



Proteomics-based dissection of stress-responsive pathways in plants

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Summary

Abiotic stress has an ability to alter the levels of a number of proteins, which may be soluble or structural in nature or which may exist before and after folding in the plant cell. The most crucial function of plant cell is to respond to stress by developing defence mechanisms. This defence is brought about by alteration in the pattern of gene expression. This leads to modulation of certain metabolic and defensive pathways. Owing to gene expression altered under stress, qualitative and quantitative changes in proteins are obvious. These proteins might play a role in signal transduction, antioxidative defence, antifreezing, heat shock, metal binding, antipathogenesis or osmolyte synthesis. A significant part of the literature shows the quantitative and qualitative changes in proteins, mainly employing western analysis, enzymatic kinetics, fraction isolation, one-dimensional SDS-PAGE electrophoresis, etc. Fortunately, recent developments in sensitivity and accuracy for proteome analysis have provided new dimensions to assess the changes in protein types and their expression levels under stress. The novel aim of this review is to do a side-by-side comparison of the proteins that are induced or overexpressed under abiotic stress, examining those from biochemical literature and the ones observed, sequenced and identified using the advanced proteomics and bioinformatic techniques.

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Introduction

Although the word 'stress' is well known to most biologists, it has proven to be a very elusive concept, and the term is used in the scientific literature in many different ways and in different contexts. Most importantly, however, stress is not

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only an attribute of the stressor (the environmental component) but also an attribute of the stressed (the biological component). Environmental stress is considered to be primarily a response to the physical features of the environment. Extrinsic stress that results from changes in abiotic factors such as temperature, climatic factors and chemical components, either naturally occurring or man-made, is regarded as the most important stress agent. In addition, biotic stresses, such as bacterial, fungal, algal and viral diseases, can also cause stress in plants. Plants have developed adaptive features against these stresses. Now, it is a well-known fact that proteins mediate these features by playing a role in directing the genome and ultimately physical features (such as in xerophytes) or by encountering stressors directly (such as antioxidant enzymes and chaperonins) or indirectly (such as a key enzyme in osmolyte synthesis).

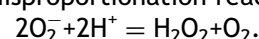
In the recent past, extensive research has resulted in impressive achievements in genome and expressed sequence tag (EST) sequencing, yielding a wealth of information for many model organisms, including the plants *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays* and *Medicago*, but genome sequence information alone is insufficient to reveal the facts concerning gene function, developmental/regulatory biology and the biochemical kinetics of life. To investigate these facts, more comprehensive approaches that include quantitative and qualitative analyses of gene expression products are necessary at the transcriptome, proteome and metabolome levels. Although transcriptome analysis using microarray and serial analysis of gene expression technologies are potential tools, mRNA and protein levels (Gygi et al., 1999; Futcher et al., 1999) cannot be correlated due to the inability of total mRNA to translate into protein, whereas proteomics provides a more direct assessment of the biochemical processes of monitoring the actual proteins performing the signalling, enzymatic, regulatory and structural functions encoded by the genome and transcriptome. Recent improvements in high-resolution two-dimensional PAGE (2-DE; Gorge et al., 1999; Zolla and Timperio, 2005), an increase in the number of sequences of protein and nucleotides, increased capabilities for protein identification utilising modern mass spectrometry methods, such as matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry; Pappin et al., 1993; Yates III, 1998a,b; Corthals et al., 2000) and valuable bioinformatic tools, have made the large-scale profiling and identification of proteins a dynamic area of renewed plant research.

During the last 15 years, research has been carried out on different plant organs (Watson et al., 2003) and on subcellular proteomes such as the chloroplast membrane (Peltier et al., 2002; Zolla et al., 2004), cell wall (Borderies et al., 2003) and nuclear envelope (Bae et al., 2003), whereas other researchers have focused on individual tissues, including seeds (Gallardo et al., 2001), mitochondria (Kruft et al., 2001; Millar et al., 2001), maize (*Z. mays*) root tips (Chang et al., 2000) and barrel medic roots (Mathesius et al., 2001), vacuoles (Carter et al., 2004), chloroplasts (Kleffmann et al., 2004) and thylakoids (Zolla et al., 2002, 2003). In addition to analysing the proteomes at subcellular and cellular levels, several studies have been carried out with the same aim, but under different extreme environmental conditions such as salinity (Kav et al., 2004), drought (Salekdeh et al., 2002), cold (Amme et al., 2006), heat (Ferreira et al., 2006), ozone (Agarwal et al., 2002), UV light (Casati et al., 2005), visible light (Kim et al., 2005), heavy metals (Ingle et al., 2005), nutrient deficiencies (Kang et al., 2004), elevated CO₂ conditions (Bae and Sicher, 2004), and fungal (Campo et al., 2004), bacterial (Jorin et al., 2006) and viral infections (Ventelon-Debout et al., 2003). These studies all together have given rise to a specific field, 'environmental proteomics'. Now, there is a good amount of work to answer about the types of proteins under- and/or overexpressed during a particular or integrative stress, their impacts on cellular metabolism and the location of the proteins. In this review, we aim to revisit the proteins with important properties, which have been shown to play a crucial role against abiotic environmental stresses directly, for stress-dependent gene regulation or as a key enzyme in the biosynthesis pathway of antistress molecules, such as osmolytes, and compare these data with those of proteins detected using the two-dimensional gel electrophoresis, spectrometry and bioinformatic tools in plants according to the said properties and environmental conditions.

Proteins respond to stress in the plant system

Superoxide dismutase (SOD)

SOD is a group of metalloenzymes that alter its activity under different environmental conditions (Bowler et al., 1992). SOD catalyses the following disproportionation reaction:

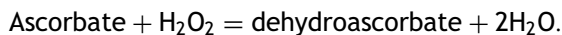


It is a highly efficient catalyst mediating a pivotal reaction in the antioxidant pathway (Foyer et al., 1994). McCord and Fridovich (1969) reported an enzymatic role for a copper-containing protein isolated from bovine albumin serum, named erythrocyprin. They were able to show that erythrocyprin can dismutate two superoxide anion radicals to hydrogen peroxide and diatomic oxygen. McCord and Fridovich (1969) named the enzyme SOD. A SOD isoform was found in chloroplasts and shown to act in concert with ascorbate peroxidase (APX), Monodehydroascorbate reductase (MDAR) and glutathione reductase (GR). In this scheme, APX reduces the H₂O₂ generated by SOD activity into H₂O. SODs are classified according to their metal cofactors, as FeSOD, MnSOD or Cu-ZnSOD. Chloroplast generally contains Cu-ZnSOD and, in a number of plant species, FeSOD (Van camp et al., 1994). MnSOD occurs in mitochondria and peroxisomes, and Cu/ZnSODs, in the chloroplast, the cytosol and possibly the extracellular space (Alscher et al., 2002).

In proteomic studies, the levels of different SOD isoforms were found up-regulated under drought (Salekdeh et al., 2002; Hajheidari et al., 2005; Taylor et al., 2005), ozone stress (Agarwal et al., 2002), high light (HL) (Nam et al., 2003), As stress (Requejo and Tena, 2005) and plant hormone treatment (Rakwal and Komatsu, 2004; Konishi et al., 2005).

Ascorbate peroxidase (APX)

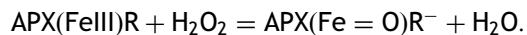
Catalase can be used to reduce hydrogen peroxide levels in the peroxisomes, but it is absent in chloroplasts. The role of catalase is filled by an ascorbate-specific peroxidase. This peroxidase uses ascorbic acid as a hydrogen donor to break down hydrogen peroxide (Asada, 2006). The reaction is as follows:



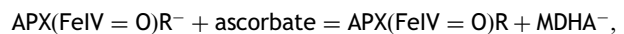
APX isoenzymes are distributed in at least four distinct cell compartments, the stroma (sAPX), the thylakoid membrane (tAPX), the mitochondria (mAPX) and the cytosol (cAPX) (Asada, 1992; Ishikawa et al., 1998). The two chloroplastic APX (Chl APX) isoenzymes have been found to be encoded by only one gene (APXII), and their mRNAs are regulated by the alternate splicing of their two 3'-terminal exons (Yoshimura et al., 1999). Different isoforms of APX behave differently under different types of stress (Yoshimura et al., 1999).

The enzyme has two cytosolic forms with a purely defensive role and a membrane-bound (27 kDa) form, which has a functional role in addition to

scavenging hydrogen peroxide (Davletova et al., 2005). The stromal form is slightly larger. The cytosolic form is present in leaf and in non-photosynthetic tissue. Although it is a homodimer, the molecular weight of its monomer is very similar to that of the stromal APX. A peroxisomal APX has also been reported, which is similar to thylakoid APX in size and is membrane bound. In the reaction catalysed by the thylakoid APX, the enzyme is initially in ferric state, which is first oxidised by H₂O₂ to produce a two-electron intermediate with the state of Fe (IV) = O and with a tryptophan radical:



This intermediate then oxidises As through two successive one-electron reactions, yielding two molecules of the MDHA radical:



Ascorbate can be regenerated from MDHA by the reaction catalysed by monodehydro ascorbate reductase (MDHAR). MDHAR are flavin nucleotide-containing enzymes found in chloroplasts (55 kDa) and in the cytosol (47 kDa), as well as in mitochondria and peroxisomes. They catalyse the reduction of MDHA to As by NAD(P)H:



The MDHA radical can also be reduced to ascorbate by photoreduced ferredoxin in the chloroplast photosystem (PS) I ///. Alternatively, it can spontaneously disproportionate to ascorbate and DHA, which can subsequently be reduced by another enzyme, DHA reductase, which regenerates ascorbate. DHA reductase, present in chloroplast stroma, reduces DHA to ascorbate by the ubiquitous cellular peptide, glutathione (GSH):

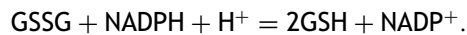


Several proteomic studies showed an up-regulation in different isoforms of APX and other peroxidases, e.g., under drought (Hajheidari et al., 2005), salinity (Yan et al., 2005), high temperature (Süle et al., 2004), Cd stress (Sarry et al., 2006b), Mn stress (Fecht-Christoffers et al., 2003) and ozone stress (Agarwal et al., 2002).

Glutathione reductase (GR)

Usually APX operates in cycle with GR. GR uses reducing equivalents derived from glucose through the pentose phosphate pathway and NADPH to generate the reduced form of GSH from the

oxidised disulphide form (GSSG) that results by the action of APX:

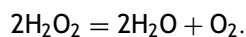


GR is mainly found in the chloroplast, cytoplasm and mitochondrion of plants. It ranges in size from around 90 to 140 kDa and usually contain two protein subunits, each with a flavin adenine dinucleotide as its active site. It appears that NADPH reduces the flavin nucleotide, which then transfers its electrons to a disulphide bridge (–S–S–) in the enzyme. The two sulphhydryl groups (–SH) that result then interact with GSSG and reduce it to GSH. The activity of GR suggests that the GSH/GSSG ratio in normal cell is kept high. The utilisation of NADPH acts as an energy sink, which may impact indirectly on the efficiency of the electron transport system. It also causes the production of a *trans*-thylakoidal proton gradient that is involved in control of electron transport (Foyer and Harbinson, 1994).

There is still insufficient literature showing up-regulation of GR under abiotic stress, suggesting an uncertain influence of abiotic stress on its expression level. However, other enzymes associated with sulphur metabolism have also been shown to be overexpression under salinity (Zörb et al., 2004), low temperature (Cui et al., 2005), As stress (Requejo and Tena, 2005), Cu stress (Smith et al., 2004) and HL (Nam et al., 2003).

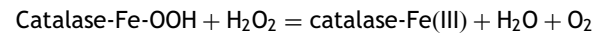
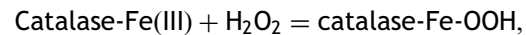
Catalase (CAT)

In both prokaryotes and eukaryotes, the presence of catalases has been shown. Catalase catalyses the dismutation of H₂O₂ to H₂O and O₂:



The sources of H₂O₂ generation are the electron transport chain of mitochondria, PS II components of chloroplasts or the P450 oxidases of the endoplasmic reticulum (ER). Intercellular sources of H₂O₂ generation include peroxisome and reactions catalysed by their associated oxidases and dehydrogenases. H₂O₂ can readily permeate cell membranes and put DNA at major risk by generating highly reactive hydroxyl radicals through the interaction of H₂O₂ with transitional metal ions such as Fe²⁺ in Fenton-type reactions. Structurally, most catalases (53–70 kDa) are made up of four protein subunits. Each individual subunit contains an Fe(III)-protoporphyrin component attached to its active site. In addition, each subunit contains an NADPH molecule. In general, catalases have high turnover rate (>40,000 molecules⁻¹), and the

reaction catalysed is as follows:



Catalases are mainly located in peroxisomes and degrade H₂O₂ without consuming cellular reducing equivalents, i.e., it is a very efficient means of removing H₂O₂ from cells (del Rio et al., 2002). Plants have isozymes of catalases. CAT-1 and CAT-2 are associated with peroxisomes, whereas CAT-3 is associated with mitochondria.

In proteomic studies, catalase was found increased under high temperature (Majoul et al., 2004), but nothing can be predicted about its level under stress until more studies come forward.

Salt-overly sensitive (SOS) pathway proteins

In SOS pathway, a myristoylated calcium-binding protein, SOS3, senses cytosolic calcium changes elicited by salt stress. SOS3 physically interacts with and activates the protein kinase, SOS2. The SOS3/SOS2 kinase complex phosphorylates and activates the transport activity of the plasma membrane Na⁺/H⁺ exchanger encoded by the *sos1* gene. Preliminary results suggest that, in addition to its transport function, SOS1 may also have a regulatory role and may even be a novel sensor for Na⁺. The SOS stress-signalling pathway was identified to be a pivotal regulator of plant ion homeostasis and salt tolerance (Hasegawa et al., 2000b; Sanders et al., 2002). This signalling pathway functionally resembles the yeast calcineurin cascade that controls Na⁺ influx and efflux across the plasma membrane (Bressan et al., 1998). Expression of an activated form of calcineurin in yeast or plants enhances salt tolerance, further implicating the functional similarity between the calcineurin and the SOS pathways (Mendoza et al., 1996; Pardo et al., 1998). The plasma membrane localised SOS1, H⁺ pump, is a P-type ATPase and is primarily responsible for pH and membrane potential gradient across this membrane (Morsomme and Boutry, 2000). A vacuolar type H⁺-ATPase and a vacuolar pyrophosphatase generate the pH and membrane potential across the tonoplast (Drozdowicz and Rea, 2001; Maeshima, 2001). The activity of these H⁺ pumps is increased by salt treatment and induced gene expression may account for some of the up-regulation (Hasegawa et al., 2000b; Maeshima, 2001). The plasma membrane H⁺-ATPase was confirmed as a salt-tolerance determinant based on analyses of phenotypes caused by the semi-dominant *aha4-1*

mutation (Vitart et al., 2001). The up-regulation of the SOS pathway proteins under salt stress leads to the salt tolerance.

Proteomic studies supporting elevation in expression of SOS proteins were carried out under salinity (Zörb et al., 2004) and ozone stress (Agarwal et al., 2002).

Methyl jasmonate (MeJA) signalling and related proteins

Methyl jasmonate (MeJA) and its free acid jasmonic acid (JA), collectively referred to as jasmonates, are important cellular regulators involved in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening and senescence. In addition, jasmonates activate plant defence mechanisms in response to insect-driven wounding, various pathogens and environmental stresses, such as drought, low temperature and salinity (Creelman and Mullet, 1997).

The way in which jasmonates regulate these processes has been studied by looking for gene expression patterns in a wide range of jasmonate-responsive physiological states. Genes up-regulated by MeJA treatment include those involved in jasmonate biosynthesis, secondary metabolism and cell-wall formation, as well as those encoding stress protective and defence proteins. By contrast, genes involved in photosynthesis, such as ribulose biphosphate carboxylase/oxygenase, chlorophyll a/b-binding protein and light-harvesting complex II, are down-regulated. The genes discovered in these experiments are under intensive analysis using functional genomics and bioinformatics approaches to better define MeJA-mediated signalling pathways and cellular responses. Methyl jasmonate formation could be one of several important control points for jasmonate-regulated plant responses. This hypothesis was tested with transgenic *Arabidopsis thaliana* overexpressing JMT, where various jasmonate-responsive genes were constitutively expressed in the absence of wounding or jasmonate treatment. Indeed, the transgenic plants showed enhanced resistance to a virulent fungal pathogen compared with non-transgenic plants (Seo et al., 2001). However, although transgenic potatoes overproducing AOS have an increased concentration of JA, transcription of jasmonate-responsive genes is not enhanced in these plants. Expression of JMT was not detected in young seedlings, but its expression initiates at the floral nectary in developing flower. In addition, expression of the gene

was induced both locally and systemically either by wounding or by treatment with MeJA. This result suggests that JMT can perceive and respond to local and systemic signals generated by external stimuli, and that the signals might include MeJA itself. Employing proteomics, Bae et al. (2003) and Majoul et al. (2004) have shown an up-regulation in levels of JA-induced proteins.

Abscisic acid (ABA) signalling and related proteins

The acclimation/adaptation process is, in large part, mediated by the plant hormone ABA (Xiong et al., 2002). Hormone level increases under common stress conditions to trigger metabolic and physiological changes. The adaptations entail changes in gene expression patterns. Numerous genes involved in the acclimation/adaptation processes are up- and/or down-regulated under stress conditions. Although not all of them are subjected to ABA regulation, expression of a large number of them is controlled by ABA. Promoter analyses of ABA/stress-responsive genes revealed that a DNA sequence element consisting of ACGTGGC is important for ABA regulation. For the past several years, researchers have been trying to identify transcription factors that regulate the expression of ABA/stress-responsive genes via the consensus element, which is generally known as 'Abscisic Acid Response Element' (ABRE). Many bZIP class DNA-binding proteins that interact with the element have been reported. Researchers have focused on the small subfamily of *Arabidopsis* bZIP proteins referred to as ABFs (ABRE-binding factors) (Choi et al., 2000), whose expression is induced by ABA and by various abiotic stresses (i.e., cold, high salt and drought) (Bae et al., 2003). To investigate their *in vivo* roles, transgenic *Arabidopsis* plants that constitutively overexpress each of them have been generated. Their phenotypes were then analysed with special attention to changes in ABA/stress responses. Each ABF displayed similar, but distinct, phenotypes. Data suggest that ABF3 is probably most important for stress tolerance among the four ABFs (ABF1, ABF2, ABF3 and ABF4). ABF3 overexpression affected the expression levels of ABA/stress-regulated genes (Kang et al., 2002).

Osmotin

One of the most extensively studied proteins that are accumulated in response to salt adaptation is osmotin, which was first identified in salt-adapted tobacco cells. At the cellular level, it accumulates

in the vacuole of salt-stressed cells. Osmotin is regulated in cells at the transcriptional level by ABA application (Singh et al., 1989), but post-transcriptional regulation has been shown to control the protein accumulation (LaRosa et al., 1992). Osmotin polypeptide sequence shows features common to maize α -amylase/trypsin inhibitor. Interestingly, the *BnD22* gene (Downing et al., 1992; Reviron et al., 1992), which is expressed in *Brassica napus* leaves in response to salt and drought, also contains the signature motif of soybean Kunitz trypsin inhibitor.

In addition to the *osmotin* gene responsible for osmoprotectant synthesis, other osmoprotectants have also been reported, the most important of which are proline and glycine betaine (Holmström et al., 2000). Proline accumulation also provides an example of osmoprotectant indicating the up-regulation of proteins and key enzymes. Under stress, the imbalance between photosynthetic light capture and NADPH utilisation in carbon fixation may alter the redox state and lead to photoinhibition. Proline synthesis, following transcriptional activation of NADPH-dependent P5C-synthetase, could provide a protective value whereby the regeneration of NADP⁺ could provide the observed protective effect.

Using a proteomic approach, Hajheidari et al. (2005) found an osmotin-like protein up-regulated in drought conditions.

Mitogen-activated protein kinase (MAPK) signalling and related proteins

MAPKs were initially identified as serine/threonine kinases. MAPKs play a key role in integrating multiple intracellular signals transmitted by various second messengers. Structurally, MAPK cascades are composed of three protein kinases [i.e., MAPKs, MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs)]. MAPKs are activated when both tyrosine and threonine residues in the TXY motif are phosphorylated by MAPKKs. MAPKKs are activated when serine and serine/threonine residues in the SXXXS/T motif are phosphorylated by MAPKKKs. The fact that cDNA-encoding MAPKs, MAPKKs and MAPKKKs have been isolated from various species of plants suggests a wider role of MAPK in cascade signalling. Both MAPK-like kinase activity and mRNA levels of the components of MAPK cascades were found to be increased, which is in agreement with their suggested role (Wrzaczek and Hirt, 2001).

It has been demonstrated that the levels of transcripts for three protein kinases, MAPKKK, MAPK and a ribosomal S6 kinase homologue,

increased markedly and simultaneously when plants were treated with mechanical stimuli, low temperature and salinity stress. This indicates that some of the MAPK cascades in plants function in transduction of signals in the presence of environmental stress and that MAPK cascades are regulated at the transcriptional and post-transcriptional levels in plants (Zhang et al., 2006).

Calcium signalling and related proteins

The changes in intercellular calcium concentration (Ca²⁺) in response to abiotic stresses are well established, suggesting that Ca²⁺ serves as a messenger in normal growth and developmental processes of plants (Reddy and Reddy, 2004). Ca²⁺ channels have been detected in the plasma membrane, vacuolar membrane, ER, chloroplast and nuclear membranes of plant cells. In response to osmotic, drought and salt stress, increased intercellular Ca²⁺ concentration mobilised from intercellular stores has been shown by Sanders et al. (2002). Intercellular and extracellular sources of Ca²⁺ and inositol phosphates have a role to play in response to salinity. In plant and animal cells phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) results in the production of inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol. There is some indirect evidence for the involvement of heterotrimeric G proteins in promoting PIP₂ hydrolysis in plants. Phosphatidylinositol-specific phospholipase C (PI-PLC) activity and genes encoding this enzyme have been characterised from plants. Plant PI-PLC hydrolyses phosphatidylinositol-4,5-bisphosphate into IP₃ and diacylglycerol with an absolute requirement for Ca²⁺ (1 μ M). It was shown that the expression of one of the phospholipase C genes (*AtPLC1*) is induced by stresses including dehydration, salinity and low temperature. Three PI-PLC isoforms (*StPLC1–3*) have been isolated from guard cell-enriched tissues of potato. Reddy (2001) has reviewed the role of Ca in signalling.

GABA-related proteins

Research into GABA (γ -amino butyric acid), a non-protein amino acid, has focused on its role as a metabolite, mainly in the context of responses to biotic and abiotic stresses. It is mainly metabolised through a short pathway called the GABA shunt, because it bypasses two steps of the tricarboxylic acid cycle (Bouche et al., 2003). Although differences in the subcellular localisation of GABA-shunt enzymes in different organisms have been

reported, such as in yeast, where succinic semi-aldehyde dehydrogenase (SSADH) is present in the cytosol, the pathway is composed of three enzymes: the cytosolic GAD and the mitochondrial enzymes GABA transaminase and SSADH. In an alternative reaction, succinic semialdehyde can be converted to GHB (γ -hydroxy butyric acid) through a GHB dehydrogenase present in animals and recently identified in plants. If GABA could activate signalling pathways in a broad range of organisms, then GABA receptors should be present/overexpressed in these organisms because the initial event leading to the activation of a cellular signalling pathway is the binding of a ligand, such as a hormone, to a specific receptor.

Other than the above-discussed proteins, there are a number of proteins that alter their concentration under the stress. Examples of such proteins are programmed cell death-related proteins and, more importantly, proteases (Bouche et al., 2003).

Types of abiotic stress in plant system and responsive proteins

A number of peptides/proteins not discussed above respond to a variety of stresses; these include heat-shock proteins (HSPs), chaparonins (CPN), phytochelatin (PCs), metallothioneins (MTs) and pathogen-related proteins (PRPs). This section provides informative summaries on them under the heads of various abiotic stresses under which these proteins show specific, not strict, response.

Water stress

Water deficits are known to alter a variety of biochemical and physiochemical processes, ranging from photosynthesis to protein synthesis and solute accumulation. Among the most common responses are electron leakage through thylakoid membranes, damage to chlorophylls, decrease in antioxidant systems, increase in production of H_2O_2 , O_2^- , lipid peroxidation (LP) and decrease in photosynthetic.

The genes induced by drought stress were identical to those induced by high levels of salinity and to some of those induced by cold stress. Many of the genes respond to ABA, and many of them do not. At least four independent signal pathways function in the activation of stress-inducible genes under drought conditions. Two of them are ABA dependent (pathways I and II), and two are ABA independent (pathways III and IV) (Shinozaki and Yamaguchi-Shinozaki, 1996). One of

the ABA-dependent pathways requires protein biosynthesis (pathway I). Many stress- and ABA-inducible genes encoding various transcription factors have now been reported. These contain conserved DNA-binding motifs, such as MYB (ATMYB2) and MYC (rd22BP1) (Abe et al., 1997). These transcription factors are thought to function in the regulation of ABA-inducible genes, such as RD22 in Arabidopsis, which responds to drought stress rather slowly after the production of ABA-inducible transcription factors (pathway I). Some observations indicate that certain MAPK cascades might function in the signal-transduction pathways in drought stress response. One of the Arabidopsis MAPK genes, ATMPK3, has been found to be induced at the mRNA level by drought, low temperature, high salinity and mechanical stress. It was demonstrated that Alfalfa MAPK, MMK4, is activated at post-translational levels by a variety of stresses including drought, low temperature and mechanical stimuli (Jonak et al., 1996).

A nine-bp conserved sequence, TACCGACAT, called drought-responsive element (DRE), is required for the regulation of the induction of RD29A under drought (Yamaguchi-Shinozaki and Shinozaki, 1994). Protein factor(s) that specifically interact with the 9-bp DRE sequence were detected in nuclear extract prepared from either dehydrated or untreated Arabidopsis plants. Five independent cDNAs for DRE/CRT-binding proteins have been cloned using the yeast one-hybrid screening method (Liu and Zhu, 1998). All DRE/CRT-binding proteins (DREBs and CBFs) contain a conserved DNA-binding motif that has also been reported in EREBP and AP2 proteins (EREBP/AP2 motif), which are involved in ethylene-responsive gene expression and floral morphogenesis, respectively (Liu and Zhu, 1998). In transgenic plants, overexpression of the DREB1A cDNA also revealed freezing and dehydration tolerance (Liu and Zhu, 1998). Over-production of 35S CaMV promoter-driven DREB1A and CBF1/DREB1B cDNAs, in transgenic plants, showed improved drought stress tolerance. The DREB1A cDNA driven by the stress-inducible rd29A promoter was expressed strongly by dehydration.

Drought stress has been found to be overcome by a novel aldose/aldehyde reductase that provides protection to transgenic plants against LP. This enzyme has been found to be active on 4-hydroxynon-2-enal, a known cytotoxic lipid peroxide degradation product in alfalfa. Synthesis of this enzyme in transgenic tobacco plants provided considerable tolerance against various forms of abiotic stress including H_2O stress (Oberschall et al., 2000).

Salt stress

The most common plant stressor in saline soils is NaCl. The observed plant responses to saline conditions generally include osmotic imbalance, which in turn leads to nutritional imbalance. Osmotic imbalances mainly result in changes in ion concentrations, particularly of potassium and Ca. At higher levels, Na⁺ and Cl⁻ have direct toxic effects on membrane structure and enzyme systems (Munns and Termaat, 1986; Ashraf and Harris, 2004). This ultimately leads to secondary stresses, such as oxidative stress, linked to production of toxic reactive oxygen intermediates characterised by LP (Qureshi et al., 2005).

The mechanisms by which plants achieve salt tolerance are exceedingly complex. Attempts to define salt tolerance at the genetic level have been difficult because of the multigenic character of the phenotypes (Shannon, 1985). It is believed that genes induced by application of salt stress are usually regulated at the transcriptional level. A few promoters of these genes have been isolated and studied, and *cis*-acting elements have been functionally identified and characterised (Yamaguchi-Shinozaki and Shinozaki, 2006). Moreover, good correlation between mRNA and protein levels has been shown for some genes (e.g., DSP 22 from *Craterostigma plantagineum* and HVA1 from barley). However, in other cases, there is a marked delay in protein induction compared with mRNA accumulation (e.g., rice SALT). On the other hand, changes in mRNA levels are not always followed by similar changes in the corresponding protein (e.g., tobacco osmotin) (LaRosa et al., 1992).

A salt-stress-associated protein from citrus, as well as an encoding gene, has been isolated. This protein was demonstrated to be a phospholipid hydroperoxide glutathione peroxidase, which had not been identified before in plants (Beeor-Tzahar et al., 1995). Salt stress induced the expression of the vacuolar h(+)-ATPase. For salinity stress tolerance in plants, the vacuolar-type (H)(+)-ATPase (V-ATPase) is of prime importance in energising Na⁺ sequestration into the central vacuole and is known to respond to salt stress with increased expression and enzyme activity (Golldack and Dietz, 2001).

Salt stress has been shown to induce the expression of a protein having a strong homology to APX in radish, while in leaves of *Vigna unguiculata* cytosolic APX was slightly reduced and chloroplastic APX unchanged. It has been reported that, in NaCl-tolerant pea cultivars, leaf mitochondrial Mn-SOD and chloroplast Cu/Zn-SOD activities increased under salt stress, while the Cu/Zn-SOD activity remained unchanged (Hernandez et al., 1995).

In the salt-sensitive cultivar, neither APX nor chloroplastic SOD was increased by salt, while the cytosolic and mitochondrial SOD even decreased. In salt-sensitive and salt-tolerant cultured citrus cells and leaf tissues, it was shown that only cytosolic Cu/Zn-SOD activity was increased by salt, whereas the activity of other isoforms was unchanged (Gueta-Dahan et al., 1997).

Low-temperature stress

It is well known that plants show varied adaptation to any specific temperature. Plants face high- and low-temperature stress. A temperature range below 10 °C comes under the term cold stress, and the temperature at which ice formation in cell takes place (0 °C and below) is called freezing stress, which causes freezing injury. Chilling injury is common in tropical and subtropical plants exposed to low but non-freezing temperatures (0–15 °C). A temperature decrease below 0 °C started the formation of ice in cells and tissue. Intracellular formation of ice is considered to be the fatal event because the intercellular fluid generally has a higher freezing point than the intracellular fluid; however, most of the injury results from the severe cellular dehydration that occurs with freezing (Webb and Steponkus, 1993).

Freeze-induced dehydration could have a number of effects that result in cellular damage, such as denaturation of proteins and precipitation of various molecules. However, the best-documented injury occurs at the membrane level. Plant species native to regions with freezing temperatures have developed mechanisms to survive these temperatures. Two basic survival mechanisms have been distinguished, avoidance of freezing (avoidance of ice formation) and tolerance to freezing (Levitt, 1980).

Much evidence supports the theory that Ca is an important second messenger in regulating the cold acclimation response. Agents blocking the Ca influx make the plants unable to cold acclimatise (Örvar et al., 2000).

Under low temperature, ABA, drought and dehydration stress at non-acclimated temperatures appear to result in enhanced freezing tolerance (Thomashow, 1998). MAPK has been found to be responsible for inducing the expression of cold-regulated genes and activating freezing tolerance mechanism (Jonak et al., 1996). Classical genetics analysis indicates that the ability of plants to cold acclimate is a quantitative trait largely involving the action of many genes and hence proteins, each with small additive effects. Plants use different

types of proteins to protect themselves against low temperature and freezing conditions. Genes encoding structural proteins, osmolyte-producing enzymes, oxidative stress scavenging enzymes and lipid desaturases have revealed the potential capacity to protect against temperature-related stresses. Dehydrin gene has been isolated and characterised from white spruce upon cold induction. Cold induction of PgDhn1 transcripts was also detected as early as 8 h after treatment (Richard et al., 2000). Antifreeze activity has been found to increase in response to cold stress in winter rye due to the accumulation of antifreeze proteins (AFPs). The pathway for regulating AFP production is independent of ABA (Griffith et al., 2005). Low-temperature-responsive genes are an important gene family. Freezing tolerance gene "*regulon*" in Arabidopsis and a family of transcriptional factor genes that control their expression have been recognised.

Among the highly expressed cold-responsive genes of Arabidopsis are *cor* (cold-regulated) genes, also designated LTI (low-temperature induced), KIN (cold-inducible), RD (response to desiccation) and ERD (early dehydration-inducible) (Thomashow, 1998, 2001). The *cor* genes comprise four gene families, each of which is composed of two genes that are physically linked in the genome in tandem array. The *cor 78*, *cor 15* and *cor6.6* gene pairs encode newly discovered polypeptides while the *cor47* gene pair encodes LEA (late embryogenic abundant) group11 proteins (also known as LEA D11 proteins), RAB proteins and dehydrins (Ingram and Bartels, 1996). Stockinger et al. (1997) isolated the first cDNA for a protein that binds to the CRTT/DRE sequence. The protein CBF1 has a mass of 24kDa, an acidic region that potentially serves as an activation domain and a putative bipartite nuclear localisation sequence. Upon exposing a plant to low temperature, however, a signal-transduction pathway is proposed to be activated, which results in modification of either ICE or an associated protein, which in turn allows ICE to induce *cbf* gene expression. The CBF proteins would then be synthesised, bind to the CRT/DRE elements in the promoters of the COR (and presumably other) genes and activate their expression.

Photosynthesis and respiration are frost-sensitive physiological activities of plants, so the proteins involved in these pathways are highly regulated. Glucanases are enzymes that belong to the group of pathogenesis-related (PR) proteins. A class 1 β -1,3-glucanase from tobacco has cryoprotective activity, and immunologically related proteins are accumulated in cabbage and spinach during frost hardening under natural conditions. In contrast to

other COR proteins, 1 β -1,3-glucanase are not induced at 4°C but probably require lower temperatures and/or freezing (Hinch et al., 1997). Evidence suggests the synthesis of cryoprotectins that bind to thylakoid membranes.

High-temperature stress

Temperature stress can have a devastating effect on plant metabolism (Suzuki and Mittler, 2006). An experiment to study the response of an organism to high temperature showed the appearance of new puffs in polytene chromosome of *Drosophila* (Ritossa and Spiegelman, 1965). Later studies confirmed that the puffs observed were the sites of vigorous RNA synthesis and that these RNAs were translated into HSPs (Ashburner and Bonner, 1979).

Thus, in response to the heat shock, certain genes that code for several proteins for the protection of the plants are expressed in the plants. These proteins, which are mostly synthesised in response to heat shock, are called HSPs. Note that, production of high levels of HSPs can also be triggered by exposure to different kinds of environmental stress. Consequently, the HSPs are also referred to as stress proteins, and their up-regulation is sometimes described more generally as part of the stress response (Lewis et al., 1999).

Molecular analysis indicates that the major classes of HSPs synthesised by plants are homologous to HSPs of other eukaryotes. Several proteins with homology of HSPs, or in some cases HSPs themselves, are also components of unstressed plant cells. The discovery that different proteins of major HSP families are found in more than one cellular compartment further indicates that HSPs (low molecular weight (LMW) and high molecular weight) play a role in basic biochemical processes. The HSPs and their homologues must perform many essential functions in both normal and stressed cells. The current hypothesis is that HSP60, HSP70 and HSP90 function to alter the conformation or assembly of other protein structures.

A number of stress protein families, including HSP90, HSP70, chaperonin 60, HSP40, the LMW stress proteins and ubiquitin, have been identified in diverse phyla (see Lewis et al., 1999). Under normal conditions, several of the major stress proteins are present at low levels and function as "molecular chaperones", key components contributing to cellular homeostasis in cells under both optimal and adverse growth conditions (see Wang et al., 2004). They are responsible for protein folding, assembly, translocation and degradation in

a broad array of normal cellular processes; they also function in the stabilisation of proteins and membranes and can assist in protein refolding under stress conditions. A wide range of proteins has been reported to have chaperone functions. Moreover, many molecular chaperones are stress proteins, and many of them were originally identified as HSPs. Thus, the names of these molecular chaperones follow their early nomenclatures and are referred to here as HSPs/chaperones. Five major families of HSPs/chaperones are conservatively recognised: the HSP70 (DnaK) family, the chaperonins (GroEL and HSP60), the HSP90 family, the HSP100 (Clp) family and the small HSP (sHSP) family. Aside from these major families, there are other proteins with chaperone functions, such as protein disulphide isomerase and calnexin/calreticulin, which assist in protein folding in the ER. Molecular Hsps/chaperones are located in both the cytoplasm and organelles, such as the nucleus, mitochondria, chloroplasts and ER. Different classes of molecular chaperones appear to bind to specific non-native substrates and states. Chaperone proteins do not covalently bind to their targets and do not form part of the final product. The two best-studied families are the chaperonins and the HSP70 family chaperones. Under conditions of environmental stress, stress proteins are involved in protecting and repairing vulnerable protein targets. Stress proteins also play a role in the lysosomal and ubiquitin protein degradation pathways, by which damaged proteins are broken down. In essence, the cellular stress response entails the orchestrated induction of key proteins that form the basis for a cell's protein repair and recycling system (Krishna, 2004). The remarkable way in which stress proteins act as catalysts of protein folding and repair is best understood by examining two major heat-inducible protein families, HSP70 and CPN60. HSP70 is a large, multi-gene family with members residing in a number of subcellular compartments including the cytoplasm, mitochondria and ER. Normally, stress70 proteins prevent incorrect folding of newly synthesised peptides by binding to the growing peptide chain and maintaining it in a loosely folded state until synthesis is complete. Another stress protein, hsp40, is suspected to interact with HSP70 and participate in this process. HSP70 disassociation, an ATP-dependent process, occurs as the protein proceeds down its folding pathway to reach its correct three-dimensional shape. Proteins that need to be distributed to other subcellular compartments are maintained in an unfolded state and escorted to that destination for translocation. Once inside the organelle, the target protein interacts

with another member of the HSP70 family that performs similar folding functions (Miernyk, 1999).

Yet another group of proteins called chaperonins carries out additional folding functions and assembly. This class of proteins includes the chaperonin 60 (CPN60) family and is found in eubacteria, mitochondria and plastids. Chaperonins assemble into large "double donut"-shaped complexes that direct the higher-level folding and the assembly of subunits into complexes (Lorentzen and Conti, 2006). Early studies on the regulation of the cellular stress response supported the notion that the synthesis of stress proteins is related to protein damage. Heat-inducible genes include a conserved sequence, referred to as the "heat-shock element", in their upstream regulatory region (Pirkkala et al., 2001).

A protein called the heat-shock factor, which binds to the heat-shock element, activates the gene. A number of studies suggest that those stress factors that cause an increase in damaged or abnormal proteins activate this process (Pirkkala et al., 2001). It has been suggested that HSP70 plays a role in this relationship and acts as a "cellular thermometer". Amino acid analogues that create abnormal proteins induce synthesis of stress protein. Biochemical conditions that alter protein conformation also affect expression of the stress response in a predictable manner. However, the most intriguing aspect of the regulation of induction of the stress proteins is that denatured proteins are both the signals that activate transcription of the stress protein genes and the substrate for the proteins themselves. While CPN60 is also normally present at low levels, in the mitochondria its abundance increases from 2 to 8 h after heat shock. Then, after heat shock, CPN60 can be seen in the nucleus associated with the nucleolus and as discrete foci. These foci are strikingly similar to the distribution of the snRNP-rich organelles called "coiled bodies", which are believed to be involved in RNA processing. Disappearance and appearance kinetics of coiled bodies by heat shock suggested peripheral labelling (Beven et al., 1995).

Induction of stress protein synthesis is highly tissue-specific. Both the temperature range of induction and the extent of induction of each stress protein appear to depend on tissue type. This tissue specificity is probably a result of two different mechanisms: differences in gene expression among specialised cell types and the extent of tissue damage. Given the current understanding of the regulation and function of the stress response, the intensity and relative concentrations of HSP70 and CPN60 should be greatest in tissues that are most

vulnerable to damage caused by a particular environmental factor.

Heavy metals

Plants respond to heavy metal toxicity in different ways (Qadir et al., 2004). Such responses include immobilisation, exclusion, chelation and compartmentalisation of the metal ions, formation of peptide metal-binding ligand PCs (Grill et al., 1985) and MTs (Cobbett and Goldsbrough, 2002) and the expression of more general stress-response mechanisms, such as ethylene and stress proteins. A large number of stress proteins have been found to be induced by heavy metal stress with a molecular mass of 10,000–70,000 Da (Delhaize et al., 1989). These mechanisms have been reviewed comprehensively by Sanita di Toppi and Gabbriellini (1999). When these systems are overloaded, oxidative stress defence mechanisms are activated. A number of metal-binding ligands have now been recognised in plants. Most of the ligands have been studied in relation to cadmium, which is one of the most important heavy metal pollutants highly hazardous to plants, even at low concentration (Xiong and Peng, 2001). In 1985, it was reported that the major Cd^{2+} ligands in Cd^{2+} -intoxicated plant cells are composed of poly(γ -glutamyl cysteinyl)glycine. In some other polypeptides, Gly is either absent or substituted with β -alanine. These types of polypeptides are termed as Class III MTs (Kojima, 1991). Such polypeptides were purified from a number of plant species and are termed as PCs (Grill et al., 1989; Rauser et al., 1991).

PCs form a family of structures with increasing repetitions of the γ -Glu-Cys dipeptide followed by a terminal Gly (γ -Glu-Cys) $_n$ -Gly, where n has been reported as being as high as 11 but is generally in the range of 2–5. They are structurally related to GSH (γ -Glu-Cys-Gly) and were presumed to be the products of the same biosynthetic pathway. These are induced by various heavy metals and even by non-metals (e.g., selenium and arsenate) (Grill et al., 1987; Delhaize et al., 1989; Gupta and Goldsbrough, 1991). The tendency of metals to induce these polypeptides in cell suspension cultures of *Rauvolfia serpentina* decreases in the order $\text{Hg} > \text{Cd}$, As , $\text{Fe} > \text{Cu}$, $\text{Ni} > \text{Sb}$, $\text{Au} > \text{Sn}$, Se , $\text{Bi} > \text{Pb}$, and Zn (Grill et al., 1987). Structure elucidation of PCs suggests that PCs were in functionally equivalent to MT proteins, and it is now apparent that plants express both of these Cys-containing metal-binding ligands.

The MTs are small, cysteine-rich proteins that have the capacity for high-affinity binding of heavy

metal ions and whose synthesis is regulated by metal ion concentrations. These properties suggest that MTs play pivotal roles in the metabolism of the relatively non-toxic essential metals (zinc and copper), as well as toxic heavy metals (cadmium). Striking features of the MTs include their high degree of amino acid sequence similarity (including conservation in the placement of cysteine residues in the molecule reflecting their function in metal binding), a conserved tripartite gene structure and their transcriptional induction by metal ions, as well as other hormonal and environmental stimuli. The precise mechanisms and biochemical pathways by which cells transduce environmental signals into transcriptional induction of the MT genes are beginning to be defined. Recent studies indicate that metal effects are exerted via positive *trans*-acting factors induced to interact with *cis*-acting DNA sequences in the promoter, in turn leading to transcriptional induction. However, the MT gene promoter is structurally complex and contains binding sites for a variety of nuclear proteins that likely regulate basal as well as induced levels of expression of these genes. There is possible involvement of post-transcriptional processes in the regulation of MT levels in the cell. Furthermore, it is likely that both PCs and MTs play relatively independent roles in metal detoxification and/or metabolism.

Ozone

As a result of anthropogenic activities, the concentration of tropospheric ozone has been elevated in the last decade. In summer, ambient ozone concentrations range between 20 and 80 nLL⁻¹, with a maximum of 200 nLL⁻¹. Ozone affects the plant health and is capable of altering the protein profile (Agarwal et al., 2002). Ozone can affect membrane permeability, enzyme activities, metabolite pools and photosynthetic activity; it also causes leaf chlorosis and necrosis. Several reactive oxygen species (ROS) generated in the cellular or subcellular loci during ozone exposure and changes in redox state and peroxide systems are responsible for this damage. Calcium, H_2O_2 , ethylene, salicylic acid (SA) and JA play roles in the signal-transduction pathway in plants under ozone stress (Rao et al., 2000).

Gene transcripts for PR proteins are reported to increase by ambient ozone concentration. Expression of genes encoding proteins of phenylpropanoid metabolism and the antioxidative system has also been observed to increase. Suppression of certain photosynthetic transcripts by ozone has also been

reported. Several ozone-affected transcripts have been published and reviewed by Sander mann et al. (1998). The expression of these 'ozone-induced' genes shows a temporal and spatial hierarchy. Some genes underwent a rapid activation, whereas others were more slowly activated. Resveratrol synthase promoter of grapevine (VST1) was described as the first ozone-responsive promoter region. Gene transcription was modulated by *trans*-acting protein factors, binding to the respective *cis*-acting elements present in the promoter sequence.

Ozone fumigation of tobacco plants resulted in increased levels of PR protein transcripts. Ozone causes selective activation of genes involved in flavonoid biosynthesis and ethylene biosynthesis. Glutathione-S-transferase transcripts are also increased in *A. thaliana* upon ozone fumigation. Elicitor responsive elements (W-boxes) and structurally related cognate DNA-binding proteins have also been characterised. The core hexamer sequence (TTGACC) is sufficient for elicitor-induced PR gene expression in maize, parsley and tobacco. Two W-box-like core sequences are essential *cis*-elements interacting with the WRKY family of *trans*-factors and are present in several pathogen-responsive promoters. These genes are also induced by other stress factors, including ozone. The *Vst1* gene is induced by pathogens and ozone. However, the ozone-responsive *Vst1* region differs from the basal pathogen-responsive promoter region (see Mahalingam et al., 2003).

UV light

Long-wavelength ultra-violet (UV) radiation (UV-A and some UV-B) reaches sea level by penetrating the atmosphere. Short-wavelength UV radiation (UV-C, <280 nm) is especially biologically hazardous as it is absorbed directly by DNA and is completely absorbed by the atmosphere. Long-wavelength UV radiation UV-A (315–400 nm) is not normally associated with injury to biological systems (Soheila and Mackerness, 2000); although it does not have a direct interaction with DNA, it can be absorbed by other cellular chromophores, whose excitation may generate potentially harmful oxidative stress.

UV-C (<280 nm) causes DNA damage; UV-B causes LP and membrane deterioration. In *A. thaliana* UV-B causes oxidative damage to proteins and elevation in antioxidant enzymes (Strid et al., 1994). There is a clear stimulation of the metabolic synthesis in cultured rose cells that have been irradiated with UV-C. Oxidised lipids appear after high doses of UV-C

and UV-B. Thus, UV radiation apparently increases the demand for a scavenging system that reduces the amount of ROS. UV-B radiation inhibits de-epoxidation of violaxanthin in isolated chloroplasts. Synergistic inhibition of photosynthesis by UV-B and visible light has been observed in algae and *B. napus*. H₂O₂ and SA are believed to be the principal signalling molecules in UV-light stress. SA was also shown to increase in response to UV-B exposure, leading in turn, as a consequence of ROS generation, to a rise in PR transcripts.

UV exposure leads to dramatic changes in gene expression in plants. There is no direct evidence for the signal that stimulates gene expression under UV stress; however, it appears that UV alters gene expression because of oxidative stress. Genes encoding enzymes of phenylpropanoid and flavonoid biosynthesis pathways [e.g., phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS)] have been reported to stimulate by UV stress (Schafer et al., 1997). Products of these pathways are important in plant protection. Arabidopsis cell suspension cultures were used to investigate the signal transduction of CHS expression by UV light. It was found that CHS transcripts accumulate in the cells within a few hours after exposure to UV (Santos et al., 1997).

The induction of proteinase inhibitor I and II genes in tomato by UV-C and UV-B was absent in a mutant defective in octadecanoid signalling, which produces the JA from linolenic acid. Hence, it was suggested that JA signalling pathway mediated the plant genomic response to UV-B. However, it was found that in Arabidopsis cells the induction of PAL by jasmonate occurs with slower kinetics than UV-B induction; moreover, the two pathways have different pharmacological properties. Thus, in the case of PAL, the UV-B induction does not appear to be mediated by jasmonate. It is, therefore, likely that several distinct pathways mediate the effects of UV-B on gene expression in plants (Soheila and Mackerness, 2000).

Besides the CHS and PAL gene transcripts, it has been found that transcripts for chloroplast proteins encoded on both the nuclear and chloroplast genomes are reduced in response to UV-B exposure (Long et al., 1998). These changes in gene expression are detectable within hours. UV-B exposure enhances both the rate of synthesis and degradation of D1 polypeptides, indicating that the primary level of UV-B control is post-transcriptional (Campbell et al., 1998). Plants have also evolved a number of protective mechanisms against UV-B-induced damage, among which is the up-regulation of various 'defence associated genes'. The most important protective mechanisms that have been studied are

UV-B attenuation by UV-B-absorbing pigments, induction of antioxidant systems and repair of induced DNA damage (Britt, 1995, 1996; Watanabe et al., 2006).

Visible light

Plants exposed to a range of fluctuating light intensities (photosynthetic photon flux) in natural environments can suffer depression in photosynthetic efficiency (photoinhibition) mainly due to oxidative damage to the PS II (Powles, 1984). At low light intensity, an increase in photosynthetic carbon fixation occurred, which varies depending on growth and light intensity, and which may lead to different susceptibilities to photoinhibition (Powles, 1984). However, above a certain threshold, carbon fixation becomes saturated and photosynthesis is incapable of using all of the energy stored by the plants. Under these conditions of excess light absorption, the chloroplast lumen becomes acidic in nature and reduces the electron transport chain, and excitation energy accumulates within the chloroplast. Excess excitation energy (EEE) could result in an increase in the singlet and triplet forms of chlorophyll and singlet oxygen, which are toxic in nature. Depletion of the NADP⁺ pool under EEE causes an increase in the rate of electron flow from the donor side of PSI to oxygen, generating ROS, such as superoxide and hydrogen peroxide (Asada, 2006).

Collective evidence from various studies has shown that the thylakoid proteome of chloroplasts contains multiple proteins involved in antioxidative defence, protein folding and repair. Moreover, proteomic studies have shown that the thylakoids contain a significant number of additional proteins, many without known function. However, it is not clear whether and how these individual proteins are coregulated in response to HL stress or how reduced cellular ascorbate content might alter this response. HL response of the thylakoid proteome in *Arabidopsis* wild type and the ascorbate-deficient mutant *vtc2-2* has been published recently (Giacomelli et al., 2006).

The main source of ROS generation in plants is the chloroplast (Asada, 2006). Furthermore, once initiated, LP becomes autocatalytic, resulting in massive membrane photodestruction (Niyogi, 1999). If these ROS are not removed immediately, they can cause damage to the cellular and molecular machinery, protein modification (Zolla and Rinalducci, 2002) and LP. To minimise the ROS effects, plants have evolved various antioxidative enzymes and LMW non-antioxidative substances,

such as ascorbate, GSH and α -tocopherol. The enzymatic antioxidants, namely SODs, convert superoxide to H₂O₂ and oxygen and exist in three isoforms (e.g., Cu/Zn SOD, Fe-SOD and MnSOD). In addition, ascorbate-GSH cycle enzymes, such as APX, dehydro ascorbate reductase, MDHAR and GR, constitute the defensive system against ROS (Asada, 2006). Superoxide radicals generated on the acceptor side of PSI are detoxified by a series of membrane-associated and stromal enzymes, including SOD and APX. In several studies, using plant transformation to increase the activities of SOD, APX or GR has improved the recovery of photosynthesis following chilling in light. Such is the case for transgenic tobacco plants with increased chloroplastic Cu/Zn SOD activity (Sen Gupta et al., 1993a, b), although the elevated activity of the native APX associated with the higher SOD activity in these plants may be most critical to their recovery of photosynthesis (Sen Gupta et al., 1993b). In fact, transgenic tobacco plants with elevated APX activity, alone, either in the chloroplast stroma or in the cytosol, exhibit increased protection of photosynthesis, as well (Allen et al., 1997). Even increasing chloroplastic GR activities in poplar leaves to increase the capacity to regenerate ascorbate via the ascorbate-GSH cycle results in a better initial recovery of photosynthesis after chilling at high photon flux densities than for the wild-type poplars (Foyer et al., 1995).

Nutrient stress

Plants require a right combination of nutrients for growth, development and reproduction. Under malnutritional conditions, there is an alteration in these parameters along with the appearance of the 'disease-like' symptoms (Shin et al., 2005). Deficiency or excess of nutrient(s) may result in stress conditions in the plant system; however, nutrient requirement varies among plants. Plant nutrients fall into two categories: macronutrients and micronutrients. Macronutrients are the elements required in relatively large amounts, such as nitrogen, potassium, sulfur, calcium, magnesium and phosphorus, whereas, micronutrients are the elements that plants need in trace amounts, such as iron, boron, manganese, zinc, copper, chlorine and molybdenum. Both macro- and micronutrients are naturally obtained through the roots. There is not much published data on the varieties of proteins up-regulated under specific nutritional conditions or in the absence of the same. However, employing the proteomic approach, Sarry et al.

(2006a) recently demonstrated the changes in levels of proteins associated with nitrogen and sulphur metabolism. It may be suggested that nutrient metabolism-associated proteins are down-regulated under nutrient-specific stress (absence or limitation).

Table 1 provides a detailed list of the proteins identified to alter their levels under different stress conditions discussed in this section of the review.

Proteomics-based identification of stress-responsive proteins under various stresses

The primary requisite for identification of stress-responsive proteins is the experimental design. For example, between two sets of the model plants, one is to be kept as a control and the other exposed to the stress for a specific duration of time. The study may be made at organellar, cellular or subcellular level. The appropriate protocols are

Table 1. List of the proteins identified to be induced or up-regulated under abiotic stress(es) or associated with nutrient metabolism as detected using 2-DIGE technology, mass spectrometry and protein identification softwares coupled with protein databases

Name of protein	Reference
<i>Drought stress</i>	
CPN21	Jorge et al. (2006)
Cu/ZnSOD	Salekdeh et al. (2002), Hajheidari et al. (2005), Taylor et al. (2005)
Hsp11, osmotin-like, peroxidase, cyclophilins, NDPK	Hajheidari et al. (2005)
Hsp22, Hsp70, Hsp90, chaperonin 10, MPP β	Taylor et al. (2005)
<i>Salt stress</i>	
Peroxidase	Yan et al. (2005)
CytP450-like, NAK (serine/threonine kinase), V-ATPase- β , β -glucosidase, glutamate ammonia ligase, ADK (adenosine kinase)	Zörb et al. (2004)
<i>Low-temperature stress</i>	
Hsp70, aldolase C-1, nucleosidase diphosphate kinase1	Imin et al. (2004)
Hsp90-like, Hsp70-like, ATMyb2, BZIP TNF, Hsc70-1, JA-induced, germin-like, HSF8, DRT102 (DNA repairing)	Bae et al. (2003)
Dehydrin (ERD10), CP29, low-temperature-induced protein 78, glycine-rich protein	Amme et al. (2006)
ATPase-CF1 α , chaperonin20, GSTII, mRNA-binding protein, Hsp70, phenyl ammonia lyase, ferritin	Cui et al. (2005)
<i>High-temperature stress</i>	
HspH, HspD, HspB, HspE, HspC, GroES1, GroES2, GroEL4, GroEL5, DnaK	Münchbach et al. (1999)
DHAR, Hsp16.9B, Hsp17.8	Süle et al. (2004)
Hsp83, Hsp80, Hsp70, V-ATPase B1, catalase, Lea, dihydroflavonol reductase, sHsp	Majoul et al. (2004)
JA-induced, Hsp82, Hsp22, Hsp17, Class II, RAN 1B, V-ATPase,	Majoul et al. (2004)
<i>Cadmium stress</i>	
V-ATPaseB, Pi49	Repetto et al. (2004)
Catalase 3, MDAR putative, peroxiredoxin, NADPH oxidoreductase, isoflavone reductase homologue P3	Sarry et al. (2006b)
AAA ATPase family, glycine rich	Aina et al. (2007)
<i>Arsenic stress</i>	
Putative GPx, CytP450, Cu-ZnSOD, ATPase, guanine-binding protein β subunit	Requejo and Tena (2005)
<i>Mn stress</i>	
Peroxidase (POC1), PR5, PR1, PR-1a, glucanase, thaumatin-like, wound-induced (TAB7)	Fecht-Christoffers et al. (2003)
<i>Copper stress</i>	
GSTs, AtGST F2, 6, 7, 8, 9, 10, 19, U19 and 20	Smith et al. (2004)
<i>Ozone stress</i>	
Ca-binding, APX, SOD, PRP (OsPR10)	Agarwal et al. (2002)

Table 1. (continued)

Name of protein	Reference
<i>High light intensity stress</i> Hsp70, 91, 92 and Hsp112, Cu/ZnSOD, APX81, 82, 83 and 137, Hsp18.6 ClassI, Hsp70-like, enolase, GPx-like29 and 70, MnSOD	Nam et al. (2003)
<i>Hormone treatment (GA₃, ABA)</i> PRs, antifungal, SALT, DHAR1, MnSOD ATPase-β, MnSOD, PR1	Konishi et al. (2005) Rakwal and Komatsu (2004)
<i>Nutrient associated proteins</i> <i>Nitrogen metabolism</i> Glutamine synthetase, glutamate ammonia lyase, α-glutamyl cysteine synthase, aspartate aminotransferase, glutamate decarboxylase1, glutamate dehydrogenase, formate dehydrogenase, carbamoylphosphate synthetase, ferridoxin-nitrite reductase, nitrilase, UTP-ammonia lyase, glutamyl-tRNA synthetase/ligase, aspartyl-tRNA synthetase	Sarry et al. (2006a)
<i>Sulphur metabolism</i> ATP sulphurylase, sulphite reductase, cysteine synthase, GS, GPx, GST, S-adenosylmethionine synthetase, adenosylhomocysteinase, methionine synthase, adenosine kinase1, methylenetetrahydrofolate reductase, thioredoxin, thioredoxin reductase	Sarry et al. (2006a)
<i>K⁺ deficiency</i> HSP81-3, Leo1-like family protein, WD domain protein-like protein, GST chain A	Kang et al. (2004)

used in order to get the best results. To extract the proteome of subcellular organelles, such as mitochondrion and chloroplast, these organelles are first isolated in pure and intact form and further ruptured in hypotonic medium. To get the protein complexes in native form from the said organelles, blue native PAGE (BN-PAGE; first dimension) is run on the protein complexes extracted in the native form under non-denaturing conditions. BN/BN-PAGE may also be run to further separate the proteins of comparatively closer molecular weights. After reduction and alkylation of the proteins in the protein complexes, the complexes are then loaded on the SDS gel (for SDS-PAGE). Thus, the subunits of protein complexes or different complexes from blue native PAGE may be separated. The molecular weight in both dimensions is calculated against the standard proteins.

For mapping the proteome, the proteins are dissolved in non-denaturing detergents and loaded on strips with an immobilised pH gradient (IPG) and subjected to an isoelectric focusing as a first-dimensional run. The focused IPG strip is then reduced and alkylated and loaded onto the SDS gel (for SDS-PAGE) for a second-dimensional run. Further, the gel is stained with Coomassie Brilliant Blue stain or silver stain. Certain other stains are now commercially available for better identifica-

tion of protein spots between the gels to be compared. Using image analyses, under- and over-expressed proteins are detected along with the proteins that are newly synthesised (or induced) and proteins that have disappeared (Fig. 1). Complications and practical hurdles in tackling the proteome (including protein interactions, solubility, etc.) and certain other methods for protein identification may be clarified by recently published review articles (Rose et al., 2004; Righetti et al., 2005).

After identification of a set of differentially expressed spots from a series of two-dimensional gels, the next step is typically to identify the cognate proteins and genes, which can be achieved in a number of ways (Gevaert and Vandekerckhove, 2000). However, MS is now the method of choice for both protein identification and characterisation. Spectrometers are coupled with advanced software that facilitate protein identification and structural analysis and to provide an instant online bridge between mass spectra and public sequence databases. For reviews describing MS instrumentation, software and techniques, see Aebersold and Mann (2003), Ferguson and Smith (2003) and Lin et al. (2003). The first step towards protein identification is excision of two-dimensional gel plugs containing the protein spots of interest, in-gel digestion with

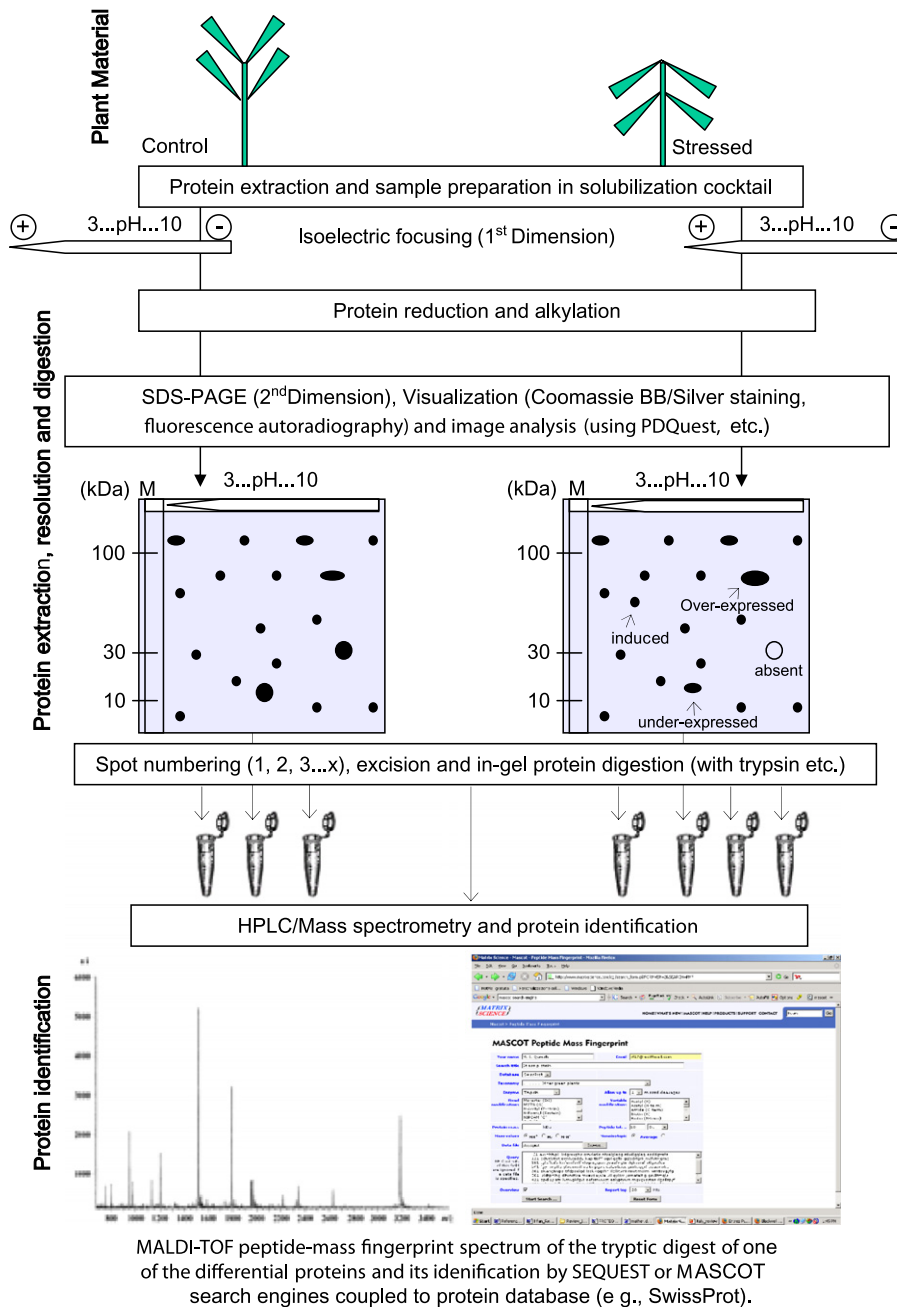


Figure 1. Demonstration of the process of the stressing plant system and protein extraction, focusing, SDS-PAGE resolution, visualisation, digestion and protein identification using advanced spectrometry and bioinformatic tools. Plant material may be, e.g., root, leaf, cultured cells, pollens, mitochondria, chloroplast, protoplasm or cell/organellar membrane.

a site-specific protease (commonly trypsin) and, finally, MS analysis of the resultant eluted peptides (Fig. 1). Two MS platforms in particular represent powerful tools for proteomic studies. The first, MALDI-TOF MS, is typically used to measure the masses of the peptides derived from the trypsinised parent protein spot, generating a 'peptide mass fingerprint' (PMF) or *de novo* sequencing by ESI MS/MS. Software package then compares the

peptide mass list with a predicted theoretical list of tryptic peptide fragments for every protein in the public databases, together with equivalent translated genomic and EST databases. In this case, the protein is identified based on the *in silico* match of experimentally determined versus predicted peptide masses, together in some cases with the apparent and predicted isoelectric point and molecular mass from the two-dimensional gels,

rather than the actual amino acid sequence. Although each PMF is usually a viable means of assigning identity to a specific protein, as a result of the variability in amino acid sequences and of the relative distribution of protease cleavage sites between proteins (Godovac-Zimmermann et al., 2005), members of protein families with a high degree of sequence similarity can also result in effectively indistinguishable PMFs. This problem is exacerbated by the fact that it is unusual for the full complement of peptides for any given protein to be ionised and detected experimentally by MALDI-TOF.

We provide an account (Table 1) of different proteins which were found to be up-regulated under the corresponding abiotic stresses, so as to give a comparative overview with the proteins discussed earlier in this review article. Proteins associated with nitrogen and sulphur metabolism as detected using proteomic tools have also been mentioned.

Concluding remarks

Stress biologists now feel comfortable with the development of excellent tools in the fields of proteomics and bioinformatics. Earlier they used enzymatic kinetics (estimation of precursor/s or product/s) to estimate the concentration of functional proteins, immunological reactions, fractionation methods and radioactive isotopes. Enzyme levels, for example, estimated in a test tube by kinetic reaction, followed by UV/visible spectrometry analysis, may be in the form of one among hundreds of spots visible on a gel. The unseen proteins are now visible. However, most of these techniques used were economical to perform, and the field of proteomics was not so developed. Now, the advancement in tools of proteomics has changed the whole scenario. Given the high throughput and high sensitivity of mass spectrometry, coupled via advanced software to protein databases, proteomics is gaining overwhelming response. The proteins from control and stressed plant parts/organs are quantified and compared by image analysis tools. The proteome map may serve in future as a reference for a specific plant part/organ, under physiological as well as stress conditions (stress proteome). It may be helpful to have a record of proteins as environmental biomarkers. The literature of proteomics is growing by leaps and bounds with reconfirmation and sequencing of previously discovered proteins. Now we have the sequence of a number of proteins in response to environmental stress. This has achieved benefits in two major areas, in the understanding of plant

metabolism and its regulatory factors, and in the development of strategies to utilise the generated knowledge/data for improved traits in plants, mainly for crop and medicinal plants via genetic/metabolic engineering after hunting a gene using reverse genetics. The understanding of plant stress physiology and of the factors that influence it is essential in order to correlate them with the changes in proteome.

Notably, the importance of studies with invisible but known proteins cannot be underestimated. In the present scenario, too, these have their own importance to prove the functional authenticity. We still have a long way to go to assess significant correlation between the data obtained from traditional methodologies of physiochemical analysis carried out *in vitro* or *in vivo* and the data obtained through proteomic studies. In this direction, however, the proof comes from the extensively studied proteins (e.g., SODs, peroxidases, MTs, HSPs and PRP), which help in combating stress (see Table 1). In conclusion, proteomics provides an excellent opportunity to study the response of plants to environmental stress and to identify stress-responsive proteins. Still, proteomics is a detailed account to assess the 'highly praised' proteins, such as GR, MAPKs, SOS components, etc., regulatory proteins and 'hidden proteome'. Further improvement with innovative additions in proteomics will surely transform most stress biologists into environmental proteomicists!

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