerance effectors (Singla-Pareek et al. 2003, Sottosanto et al. 2004, Taji et al. 2004).

With the availability of complete *Arabidopsis thaliana* and *Oryza sativa* (and from large number of other plant species in the form of unpublished database) genome sequence information, newer and exciting directions are emerging for unearthing details on stress biology. Salt-stress-related transcriptome analysis has been undertaken by a large number of workers (Kawasaki et al. 2001, Kreps et al. 2002, Oono et al. 2003, Rabbani et al. 2003, Sahi et al. 2003, Seki et al. 2002). Random sequencing of salt-stress cDNA libraries has generated vast database on salt-stress-related expressed sequence tags (ESTs) (Richmond and Somerville 2000, Rudd 2003). In specific cases, cDNAs have been normalized or subtracted to specifically address the salt-regulated clones (Gong et al. 2001, Reddy et al. 2002, Sahi et al. 2003). Microarray and macroarray-based transcriptional profiling has given quantitative information about the expression levels of a large number of genes simultaneously. Transcriptional profiling data from *A. thaliana* and *O. sativa* suggest that metabolic readjustments is the hallmark of the salt-stress response (Kawasaki et al. 2001, Kreps et al.

2002, Oono et al. 2003, Rabbani et al. 2003, Seki et al. 2002, Sottosanto et al. 2004). Further progress on the transcripts associated with salt tolerance has been paved using the comparative genomics approach. Comparative stress genomics essentially means that various commonalities and differences in expression patterns of different genes relative to populations that differ in stress tolerance are scored. This approach appears highly valuable for unveiling the key genetic contributors to the complex physiological processes involved in salt-tolerance trait (Bressan et al. 2001). Taji et al. (2004) noted that fewer number of genes are induced by 250 mM NaCl stress in *Thellungiella halophila* (salt cress; a wild salt-tolerant relative of *Arabidopsis*), in contrast to *Arabidopsis*. It was emphasized that stress tolerance of salt cress may be due to constitutive over-expression of several genes that function in stress tolerance and that are stress inducible in *Arabidopsis*. Sottosanto et al. (2004) showed that *Atnhx1* knockout transcriptome responded appreciably different from the wild-type *Arabidopsis* plants both under unstressed and salt-stressed conditions. The latter work showed that apart from ion homeostasis, *AtNHX1* has important role to play in intracellular vesicular trafficking, protein targeting and several other cellular processes. Larger spectrum of gene expression changes noted between *Atnhx1* knockout mutant and wild-type plants by Sottosanto et al. (2004) emphasize that salt-sensitive and tolerant phenotypes differ markedly in their genetic machinery. An important conclusion that emerged from the comparison of transcription between *Atnhx1* and wild-type *Arabidopsis* plants is that changing levels of a single protein (*AtNHX1* in this case) can affect the expression of a large range of plant genes. It would be worthwhile to unveil how the transcriptomes have been affected in other single-protein alteration experiments done so far, but unfortunately this has not been analyzed to a great extent. Kawasaki et al. (2001) also observed that the gene expression response in salt-stress-related contrasting rice plants is both qualitative and quantitative. Sahi et al. (2003) provided further evidence that a large number of constitutive and stress-regulated gene expression differences underlie the response of three contrasting rice types to salt stress. Very recently, Shiozaki et al. (2005) also echoed essentially the same conclusions, in showing that contrasting rice types differed in expression of a large number of cDNA clones.

Detailed work on cDNA clones/ESTs reported from salt-stressed libraries showed that transcripts upregulated in salt stress belong to a variety of functionality classes such as RNA metabolism, transcription, hormone-related functions, signaling, translational machinery, transport proteins, osmoprotectants, ROS scavengers,

Table 1. Major categories of proteins that appear related to salt-stress response in plants.

Functionality class	Selected examples	Possible role in stress	References
Signaling molecules	Receptor like protein kinases, MAP kinases, histidine kinases, protein phosphatase 2C, G-protein coupled receptor protein, AAA-type ATPase, calmodulin, calcineurin, EF-hand containing proteins, serine-threonine protein kinase, ADPRFs	Stress signal transduction and gene expression	Pardo et al. (1998), Saijo et al. (2000), Cardinale et al. (2002), Ulm et al. (2002)
Transcriptional and post-transcriptional machinery	DREB, EREBP, MYB, MYC and Zn-finger transcription factors, RING finger proteins, MADS box proteins, homeodomain leucine zipper, CBF, TATA-binding protein, General Control Nonrepressed (GCN)-like proteins, glycine-rich and zinc finger RNA-binding proteins, RNA polymerase, splicing factors, micro RNAs	Transcriptional regulation of stress gene expression, transcript stability, turnover, processing	Lee et al. (2001), Park et al. (2001), Cooper et al. (2003), Sanan-Mishra et al. (2005)
Translational machinery	Ribosomal proteins, translation initiation and elongation factors, t-RNA synthetases	Stress-regulated protein translation, selective translation, transport, localization	Wood and Oliver (1999), Wood et al. (2000)
Protein folding	F-box, WW, WD40, Postsynaptic density protein, Disc-large, Zo1 (PDZ), Tetraatricopeptide repeat (TPR)-domain-containing proteins, HSPs, PPlases, DnaJ, DnaK like proteins, calreticulin	Maintenance of protein structures, protein folding, preventing protein denaturation, Protein sorting, targeting	Sun et al. (2001)
Protein turnover	Polyubiquitins, ubiquitin conjugating enzymes and ligases, components of the proteasome pathway, proteases, protease inhibitors	Regulation of protein metabolism, targeted protein degradation in response to stress	Khedr et al. (2003), Moon et al. (2004)
Osmoprotectants	Proteins encoding for enzymes that govern levels of proline (pyroline carboxylate reductase, proline oxidase), glycinebetaine (choline oxidase), trehalose (TPS), mannitol (mt1D) and sorbitol (sac B); LEA, cor, dehydrins, WSP (water stress proteins)	Osmotic adjustment, protection of cellular structures and macromolecules	Tarczynski et al. (1993), Kavi Kishore et al. (1995); Nomura et al. (1998)
Transport protein	Water, amino acid, sugar and metal transporters, aquaporins, membrane proteins, antiporters, ion channels, sulphate transporters, ABC-type transporters, amino acid permease, Na ⁺ and K ⁺ transporters, plasma membrane and vacuolar ATPases, TIP	Ion homeostasis during stress, compartmentalization of solutes and amino acids	Apse et al. (1999), Gisbert et al. (2000) Shi et al. (2000), Zhang and Blumwald (2001) Zhang et al. (2001)
ROS scavengers, cell death, senescence and ageing	SOD, peroxidases, oxido-reductases, PAL, catalase, glutathione S-transferase, cytochrome c-oxidase, glyoxalase, cyclin H1, histones, tumor suppressors	Detoxification of free oxygen radicals, cell death, hypersensitive response	Roxas et al. (1997), Veena and Sopory (1999)
Metal-binding proteins	Metallothionin, ferritin, Cu- and Zn-binding proteins, calmodulin	Affecting cellular metabolism, metal ion homeostasis, acting as cofactors for critical reactions, signaling, metal toxicity, secondary stress responses, oxidative stress	Kawasaki et al. (2001), Sahi et al. (2003)
Photosynthesis	Chlorophyll <i>a/b</i> -binding protein, photosystem I subunit PS-I like protein, ATP sulphurylase, rubisco activase	Regulation of photosynthesis	Kawasaki et al. (2001), Sahi et al. (2003)

Table 1. Continued

Functionality class	Selected examples	Possible role in stress	References
Defense-related proteins	WRKY family of transcription factors, chitinase, glucanases, protease inhibitors, myrosinase-binding protein, other PR proteins like thaumatin	Protection against biotic stress including viral, bacterial and fungal infestation	Reymond et al. (2000), Cheong et al. (2002)
Hormone-related proteins	Zeaxanthin epoxidase, <i>gda-1</i> (GA-induced gene), <i>asr-1</i> (abscisic acid responsive), ACC Synthase, ABI-3 interacting protein, allene oxide synthases, Nucleosidediphosphate Kinase (NDPK), arginine decarboxylase, glucosyltransferases, mannosyltransferases, methyl and acetyl transferases, choline kinase, lipoxygenase, fatty acid desaturase, GAPDH, lipase, ferredoxin nitrite reductase, aldolase, enolase, alanine transaminases, methionine synthase, asparagine synthetase, tryptophan synthase, acetohydroxyacid synthase, NADP-ME, fructose bis-phosphatase, malate dehydrogenase, enzymes of the photorespiratory and pyruvate cycle pathways, acetyl Co-A synthetase, phenylpropanoid pathway	Hormonal homeostasis and gene expression	Dombrowski (2003) Kalifa et al. (2004)
General metabolism		Overall cellular function, housekeeping metabolic pathways carbohydrate, fatty acid and protein synthesis and modifications membrane fluidity, nitrogen metabolism, carbon and nitrogen fixation	Hoshida et al. (2000), Jeong et al. (2001)
Unclassified proteins	Hypothetical and putative proteins which includes genes encoding proteins with uncharacterized domains and tissue specific genes	Unknown	Sahi et al. (2003), Shiozaki et al. (2005)

ATPase, adenosine triphosphatase; CBF, C-repeat binding factor; DREB, dehydration-responsive element-binding protein; EREBP, ethylene responsive element-binding proteins; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSP, heat-shock protein; LEA, late-embryogenesis-abundant proteins; MAP, microtubule-associated protein; MYB, myeloblastosis; MYC, myelocytomatosis; PAL, phenylalanine ammonia lyase; PDIase, protein disulphide isomerases; PPIases, peptidyl prolyl *ds-trans* isomerases; PR, pathogenesis related; RING, really interesting gene; ROS, reactive oxygen species; SOD, superoxide dismutase

cell death and ageing, photosynthesis, general metabolism, protein transport/turnover, metal-binding proteins, protein-protein interactions and folding, defense-related functions, other stress proteins, and several unclassified proteins. Table 1 lists specific examples of the proteins encoded by transcripts from the above-mentioned categories that have been noted to be associated with the response of cells to salt stress. From the large body of work emanating from transcriptional profiling data, it appears that apart from the biosynthesis of osmolytes and ion transporters, proteins/pathways linked with maintenance and selective action of transcriptional and translational functions are associated with plant salt-stress response. Selected examples of this category include transcription factors (TFs), RNA helicase proteins, glycine-rich (GR) RNA-binding protein (RBPs) (GR-RBP), protein translation and turnover components (eukaryotic translation initiation and elongation factors, proteases and protease inhibitors), and chaperones and foldases (like heat shock proteins and peptidyl prolyl *cis-trans* isomerases).

Major alterations in transcriptional and post-transcriptional activities are noted to accompany response of plants to salt stress. The salt-stress response is under active genetic control, thus involving activation of large number of specific genes concomitant to the repression in activity of a large number of house-keeping genes. Battery of regulatory molecules such as TFs (including different classes of DNA-binding proteins like dehydration-response element/C-repeat, Myb and Myc proteins, and proteins containing bZIP, Zn-finger, or AP2 domains) appears to be principal genetic determinants in salt-stress transcriptional profiles (Kawasaki et al. 2001, Mukhopadhyay et al. 2004, Oono et al. 2003, Rabbani et al. 2003, Sahi et al. 2003, Sottosanto et al. 2004). Basic helix loop helix (bHLH) and myeloblastosis TFs were reported to function as transcriptional activators of abscisic acid signaling in plants (Abe et al. 2003). MCM1, AGAMOUS, DEFICIENS, SRF (MADS) box TFs appear important in salt-stress networking in plants (Cooper et al. 2003). The functional validation of the role of several different TFs in imparting stress tolerance has been done employing transgenic plants in specific instances (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Kasuga et al. 2004, Kim et al. 2001, Kim et al. 2004, Park et al. 2001). Transcript synthesis, stability, and localization are emerging as an essential component of plant-stress responses. RNA helicase-like protein has been shown to be an early regulator of plant-chilling and freezing tolerance in *Arabidopsis* (Gong et al. 2002). It was recently reported that mutant plants lacking DEAD box RNA helicase are heat sensitive. Mutation in this gene caused change in total cellular levels of several

cold responsive gene transcripts (Gong et al. 2005). Transgenic tobacco plants over-expressing pea DNA helicase showed higher accumulation of Na⁺ in the old leaves and negligible levels in seeds of T₁ plants as compared with wild-type plants (Sanan-Mishra et al. 2005). RBPs are turning out to be an important aspect of plant salt-stress response. Most of the stress cDNA libraries showed redundancy of genes corresponding to various RBPs including GR and Zn-finger RBPs, splicing factors, and several other snRNPs and hnRNPs (Agarwal and Grover 2005). SR-rich-splicing factors have been implicated in salt tolerance (Forment et al. 2002). Transcripts for GR-RBP were shown to be upregulated by low-temperature stress, and the germination and seedling growth of the loss-of-function mutants of *Arabidopsis* GR-RBP was retarded. On other hand, over-expression of this protein in *Arabidopsis* showed earlier germination and better seedling growth, and the transgenic plants were more freezing tolerant (Kim et al 2005).

Regulation of the translational machinery also appears to be an important component of the cellular-stress response (Bailey-Serres 1999). Water deficit induces rapid changes in the cell polyribosomes. A putative regulatory role of specific polysome-associated proteins in stress-induced translational control has been proposed. Formation of mRNP (messenger ribonucleoprotein) complexes and polysomal retention of transcripts for ribosomal proteins RPS14, RPS16, and RPL23 were correlated with desiccation response in *Tortula ruralis* (Wood and Oliver 1999, Wood et al. 2000). Active conservation of the polyribosomes during desiccation has been associated with high-level stress tolerance in plants (Bartels and Salamini 2001, Bensen et al. 1988). Regulation of the protein degradation machinery is thought to play critical role(s) in plant-stress response (Khedr et al. 2003). Redundancy noted for different classes of proteases and protease inhibitors in salt and water-stress libraries would indicate that regulated protein degradation is an important stress response in plants. Because denatured proteins are toxic to the cells, they need immediate removal. E3 ubiquitin ligase and the really interesting new gene (RING) finger proteins are the key components of the ubiquitin proteasome pathway (Freemont 2000, Moon et al. 2004). The expression of genes encoding RING finger protein was rapidly increased during stress, and these are thought to be involved in rapid degradation of regulatory proteins (Lee et al. 2001, Salinas-Mondragon et al. 1999). Representation of peptidyl prolyl *cis-trans* isomerases (PPIases), protein disulphide isomerases (PDIases), and chaperones (Hsp, DnaK, DnaJ) encoding transcripts along with their salt-regulated expression shows that protein folding is important parameter in salinity. Selected Hsp have been shown to be important for imparting salt tolerance in

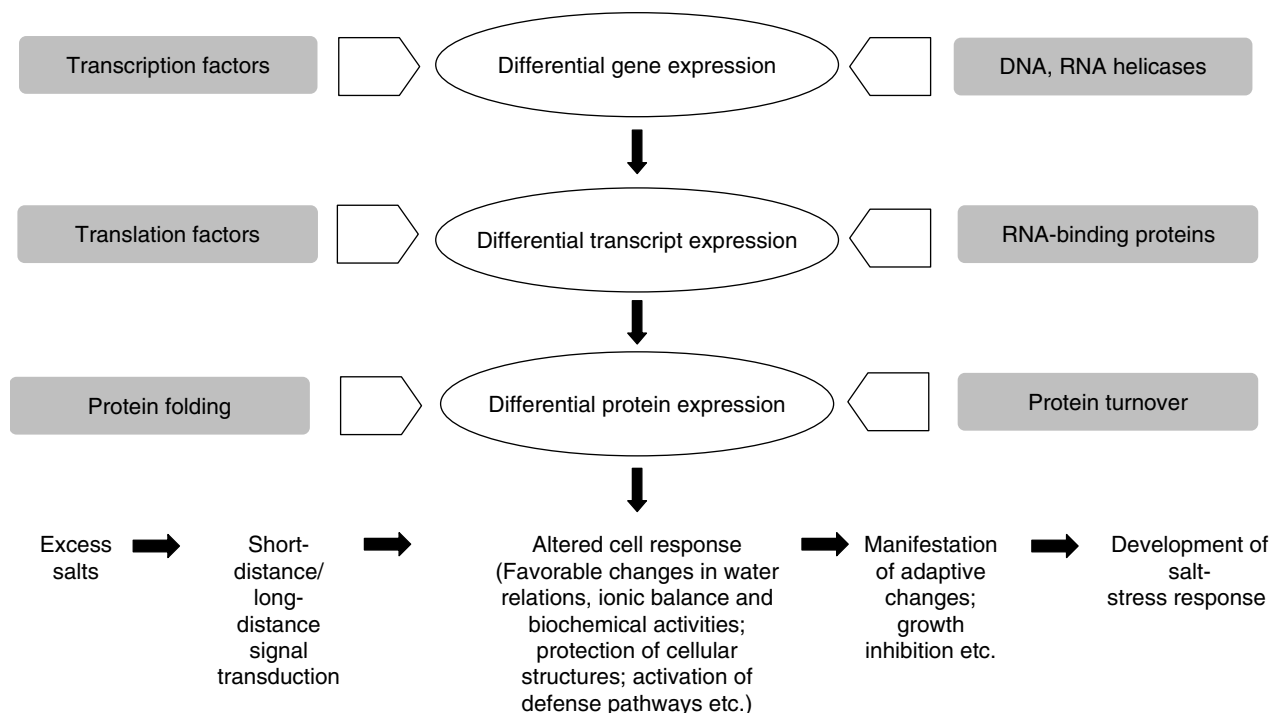


Fig. 1. Model showing several novel categories of proteins (in gray-coloured boxes) that appear important in governing increased salt-tolerance phenotype in plants. The evidence for the involvement of these proteins in imparting salt-stress tolerance has come through their salt-regulated characteristic and genetic experiments (see text for details). The proteins such as those regulating levels of osmolytes and controlling ion homeostasis which have already been shown to be important in governing salt-tolerant phenotype are not shown here.

plants (Sun et al. 2001). Likewise, there is evidence that transcripts encoding PPIase are regulated by salt stress in maize and bean plants (Marivet et al. 1994).

Transgenic plants overexpressing genes involved in osmolyte production showed enhanced salt-stress tolerance. Ectopic over-expression of ion transporters resulted in a novel way of sequestering excess Na^+ levels to cause increased salt-tolerant phenotype. Transcriptome analysis suggests that genes associated with regulation of RNA and protein metabolism appear to have an utmost significance in regulating salt-stress tolerance (Fig. 1). Microarray analysis has clearly shown that transcripts encoding RBPs, helicases, cyclophilins, F-box proteins, dynamin-like proteins, and ribosomal proteins are linked to salt-stress response in *Arabidopsis* (Sottosanto et al. 2004). Thus, there appears to be a coordinated action of several ribosomal proteins, RBPs, and translation initiation and elongation factors along with several accessory proteins that regulate stress-associated translation in controlling various cellular adaptations during salt stress. According to Fedoroff (2002), RNA metabolism and modification appears to be an important and well-conserved stress-response pathway in yeast, animal as well as in plant systems. Further characterization of these genes by analyzing their protein expression and by

altering their levels of expression in varied homologous and heterologous systems and through analysis of requisite knockout mutants is the need of the hour.

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