Chapter 2
Transgenic Plants for Abiotic Stress Resistance

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2.1 Introduction

Modern agricultural crop production relies on the growth of a few of the world's plant species selected for their superior qualities and suitability as food, animal feed, fiber or industrial end uses. Centuries of selection and, more recently, scientific breeding for adaptation to biotic and abiotic stresses have been necessary to improve yield, yield stability, and product quality in agricultural species.

Nevertheless, abiotic stresses remain the greatest constraint to crop production. Worldwide, it has been estimated that approximately 70% of yield reduction is the direct result of abiotic stresses (Acquaah 2007). The ever increasing pressure put on agricultural land by burgeoning human populations has resulted in land degradation, a cultivation shift to more marginal areas and soil types, and heavier requirements for agricultural productivity per unit area. Additionally, climate change has exacerbated the frequency and severity of many abiotic stresses, particularly drought and high temperatures, with significant yield reductions reported in major cereal species such as wheat, maize, and barley (Lobell and Field 2007). In many parts of the world, rainfall has become less predictable, more intense, and, due to increasing temperatures, subject to higher evapotranspiration. For example, in the major grain growing areas of eastern Africa, the predominant rainy season is starting later and ending earlier (Segele and Lamb 2005) with longer dry spells in between (Seleshi and Camberlin 2006).

Agricultural practices to improve crop productivity per unit area have, in many cases, accelerated the rate of land degradation, with particularly marked effects in irrigated areas. Irrigation has led to salinity across large tracts of agricultural land,

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with cases, such as in India, where it has reportedly led to the loss of seven million hectares from cultivation (Martinez-Beltran and Manzur 2005). In Australia, almost eight million hectares of land are under threat of dryland salinity (Munns et al. 2002). Higher yields are also only sustainable with higher nutrient use, and the heavy demand for fertilizers has caused rising costs for farmers worldwide. The environmental and economic consequences of increased nutrient use have been widely reported. For sustainability of crop production, there is a need to reduce the environmental footprint of food and fiber production, and nutrient use efficient crops are highly sought after.

Transgenic approaches are one of the many tools available for modern plant improvement programs. Gene discovery and functional genomics projects have revealed multitudinous mechanisms and gene families, which confer improved productivity and adaptation to abiotic stresses. These gene families can be manipulated into novel combinations, expressed ectopically, or transferred to species in which they do not naturally occur or vary. Hence, the ability to transform the major crop species with genes from any biological source (plant, animal, microbial) is an extremely powerful tool for molecular plant breeding. Transgenic plants can be used as sources of new cultivars (or their germ plasm as new sources of variation in breeding programs) and they are also extremely useful as proof-of-concept tools to dissect and characterize the activity and interplay of gene networks for abiotic stress resistance.

In this chapter, we will outline the major yield-limiting abiotic stresses faced by crop plants: drought, salinity, cold, nutrient deficiency, and metal toxicity. Within each section, we will then cite specific examples of transgenic crop approaches to overcome these stresses and also discuss a number of conserved plant stress response mechanisms, which have been demonstrated to confer better adaptation to a number of different abiotic stresses.

2.2 Water Scarcity and Agriculture

Drought is the most significant environmental stress on world agricultural production (Tuberosa and Salvi 2006; Cattivelli et al. 2008) and enormous effort is being made by plant scientists to improve crop yields in the face of decreasing water availability. During the twentieth century, the world's population tripled from approximately 1.65 to 5.98 billion and population projections of 8.91 and 9.75 billion are expected to occur by 2050 and 2150, respectively. Developing countries in Africa and Asia account for approximately 80% of this growth and, with an estimated 800 million people in these countries already undernourished, the Food and Agriculture Organization (FAO) of the United Nations predicts that a 60% increase in world food production over the next two decades is required in order to sustain these populations.

Agriculture accounts for approximately 70% of global water use and irrigation accounts for up to 90% of total water withdrawals in arid nations (World Water

Council 2008; FAO 2009a). Approximately, 40% of all crops produced in developing countries are grown on irrigated arable land, which accounts for only 20% of the total arable land in these nations (FAO 2009c). The water withdrawal requirement for irrigation is expected to increase by 14% in developing countries by 2030 and strategies to decrease this demand by developing crops that require less irrigation will, therefore, play a vital role in maintaining world food supply.

The area of plant drought tolerance research and improvement encompasses an enormous range of environmental, genetic, metabolic, and physiological considerations and an exhaustive discussion of all available avenues for developing drought-resistant crop varieties is beyond the scope of this chapter. Rather, this section attempts to provide an overview of some of the genetic mechanisms that have been manipulated in order to develop transgenic crops with improved drought tolerance and focuses on research that has involved long-term and field-based drought stress treatments performed on agronomic and horticultural crop species at yield determining plant life stages.

2.2.1 Improving Drought Tolerance in Agricultural Crops

All plants require water to complete their life cycle, with most plant cells consisting of at least 70% water on a fresh weight basis. When insufficient water is available, plant water status is disrupted, which causes imbalances in osmotic and ionic homeostasis, loss of cell turgidity, and damage to functional and structural cellular proteins and membranes. Consequently, water-stressed plants wilt, lose photosynthetic capacity, and are unable to sequester assimilates into the appropriate plant organs. Severe drought conditions result in reduced yield and plant death.

The overall aim of genetically improving crops for drought resistance is to develop plants able to obtain water and use it to produce sufficient yields for human needs under drought conditions. While advances have been made in developing crops that are genetically improved with traits such as herbicide and pesticide resistance, attempts to improve plant drought resistance have been hindered by the complexity of plant drought resistance mechanisms at the whole plant, cellular, metabolic, and genetic levels. Interactions between these mechanisms and the complex nature of drought itself, adds another layer of intricacy to this problem.

2.2.2 Complexity of Drought and Plant Responses to Drought Stress

Drought (nonavailability of water for crop growth) and water deficit (insufficient plant water status) are variable, complex, and recurring features in most parts of the

world. Even in areas with very high precipitation, many crops are likely to experience a certain level of water deficit at some stage during the growing cycle.

Elucidation of plant drought resistance and response mechanisms has been compounded by the variable levels and forms of drought. Drought can be spatially and temporally variable; terminal, short-term, or sporadic; severe, moderate, or minor; and can occur at rates ranging from very sudden to gradual. In addition, the effects of drought and water deficit on crop productivity vary for different crops, macro- and microenvironments across a single field, plant life stages, and the plant material to be harvested. Furthermore, the effects of drought on crop productivity are often compounded by associated stresses such as heat, salt, and nutrient stress.

2.2.3 Plant Drought Resistance and Response

Plant drought resistance mechanisms can be broadly grouped into avoidance or tolerance mechanisms. Drought avoidance mechanisms are associated with physiological whole-plant mechanisms such as canopy resistance and leaf area reduction (which decrease radiation adsorption and transpiration), stomatal closure and cuticular wax formation (which reduce water loss), and adjustments to sink-source allocations through altering root depth and density, root hair development, and root hydraulic conductance (Beard and Sifers 1997; Rivero et al. 2007).

Drought tolerance mechanisms are generally those that occur at the cellular and metabolic level. These mechanisms are primarily involved in turgor maintenance, protoplasmic resistance, and dormancy (Beard and Sifers 1997). Plants respond to water-limiting conditions by altering the expression of a complex array of genes (Fig. 2.1) and, although elucidation of biochemical pathways associated with many of these genes has been the focus of an enormous amount of research over the last two to three decades, the mechanisms by which these genes and their products interact remains relatively poorly understood.

Abiotic stress leads to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001). Drought, salinity, extreme temperatures, and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano et al. 1999; Zhu 2001). Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins (Smirnoff 1998). As a consequence, these diverse environmental stresses often activate similar cell signaling pathways (Knight 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2001, 2002) and cellular responses, such as the production of stress proteins, upregulation of antioxidants and accumulation of compatible solutes (Vierling and Kimpel 1992; Zhu et al. 1997; Cushman and Bohnert 2000; Wang et al. 2003b).

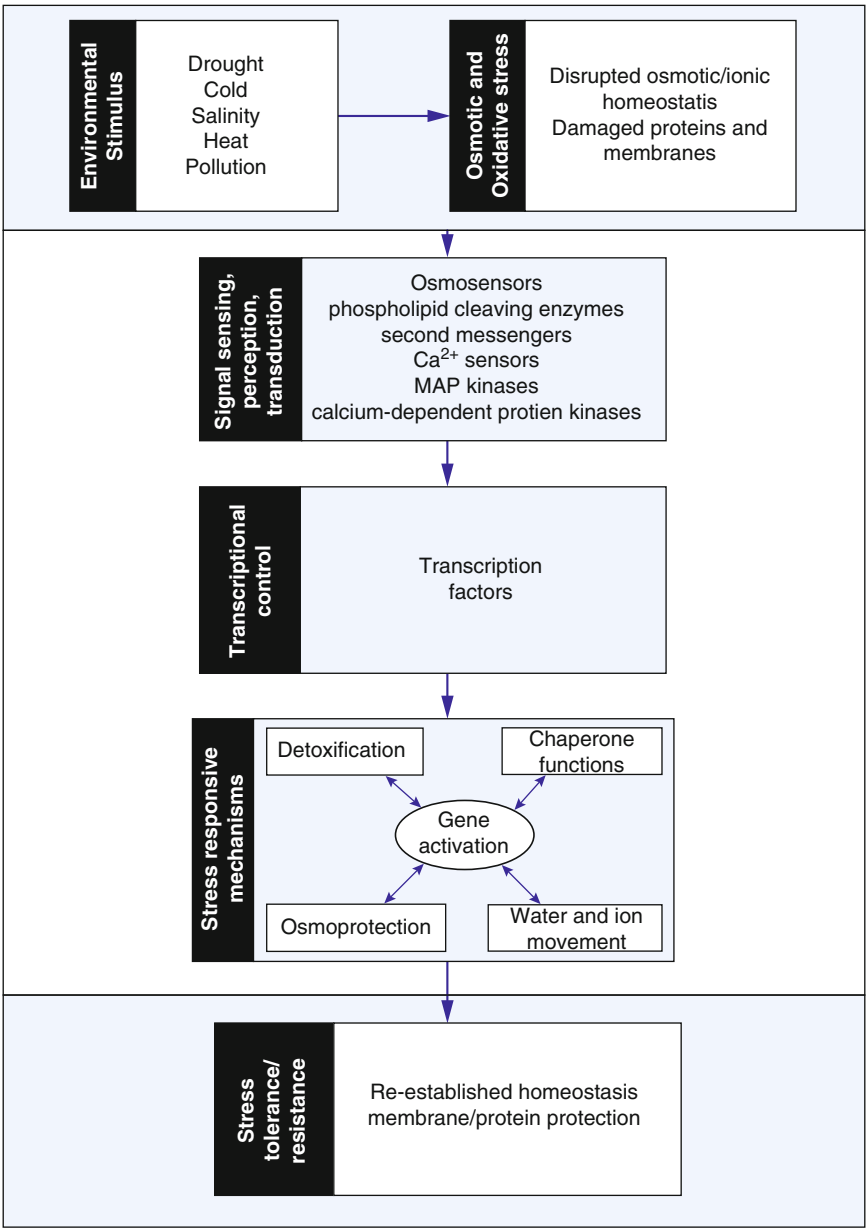

Fig. 2.1 Plant responses to abiotic stress

2.2.4 The Genetic Basis of Drought Tolerance

Expression studies have shown that drought-specific genes can be grouped into three major categories: (1) Genes involved in signal transduction pathways (STPs) and transcriptional control; (2) Genes with membrane and protein protection functions; and (3) Genes assisting with water and ion uptake and transport (Vierling 1991; Ingram and Bartels 1996; Smirnoff 1998; Shinozaki and Yamaguchi-Shinozaki 2000).

Plants adapt to drought conditions by tightly regulating specific sets of these genes in response to drought stress signals, which vary depending on factors such as the severity of drought conditions, other environmental factors, and the plant species (Wang et al. 2003b). To date, successes in genetic improvement of drought resistance have involved manipulation of a single or a few genes involved in signaling/regulatory pathways or that encode enzymes involved in these pathways (such as osmolytes/compatible solutes, antioxidants, molecular chaperones/osmoprotectants, and water and ion transporters; Wang et al. 2003b). The disadvantage of this is that there are numerous interacting genes involved, and efforts to improve crop drought tolerance through manipulation of one or a few of them is often associated with other, often undesirable, pleiotropic and phenotypic alterations (Wang et al. 2003b). These complex considerations, when coupled with the complexity of drought and the plant–environment interactions occurring at all levels of plant response to water deficit, illustrate that the task plant researchers are faced with in engineering drought resistant crops is dauntingly multi-faceted and extremely difficult.

2.2.5 Engineering Improved Drought Avoidance in Crops

Most transformation studies to improve plant drought resistance have produced transformants that display a variety of both tolerance and avoidance traits. An exception was demonstrated by Rivero et al. (2007) who manipulated a leaf senescence gene. Leaf senescence is an avoidance strategy and is accelerated in drought-sensitive plants to decrease canopy size. In crop plants, accelerated senescence is often associated with reduced yield and is thought to be the result of an inappropriately activated cell death program. Therefore, suppression of drought-induced leaf senescence in tobacco plants was investigated as a tool to enhance drought resistance. Transgenic plants were developed by expressing isopentyl transferase (IPT), a key enzyme in the biosynthesis of cytokinin (a leaf senescence inhibitor) under the control of the senescence-associated receptor protein kinase promoter (PSARK). The *SARK* gene, which is induced during late maturation and drought and decreased during senescence development, encodes a maturation/-sensescence-dependent protein kinase. Although transgenic plants wilted under a 15-day glasshouse drought stress treatment, senescence did not occur and a reduced

photosynthetic capacity was maintained. Following rewatering, transgenic plants recovered full leaf turgor and resumed growth and maximum photosynthetic capacity, while the control plants were unable to recover from the drought stress. Water use efficiency (WUE) of the transgenic plants was also markedly higher than wild-type (WT) plants, and was two to three times higher after rewatering than before the drought treatment. An experiment to assess whether the transgenic plants could produce significant yields under water-limiting conditions determined that biomass and seed yield were not as affected in transgenic plants than in WT controls, although this result was not significant (Rivero et al. 2007).

Other drought avoidance/whole-plant traits that have been investigated include stay-green and cuticular biosynthesis. Stay-green is a variable and quantitative trait, which generally refers to delayed senescence. It has not yet been used to successfully produce transgenic plants with increased drought resistance in the field. Cuticular biosynthesis was investigated by transgenic expression of *AtMYB41*, which encodes an R2R3-MYB transcription factor (TF) in *Arabidopsis*. *AtMYB41* is expressed at high levels in response to drought, abscisic acid (ABA; Sect. 2.2.6.1), and salt treatments, and was demonstrated to have a role in cell expansion and cuticle deposition. The transformation of *Arabidopsis* with *AtMYB41* was associated with undesirable pleiotropic phenotypes including dwarfism, enhanced sensitivity to desiccation, and enhanced permeability of leaf surfaces (Cominelli et al. 2008).

2.2.6 Improving Plant Drought Tolerance

2.2.6.1 Absicisic Acid and Transcriptional Regulation

The plant hormone ABA regulates the plant's adaptive response to environmental stresses such as drought, salinity, and chilling via diverse physiological and developmental processes. ABA has functional roles ranging from seed maturation processes to lateral root development (McCourt and Creelman 2008; Wasilewska et al. 2008). Under abiotic stress, ABA induces stomatal closure, reduces water loss via transpiration, and induces gene expression (Chandler and Robertson 1994). Gene expression and biochemical studies into ABA synthesis in *Arabidopsis* and some other model plants have largely elucidated the basic ABA biosynthetic pathway (Schwartz et al. 2003) and many of the key enzymes involved in ABA synthesis have been investigated transgenically in relation to improving plant drought tolerance. For example, transgenic *Arabidopsis* plants constitutively overexpressing the zeaxanthin epoxidase gene, *AtZEP*, which encodes an enzyme required for an initial step in ABA synthesis from isopentyl diphosphate (IPP) and β-carotene (Schwartz et al. 2003) showed increased tolerance to drought and salinity stress. The increased drought stress tolerance was attributed to increased leaf and lateral root development, longer primary roots, higher fresh weight, and increased survival compared with control plants following drought treatment.

Furthermore, compared with WT plants, the rate of water loss was lower, the levels of ABA were higher, the expression of stress responsive genes such as *Rd29a* was much higher, and stomatal aperture was smaller under salt and/or drought stress. Overall, the increased stress resistance was attributed to increased ABA levels in response to osmotic stress, which resulted in enhanced expression of ABA-responsive stress-related genes (Park et al. 2008).

Many of the drought stress response pathways that have been identified to date appear to be under transcriptional regulation and ABA plays a key role in this process (Fig. 2.2). Transcriptional regulation involves interaction between TFs and specific *cis*-acting elements located within or near the promoter region upstream of expressed genes. Figure 2.2 shows links between responses to low temperature and dehydration stress at the transcriptional level. It can be seen that ABA is involved in both types of abiotic stress.

Many transcriptional responses to drought stress have been well characterized and are classified as being ABA-dependent, ABA-independent, or both. ABA is

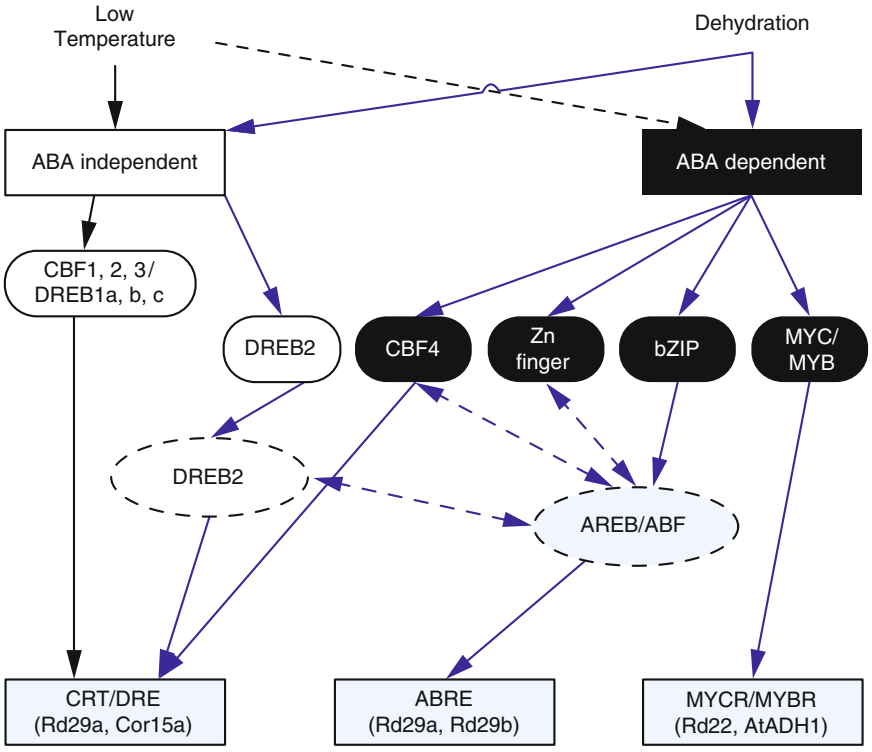

Fig. 2.2 Plant transcriptional processes induced by dehydration and low temperature stress. Displayed are transcription factors (*rounded rectangles*) both ABA-dependent (*shaded*) and ABA-independent (*unshaded*), posttranscriptional modification such as phosphorylation (*elipses*), transcription factor binding sites and representative promotors (*rectangles*), possible regulation points (*dotted arrows*), and possible cross-talk (*bidirectional arrows*)

often used in stress and stress acclimation studies because it is produced in response to stress. ABA induces expression of many genes that are also induced by drought, cold, and salinity when applied exogenously (Shinozaki and Yamaguchi-Shinozaki 1996). There are two types of ABA-dependent transcription. The "direct" pathway involves *cis*-acting ABA-responsive elements (ABREs), which are directly activated by binding with TFs such as basic-domain leucine zipper (bZIP)-type DNA-binding proteins (Shinozaki and Yamaguchi-Shinozaki 1996; Kobayashi et al. 2008). Alternatively, the "indirect" ABA-dependent transcription pathway involves other *cis*-acting elements, such as MYC and MYB. These elements are activated through binding with ABA- or drought-inducible TFs, such as basic helix–loop–helix (bHLH)-related protein AtMYC2 and an MYB-related protein, AtMYB2 (Abe et al. 2003). An example of the indirect pathway can be seen in the expression of *rd22* from *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 1996).

Some genes are induced by drought stress but are not expressed in response to exogenous ABA applications and these genes are the product of ABA-independent STPs. One such gene is *rd29a* (also known as *lti78* and *cor78*). Yamaguchi-Shinozaki and Shinozaki (Yamaguchi-Shinozaki and Shinozaki 1994) identified a dehydration-responsive element (DRE) in the promoter region of *rd29a* and the DRE-binding (DREB) protein transcription pathway has since been explored for its important roles in drought, cold, and salinity stress (Shinozaki and Yamaguchi-Shinozaki 1996; Qin et al. 2007). Several C-repeat (CRT) binding factor (CBF)/DREB proteins have now been identified from the promoter regions of other stress-inducible *Arabidopsis* genes, such as *cor15a*, *kin1*, *cor6.6* and *cor47*/*rd17,* and the CBF/DREB pathway has been shown to be conserved across species (Benedict et al. 2006; Pasquali et al. 2008). CBF/DREB1 and DREB2, belong to the ethylene-responsive element/apetela 2 (ERE/AP2) TF family; their expression is induced by cold or drought stress and both activate expression of genes possessing a CRT/DRE *cis*-element (Stockinger et al. 1997; Liu et al. 1998). Likewise, DREB2A positively regulates expression of many abiotic stress-related genes possessing DRE sequences in their 5'-upstream regions. *DREB2A* overexpression in *Arabidopsis* confers significant drought tolerance in transgenic plants (Sakuma et al. 2006a, b). DREB genes have been used in transformation of several crops, including wheat and rice, in attempts to increase drought tolerance (Chen et al. 2008; Kobayashi et al. 2008).

Although DREs are *cis*-acting elements that were first thought to activate ABA-independent stress-responsive gene expression, some are also implicated in ABA-dependent expression (Shinozaki and Yamaguchi-Shinozaki 2000). *CBF4* is an apparent homolog of the CBF/DREB1 proteins that is thought to be a critical regulator of gene expression in drought stress signal transduction. The action of CBF4 is thought to be through its binding with CRT/DRE elements in promoter regions of drought- and cold-inducible genes (Haake et al. 2002). *CBF4* gene expression has been shown to be upregulated in response to drought and ABA; however, constitutive expression of *CBF4* was found to result in expression of cold- and drought-induced genes under nonstress conditions and this was associated with retarded growth, shorter petioles, darker green leaves, and delayed time to

flowering in *Arabidopsis* seedlings (Haake et al. 2002). Another study showed that *CBF4* expression was induced by salt, but not by drought, cold, or ABA (Sakuma et al. 2002). Similar observations, and observations of higher levels of soluble sugars and proline, have been recorded during many *CBF* overexpression studies, which suggest that the use of constitutively expressed *CBF*/*DREB* genes may not be applicable to the development of crops with improved drought tolerance. It is thought that the use of stress-inducible promoters that have low expression levels under non-stress conditions could be used in conjunction with *CBF* genes to alleviate the retarded growth observed in CBF overexpression studies (Zhang et al. 2004).

Many studies have illustrated the potential of manipulating *CBF*/*DREB* genes to confer improved drought tolerance. For example, overexpression of *CBF1*/*DREB1B* from *Arabidopsis* was able to improve tolerance to water-deficit stress in tomato. Furthermore, when driven by three copies of an ABA-responsive complex (ABRC1) from the barley *HAV22* gene, transgenic tomato plants expressing *CBF1* exhibited enhanced tolerance to chilling, water deficit, and salt stress, and maintained normal growth and yield under normal growing conditions when compared with control plants (Lee et al. 2003a). Other studies have also found that expression of *CBF*/*DREB* genes under stress-inducible promoters result in transgenic plants that do not express detectable levels of these genes under non-stress conditions, minimizing growth retardation and other adverse effects (Al-Abed et al. 2007).

The CRT/DRE motif also acts as one of the binding sites for the ERF family of TFs (Trujillo et al. 2008). A novel ERF from sugarcane, *SodERF3*, was found to enhance salt and drought tolerance when overexpressed in tobacco plants. Under drought treatment, transgenic plants were significantly taller than controls and were able to flower under an extended growth period. Furthermore, the absence of observable differences in height, number of leaves, leaf area, leaf weight, and stalk weight between transgenic and control plants illustrates that this gene has potential for engineering drought stress tolerance in plants (Trujillo et al. 2008).

Other TFs involved in mediation of ABA-dependent and ABA-independent signal transduction and gene expression include NAC, WRKY, RING finger, and zinc-finger TFs (Seki et al. 2003; Zhang et al. 2004; Chen et al. 2006). Nelson et al. (2007) showed that constitutive expression of a TF from the nuclear factor (NF-Y) family, *AtNF-YB1*, which belongs to the CCAAT-binding TF family, improved performance of *Arabidopsis* under drought conditions. Consequently, an orthologous maize TF gene, *ZmNF-YB2*, was constitutively expressed in maize. Transgenic lines were exposed to both glasshouse-based and field-based drought stress treatments. Transgenic lines exhibited less wilting and faster recovery and re-established growth more rapidly than WT (on average) under glasshouse-based drought treatment. Transgenic lines subjected to field-based drought stress at the late vegetative stage exhibited superior health, higher chlorophyll indices and photosynthetic rates, lower leaf temperatures, higher stomatal conductance, and less yield reduction than WT plants. Furthermore, under favorable conditions, transgenic plants were greener, flowered 1–3 days earlier, and had slightly compressed internodes. Most importantly, the stress adaptation response contributed to a yield advantage in

transgenic maize grown within drought environments, suggesting that *ZmNF-YB2* has a realistic application for use in commercial agriculture under severe water-limiting conditions (Nelson et al. 2007).

Another TF that has been manipulated in order to increase plant drought tolerance is the *HARDY* (*HRD*) gene, which has been linked to increased transpiration efficiency related to stomatal adjustment. *HRD* is an AP2/ERF-like TF isolated from *hrd*-dominant (*hrd-D*) *Arabidopsis* mutants, which displayed vigorous rooting and dark green leaves that were smaller and thicker than WT plants. Karaba et al. (2007) isolated the *HRD* gene and constitutively expressed it in *Arabidopsis* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The thicker leaves, higher root density, and increased root strength were associated with abundant chloroplasts, increased secondary and tertiary root initiation and proliferation, and extra corticle cell layers and more compact, stele bearing vascular tissue, respectively. Furthermore, the mutants survived longer periods of drought stress and could reach full maturity under high levels of salt stress. *HRD* was also constitutively expressed in rice and transgenic plants displayed no reduction in growth, seed yield, or germination, but had significantly increased leaf canopy with more tillers under normal greenhouse conditions compared with WT controls. Under drought stress, the transgenic plants were of deeper green color (attributable to increased number of bundle sheath cells), displayed distinctive drought tolerance and lower stomatal conductance, had higher net carbon assimilation and photosynthetic rates, and possessed higher root biomass (Karaba et al. 2007).

Recently, a novel drought-tolerant gene, *HDG11*, which encodes a protein from the homeodomain (HD)-START TF family (also known as the Class IV HD-leucine zipper TF family) was identified in *Arabidopsis* and was found to confer drought resistance via enhanced root growth and decreased stomatal density when constitutively overexpressed in transgenic tobacco (Yu et al. 2008). The constitutive expression of the gene was not associated with retarded growth or any other observable deleterious phenotypic effects and, the gene was also shown to *trans*-activate a number of other genes involved in the drought stress response including *ERECTA* (Sect. 2.2.6.2.1; Yu et al. 2008). *SNAC1* from rice has also been shown to have *trans*-activation activity. NAC TFs comprise a large gene family with proteins exhibiting a highly conserved N-terminal DNA-binding domain and a diversified C-terminal domain. NAC was derived from the names of the first three described proteins containing the DNA-binding domain, namely, NAM (no apical meristem), ATAF1-2, and CUC2 (cup-shaped cotyledon; Souer et al. 1996; Aida et al. 1997). NAC is a plant-specific TF family with diverse roles in development and stress regulation. When constitutively overexpressed in rice, *SNAC1* was found to significantly improve plant resistance to severe drought stress during reproductive and vegetative growth and was not associated with any negative phenotypic effects or yield penalty (Hu et al. 2006). Transgenic plants were more sensitive to ABA and closed more stomata than WT plants but maintained continual photosynthetic activity. There was no difference between root morphologies of transgenic and WT plants indicating that the improved drought resistance was not related to increased root-water uptake.

The WRKY superfamily of plant TFs has a conserved sequence (WRKYGQK) at their N-terminal ends (Wu et al. 2008b). Transgenic rice seedlings, expressing *OsWRKY11* under the control of a rice heat shock protein (HSP) promoter, HSP101, were shown to survive longer and lose less water under a short, severe drought treatment, than WT plants (Wu et al. 2008b). A TFIIIA-type zinc-finger protein gene, *ZFP252*, was also found to confer improved drought stress resistance in rice. Young transgenic rice plants overexpressing *ZFP252* survived longer, displayed less relative electrolyte leakage, and accumulated more compatible osmolytes than WT plants or plants with *ZFP252* knocked out during a 14-day period of drought stress (Xu et al. 2008a). A salt- and drought-induced RING-finger protein, SDIR1, was found to confer enhanced drought tolerance to tobacco and rice (Zhang et al. 2008b). *Arabidopsis* E3 ligase SDIR1 is a positive regulator in ABA signal transduction. Tobacco and rice plants constitutively overexpressing the *SDIR1* gene displayed less leaf wilting and rolling, longer survival, and improved recovery under drought conditions than control plants. The mechanism of drought tolerance was thought to be due to decreased stomatal aperture, which increased transpiration efficiency of transgenic plants.

Some genes have been shown to suppress expression of drought-response transcription pathways. For example, Jiang et al. (2008) recently characterized *SAZ*, an *Arabidopsis* gene from the SUPERMAN (SUP) family of plant-specific zinc-finger genes, which encode proteins containing single C_2H_2-type zinc-finger motif with a conserved short amino acid sequence and a class II ERF-associated amphiphilic repression (EAR) motif-like TF domain at the carboxy-terminal region. *SAZ* was found to be rapidly downregulated in response to drought and other abiotic stresses and *SAZ* gene knockouts resulted in elevated expression of the ABA-responsive genes *rd29B* and *rab18* under stressed and unstressed conditions. This shows that gene knockouts and gene silencing may also be applicable to the development of crops with improved drought resistance.

2.2.6.2 Signal Sensing, Perception, and Transduction

Prior to transcriptional activation of genes, drought stress signals are received and messages conveyed to the appropriate components of the downstream pathway (Xiong and Ishitani 2006). In general, STPs involve perception of stress by specific receptor molecules, which vary in identity, structure, perception, signal relay mechanism, and location within the cell (Xiong and Ishitani 2006). Plant stress STPs often involve secondary messengers, which may modify signals (often via reversible protein phosphorylation) prior to conveying them from receptor molecules to the activators of the appropriate gene expression pathway (Xiong and Ishitani 2006). Other molecules may also be involved in stress STPs and the functions of these include recruitment and assembly of signaling complexes, targeting of signaling molecules, and regulation of signaling molecule lifespan (Xiong and Ishitani 2006).

The major molecules involved in drought stress signal sensing, perception, and transduction include receptor molecules/osmosensors, phospholipid-cleaving enzymes (PLEs), reactive oxygen species (ROS), mitogen-activated protein kinases (MAPK), and Ca^{2+} sensors.

Receptor Molecules/Osmosensors

Receptor molecules/osmosensors are the initial stress signal perceivers and they convey the signal to the appropriate molecule to initiate STPs. On the basis of analyses of plants and other species, receptor molecules are thought to include receptor-like kinases, two-component receptors, receptor tyrosine kinases, G-protein-coupled receptors, iontropic channel-related receptors, histidine kinases, and nuclear hormone receptors. Receptor molecules that have been identified to date in plants include: ROP10, a small G protein from the ROP family of Rho GTPases, that negatively regulates ABA response in *Arabidopsis* (Zheng et al. 2002); ATHK1, a putative homolog of the yeast SLN1, which is a functional histidine kinase feeding into the HOG MAPK pathway (Urao et al. 1999; Reiser et al. 2003); NtC7, a receptor-like membrane protein from tobacco (Tamura et al. 2003); and Cre1, a putative cytokinin sensor and histidine kinase from *Arabidopsis* (Reiser et al. 2003).

The *ERECTA* gene from *Arabidopsis* is a putative leucine-rich repeat receptor-like kinase (LRR/RLK). It was the first gene to be shown to act on the coordination between transpiration and photosynthesis (Masle et al. 2005). *ERECTA* was analyzed by its transgenic expression in null-mutants and was shown to have roles in lowering stomatal conductance, controlling leaf photosynthesis and organogenesis, modulation of cell expansion, cell division, cell–cell contact, cell–cell and tissue–tissue signaling, cell proliferation, and inflorescence differentiation. Owing to the range of traits attributed to *ERECTA* expression, *ERECTA* is thought to act as a master gene in transpiration regulation (Masle et al. 2005). No known studies have yet involved transgenic expression of *ERECTA* in economic crops; however, initial studies suggest that this gene may be useful in the design of crops with improved transpiration efficiencies, reduced stomatal limitations, and increased yield potentials.

Phospholipid-Cleaving Enzymes

PLEs degrade phospholipid membranes, catalyzing the release of lipid and lipid-derived secondary messengers (Chapman 1998; Sang et al. 2001). Phospholipases C (PLC) and D (PLD) are both involved in ABA-mediated signal transduction and drought stress tolerance perception in plants. Phosphatidic acid (PtdOH), a product of the PLC and PLD pathways, is also important in the signaling process (Bartels et al. 2007; Wang et al. 2008a).

Wang et al. (2008a) successfully produced maize plants constitutively overexpressing *ZmPLC1*, a phospholipase catalyzing the hydrolysis of 4,5-bisphosphate to form diacylglycerol (DAG) and inositol 1,4,5-trisphosphate, the products of which are the second messengers Ca^{2+} and PtdOH, respectively. This pathway is important in a wide variety of abiotic stress-responsive processes. Transgenic maize plants carrying the *ZmPLC1* gene were shown to have increased photosynthetic activity, reduced anthesis to silking interval (ASI; an indicator of maize yield potential), better recovery, and higher grain yield than WT plants when subjected to 21 days of drought stress at the ten-leaf stage. Because there was no significant difference between stomatal conductances of WT and transgenic plants, the higher photosynthetic rate was attributed to better photochemical activity rather than the improved guard cell signaling. This has also been demonstrated in other studies (Staxen et al. 1999; Hunt et al. 2003; Mills et al. 2004). Sang et al. (2001) showed that overexpression of PLD results in enhanced sensitivity of transgenic tobacco and plays a key role in controlling stomatal movements and plant response to water stress.

Reactive Oxygen Species

ROS are generated in plants as photoreaction and cellular oxidation byproducts under normal conditions and can cause cellular damage under water deficit when they accumulate to toxic levels. Some of these species also have important roles in early stress response through activation of cellular defense mechanisms and mitigation of cellular damage. While plant mechanisms must be in place to detoxify high levels of ROS that occur under drought, low levels of these beneficial ROS must also be maintained. Those ROS known to have important signaling roles in plant stress STPs include nitric oxide (NO) and hydrogen peroxide (H_2O_2).

Mitogen-Activated Protein Kinases

MAPKs are enzymes that catalyze reversible phosphorylations, important for relaying signals. They function via cascades, which involve sequential phosphorylation of a kinase by its upstream kinase (Xiong and Ishitani 2006). Recently, the MKK2 pathway was identified in *Arabidopsis* as having involvement in cold and osmotic stress signal transduction. An example of a MAPK having specific involvement in drought and salt stress is the p44MMK4 kinase from alfalfa (*Medicago sativa*; Jonak et al. 1996). Phosphatases involved in the sequential phosphorylation of MAPKs and other protein kinases are also important for stress signaling. For example, the ABI1 and AB12 proteins from *Arabidopsis* have been shown to act in a negative regulatory feedback loop of the ABA signaling pathway (Merlot et al. 2001). Some MAPK and MAPKK proteins have also been shown to activate the Rd29a stress pathway in *Arabidopsis* (Hua et al. 2006). Other protein kinases involved in stress signaling include calcium-dependent protein kinases (CDPKs),

kinases from the SNF1 family of protein kinases, and serine-threonine-type protein kinases (Xiong and Ishitani 2006; Bartels et al. 2007).

Ca^{2+} Sensors

Ca^{2+} sensors are important for coupling extracellular signaling to intercellular responses and comprise calmodulin (CaM) and CaM-related proteins (Sneddon and Fromm 1998; Sneddon and Fromm 2001), calcineurin B-like proteins (CBL; also known as SCaBP/SOS3-like calcium-binding proteins; Kudla et al. 1999), and CDPKs (Harmon et al. 2000). Ca^{2+} sensors that have been attributed with roles in drought tolerance in plants include the *CBL1* gene (Kudla et al. 1999) and the AtCAMBP25 protein (Perruc et al. 2004) from *Arabidopsis*.

2.2.6.3 Stress-Responsive Mechanisms

The outcome of stress signal perception, transduction, and transcriptional up- or downregulation of genes is the production of molecules with various plant protection, repair, and stabilization functions. These molecules can be broadly grouped into five functional groups: (1) detoxification; (2) chaperoning; (3) late embryogenesis abundant (LEA) protein functions; (4) osmoprotection; and (5) water and ion movement.

Detoxification

To prevent stress injury, cellular ROS need to remain at nontoxic levels under drought stress. Antioxidants involved in plant strategies to degrade ROS include: (1) enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase; and (2) nonenzymes such as ascorbate, glutathione, carotenoids, and anthocyanins (Wang et al. 2003b). Some proteins, osmolytes, and amphiphilic molecules also have antioxidative functionality (Bowler et al. 1992; Noctor and Foyer 1998).

Chaperoning

Chaperone functions involve specific stress-associated proteins, which are responsible for protein synthesis, targeting, maturation and degradation, and function in protein and membrane stabilization, and protein renaturation. HSPs, which can be divided into five conserved families, have been shown to have particularly important stress-related chaperone functions in plants (Hendrick and Hartl 1993; Boston et al. 1996; Hartl 1996; Waters et al. 1996; Torok et al. 2001). HSPs, which are induced by heat, have been implicated in plant cell protection mechanisms under drought stress. Protein denaturation occurs under drought stress because decreased

cellular volume increases the likelihood of degradative molecular interactions (Cho and Hong 2006). HSPs maintain or repair companion protein structure and target incorrectly aggregated and non-native proteins for degradation and removal from cells (Cho and Hong 2006). One such protein, *NtHSP70-1*, was constitutively overexpressed in tobacco to ascertain its role in plant drought response and tolerance (Cho and Hong 2006). The drought tolerance of transgenic seedlings was increased and their optimum water content was maintained after progressive drought stress (Cho and Hong 2006). Few other studies have involved transforming plants with HSPs; however, *HSP24* from *Trichederma harzianum* was found to confer significantly higher resistance to salt, drought, and heat stress when constitutively expressed in *Saccharomyces cerevisiae* (Liming et al. 2008).

Late Embryogenesis Abundant Protein Functions

LEA proteins are produced in response to dehydration stress and function in water status stabilization, protection of cytosolic structures, ion sequestration, protein renaturation, transport of nuclear targeted proteins, prevention of membrane leakage, and membrane and protein stabilization. LEA and LEA-type genes are found universally in plants. They accumulate in seeds during the late stages of embryogenesis and are associated with the acquisition of desiccation tolerance under drought, heat, cold, salt, and ABA stress (Sivamani et al. 2000; Bartels et al. 2007). They are also present in the biomass tissue of resurrection plants and are upregulated in many desiccation-sensitive plants in response to drought stress (Bartels et al. 2007). LEA proteins are divided into groups based on conserved sequence motifs (Zhang et al. 2000; Wise 2003). Five of these groups have been characterized at the molecular and structural level (Table 2.1); however, recent research indicates that additional groups of LEA and LEA-like proteins are still being identified (Park et al. 2003; Wang et al. 2006; March et al. 2007). Common

Table 2.1 The five groups of LEA proteins

LEA group	Description
Group 1	Contain a 20-amino acid motif and are represented by the wheat Em protein, for which gene homologs have been identified in a wide range of plant species
Group 2 (dehydrins)	The most extensively studied group. They contain a lysine-rich 15-amino acid motif (K-segment; EKKGIMDKIKEKLPG), which is predicted to form an amphipathic α-helix, a tract of contiguous serine residues and a conserved motif containing the consensus sequence DEYGNP in the N-terminal section of the protein
Group 3	Contain a characteristic repeat motif of 11 amino acids, which have been predicted to form an amphipathic α-helix with possibilities for intra- and inter-molecular interactions
Group 4	Have a conserved N-terminus, which is proposed to form α-helices and a diverse C-terminal region with a random coil structure
Group 5	Contain more hydrophobic residues than the other groups, are insoluble after boiling, and are likely to adopt a globular structure

Source: Bartels and Salamini (2001), Ramanjulu and Bartels (2002)

features of LEA proteins generally include hydrophilicity (Garay-Arroyo et al. 2000; Park et al. 2003), heat stability (Close and Gallagher-Ludeman 1989; Ceccardi et al. 1994; Houde et al. 1995; Thomashow 1998, 1999), and transcriptionally regulated and ABA-responsive gene expression (Close and Gallagher-Ludeman 1989). It is generally assumed that they play a role in water-deficit tolerance and the possible functions of LEA proteins include binding and replacement of water (Dure 1993), ion sequestration (Bray 1993), maintenance of protein and membrane structure (Baker et al. 1988), molecular chaperones (Close 1996), membrane stabilization (Koag et al. 2003), and nuclear transport of specific molecules (Goday et al. 1994). One class of LEAs, the dehydrins, which have detergent and chaperone-like properties, stabilize membranes, proteins, and cellular compartments (Close 1996).

LEA genes have been manipulated in many plants in order to increase drought resistance. For example, a wheat dehydrin, DHN-5, was ectopically overexpressed in *Arabidopsis* and transgenic plants displayed superior growth, seed germination rate, water retention, ion accumulation, more negative water potential, and higher proline contents than WT plants under salt and/or drought stress (Brini et al. 2007a).

The barley (*Hordeum vulgare* L.) group 3 LEA gene, *HVA1* was constitutively overexpressed in rice plants to increase drought tolerance. Transgenic plants displayed significantly increased tolerance to water deficit and salinity, which was associated with higher growth rates, delayed onset of stress damage symptoms, and improved recovery following stress removal (Xu et al. 1996). A more recent study involving the overexpression of this gene in rice showed that transgenic plants had significantly higher relative water content (RWC), improved turgor, less reduction in shoot and root growth, and improved cell membrane stability under prolonged drought conditions. It was found that *HVA1* did not function as an osmolyte and that membrane protection was the mechanism, which inferred drought resistance in rice plants (Chandra Babu et al. 2004). *HVA1* was also expressed in Basmati rice under control of either a constitutive rice promoter or a stress-inducible promoter. Transgenic plants exhibited increased stress tolerance in terms of cell integrity and growth, and it was found that inducible expression of *HVA1* resulted in transgenic plants that were able to grow normally under nonstress conditions (Rohila et al. 2002). Transgenic wheat plants expressing *HVA1* displayed more root fresh and dry weights, and shoot dry weight than WT plants under water-deficit conditions (Sivamani et al. 2000). Similarly, *HVA1* overexpressing transgenic mulberry, *Morus indica*, exhibited improved cellular membrane stability, photosynthetic yield, less photo-oxidative damage, and superior WUE than WT plants under salt and drought stress (Lal et al. 2008). The discussion of expression of *HVA1* in mulberry will be continued in Sect. 2.3.5.

Other group 3 LEA genes that have been manipulated in order to improve plant drought tolerance include a *Brassica napus* group 3 LEA gene, which conferred improved salt and drought tolerance when constitutively expressed in Chinese cabbage (Park et al. 2005a), and TaLEA3 from wheat, which increased RWC, leaf water potential, and relative average growth rate of transgenic plants compared to WT plants under drought stress when constitutively overexpressed in the perennial grass *Leymus chinensis* (Wang et al. 2008b). Two group 4 LEA proteins,

BhLEA1 and BhLEA2 from the resurrection plant *Boea hygrometrica*, conferred improved drought tolerance in transgenic tobacco. This was associated with plant cell protection and increased membrane and protein stability during dehydration (Liu et al.). A novel LEA gene from *Tamarix androssowii* also conferred increased drought tolerance when expressed in transgenic tobacco (Wang et al. 2006).

Osmoprotection

Osmoprotection involves the upregulation of compatible solutes (osmolytes) that function primarily to maintain cell turgor, but are also involved in antioxidation and chaperoning through direct stabilization of membranes and/or proteins (Yancey et al. 1982; Bohnert and Jensen 1996; Lee et al. 1997; Hare et al. 1998; McNeil et al. 1999; Diamant et al. 2001). Compatible solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations. The three major groups of compatible solutes are amino acids (such as proline), quaternary amines (glycine betaine (GlyBet), polyamines, and dimethylsulfoniopropionate), and polyol/sugars (such as mannitol, galactinol, and trehalose; Wang et al. 2003b). Many genes involved in the synthesis of these osmoprotectants have been explored for their potential in engineering plant abiotic stress tolerance (Vinocur and Altman 2005).

GlyBet and trehalose act as osmoprotectants by stabilizing quaternary structures of proteins and highly ordered states of membranes. Mannitol serves as a free-radical scavenger. Proline serves as a storage sink for carbon and nitrogen and a free-radical scavenger. It also stabilizes subcellular structures (membranes and proteins), and buffers cellular redox potential under stress. Many crops lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress tolerant organisms. It is believed that osmoregulation would be the best strategy for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity, and high temperature. Therefore, a widely adopted strategy to develop stress-tolerant crops has been to engineer or overexpress certain osmolytes in plants (Bhatnagar-Mathur et al. 2008).

GlyBet is a compatible solute that has been extensively studied for its role in drought stress response and increasing the levels of GlyBet in plants via genetic engineering has enhanced the drought tolerance of many model plants (Sakamoto and Alia 1998; Sakamoto and Murata 2000; Mohanty et al. 2002). A two-step enzymatic process accomplishes production of GlyBet in plants. The first step involves conversion of choline to betaine aldehyde by choline monoxygenase (CMO), a stromal enzyme with a Rieske-type (2Fe-2S) center (Brouquisse et al. 1989), and the second step involves betaine aldehyde dehydrogenase (BADH), a nuclear-encoded chloroplast stromal enzyme, which converts betaine aldehyde to GlyBet (Weigel et al. 1986). Quan et al. (2004) reported one of the first attempts to increase the GlyBet expression levels of maize by overexpressing the *betA* gene, which encodes choline dehydrogenase (CHO), another key enzyme in the choline–betaine aldehyde reaction (Zhang et al. 2008a). The study showed that

transgenic maize plants were more drought tolerant than WT plants at three different life stages, including the ten-leaf-flowering stage, and also that yields of transgenic plants were less affected by drought stress than WT.

Tobacco lacks GlyBet; however, it possess some BADH activity and the transfer of CMO is, therefore, a means of installing the GlyBet pathway in tobacco. Furthermore, because conversion of choline to GlyBet occurs in the chloroplast, it is also possible to use chloroplast genetic engineering to transfer CMO into GlyBet non-accumulators (Zhang et al. 2008a). Zhang et al. (2008a) transformed tobacco with a gene for CMO from beetroot via chloroplast genetic engineering and found that the transgenic plants accumulated GlyBet in leaves, roots, and seeds, and exhibited improved tolerance to toxic choline levels and salt and drought stress. GlyBet accumulation in the chloroplasts may be more effective than in other organelles, such as the nucleus, for abiotic stress protection because of protection and stabilization of chloroplast proteins, membrane, and photosynthesis systems under stress conditions (Zhang et al. 2008a).

Lv et al. (2007) found that transgenic cotton plants constitutively overexpressing *betA* had increased RWCs, increased photosynthesis, better osmotic adjustment, decreased percentage of ion leakage, decreased lipid membrane peroxidation, and increased yield in response to drought stress at the seedling, squaring, and anthesis stages.

Water and Ion Movement

Water and ions move through plants via transcellular and intracellular pathways. Aquaporins (major intrinsic proteins; MIPs), which are either tonoplast- (TIP) or plasma membrane- (PIP) localized, facilitate water, glycerol, small molecule, and gas transfer through membranes and, therefore, have a role in water homeostasis (Bartels et al. 2007). Active transport of solutes into the cell and cellular organelles, particularly the vacuole, is another means of cell turgor maintenance as increased solute potential facilitates the passive movement of water into cells and cellular compartments (Li et al. 2008).

Successful attempts made in engineering plants expressing genes for enzymes involved in proton pumps that generate energy for tonoplast transport of solutes into vacuoles include the overexpression of the *Arabidopsis* H^+-pyrophosphatase (H^+-PPase; *AVP1*) in *Arabidopsis* (Gaxiola et al. 2001), upregulation of *AVP1* in tomato (Park et al. 2005c), heterologous expression of the *Thellungiella halophila* vacuolar-H^+-PPase (V-H^+-PPase; *TsVP*) in tobacco (Gao et al. 2006), and overexpression of the wheat Na^-H^+ antiporter, *TNHX1*, and H^+-PPase, *TVP1*, in *Arabidopsis* (Brini et al. 2007b; Li et al. 2008). In all the cases, the transgenic plants displayed superior drought and/or salinity resistance compared with WT plants with resistance being attributed to mechanisms such as increased vacuolar H^+ to drive secondary uptake of ions into the vacuole and more enhanced development and robustness of root systems (Gaxiola et al. 2001; Li et al. 2005a; Park et al. 2005c; Gao et al. 2006; Brini et al. 2007a, b). Recently, Li et al. (2008) reported that

heterologous expression of the potassium-dependent *TsVP* gene from the halophyte *T. halophyta* in maize under the control of the *maize* ubiquitin promoter could infer drought tolerance. Under drought stress, transgenic plants had a higher percentage of seed germination, better-developed root systems, more biomass, increased solute accumulation, less cell membrane damage, less growth retardation, shorter ASI, and much higher grain yields than WT plants.

Attempts have also been made to improve drought tolerance of plants by altering the expression of aquaporins (Aharon et al. 2003; Porcel et al. 2005; Yu et al. 2005; Peng et al. 2006; Jang et al. 2007; Cui et al. 2008; Miyazawa et al. 2008; Zhang et al. 2008c). Aquaporins facilitate transport of water and other small solutes and ions across membranes via the apoplastic route (Aharon et al. 2003; Cui et al. 2008; Jang et al. 2007; Peng et al. 2006; Zhang et al. 2008c). Research into the role of aquaporins in plant drought tolerance has shown that various aquaporins function differently depending on the severity and type of stress. For example, some aquaporins, such as the *Arabidopsis Rd28*, and rice *RWC3*, are upregulated under drought stress and others, such as *NtQP1* and *AtPIP1*, remain unchanged under drought stress (Cui et al. 2008). Additionally, some aquaporins genes, such as *AtPIP1b* have been shown to diminish the drought tolerance capability of some plants, while others, such as the *Vicia faba PIP1*, *Panax ginseng PgTIP1*, *Brassica napus BnPIP1*, and *Brassica juncea BjPIP1*, have been shown to improve drought tolerance (Aharon et al. 2003; Yu et al. 2005; Peng et al. 2006; Cui et al. 2008; Zhang et al. 2008c). There is also evidence that overexpression of aquaporins in some plants causes them to respond differently to different stresses. For example, Jang et al. (2007) found that *Arabidopsis* and tobacco plants overexpressing *Arabiopsis PIP's* displayed enhanced water flow and improved germination under cold stress, but exhibited rapid water loss, retarded seedling growth, and inferior germination under drought conditions. It is therefore thought that different aquaporin isoforms are associated with different physiological processes and that plants respond to drought conditions either by increasing aquaporin expression, which facilitates water movement (especially into the tonoplast in order to maintain cell-turgor) or downregulating aquaporin expression to avoid excessive water loss (Aharon et al. 2003; Peng et al. 2006). Overexpression of aquaporins has also been implicated in conferring heavy metal tolerance to transgenic plants by alleviation of metal ion-induced water deficit and oxidative damage caused by metal ions (Zhang et al. 2008c).

2.3 Engineering Salt Tolerance in Plants

2.3.1 Impacts of Salinity on Agricultural Production

The damaging effects of salt accumulation in agricultural soils have severely affected agricultural productivity in large swathes of arable land throughout the world. Salt-affected land accounts for more than 6% of the world's total land area

(FAO 2009c) and is distributed largely amongst coastal salt marshes or inland desert sands. These have primarily arisen naturally through mineral weathering (which leads to the release of soluble salts such as chlorides of calcium, magnesium and sodium, and, to a lesser extent, sulfates and carbonates) or wind and rain deposition of oceanic water (Szabolcs 1989; Munns and Tester 2008; FAO 2009b).

Secondary salinization occurs when irrigation and tree clearing of agricultural land cause water tables to rise and concentrate salts in the root zone (Rengasamy 2006). Approximately 20% of the world's irrigated land, from which one-third of the world's food supply is produced, is presently affected by salinity (Ghassemi et al. 1995). With the expected increase in world population, the loss of arable land due to salinity presents a serious challenge to food sustainability and productivity.

Removal of salts from the root zone (reclamation) is perhaps the most effective way to ameliorate the detrimental effects of salinity; however, this is a slow and expensive process. The use of plant breeding and genetic engineering technologies to alter the salt tolerance of crops will, therefore, play an important role in maintaining global food production in the future.

2.3.2 *Improving Salinity Tolerance of Agricultural Crops*

Plants have evolved a complex adaptive capacity to perceive and respond to salt stress. The existence of salt-tolerant flora (halophytes) and differences in salt tolerance between genotypes within the salt-sensitive plant species (glycophytes) give rise to the belief that salt tolerance has a genetic basis (Yamaguchi and Blumwald 2005).

As for drought, efforts to improve the salt tolerance of crops have met with limited success because of the physiological and genetic complexity of the trait. Salinity tolerance is a multi-genic trait, with quantitative trait loci (QTL) identified in barley, wheat, soybean, citrus, rice, and tomato (Flowers and Flowers 2005; Jenks et al. 2007). Genetic approaches currently being used to improve salinity tolerance include the exploitation of functional genomics, bioinformatics, and natural genetic variations, either through direct selection in stressful environments or through the mapping of QTLs and subsequent marker-assisted selection (Yamaguchi and Blumwald 2005), or the generation of transgenic plants (Vij and Tyagi 2007).

2.3.3 *Physiological Effects of Salinity on Plants and Salinity Tolerance Mechanisms*

Salinity imposes a variety of stresses on plant tissues. Two of these are osmotic stress, which results from the relatively high soil solute concentrations, and ion

cytotoxicity. The decreased rate of leaf growth that occurs after an increase in soil salinity is primarily due to the osmotic effect of the salt around the roots, which inhibits plant water uptake and causes leaf cells to lose water. However, this loss of cell volume and turgor is transient and reductions in cell elongation and also cell division lead to slower leaf appearance and smaller final size over the longer term (Bartels and Sunkar 2005; Munns and Tester 2008).

Under prolonged salinity stress, inhibition of lateral shoot development becomes apparent within weeks and, within months, there are effects on reproductive development, such as early flowering and reduced floret number. Concomitantly, older leaves may die while the production of younger leaves continues. The cellular and metabolic processes involved are similar to those occurring in drought-affected plants and are responses to the osmotic effect of salt (Yeo et al. 1991; Munns and Tester 2008).

Ion cytotoxicity occurs when salt accumulates to toxic concentrations in fully expanded leaves (which, unlike younger leaves, are unable to dilute high salt concentrations), causing leaf death. Replacement of K^+ by Na^+ in biochemical reactions leads to conformational changes and loss of protein function, as Na^+ and Cl^- ions penetrate hydration shells and interfere with noncovalent interactions between amino acids. If the rate of leaf death generated by ion cytotoxicity is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate requirement of young leaves, which further reduces their growth rate (Munns and Tester 2008).

Halophytes, though taxonomically widespread, are relatively rare amongst the flowering plants and virtually all crop plants are glycophytes (Flowers and Flowers 2005). However, there is considerable variability in the tolerance of glycophytes to salt. Munns and Tester (2008), categorize salinity tolerance under three broad categories: (1) Tolerance to osmotic stress, which immediately reduces cell expansion in root tips and young leaves, and causes stomatal closure; (2) Na^+ exclusion from leaf blades, which ensures that Na^+ remains at nontoxic concentrations within leaves; and (3) Tissue tolerance to Na^+ or Cl^-, which requires compartmentalization of Na^+ and Cl^- at the cellular and intracellular level to avoid accumulation of toxic concentrations within the cytoplasm.

2.3.4 Salt Tolerance Using Transgenic Approaches

2.3.4.1 Osmoprotectants

Osmoprotectants were discussed previously (Sect. 2.2.6.3.4) in relation to their use in developing drought-tolerant crops and the transfer of GlyBet intermediates have improved the drought and salt tolerance of transgenic plants in many cases. Mohanty et al. (2002) demonstrated that *Agrobacterium*-mediated transformation of an elite *indica* rice cultivar to increase GlyBet synthesis through the incorporation of the *codA* gene, which encodes choline oxidase, was an effective way to

improve salinity tolerance. Challenge studies performed with R1 plants by exposure to salt stress for one week, followed by a recovery period, revealed that in some cases more than 50% of the transgenic plants could survive salt stress and set seed whereas WT plants failed to recover. A more recent example of enhanced GlyBet synthesis experiments involved transformation of maize with the *BADH* gene, introduced by the pollen-tube pathway (Wu et al. 2008a). Transgenic lines were examined for tolerance to NaCl by induced salt stress and, after 15 days of treatment, most transgenic seedlings survived and grew well, whereas WT seedlings wilted and showed loss of chlorophyll.

The amino acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses (Kavi Kishore et al. 2005; Ashraf and Foolad 2007). In plants, the precursor for proline biosynthesis is l-glutamic acid. Two enzymes, pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), play major roles in the proline biosynthetic pathway (Delauney and Verma 1993; Ashraf and Foolad 2007). Su and Wu (2004) established that the rate of growth of transgenic rice plants expressing mothbean Δ^1-pyrroline-5-carboxylate synthetase (*p5cs*) cDNA under either a constitutive or stress-inducible promoter led to the accumulation of *p5cs* mRNA and proline in third-generation (R2) transgenic rice seedlings. Significantly higher salinity and water-deficit stress tolerance of R2 seedlings were attributed to faster growth of shoots and roots in comparison with non-transformed plants. Stress-inducible expression of the *p5cs* transgene showed significant advantages over constitutive expression in increasing the biomass production of transgenic rice grown in soil under stress conditions. The osmoprotectant role of proline has been verified in other plants such as potato, where salt tolerance, measured by comparing tuber yield of transgenic lines cultivated in a greenhouse and watered with saline water to that of plants watered with normal tap water, had a less significant effect on tuber yield of transgenic plants than WT (Hmida-Sayari et al. 2005).

Polyamines, including spermidine (Spd, a triamine), spermine (Spm, a tetramine), and their obligate precursor putrescine (Put, a diamine), are aliphatic amines widely present in living organisms. The polyamine biosynthetic pathway is depicted in Fig. 2.3. Recently, it has been demonstrated that plant polyamines are involved in the acquisition of tolerance to such stresses as high and low temperatures, salinity, hyperosmosis, hypoxia, and atmospheric pollutants (Liu et al. 2007). Furthermore, genetic transformation of several plant species with polyamine biosynthetic genes encoding arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), or Spd synthase (SPDS) led to improved environmental stress tolerance (Liu et al. 2007). He et al. (2008) tested transgenic apple engineered with (SPDS)-overexpressing transgenic European pear *(Pyrus communis* L. "Ballad") for changes in enzymatic and nonenzymatic antioxidant capacity in response to NaCl or mannitol stress. Their research revealed that transgenic plants accumulated more Spd than WT. The transgenic line contained higher antioxidant enzyme activities (less malondialdehyde and H_2O_2) than the WT, implying that it suffered from less injury and enhanced enzymatic and nonenzymatic antioxidant capacity (He et al. 2008).

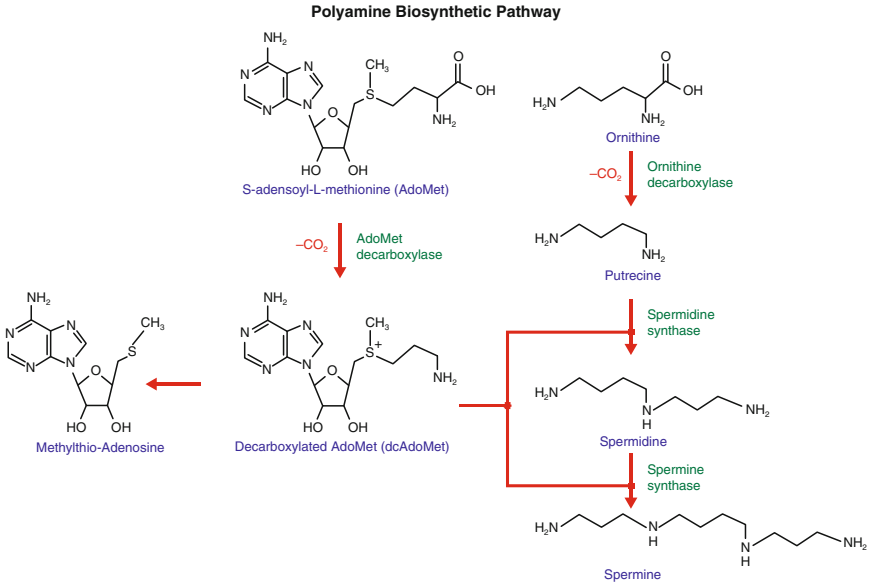

Fig. 2.3 Polyamine biosynthesis

Mannitol is a primary photosynthetic product that is associated with exceptional salt tolerance. In celery, mannitol metabolism is clearly altered by salt stress, with several lines of evidence indicating a connection between mannitol and salt tolerance, including increases in the capacity for mannitol biosynthesis and accumulation and decreases in catabolism (Williamson et al. 2002). Sickler et al. (2007) showed that *Arabidopsis* plants transformed with celery's mannose-6-phosphate reductase (*M6PR*) gene produced mannitol and grew normally in the absence of stress. However, in the presence of salt stress, daily analysis of the increase in growth (fresh and dry weight, leaf number, leaf area per plant, and specific leaf weight) over a 12-day period showed less effect of salt on transformants than WT plants. The daily energy use efficiency for photochemistry by photosystem 2 (PSII) was also measured and demonstrated that, unlike transformed plants, which were not affected, WT plants treated with 100 mM NaCl displayed a reduction in PSII yield after 6 days with a 50% loss in yield after 12 days. Similarly, under atmospheric levels of CO_2, growth with 200 mM NaCl caused an increase in sub-stomatal levels of CO_2 in WT plants but not in transformants.

Trehalose is a disaccharide sugar widely distributed in bacteria, fungi, insects, plants and invertebrate animals. In microbes and yeast, trehalose is produced from glucose by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), and functions in sugar storage, metabolic regulation, and protection against abiotic stress (Strom and Kaasen 1993; Wiemken 1990). Trehalose acts as a compatible solute, protecting membranes and proteins and conferring desiccation

tolerance on cells in the absence of water (Crowe et al. 1984). Ge et al. (2008) illustrated the protective role of trehalose in higher plants. Expression analysis demonstrated that *OsTPP1* isolated and cloned from rice, was initiated and transiently upregulated after salt, osmotic, and ABA treatments but slowly upregulated under cold stress. *OsTPP1* overexpression in rice enhanced salt and cold stress tolerance. Tolerance of transgenic plants to abiotic stress was examined by observing 2-week-old seedlings exposed to salt. Following one week of exposure, seedlings exhibited salt-induced damage symptoms such as wilted leaves. However, after prolonged salt treatment, transgenic lines were more vigorous and displayed increased leaf greenness and viability over control plants.

Generally, two broad themes have emerged from the results of attempts to engineer overexpression of osmoprotectants. The first is that metabolic limitations have been encountered in generating absolute levels of target osmolytes, especially when compared with salt-tolerant halophytes and the second is that the degree to which transformed plants are able to tolerate salinity stress is not necessarily correlative with the levels of osmoprotectants attained.

2.3.4.2 Transporter Genes

Mechanisms that confer salt tolerance vary with the plant species; however, the ability to maintain low cytosolic Na^+ is thought to be one of the key determinants of plant salt tolerance (Tester and Davenport 2003). Salt "inclusion" and "exclusion" are recognized as different mechanisms by which higher plants tolerate salinity. The functional removal of Na^+ from the cytoplasm of plant cells and the maintenance of low cytosolic Na^+ concentrations under salinity conditions (Blumwald et al. 2000) is accomplished by either pumping Na^+ out of cells (plasma membrane antiporter) or into vacuoles (vacuolar antiporter) in exchange for H^+. Na^+/H^+ antiporter activity is driven by the electrochemical gradient of protons (H^+) generated by the H^+ pumps (H^+-ATPase) in the plasma membrane or the tonoplast (Chinnusamy and Zhu 2003; Tester and Davenport 2003). In *Arabidopsis,* active exclusion of Na^+ is mediated by the plasma membrane-localized Na^+/H^+ antiporter, AtSOS1 (Shi et al. 2003). In contrast, the sequestration of excess Na^+ into the vacuole is mediated by the vacuolar membrane-localized Na^+/H^+ antiporter, AtNHX1 (Gaxiola et al. 1999; Shi et al. 2008). In a similar way, overexpression of the *S. cerevisiae HAL1* gene (Gaxiola et al. 1992) conferred salt tolerance in yeast by increasing intracellular K^+ and decreasing Na^+ levels.

The successful use of transporter genes has been demonstrated in several plants. He et al. (2005) created transgenic cotton plants expressing *AtNHX1* and found that transgenic plants generated more biomass and produced more fibers under salt stress in a greenhouse. It was suggested that the increased fiber yield was due to superior photosynthetic performance and higher nitrogen assimilation rates observed in the transgenic plants compared with WT. Interestingly, the researchers demonstrated that field-grown irrigated *AtNHX1*-expressing cotton plants produced higher fiber yields (fiber plus seeds) than WT, with an average increase of more

than 25% per line. Furthermore, the fibers produced by transgenic plants were generally more uniform, stronger, and longer than those of WT. Similarly, Chen et al. (2007) engineered maize plants overexpressing the rice *OsNHX1* gene. Transformants accumulated more biomass under greenhouse-based salt stress. Higher Na^+ and K^+ content was observed in transgenic leaves than in WT when treated with 100–200 mM NaCl, while the osmotic potential and the proline content in transgenic leaves was lower than in WT. Salt stress field trials revealed that the transgenic maize plants produced higher grain yields than WT plants at the vegetative growth stage.

Biochemical studies suggest that Na^+/H^+ exchangers in the plasma membrane of plant cells contribute to cellular sodium homeostasis by transporting Na^+ ions out of the cell (Qiu et al. 2002). *SOS*1 encodes a plasma membrane Na^+/H^+ exchanger in *Arabidopsis* (Qiu et al. 2002) and the important role of the plasma membrane Na^+/H^+ exchangers for plant salt tolerance was supported by the finding that overexpression of *SOS*1 improved plant salt tolerance (Shi et al. 2003). Zhao et al. (2006) demonstrated that expressing the plasma membrane Na^+/H^+ antiporter *SOD2* from yeast (*Schizosaccharomyces pombe*) in transgenic rice also increased salt tolerance. Transgenic plants accumulated more K^+, Ca^{2+}, and Mg^{2+} and less Na^+ in their shoots compared with non-transformed controls. Moreover, measurements on isolated plasma membrane vesicles derived from the *SOD2* transgenic rice plant roots showed that the vesicles had enhanced P-ATPase hydrolytic activity as well as being able to maintain higher levels of photosynthesis and root proton exportation capacity. Martinez-Atienza et al. (2007) identified an *AtSOS1* homolog, *OsSOS1*, in rice, which demonstrated a capacity for Na^+/H^+ exchange in plasma membrane vesicles of yeast (*S. cerevisiae*) cells and reduced their net cellular Na^+ content. *OsSOS1* was also shown to suppress the salt sensitivity of an *sos1-1* mutant of *Arabidopsis*.

In relation to the introduction of genes that modulate cation transport systems, many researchers have sought to employ the overexpression of the *S. cerevisiae HAL1* gene, which has conferred salt tolerance in yeast by facilitating intracellular K^+ accumulation and decreasing intracellular Na^+ (Gaxiola et al. 1992; Rios et al. 1997). Rus et al. (2001) established ectopic expression of *HAL1* in transgenic tomato plants, and showed that transformants were able to minimize the reduction in fruit production caused by salt stress. Maintenance of fruit production by transgenic plants was correlated with enhanced growth under salt stress of calli derived from the plants. The *HAL1* transgene enhanced water and K^+ contents in leaf calli and leaves in the presence of salt, which indicates that, similar to the yeast gene, plant *HAL1* functions by facilitating K^+/Na^+ selectivity under salt stress. Ellul et al. (2003), utilizing an optimized *Agrobacterium*-mediated gene transfer protocol, developed *HAL1*-expressing watermelon (*Citrullus lanatus*). Salt tolerance of transgenic plants was confirmed in a semi-hydroponic system on the basis of the higher growth performance of transgenic lines compared to control plants. The halotolerance observed supports the potential usefulness of the *HAL1* gene as a molecular tool for genetic engineering salt-stress protection in other crop species.

2.3.4.3 Detoxifying Genes

The mechanisms of plant detoxification of ROS under drought stress were introduced in Sects. 2.2.6.2 and 2.2.6.2.3. As an antioxidant enzyme, glutathione peroxidase (GPX) reduces hydroperoxides in the presence of glutathione to protect cells from oxidative damage, including lipid peroxidation (Maiorino et al. 1995). Gaber et al. (2006) generated transgenic *Arabidopsis* plants overexpressing GPX-2 genes in cytosol (*AcGPX2*) and chloroplasts (*ApGPX2*). The activities toward α-linolenic acid hydroperoxide in *ApGPX2*- and *AcGPX2*-expressing plants were 6.5–11.5 and 8.2–16.3 nmol min^{-1} mg $protein^{-1}$, respectively, while no activity was detected in the WT plants. Both transgenic lines showed enhanced tolerance to oxidative damage caused by the treatment with H_2O_2, Fe ions, or methylviologen (MV) and environmental stress conditions, such as chilling with high light intensity, high salinity or drought. The degree of tolerance of the transgenic plants to all types of stress was correlated with the levels of lipid peroxide suppressed by the overexpression of the GPX-2 genes.

SOD is the first enzyme in the enzymatic antioxidative pathway and halophytic plants, such as mangroves, reported to have a high level of SOD activity. SOD plays a major role in defending mangrove species against severe abiotic stresses. Prashanth et al. (2008) further characterized the *Sod1* cDNA (a cDNA encoding a cytosolic copper/zinc SOD from the mangrove plant *Avicennia marina*) by transforming it into rice. Transgenic plants were more tolerant to MV-mediated oxidative stress in comparison to WT and withstood salinity stress of 150 mM of NaCl for a period of 8 days while WT plants wilted at the end of the hydroponic stress treatment. Pot-grown transgenic plants tolerated salinity stress better than the WT when irrigated with saline water.

In plant cells, APXs are directly involved in catalyzing the reduction of H_2O_2 to water, which is facilitated by specific electron donation by ascorbic acid. APXs are ubiquitous in plant cells and are localized in chloroplasts (Takahiro et al. 1995), peroxisomes (Shi et al. 2001), and cytosol (Caldwell et al. 1998). Xu et al. (2008b) transformed *Arabidopsis* plants with a pAPX gene from barley (*HvAPX1*). The transgenic line was found to be more tolerant to salt stress than the WT. There were no significant differences in Na^+, K^+, Ca^{2+}, and Mg^{2+} contents and the ratio of K^+ to Na^+ between pAPX3 and WT plants, which indicated that salt tolerance in transgenic plants was not due to the maintenance and re-establishment of cellular ion homeostasis. However, the degree of H_2O_2 and lipid peroxidation (measured as the levels of malondialdehyde) accumulation under salt stress was higher in the WT than in transgenic plants. The mechanism of salt tolerance in transgenic plants was explained by a reduction of oxidative stress injury.

Apart from catalase and various peroxidases and peroxiredoxins (Dietz 2003), four enzymes, APX, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase (GR), are involved in the ascorbate-glutathione cycle, a pathway that allows the scavenging of superoxide radicals and H_2O_2 (Asada 1999). Most of the ascorbate-glutathione cycle enzymes are located in the stroma, cytosol, mitochondria, and peroxisomes (Jimenez et al. 1998). APX and GR, the first and

last enzymes in this cycle, respectively, are responsible for H_2O_2 detoxification in green leaves (Foyer et al. 1994). GR has a central role in maintaining the reduced glutathione (GSH) pool during stress (Pastori et al. 2000).

Lee and Jo (2004) introduced *BcGR1*, a Chinese cabbage gene that encodes cytosolic GR into tobacco plants via *Agrobacterium*-mediated transformation. Homozygous lines containing *BcGR1* were generated and tested for their acquisition of increased tolerance to oxidative stress. When ten-day old transgenic tobacco seedlings were treated with 5 to 20 μM MV, they showed significantly increased tolerance compared to WT seedlings. The most drastic difference was observed at a concentration of 10 μM MV. In addition, when leaf discs were subjected to MV, the transgenic plants were less damaged than the WT with regard to their electrical conductivity and chlorophyll content.

2.3.5 Late Embryogenesis Abundant (LEA) Proteins

The LEA proteins were introduced in Sect. 2.2.6.3.3 in relation to their use in improving plant drought tolerance. These proteins have also been used in engineering salt-tolerant crops. Park et al. (2005b) introduced a *B. napus* LEA protein gene, *ME-leaN4* (Wakui and Takahata 2002) into lettuce (*Lactuca sativa* L.) using *Agrobacterium*-mediated transformation. Transgenic lettuce demonstrated enhanced growth ability compared with WT plants under salt- and water-deficit stress. After 10-day growth under hydroponic 100 mM NaCl conditions, average plant length and fresh weight of transgenic lettuce were higher than those of WT and the increased tolerance was also reflected by delayed leaf wilting caused by water-deficit stress.

Brini et al. (2007a) analyzed the effect of ectopic expression of dehydrin (Dhn-5; Table 2.1) cDNA in *Arabidopsis* under salt and osmotic stress. When compared to WT plants, the Dhn-5-expressing transgenic plants exhibited stronger growth under high concentrations of NaCl or water deprivation, and showed a faster recovery from mannitol treatment. Leaf area and seed germination rate decreased much more in WT than in transgenic plants subjected to salt stress. Moreover, the water potential was more negative in transgenic than in WT plants and the transgenic lines had higher proline contents and lower water loss rates under water stress. Na^+ and K^+ also accumulated to a greater extent in the leaves of the transgenic plants.

Lal et al. (2008) reported the effects of overexpression of the *HVA1* gene in mulberry under a constitutive promoter. *HVA1* is a group 3 LEA (Table 2.1) isolated from barley aleurone layers and has been found to be inducible by ABA. Transgenic plants were subjected to simulated salinity and drought stress conditions to study the role of *HVA1* in conferring tolerance. Using leaf discs as explants, growth performance under salt-stress and water-deficit conditions were carried out from 8- to 10-month-old transgenic plants. Leaf discs of uniform size were cut and used for simulated salt and water-deficit stress treatments for different durations. After the stress treatments, leaf discs were analyzed for proline content,

photosynthetic yield, RWC, and cellular membrane stability (CMS). Transgenic plants showed better CMS, photosynthetic yield, less photooxidative damage, and better WUE as compared with the nontransgenic plants under both salinity and drought stress. Under salinity stress, transgenic plants showed a manyfold increase in proline concentration over WT plants and, under water-deficit conditions, proline accumulated only in the nontransgenic plants. Results also indicated that the production of *HVA1* proteins enhanced the performance of transgenic mulberry by protecting plasma and chloroplast membrane stability under abiotic stress.

2.3.6 Transcription Factors

The importance of TFs in plant stress response was discussed in Sect. 2.2.5. In addition to binding to *cis*-acting elements in the promoters of environmental stress-responsive genes, TFs can activate and repress gene expression through interactions with other TFs, thus playing a central role in plant response to environmental stresses (Chen and Zhu 2004). It is generally accepted that activation or ectopic expression of a specific TF can result in expression of many functional genes related to stresses.

CBF/DREBs are key regulatory factors that function primarily in freezing tolerance by activating a network of target genes (Fowler and Thomashow 2002; Maruyama et al. 2004). Oh et al. (2007) isolated a barley gene, *HvCBF4,* whose expression is induced by low temperature stress. Transgenic overexpression of *HvCBF4* in rice resulted in an increase in tolerance to drought, high-salinity, and low temperature stresses without stunting growth. Interestingly, under low temperature conditions, the maximum photochemical efficiency of PSII in the dark-adapted state in *HvCBF4* plants was higher by 20 and 10% than that in non-transgenic and *CBF3*/*DREB1A*-expressing plants, respectively. Using the 60K Rice Whole Genome microarray, 15 rice genes were identified that were activated by HvCBF4. When compared with 12 target rice genes of CBF3/DREB1A, 5 genes were common to both HvCBF4 and CBF3/DREB1A, and 10 and 7 genes were specific to HvCBF4 and CBF3/DREB1A, respectively. Results suggested that *CBF*/*DREBs* of barley acted differently from those of *Arabidopsis* in transgenic rice.

The NAC family of TFs (Sect. 2.2.6.1) has applicability for generating salt-tolerant crops. Hu et al. (2008) characterized a stress-responsive NAC gene (*SNAC2*) isolated from upland rice for its role in stress tolerance. Northern blot and *SNAC2* promoter activity analyses demonstrated that *SNAC2* expression was induced by drought, salinity, cold, wounding and ABA treatment. The *SNAC2* gene was overexpressed in *japonica* rice to test the effect on improving stress tolerance. To test salinity tolerance, germinated positive transgenic and WT seeds were transplanted on Murashige and Skoog (MS) medium containing 150 mM NaCl and the normal MS medium without NaCl as a control. Under saline conditions, transgenic seedlings grew faster and their shoots were significantly longer than WT after 20 days. However, there was no difference in root length or root numbers

between transgenic and WT seedlings grown under saline conditions and no difference in growth performance was observed between transgenic and WT seedlings in the normal MS medium. Hu et al. (2008) also evaluated germination ability of transgenic lines harboring *SNAC2* under salt-stress conditions. After 4 days of germination on the medium containing 150 mM NaCl, only 40% of WT seeds were poorly germinated, whereas more than 70% of transgenic seeds germinated efficiently. In the MS medium, more than 90% of both transgenic and WT seeds germinated well and there was no significant difference in germination rates, suggesting that overexpression of *SNAC2* does not affect seed germination under normal conditions. The significantly higher germination rate of transgenic seeds than that of WT under saline conditions further supported the improved salt tolerance of *SNAC2*-overexpressing plants. DNA chip profiling analysis of the transgenic plants revealed many upregulated genes related to stress response and adaptation such as peroxidase, ornithine aminotransferase, heavy metal-associated protein, sodium/hydrogen exchanger, HSP, GDSL-like lipase, and phenylalanine ammonia lyase. This data suggests that SNAC2 is a novel stress-responsive NAC TF that possesses potential utility in improving stress tolerance of rice.

The TFIIIA-type zinc-finger proteins, first discovered in *Xenopus*, represent an important class of eukaryotic TFs (Miller et al. 1985). More than 40 TFIIIA-type zinc-finger protein genes have been identified from various plants, including petunia, soybean, *Arabidopsis,* and rice (Kim et al. 2001; Sugano et al. 2003; Mittler et al. 2006; Huang and Zhang 2007) and these genes have been shown to be induced by various abiotic stresses. Xu et al. (2008a) have recently reported the functional analysis of *ZFP252* (a salt and drought stress responsive TFIIIA-type zinc-finger protein gene from rice), using gain- and loss-of-function strategies. They discovered that overexpression of *ZFP252* in rice increased the amount of free proline and soluble sugars, elevated the expression of stress defense genes, and enhanced rice tolerance to salt and drought stresses compared with *ZFP252* antisense and non-transgenic plants. Their findings suggest that *ZFP252* plays an important role in rice response to salt and drought stresses and is useful in engineering crop plants with enhanced drought and salt tolerance (Xu et al. 2008a).

2.3.6.1 Signal Transduction Genes

Plant salt-stress-response genes are involved in many plant cellular processes, including physiological metabolism, cell defense, energy production and transportation, ion transfer and balance, and cell growth and division. These genes function through certain coordination mechanisms to maintain the normal growth of plants under salt stress. As discussed previously, components of the STP may also be shared by various stress factors such as drought, salt, and cold (Shinozaki and Yamaguchi-Shinozaki 1999).

Signal molecules, H_2O_2 and NO, are involved in the ABA-induced stomatal closure and gene expression and activities of antioxidant enzymes (Zhang et al. 2006, 2007a). ABA-induced H_2O_2 production mediates NO generation, which in

turn activates MAPK and results in upregulation of the expression and activities of antioxidant enzymes (Zhang et al. 2007a). The importance of ABA in plant environmental stress responses was discussed in Sect. 2.2.6.1. The oxidative cleavage of *cis*-epoxycarotenoids by 9-*cis*-epoxycartenoid dioxygenase (NCED) is the key regulatory step of ABA biosynthesis in higher plants. Overexpression of *SgNCED1* in transgenic tobacco plants resulted in 51–77% more accumulation of ABA in leaves (Zhang et al. 2008d). Transgenic tobacco plants were shown to have decreased stomatal conductance and transpiration and photosynthetic rates and increased activities of SOD, catalase, and APX activities. H_2O_2 and NO in leaves were also induced in the transgenic plants. Compared with WT, the transgenic plants displayed improved growth under 0.1 M mannitol-induced drought stress and 0.1 M NaCl induced salinity stress. It was suggested that the ABA-induced H_2O_2 and NO generation upregulates stomatal closure and antioxidant enzymes and, therefore, increases drought and salinity tolerance in transgenic plants.

Salt stress is known to trigger a rapid and transient increase of free calcium concentration in plant cells (Knight 2000; Pauly et al. 2000). As such, Ca^{2+} signaling processes are one of the earliest events in salt signaling and may play an essential role in the ion homeostasis and salt tolerance in plants (Zhu 2003; Reddy and Reddy 2004). CBLs represent a unique family of calcium sensors in plants and function as a positive regulator in the salt-stress-response pathway. Extensive studies have progressed toward understanding of *Arabidopsis CBLs*, yet knowledge of their functions in other plant species is still quite limited. Wang et al. (2007a) have reported the cloning and functional characterization of *ZmCBL4*, a novel CBL gene from maize. *ZmCBL4* encodes a putative homolog of the *Arabidopsis* CBL4/SOS3 protein. Under normal conditions, *ZmCBL4* was shown to be expressed differentially at a low level in various organs of maize plants and its expression was regulated by NaCl, LiCl, ABA and PEG treatments. Expression of 35S::*ZmCBL4* not only complemented the salt hypersensitivity in an *Arabidopsis sos3* mutant, but also enhanced the salt tolerance in *Arabidopsis* WT plants at the germination and seedling stages. *ZmCBL4*-expressing *Arabidopsis* lines accumulated less Na^+ and Li^+ as compared with WT plants. Wang et al. (2007a) concluded that the maize CBL gene functions in salt-stress-elicited calcium signaling and thus in maize salinity tolerance.

2.4 Engineering Cold Tolerance in Plants

2.4.1 Impacts of Cold Stress on Agricultural Production

Agricultural borders for crop species are defined geographically by occurrences of low temperatures and frost, which cause severe yield losses in marginal areas. Approximately two-thirds of the world's landmass is annually subjected to temperatures below freezing and half to temperatures below –20°C.

Most crops of tropical origin, as well as many of subtropical origin, are sensitive to chilling temperatures. Amongst the major world food crops, maize and rice are sensitive to chilling temperatures and yield loss or crop failure of these species can occur at temperatures below 10°C. Many other crops, such as soybean, cotton, tomato, and banana, are injured at temperatures below 10–15°C (Lynch 1990). The temperature below which chill injury can occur varies with species and regions of origin and ranges from 0 to 4°C for temperate fruits, 8°C for subtropical fruits, and about 12°C for tropical fruits (Lyons 1973).

Cold acclimation, also known as cold hardening, describes an increase in tolerance over time to cold temperatures and cellular desiccation in response to conditions such as cold temperature, short photoperiods, and mild drought and results from changes in gene expression and physiology (Xin and Browse 2000; Kalberer et al. 2006). Most temperate plants can cold-acclimate and acquire tolerance to extracellular ice formation in their vegetative tissues. Winter-habit plants such as winter wheat, barley, oat, rye, and oilseed rape have a vernalization requirement, which allows them to survive freezing stress as seedlings during winter. However, after vernalization and at the end of the vegetative phase, the cold acclimation ability of winter cereals gradually decreases, making them sensitive to freezing injuries (Fowler et al. 1996; Chinnusamy et al. 2007). Therefore, it is not surprising that the impacts of cold stress on plant life have been comprehensively studied. Many attempts have been made to improve cold resistance of important crop plants; however, progress in achieving frost hardiness of plants either by classical breeding or by gene transfer is difficult because of the fact that cold resistance is not a quality conferred by the product of one gene, but, as for most abiotic stress tolerance mechanisms, is quantitative in nature (Mahajan and Tuteja 2005).

2.4.2 Physiological Effects of Cold Stress on Plants and Cold Tolerance Mechanisms

The symptoms of chilling-induced stress injury in cold-sensitive plants are variable and generally manifest within 48 to 72 h of stress exposure. Observed phenotypic symptoms in response to chilling stress include reduced leaf expansion, wilting, chlorosis, and necrosis (Mahajan and Tuteja 2005). Chilling also severely inhibits plant reproductive development, with species such as rice displaying sterility when exposed to chilling temperatures during anthesis (Jiang et al. 2002). The extent of plant damage caused by exposure to low temperature depends on factors such as the developmental stage, the duration and severity of the frost, the rates of cooling (and rewarming), and whether ice formation takes place intra- or extra-cellularly (Beck et al. 2004).

Chilling stress (<20°C) is a direct result of low temperature effects on cellular macromolecules, which leads to slowing of metabolism, solidification of cell

membranes, and loss of membrane functions. This kind of damage results primarily from loss of function of biomembranes associated with decreased fluidity and inactivation or deceleration of membrane-bound ion pumps. Absorbance of light energy, which occurs independently of temperature, results in oxidative stress if metabolism cannot keep pace with the excitation of the photosynthetic components. Freezing stress (<0°C), which causes extracellular ice-crystal formation, freeze-induced dehydration, and concentration of cell sap, has major mechanical impacts on cell walls and plasma membranes and leads to cell rupture (Margesin et al. 2007).

Generally, freezing results in loss of membrane integrity and solute leakage. The integrity of intracellular organelles is also disrupted under freezing stress and leads to loss of compartmentalization and reduction and impaired photosynthesis, protein assembly, and general metabolic processes. The primary environmental factors responsible for triggering increased tolerance against freezing are collectively known as "cold acclimation" (Mahajan and Tuteja 2005).

Frost resistance can be achieved by two main mechanisms: (1) avoidance of ice formation in tissues; or (2) tolerance of apoplastic extracellular ice. An individual plant may employ both mechanisms of frost resistance in different tissues (Sakai and Larcher 1987; Margesin et al. 2007). A key function of cold acclimation is to stabilize membranes against freezing injury through mechanisms such as adjustment of lipid composition and accumulation of protective sugars, hydrophilic and LEA proteins, and antioxidants (Thomashow 1999).

2.4.3 Cold Tolerance Using Transgenic Approaches

2.4.3.1 ROS Detoxifying Substances

The mechanisms of ROS accumulation and detoxification were discussed in Sects. 2.2.6.2 and 2.3.4.3. The decrease in the amount of unsaturated fatty acids during lipid peroxidation elevates membrane viscosity, promotes lipid transition from a liquid crystalline phase to a gel phase, raises proton permeability of membranes, diminishes membrane electric conductance, and causes inactivation of membrane-localized enzymes. The adaptation of plant cells to low temperature is based on their ability to maintain saturation of fatty acids in membrane lipids, thus modifying membrane fluidity (Szalontai et al. 2003).

Demin et al. (2008) examined the role of Δ12-acyl-lipid desaturase in plant resistance to hypothermia-induced oxidative stress. The study focused on modulation of free-radical processes occurring at low temperature in leaf cells of potato plants transformed with the gene for Δ12-acyl-lipid desaturase from cyanobacterium. Plants were grown in vitro on MS agar medium containing 2% sucrose. During hypothermia (–9°C), the rate of O_2^- generation and H_2O_2 concentration decreased significantly. In addition, the contents of both primary products (conjugated dienes and trienes) and secondary products (malonic dialdehyde) of lipid

peroxidation were lower in transformed plant leaves than in leaves of WT plants. It was hypothesized that insertion of Δ12-acyl-lipid desaturase into the plant genome stabilizes the composition and physical properties of biomembranes by promoting polyunsaturation of fatty acids, which averts the accelerated generation of O_2^- and suppresses lipid peroxidation during hypothermia.

Evidence suggests that ROS are the cause of photosystem 1 (PSI) inactivation in chilling-sensitive plants during cold stress in the light, and that H_2O_2 accumulation is a major factor leading to the decline in PSI activity (Sonoike 1996). Cotton is considered to be a chilling-sensitive species; its photosynthetic performance and ability to rapidly recover photosynthetic activity following chilling in the light diminish considerably with time of exposure (Koniger and Winter 1993). Kornyeyev et al. (2003) compared the photosynthetic performance between leaf discs of WT cotton and transgenic cotton overexpressing chloroplast stroma-based APX_+ plants during exposure to 10°C and 500 μmol photons m^{-2} s^{-1}. They showed that APX_+ leaves did not exhibit as large an increase in cellular H_2O_2 as WT leaves shortly after the imposition of the chilling treatment. In addition, APX_+ leaves exhibited slightly but significantly less PSI and PSII photoinhibition.

The electron transport chain (ETC) of plant mitochondria has also been researched in relation to production of freeze-tolerant transgenic plants. Plant ETCs have unique features compared with other eukaryotes, including the ubiquitous presence of a terminal alternative oxidase (AOX) that competes for electrons with the standard cytochrome (Cyt) pathway (Finnegan et al. 2004). The maintenance of ETC redox balance is critical because electron input in excess of ETC capacity can be responsible for the production of ROS, particularly O_2^- and H_2O_2. It has been proposed that AOX may be an important factor in allowing plants to tolerate chilling-induced ROS damage to membrane phospholipids (Purvis and Shewfelt 1993). Evidence suggests that the AOX pathway of plant mitochondria uncouples respiration from mitochondrial ATP production and may ameliorate plant performance under stressful environmental conditions, such as cold temperatures, by preventing excess accumulation of ROS (Wagner et al. 1998). Fiorani et al. (2005) tested this model in whole tissues by growing *AtAOX1a*-transformed *Arabidopsis* plants at 12°C. Twenty-one days after sowing, antisense mutants showed (on average) a 27% reduction in leaf area and 25% smaller rosettes versus 30% increased leaf area and 33% larger rosette size for overexpressing lines compared with WT plants. These results demonstrate that AOX activity plays a role in shoot acclimation to low temperature in *Arabidopsis*.

2.4.3.2 Membrane Modifications

Chilling-resistant plants have a greater abundance of unsaturated fatty acids than chilling-sensitive plants and it has been shown that the proportion of unsaturated fatty acids increases during acclimation to cold temperature (Palta et al. 1993). This modification allows membranes to remain fluid by lowering the temperature at which the membrane lipids experience a gradual phase change from fluid to

semi-crystalline. Thus, desaturation of fatty acids provides protection against damage from chilling temperatures (Khodakovskaya et al. 2006). Khodakovskaya et al. (2006) developed a cold-inducible genetic construct cloned using a chloroplast-specific omega-3-fatty acid desaturase gene (*FAD7*) under the control of a cold-inducible promoter (*cor15a*) from *Arabidopsis* and expressed it in young tobacco plants. When seedlings were exposed to low-temperature (0.5, 2, or 3.5°C) for up to 44 days, survival within independent *cor15a-FAD7* transgenic lines (40.2%–96%) was far superior to the WT (6.7%–10.2%). In addition, the major trienoic fatty acid species remained stable in cold-induced *cor15a-FAD7* plants under prolonged cold storage while the levels of hexadecatrienoic acid (16:3) and octadecatrienoic acid (18:3) declined in WT plants under the same conditions (79 and 20.7%, respectively). Electron microscopy showed that chloroplast membrane ultrastructure in *cor15a-FAD7* transgenic plants was unaffected by prolonged exposure to cold temperatures. In contrast, WT plants experienced a loss of granal stacking and disorganization of the thylakoid membrane under the same conditions.

In higher plants, the most abundant lipids of thylakoid membranes are glycolipids (monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG)). PG is the only phospholipid in thylakoid membranes. The chilling resistance of higher plants is apparently closely correlated with the level of *cis*-unsaturated fatty acids in PG from chloroplast membranes (Nishida and Murata 1996). Chilling-resistant plants contain a large proportion of *cis*-unsaturated fatty acids at the *sn-1* position of PG and there are few *cis*-unsaturated fatty acids in chilling-sensitive plants. The *sn-2* position is occupied mainly by saturated and *trans*-unsaturated fatty acids (Bertrams and Heinz 1981) and hence the content of *cis*-unsaturated fatty acids at the *sn-1* position of PG determines plant chilling resistance. The dominant factor that determines the level of *cis*-unsaturated fatty acids in PG is the substrate selectivity of glycerol-3-phosphate (G3P) acyltransferase (GPAT: EC2.3.1.15) in chloroplasts, which catalyzes the first step of glycerolipid biosynthesis by transferring the acyl group of acyl-(acyl carrier protein; ACP) to the *sn-1* position of G3P to yield 1-acylglycerol-3-phosphate (lysophosphatidate; LPA; (Roughan and Slack 1982)). GPAT from chilling-resistant plants prefers oleoyl-ACP (18:1-ACP) to palmitoyl-ACP (16:0-ACP) as a substrate resulting in a high level of *cis*-unsaturated fatty acids in PG. The enzymes from chilling-sensitive plants hardly distinguish 18:1-ACP from 16:0-ACP resulting in a low level of *cis*-unsaturated fatty acids at the *sn-1* position of PG (Weber et al. 1991). In this way, fatty acids remain saturated, which renders the plants sensitive to chilling stress. Sui et al. (2007) isolated a tomato GPAT gene (*LeGPAT*), which despite the chilling sensitivity of tomato exhibited selectivity to 18:1 over 16:0. Overexpression of *LeGPAT* increased total activity of LeGPAT and *cis*-unsaturated fatty acids in the thylakoid membrane. Chilling treatment (4°C for 4 h) induced less ion leakage from the transgenic plants than from the WT. The photosynthetic rate and the maximal photochemical efficiency of PSII (Fv/Fm) in transgenic plants decreased more slowly during chilling stress and recovered faster than WT plants under optimal

conditions. These results indicate that overexpression of *LeGPAT* increased the levels of PG *cis*-unsaturated fatty acids in thylakoid membranes, which was beneficial for the recovery of chilling-induced PSI photoinhibition in tomato.

2.4.3.3 LEA and Chaperoning Modifications

Several proteins are expressed in plants upon exposure to low temperature. These are either located in the cytosol or secreted to the apoplast. They have various putative functions, including cryoprotection, altered lipid metabolism, protein protection, desiccation tolerance, and sugar metabolism (Hiilovaara-Teijo and Palva 1999; Margesin et al. 2007). During cold acclimation, several stress proteins that may function as chaperones and membrane stabilizers during freeze dehydration are expressed in the cytosol (Puhakainen et al. 2004). Of the many low temperature-responsive genes characterized to date, several have been predicted to encode proteins with the characteristics of the dehydrin class of LEA proteins (Table 2.1). Houde et al. (2004) reported an improvement of the selection procedure and the transfer of the wheat *Wcor410a* acidic dehydrin gene to strawberry. The WCOR410 protein was expressed in transgenic strawberry at a level comparable with that in cold-acclimated wheat. Freezing tests showed that cold-acclimated transgenic strawberry leaves had a 5°C improvement in freezing tolerance over WT leaves or transformed leaves not expressing the WCOR410 protein. However, no difference in freezing tolerance was found between the different plants under non-acclimated conditions, suggesting that the WCOR410 protein needs to be activated by another factor induced during cold acclimation. The data demonstrated that WCOR410 protein prevents membrane injury and greatly improves freezing tolerance in leaves of transgenic strawberry.

HSPs (Sect. 2.2.6.1) accumulate in response to low temperature. The HS response in plants has been extensively investigated (Waters et al. 1996). Plants synthesize predominantly small (15–30 kDa) HSPs (sHSPs) during the heat-shock response, and it has been suggested that the accumulation of sHSPs is correlated with thermotolerance (Vierling 1991). Guo et al. (2007), characterized a sweet pepper cDNA clone, *CaHSP26* encoding the chloroplast (CP)-sHSP, with regard to its sequence, response to various temperatures, and function in transgenic tobacco plants. Expression of the *CaHSP26* gene showed that the mRNA accumulation of *CaHSP26* was induced by heat stress. Higher transcript levels were observed when sweet pepper leaves were treated at 42°C for 3 h. However, the expression of the *CaHSP26* gene was not induced by chilling stress (4°C) in the absence of HS and the transcripts were detected at 48 h at 4°C after HS while not at 25°C. The photochemical efficiency of PSII (Fv/Fm) and the oxidizable P700 in transgenic tobacco overexpressing *CaHSP26* were higher than that in WT tobacco during chilling stress under low irradiance. These results suggest that the CP sHSP protein plays an important role in the protection of PSII and PSI during chilling stress under low irradiance.

2.4.3.4 Osmoprotectants/Compatible Solutes

Long-term acclimation to the cold and winter survival in herbaceous plants is strongly correlated with the recovery of photosynthesis at low temperature and the maintenance of soluble carbohydrate reserves (Stitt and Hurry 2002; Strand et al. 2003), namely, through upregulation of sucrose biosynthesis. Strand et al. (2003) tested this hypothesis by comparing the acclimation responses of WT *Arabidopsis* with transgenic plants overexpressing sucrose phosphate synthase (over-sps) or with antisense repression of either cytosolic fructose-1,6-bisphosphatase (antifbp) or sucrose phosphate synthase (antisps). Plants overexpressing sucrose phosphate synthase showed improved photosynthesis and increased flux of fixed carbon into sucrose when shifted to 5°C, whereas both antisense lines showed reduced flux into soluble sugars relative to WT plants. The improved photosynthetic performance by the overexpressing sps plants was associated with an increase in freezing tolerance relative to WT (–9.1 and –7.2°C, respectively). In contrast, both antisense lines showed impaired development of freezing tolerance (–5.2 and –5.8°C for antifbp and antisps, respectively). Similarly, metabolic engineering for the biosynthesis of fructans is a potential strategy for increasing water-stress tolerance. Fructans are a class of water-soluble fructose polymers based on sucrose, which accumulate in many bacterial and plant species serving as an important storage carbohydrate and protecting plants against water deficit caused by low matrix potential, salinity, or low temperatures (Spollen and Nelson 1994). In plants, fructans are synthesized in vacuoles from sucrose by the action of two or more different fructosyltransferases, including sucrose:sucrose 1-fructosyltransferase (1-SST), sucrose:fructan 6-fructosyltransferase (6-SFT), fructan:fructan 1-fructosyltransferase (1-FFT), and fructan:fructan 6G-fructosyltransferase (6G-FFT; Vijn and Smeekens 1999). Kawakami et al. (2008) used rice, which is highly sensitive to chilling temperatures and is not able to synthesize fructans, to study the effect of fructan biosynthesis against water stress. Two wheat fructan-synthesizing enzymes, 1-SST, encoded by *wft2*, or 6-SFT, encoded by *wft1*, were introduced into rice plants. The transgenic seedlings with *wft2* accumulated significantly higher concentrations of oligo- and polysaccharides than nontransgenic rice seedlings, and exhibited enhanced chilling tolerance (11-day exposure to 5°C). The oligo- and polysaccharide concentrations of seedlings expressing *wft1* were visibly lower than those of lines expressing *wft2*, and no correlation between oligo- and polysaccharide concentrations and chilling tolerance was detected in *wft1*-expressing rice lines. The results suggest that transgenic rice lines expressing wheat-derived fructosyltransferase genes accumulated large amounts of fructans in mature leaf blades and exhibited enhanced chilling tolerance at the seedling stage.

2.4.3.5 Transcription Factors

Cold acclimation in plants is a complex process involving changes in the expression of numerous cold-responsive (*COR*) genes (Chinnusamy et al. 2006). This results in

modification of plant cell structural, biochemical, and photosynthetic properties that facilitate an increase in the plant's freezing stress tolerance. Regulatory factors influencing expression of *COR* genes and/or freezing tolerance have been identified over the last decade (Chinnusamy et al. 2006). The cold-induced *CBF* transcriptional regulatory factor *CBF1-3* controls the cold-responsive expression of a major regulon of *COR* genes that increases plant freezing tolerance (van Buskirk and Thomashow 2006). Ectopic expression of *CBF* transgenes under warm conditions activates a suite of *COR* genes without cold stimulus, increasing plant freezing tolerance and inducing biochemical and structural alterations normally observed during exposure to cold. Pino et al. (2008) studied the effect of ectopic *AtCBF* overexpression on physiological alterations that occur during cold exposure in frost-sensitive (*Solanum tuberosum)* and frost-tolerant (*S. commersonii*) potato. Relative to WT plants, ectopic *AtCBF1* overexpression enhanced expression of *COR* genes without a cold stimulus in both species and imparted a significant increase in freezing tolerance gain in both species (2°C in *S. tuberosum* and up to 4°C in *S. commersonii*). Transgenic *S. commersonii* displayed improved cold acclimation potential, whereas transgenic *S. tuberosum* was still incapable of cold acclimation. During cold treatment, leaves of WT *S. commersonii* showed significant thickening resulting from palisade cell lengthening and intercellular space enlargement, whereas those of *S. tuberosum* did not. Ectopic *AtCBF1* activity induced these same leaf alterations in the absence of cold in both species. In transgenic *S. commersonii*, *AtCBF1* activity also mimicked cold treatment by increasing proline and total sugar contents in the absence of cold. Relative to WT, transgenic *S. commersonii* leaves were darker green, had higher chlorophyll and lower anthocyanin levels, greater stomatal numbers, and displayed greater photosynthetic capacity, suggesting higher productivity potential. These results suggest that an endogenous *CBF* pathway is involved in many of the structural, biochemical, and physiological alterations associated with cold acclimation in potato.

The cuticle is one of the most important barriers for all terrestrial plants and mitigates damage to above-ground biomass caused by low humidity and other biotic and abiotic stresses (Jenks and Ashworth 1999). The important physiological and ecological functions of the cuticle (which include control of transpiration and leaching and facilitation of foliar penetration of pesticides) are related to the presence of cuticular waxes that are embedded in or deposited on the cutin matrix (Kunst and Samuels 2003). Increased accumulation of cuticular waxes in leaves has been achieved through the overexpression of TF genes such as *WIN1/SHN1* in *Arabidopsis* (Broun et al. 2004) and *WXP1* in alfalfa (Zhang et al. 2005). Elevated leaf cuticular wax deposition led to significant improvement in drought tolerance of the transgenic plants (Broun et al. 2004; Zhang et al. 2005). Zhang et al. (2007b) reports the functional characterization of two putative ERF TF genes *WXP1* and its paralog *WXP2* from *Medicago truncatula*. Transgenic expression of *WXP1* and *WXP2* in *Arabidopsis* led to significantly increased cuticular wax deposition on leaves of 4- and 6-week-old transgenic plants. Differences in the accumulation of various wax components, as well as their chain length distributions, were found in the *WXP1* and *WXP2* plants. Analysis of fresh weight loss from detached leaves

revealed that the transgenic leaves retained more water than controls. Both *WXP1* and *WXP2* transgenic plants showed significantly enhanced whole-plant drought tolerance. Analysis of freezing tolerance at the whole-plant level and measurement of electrolyte leakage from detached leaves revealed that the *WXP1* plants had increased freezing tolerance while the *WXP2* plants were more sensitive to low temperature when compared to the control. Transgenic expression of *WXP1* had no obvious effects on plant growth and development; however, the expression of *WXP2* led to slower plant growth. These results indicate that *WXP1* is a useful candidate gene for improving plant drought and freezing tolerance by genetic transformation.

2.5 Nutrient Deficiency and Nutrient Use Efficiency

In all, plants require 17 essential elements, 14 of which are taken up in inorganic forms by the roots. The absence or paucity of any one of these essential elements will commonly lead to plant death or inability to complete its life cycle. In the presence of nutrient deficiencies, even at asymptomatic levels, crop performance, yield, and quality are frequently compromised. Hence, fertilizer and other soil ameliorations are essential to ensuring food security and the sustainability of agriculture, and these add a major economic and potentially environmental burden to crop production. The ability to improve and enhance the efficiency of nutrient uptake and utilization in major crop plants has become a major objective in modern plant improvement. Interestingly, different species have evolved differential mechanisms for improving their ability to scavenge essential elements at low concentrations in the soil. In this section, we will discuss the ways in which biotechnological intervention can be applied to improve the ability of crop plants to survive and produce yield in nutrient-poor environments. The discussion will focus on the most limiting nutrient, nitrogen, with some discussion of phosphorus and iron, which are particularly susceptible to soil pH variation.

2.5.1 Nitrogen

Nitrogen (N) is quantitatively the most important nutrient that plants obtain from the soil (Paungfoo-Lonhienne et al. 2008), with an estimated 10^{11} tons of N fertilizer applied annually worldwide (Lea and Azevedo 2006). The total cost of this agricultural input exceeds US $50 billion. In general, however, plants are only able to acquire 30–40% of this applied N, with significant losses occurring through leaching, denitrification, and the volatilizationz of ammonium to the atmosphere (Lea and Azevedo 2006).

Nitrogen assimilation is the process by which inorganic N forms (typically nitrate) are reduced to ammonium, and then converted into organic forms (amino

acids) for transportation and use by the plant. Nitrate is the main form of N taken up by plants and is reduced in the plant through the combined action of the enzymes, nitrate reductase (NR) and nitrite reductase. It has long been believed that this step is the major control point in nitrate assimilation and it has been shown that expressing a constitutive NR as opposed to a fully regulated NR demonstrates accumulation of high concentrations of asparagine and glutamine (Good et al. 2004; Lea and Azevedo 2006).

Inorganic N is then converted into organic amino acids by the GS-GOGAT cycle, and these amino acids are used to transport N around the plant. The major N transport amino acids are glutamate, glutamine, aspartate, and asparagine, with differences in importance among different phyla and also environmental conditions. Ammonium is incorporated into glutamine through the enzyme glutamine synthetase (GS). This places the ammonium molecule initially into the amide position of the glutamine molecule from where the N is relocated to the α-amino position of glutamate through the action of glutamate synthase (GOGAT) (Glass et al. 2002; Good et al. 2004; Lea and Azevedo 2006). The GS-GOGAT has been considered a primary target for the manipulation of nitrogen use efficiency (NUE).

NUE is usually described as unit biomass per unit of N present in the plant or the yield of N in the plant per unit of available N in the soil (Bushoven and Hull 2001; Lea and Azevedo 2006). NUE is dependent on physiological traits such as uptake and assimilation of N (Good et al. 2004). While there are other processes that use N in plants, it is these two traits that control NUE. The ability to uptake and transport N throughout the plant is, along with photosynthetic efficiency and WUE, one of the major determinants of plant survival, growth, and yield. In addition, the mobilization of organic N into fruits and seeds is a major determinant of yield and product quality, especially in cereal and legume grains, where the protein content determines the nutritional and end-use qualities.

N nutrients are powerful signaling molecules within the plant (Vidal and Gutierrez 2008). Nitrate controls the expression of thousands of genes involved in many plant processes, with some genes responding to nanomolar concentrations (Wang et al. 2003a, 2007b). This includes the nitrate transporter molecules (for an introduction to transporter genes, see the description in Sect. 2.3.4.2). The nitrate transporters, NRT1.2 and NRT2.1, are not only involved with N uptake, but they are also sensor molecules, and have been shown to increase N uptake rate at low rhizosphere concentration.

It is known that root branching occurs at higher frequency in regions of the rhizosphere with local N-enrichment (Scott Russell 1977). The transporter NRT1.1 is involved in signaling, which leads to colonization of nitrate-rich patches in the soil by promoting localized root proliferation in concert with ANR1, a MADS-box TF gene not yet fully functionally characterized (Remans et al. 2006). Both these genes have regulatory sequences which direct reporter gene expression in root primordia and root tips.

Amino acid synthesis and transport genes have been manipulated to alter the NUE in numerous plants. For example, the overexpression of a bacterial asparagine synthetase gene in the leaves of lettuce was shown to affect N status (Giannino

et al. 2008). Early vegetative growth rate of these transgenic lettuce plants was approximately 1.3-fold higher for the first 35 days post-germination, accompanied by higher leaf number and leaf area. However, these plants also attained reproductive phase earlier, which was not advantageous for a leaf vegetable. Similar effects have been reported in transgenic oilseed rape (Seiffert et al. 2004) and *Lotus* (Vincent et al. 1997). The transgenics had approximately twofold more aspartate and asparagine than WT, and interestingly also had elevated levels of glutamine with no effect on glutamate concentration. Leaf assays also revealed a lower level of free nitrate in the transgenic lettuce lines, a phenomenon previously reported in lettuce overexpressing a nitrate reductase gene (Curtis et al. 1999). The authors suggested that these plants may be suitable for breeding new quick-harvest lettuce varieties with lower N fertilizer requirement, or enhanced NUE.

2.5.2 Phosphorus

Worldwide, it is estimated that 5.7 billion hectares of land lack sufficient quantities of plant-available Phosphorus (P) (Batjes 1997). P deprivation in plants has been widely studied, as P becomes limiting to plant productivity with falling soil pH, and tends to bind very tightly with soil. In mildly acid soils with pH < 6, soil P rapidly becomes immobile and forms insoluble compounds, commonly with Fe and Al. Even in soils with abundant P, usually only about 1% of the soil P is actually in a readily available, soluble form, and over 90% is generally bound tightly to soil particles in organic and inorganic forms, which require mineralization before they become plant available.

P uptake and transporter genes are generally regarded as high or low affinity, with the high affinity genes becoming more important for scavenging nutrient from the soil as P becomes limiting. Many Australian soils are particularly P-deficient and, as a result, many native Australian plants have evolved different mechanisms to overcome this with high-affinity P transporters, association with vesicular-arbuscular mycorrhiza, the secretion of organic acids into the rhizosphere, and the formation of proteoid roots (Lambers et al. 2006).

Attempts to overexpress high affinity P transporters have met with mixed success. Overexpression of high-affinity transporter genes in barley did not have any effect on P uptake or plant productivity and growth (Rae et al. 2004), despite having demonstrated that these genes increased P uptake in transgenic rice callus cultures. However, the overexpression of homologous high-affinity P transporter genes in rice enabled the plants to accumulate twice as much P as WT rice, and the resulting phenotype produced more tillers (Seo et al. 2008). Further research has also demonstrated that many plant phosphate transporters have complex interactions with vesicular arbuscular mycorrhizae (Glassop et al. 2007).

Plants have been demonstrated to alter the rhizosphere with specific exudates, commonly organic acids or enzymes, to improve the availability of nutrients such as phosphate. The exudation into the rhizosphere of acid phoaphatase has led to

improved biomass and P accumulation in *Arabidopis* (Xiao et al. 2007) and rice (Park et al. 2007). Similar results have been achieved with the ectopic expression of phytase in root tissues of potato (Hong et al. 2008).

2.5.3 Iron

Iron is not only an essential plant nutrient, but its deficiency, known as anemia, also represents the world's most frequent and debilitating human mineral nutrient deficiency (Wintergerst et al. 2007). Hence, improving the ability of plants to uptake, translocate and store iron in bioavailable forms is not only a plant health and productivity issue, it is a major human and animal nutritional target.

Plants have the ability to sense low-iron conditions, which induces a coordinated response. The predominant form of iron in aerobic soils is Fe(III), and plants must possess strategies to utilize this form. Most plants use what has become widely known as Strategy I, whereby they acidify the rhizosphere with the induction of a specific proton pump which solubilizes more Fe, and then, by the production of Fe chelate reductase, convert Fe(III) to Fe(II), which is then transported into the cell by a membrane-bound Fe(II) transporter.

Grasses and cereals, however, utilize Strategy II, which involves the production and secretion of a specialized group of chemicals, known as phytosiderophores (PS) into the rhizosphere. PS are a form of mugineic acids, which form strong chelates with Fe(III), and help to solubilize them for plant uptake by specialized protein forms, known as the Yellow Stripe proteins (von Wiren et al. 1994; Curie et al. 2001), which are actually Fe(III) transporter genes.

Interestingly, rice is a special case and appears to be able to utlilize both strategies of iron uptake. Unusually, among the grasses rice has an efficient Fe(II) uptake system and does not produce much PS (Mori et al. 1991). Rice has been engineered to produce larger quantities of PS, and the transgenic lines were more tolerant to Fe-deficient soils (Suzuki et al. 2008). Other successful approaches to improve Fe uptake of transgenic rice in alkaline soils have included the overexpression of Fe chelate reductase genes (Ishimaru et al. 2007) and barley nicotianamine amino transferase genes (Takahashi et al. 2001).

2.6 Engineering Metal Toxicity Tolerance in Plants

2.6.1 Heavy Metal Contaminated Soil

Soils naturally consist of varying high levels of heavy metals; however, concentrations in soils are greatly increased where humans have extracted them and used them for industrial purposes (Greger 1999; Harmsen 2002; UN-Oceans 2008).

Heavy metals, especially those that are present from weathering of parent rock, are usually bound or immobile in soils and their removal via leaching or plant accumulation is slow and inefficient. As a consequence, metals accumulate in soils (Haygarth and Jones 1992). Heavy metals usually exist as cations under biological conditions and form complexes with soil sediments and colloids, which are made up of negatively charged organic substances and inorganic clay particles. Formation of this complex is a slow process and, because unbound metals are bioavailable and may deleteriously affect agricultural products or leach into groundwater, areas where human activities have led to elevated soil metal content are a socioeconomic as well as an environmental concern (Greger 1999; Harmsen 2002).

Common heavy metal soil pollutants include arsenic, cadmium, chromium, copper, nickel, lead, and mercury (UN-Oceans 2008). Cd and Hg are particularly concerning because they are widespread and have no known function in human metabolism (Nordberg et al. 2002; UN-Oceans 2008).

2.6.2 Engineering Heavy Metal Tolerance in Plants

Few known studies have focused on engineering heavy metal tolerance in plants. It is assumed that this is due to the complex interactions of stress factors, which mean that many studies focusing on improving other types of abiotic stress, such as osmotic and low temperature stress, include analysis of improved tolerance to one or a few heavy metals. For example, Zhang et al. (2008c) isolated an aquaporin gene *BjPIP1* from the heavy metal hyperaccumulator Indian mustard, which is upregulated in leaves under drought, salt, low temperature, and heavy metal stress. Constitutive expression of *BjPIP1* in tobacco decreased water loss rate, transpiration rate, and stomatal conductance of transgenic plants compared to WT under osmotic stress. On exposure to Cd, transgenic plants displayed enhanced Cd resistance of root growth and lowered transpiration rates and stomatal conductance, increased activities of antioxidative enzymes, lower levels of electrolyte leakage, and lower malondialdehyde content. The study suggested that the increased heavy metal resistance was conferred by maintaining reasonable water status in transgenic plants. Other proteins that have been implicated in conferring heavy metal tolerance include LEA proteins and cation-efflux transport proteins (Zhang et al. 2007c). When Koh et al. (2006) introduced a yeast Cd factor (YCF1), which sequesters glutathione chelates of heavy metals and xenobiotics into vacuoles, into *Arabidopsis* in order to improve heavy metal tolerance, transgenic plants were found to have improved salt tolerance as well (Zhang et al. 2007c). While the use of genetic engineering to develop crops with improved heavy metal tolerance remains relatively unexplored, a related field is the use of genetic engineering to develop plants with increased ability to uptake and accumulate metals in order to remove the soil contamination in a process known as phytoremediation. Many of the plant characteristics required to confer metal accumulation ability also confer heavy metal tolerance and, as such, plants engineered with enhanced phytormediation

capabilities inevitably possess enhanced heavy metal tolerance as well. Unlike engineering plant heavy metal tolerance, this field has been the focus of many studies over the last decade. Therefore, the remainder of this chapter will briefly discuss the use of transgenic technology to improve the phytoremediation capabilities of plants.

2.6.3 Soil Heavy Metal Management and Remediation Options

There are many management and remediation options available to prevent entry of soil heavy metals into the human food chain via agricultural crops. These include: prevention, agronomic management, and physical remediation. These options, while contributing to reductions in human exposure to some heavy metals, can be unrealistic, labor intensive, and prohibitively expensive (McLaughlin et al. 1999; Ensley 2000; Glass 2000; Clarkson 2002; Madden et al. 2002; Prasad 2002; Robinson et al. 2003; Song et al. 2003).

2.6.3.1 Phytoremediation: Engineering Crops to Increase Heavy Metal Uptake

Phytoremediation is a biological technology, which utilizes the physiology of plants to extract and detoxify soil and water pollutants (Kramer and Chardonnens 2000; Rosselli et al. 2003). The use of plants for contaminated land reclamation is based on the existence of metal hyperaccumulator plants, which are capable of growing on metal contaminated soils and accumulating extremely high levels of heavy metals in their harvestable tissue (Robinson et al. 2003). The major metal tolerance mechanisms identified in heavy metal accumulator plants include: (1) cell wall metal ion binding; (2) inhibition of metal ion transport across plasma membranes; (3) active metal ion efflux from cells; (4) chelation and detoxification of metal ions; and (5) compartmentalisation of metal ions in organelles (Bargagli 1998).

The several types of phytoremediation include: phytostabilization (the use of plants to immobilize metals in the environment to prevent them from leaching into groundwater (Prasad 2002)), phytodegradation (the use of plants to degrade contaminants in the root zone), phytoextraction, and phytovolatization. Phytoextraction and phytovolatilisation are particularly important for metal phytoremediation. Phytoextractor plants remove metal ions from soils and store them in above-ground tissue using the photosynthetically driven processes of soil-ion extraction and translocation. Normal harvesting processes enable easy removal of contaminants and their responsible disposal. Phytovolatilization is similar to phytoextraction but, rather than be stored in biomass, extracted elements are converted into less toxic, volatile forms via plant metabolic pathways and released into the atmosphere (Kramer and Chardonnens 2000; Robinson et al. 2003).

2.6.4 Phytovolatilization of Soils Contaminated with Hg

Relatively low exposure to Hg and mercurial compounds can cause severe detrimental physiological effects in all biological organisms. Atmospheric Hg increased from approximately 2×10^6 kg to 4×10^6 kg over the twentieth century because of Hg use in the chemical, medical, paper, mining, and defense industries (Bizily et al. 2000). Environmental Hg is present as Hg(II), elemental Hg (Hg(0)), and organomercurial compounds ($R\text{-}Hg^+$) such as $CH_3\text{–}Hg^+$. Toxicity symptoms between these forms differ; $CH_3\text{–}Hg^+$ is the most dangerous because it is lethal at doses two to three orders of magnitude lower than Hg(0) or Hg(II), diffuses passively through biological membranes, is highly reactive with biological compounds, has a long retention time in the body, and is biomagnified through the food chain (Rugh et al. 2000).

There are no known naturally occurring plants that are able to detoxify or hyperaccumulate Hg (Pilon-Smits and Pilon 2002). Therefore, a bacterial Hg volatilization pathway has been used in the development of Hg phytoremediators (Pilon-Smits and Pilon 2002). Bacterial colonies that metabolically convert Hg(II) and $R\text{-}Hg^+$ compounds into the less toxic Hg(0) have been discovered inhabiting Hg-contaminated sites. The volatilization pathway is conferred by the presence of the *mer*-operon, which encodes a set of genes involved in the detection, mobilization, and enzymatic detoxification of $R\text{-}Hg^+$ compounds via two main reactions, which are catalyzed by organomercurial lyase (MerB) and mercuric ion reductase (MerA) (Rugh et al. 1998). A modified form of the MerA gene, *merA9*, was transferred into *Arabidopsis* and tobacco and transgenic plants were found to be resistant to 50 μm Hg(II) (Rugh et al. 2000). Expression of the same gene in the forest species, *Liriodendron tulipifera* (yellow poplar) resulted in transgenic plants, which thrived in solution containing 50 μm Hg(II) and volatized Hg(0) at a rate tenfold higher than WT controls (Kramer and Chardonnens 2000). MerA has also been expressed in the eastern cottonwood (*Populus deltoids*) with similar success. The transformation of *Arabidopsis* with constructs of both the *merA* and *merB* genes resulted in transgenic plants able to tolerate media containing 40-fold higher $CH_3\text{–}Hg^+$ levels than WT plants and were able to volatilize Hg at a rate severalfold higher than WTs (Bizily et al. 2000). Tobacco was also transformed with MerA and MerB via the chloroplast genome and transgenic plants grew well with root Hg concentrations up to 2,000 μg g^{-1}, accumulated $R\text{-}Hg^+$ compounds and inorganic mercurials to levels surpassing soil concentrations and displayed a 100-fold increase in the efficiency of shoot Hg accumulation over WT plants (Hussein et al. 2007). MerA and MerB have also been expressed simultaneously in transgenic poplars and eastern cottonwood trees (Lyyra et al. 2007; Young et al. 2007). Transgenic poplars were tolerant to 50 μM $HgCl_2$ and 2 μM CH_3HgCl in culture; however, a high level of variation in Hg-tolerance was observed in transgenic plants (Young et al. 2007). Transgenic eastern cottonwood trees were highly resistant to $R\text{-}Hg^+$ compounds and detoxified them two to three times more rapidly than controls (Lyyra et al. 2007).

2.6.5 *Phytoextraction of Soils Contaminated with Cd*

Plants able to tolerate high Cd levels do so via exclusion (reducing metal uptake by cell wall binding) or intracellular compartmentalization (binding of metals with detoxifying ligands within the cell and the sequestration of these complexes in organelles where the metals cannot interact with the plants metabolic processes (Robinson et al. 1994)). The two major heavy metal detoxifying ligands in plant cells are the metallothioneins (MTs) and the phytochelatins (PCs; Cobbett and Goldsborough 2000). Class I and II MTs are low molecular weight metal-binding products of mRNA translation. PCs are represented by the class III MTs and are products of enzymatic reactions (Cobbett and Goldsborough 2002).

2.6.5.1 Metallothioneins

It has been difficult to analyze plant metallothionein (MT) proteins because they are unstable under aerobic conditions; however, isolated and cloned mammalian MT genes have been assessed for their ability to increase metal tolerance when expressed in transgenic organisms. Table 2.2 lists the findings of some of these studies. These results, obtained in laboratory trials, demonstrate that expression of mammalian MTs in plants can provide protection against toxic effects of heavy metal ions such as Zn^{2+}, Cd^{2+}, and Hg(II); but it has not yet been possible to achieve similar results in the field (Kramer and Chardonnens 2000).

2.6.5.2 Phytochelatins

Phytochelatins (PCs) were first discovered in plants and are structurally related to GSH, which is thought to act as the substrate for PC synthesis by PC synthase (PCS). Various methods have been employed to enhance the effectiveness of PC-assisted metal detoxification. Indian mustard was engineered to express the *E. coli* GSH synthesis gene, *gsh2*, and transgenic plants displayed increased GSH and PC levels and a 25% increase in shoot Cd concentration (Zhu et al. 1999). The enzyme that catalyzes synthesis of the GSH precursor, γ-glutamylcysteine synthase (GCS), was also expressed in *B. juncea* and shoot Cd concentrations were increased by 40–90% in the transformed plants (Kramer and Chardonnens 2000).

A variety of PC genes have been isolated from different species and over-expressed in endogenous or exogenous species to determine their potential for Cd phytoremediation. The most extensively studied PCS gene is *AtPCS1* from *Arabidopsis*, which has been found to play roles in increased tolerance and/or accumulation of Cd and As (Gasic and Korban 2007b). However, depending on the expression level, transfer of PCS genes have also been associated with decreased Cd accumulation and Cd hypersensitivity (Lee et al. 2003b, c; Li et al.

Table 2.2 Genetic transfer of MT genes for improved phytoremediation capabilities of plants

Transformed species	Transgene(s)	Findings	References
Eschericia coli	Human MT genes	Increased metal adsorption	Kramer and Chardonnens (2000)
Ralstonia eutrophia	Mouse MTs genes	Enhanced Cd immobilizing ability when expressed on cell surface	Valls et al. (2000)
Tobacco	Mammalian MT gene	Root-shoot Cd transport is reduced	Kramer and Chardonnens (2000)
Brassica oleracea	Yeast MT gene, *CUP1*	Increased leaf Cd accumulating ability	Kramer and Chardonnens (2000)
Tobacco	Construct containing *CUP1* and an additional metal-binding domain	Up to 90% increased Cd accumulation in harvestable parts without any visible difference in growth characteristics	Macek et al. (2002)
Sunflower	*CUP1*	Enhanced Cd tolerance when expressed at the callus stage	Watanabe et al. (2005)
Tobacco	*Arabidopsis* MT gene, *MT2aI*	Expression strongly induced by Cu^{2+}, Zn^{2+}, and Cd^{2+} suggesting the promoter has specificity for heavy metal stress	Tonkovska et al. (2003)
Tobacco	Construct encoding a poyhistidine cluster with a yeast MT	45–75% increase in Cd accumulation in harvestable plant parts and increased resistance to Cd-induced stress	Pavlikova et al. (2004a, b)
Arabidopsis	Garlic MT gene	Stronger Cd tolerance and higher Cd accumulation	Zhang et al. (2006)
Arabidopsis	*Brassica juncea* MT gene, *BjMT2*,	Increased tolerance to Cu^{2+} and Cd^{2+} based on shoot growth and chlorophyll content and decreased root growth in the absence of heavy metal exposure	Zhigang et al. (2006)
Tobacco	*Silene vulgaris* L. MT gene	Signigicantly increased Cd accumulation in roots and leaves	Gorinova et al. (2007)
Arabidopsis	*Brassica rapa* MT gene	Chloroplast target of gene resulted in detoxification of Cd and H_2O_2	Sun et al. (2007)

2004, 2005b; Gasic and Korban 2007a). Other studies have shown that *AtPCS1* can lead to increased Cd tolerance and accumulation in roots but not translocation of Cd into harvestable tissue (Pomponi et al. 2006). PCS genes from *Cyndon dactylon* (*CdPCS1*) and wheat (*TaPCS1*) have also been transformed into tobacco and have led to increases in leaf accumulation of Cd and other heavy metals (Li et al. 2006; Martinez et al. 2006). Tobacco transformed with *TaPCS1* was grown in the field to

assess its Cd-accumulating ability and it was found that transgenic plants could accumulate more heavy metals and 100 times more biomass on contaminated soils than the hyperaccumulator *Thlaspi caerlescens* (Martinez et al. 2006). Another approach was employed by Song et al. (2003) and was aimed at increasing the efficiency of metal transport across the tonoplast. In this study, *YCF1* from *S. cerevisiae* was overexpressed in *Arabidopsis* and transformed plants displayed increased tolerance to Cd and Pb and improved vacuolar Cd sequestration. Many studies have also shown that dual- or multiple-gene expression using constructs with combinations of PC synthesis genes such as GCS, GSH synthase, ATP sulfurylase, and serine acetyltranferase may be the most promising route toward the development of a useful Cd phytoremediator and a phytoremediator useful for removal of mixtures of heavy metals from soils (Bennett et al. 2003; Wawrzynski et al. 2006; Guo et al. 2008; Reisinger et al. 2008).

2.7 Conclusion and Future Directions

This review has briefly summarized some of the stress resistance mechanisms that have been targeted for manipulation in the endeavor to develop crop plants with improved resistance to drought, salinity, cold, nutrient deficiencies, and metal toxicities. In the majority of cases, there are many knowledge gaps that need to be filled prior to development and release of these crops. Water-limiting environments are the most widespread form of stress, and also the least amenable to rapid advances in resistance. Reasons for this include the incomplete knowledge regarding plant drought responses, lack of field trials and drought stress treatments which are truly reflective of climatic conditions, the lack of site and crop specificity of drought tolerance studies, and the lack of integration of disciplines.

The last century of breeding effort and crop physiology studies have led to increases in the economic yield of most major crop species and have elucidated many traits that are associated with plant adaptability to drought-prone environments (such as small plant size, reduced leaf area, early maturity, and prolonged stomatal closure). However, many attempts to improve drought tolerance through breeding have been associated with reduced yield potential (Cattivelli et al. 2008). Nevertheless, enormous advances should have been make in the understanding of the physiological and molecular responses of plants to water deficit through breeding and physiological studies, and scientists in these areas will continue to have a fundamental role in the development of transgenic drought-resistant crops. One of the greatest limitations in drought stress tolerance breeding has been the fact that drought takes many varied forms. Depending on the crop and the season, water stress may be experienced in early vegetative stages, during transition to flowering and even post-anthesis. In some cases, this may be a terminal stress or, more commonly, a recurring event broken by sporadic rainfall precipitating a recovery of the whole plant, sometimes with reduced yield potential as a result of conservative plant cell survival responses.

It is entirely possible that the development of transgenic crops with improved abiotic stress tolerance may lead to unforeseen outcomes, both positive and negative. The development of "super varieties" that offer promising solutions to the problems of water deficit or salinity stress, may also give rise to problems associated with high-input agriculture in monoculture situations. Additionally, this review has shown that there are a plethora of genetic components that may be manipulated in order to confer improved abiotic stress tolerance of crops and that there is, therefore, unlikely to be a single gene that will result in a suitable "drought-tolerant" or "salt-tolerant" variety for all conditions. Consequently, it may be beneficial to trial novel technologies such as overexpression of multiple genes known to be involved in the adaptation to stress and introgression of these genes in random combinations into many cultivars of the one crop prior to field release. This may increase the stability of these genes and allow specific environments to "naturally select" the transgenes that are most suitable for their particular climatic conditions in the same way that increased natural genetic diversity does so in natural populations.

Salt tolerance is a physiologically complex trait and halophytes and less tolerant plants show a wide range of adaptations to salt stress. Attempts to enhance tolerance have involved conventional breeding programs, the use of in vitro selection, physiological trait pooling, interspecific hybridization, the use of halophytes as alternative crops, the use of marker-aided selection, and the use of transgenic plants. The assessment of salt tolerance in transgenic experiments as described above has mostly been carried out using a limited number of seedlings or mature plants in laboratory experiments. In most cases, experiments were carried out in greenhouse conditions where the plants were not exposed to conditions that prevail in high-salinity soils (e.g., alkaline soil pH, high diurnal temperatures, low humidity, presence of sodic salts, and elevated concentrations of selenium and/or boron (Yamaguchi and Blumwald 2005)). Therefore, the salt tolerance of plants needs to be evaluated under field conditions and, more importantly, salt tolerance needs to be evaluated as a function of yield. The evaluation of field performance under salt stress is difficult because of the variability of salt levels in field conditions (Daniells et al. 2001) and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity and water loss due to transpiration. Evaluating tolerance is further complicated because of variation in sensitivity to salt during plant life cycles. According to Flowers and Flowers (2005), conventional breeding programs have rarely delivered enhanced salt tolerance, while wide crossing to achieve salt tolerance generally reduces yield to unacceptably low levels (Flowers and Flowers 2005). There has been success using physiological criteria as the basis of selection for rice (Dedolph and Hettel 1997) and such an approach has been advocated for wheat (Munns et al. 2002). According to Flowers and Flowers (2005) recent analysis has shown that, while it is possible to produce a wide range of transgenic plants where some aspect of a trait relating to salt tolerance is altered, none or few transgenic plants have been tested in the field and few claims for success meet minimal criteria required to demonstrate enhanced tolerance (Flowers 2004).

While adaptation to stress under natural conditions has some ecological advantages, the metabolic and energy costs may sometimes mask and limit its benefit to agriculture and result in yield penalty. An ideal genetically modified crop should possess a highly regulated stress-response capability that does not affect crop performance when stress is absent. In this respect, conventional breeding and selection techniques will continue to make a contribution (Wang et al. 2001). As a result of this, transgenic approaches to plant improvement are best regarded as a means of widening genetic variation. Transgenic plants will nevertheless continue to be extremely useful tools in basic plant science research, and will lead to improved understanding of the gene networks and molecular physiology of plant responses to abiotic stresses.

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