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Molecular and Genetic Analysis of Abiotic Stress Resistance of Forage Crops

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1. Introduction

Abiotic stress, brought about by salinity, drought, extreme temperatures and oxidative stress are serious threats to agriculture and result in a huge reduction of production. Drought and salinity are becoming major threats throughout the world. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and development (Wang et al., 2001). In order to survive from these harsh stresses, forage plants have developed precise and complicated tolerance mechanisms at the morphological, physiological and molecular levels.

Under serious threat, forage plants can change the shape of the leaves and roots to decrease the water loss. Some plants have evolved special structures, such as salt glands to excrete salt. At the physiological level, respiratory, photosynthesis metabolism and osmotic adjustments etc. all change in order to resist stress (Chaves, 1991; Sheng, 2010; Yang et al., 2007). In fact, all of these changes are related to gene expression. The complex plant response to abiotic stress involves many genes and molecular mechanisms. In the past several decades, multiple genes responding to drought, salt, low-temperature and oxidative stress have been identified. These genes are divided into two groups (Shinozaki et al., 2003). The first group functions to directly protect the plant against stress, involving key enzymes for osmolyte biosynthesis, LEA (late embryogenesis abundant) proteins, detoxification enzymes and enzymes involved in many metabolic processes. The other group consists of contained protein factors involved in the regulation of signal transduction, including various transcription factors, proteins kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (Yamaguchi-Shinozaki & Shinozaki, 2006). Genes have been used extensively to improve the stress-tolerance in crop and forage crops. The investigation of stress-tolerance genes will increase our knowledge of tolerance mechanisms, which could in turn be used to promote improvements in forage crop plants tolerance.

2. The morphological response to abiotic stress

Plants often produce a visible response to certain types of environmental stress. Under drought stress, the leaf morphology changes in order to retain water and increase the water use efficiency in forage crops plants. In general, plants decrease the leaf area to limit water loss (Turner, 1979). Nobel investigated the relationship between leaf structure and water use

efficiency under water deficit, and found that if other conditions are invariant. The mesophyll cells become smaller per unit area under water deficit, conversely the larger the area of mesophyll cells, the higher is the water use efficiency (Noble, 1980). In an earlier study, tall fescue and turfgrasses were seen to rely primarily on a deep and extensive root system for drought tolerance because the longer root system had greater volume and surface area in contact with the soil, facilitating water and nutrient uptake under drought stress (Qian et al., 1997).

Facing salinity stresses, plants evolved special structures to survive. *Mesembryanthemum crystallinum* is a salt-secreting plant and possesses epidermal bladder cells in its aerial parts, which store Na^+ (Adams et al., 1998). While *Tamarix aphylla* uses a salt gland to excrete salt (Thomason et al., 1969).

Annual plants can escape drought by maturing before stress becomes severe. Some Fescue grass cultivars avoid drought stress through changes in leaf and root morphology (reducing the transpiration surface area and closing stomata) and probably through osmotic adjustment maintain sufficient turgor pressure in the growing zone for leaf elongation (Wang & Burghrara, 2008).

3. The physiological response to abiotic stress

Serious environmental threats stimulates forage and turf grassess to produce a physiological response. Photosynthesis and cell growth are the primary processes which are affected by stress (Chaves, 1991; Munns, et al., 2006). Under such stress, the rate of photosynthesis and assimilation of products of forage crops decreases remarkably, resulting in a slow down of growth. In a study of eight grass forage plants, chlorophyll content was found to decrease with increased water stress (Yang et al., 2007). Low-temperature has been found to decrease the chlorophyll content in *Poa pratensis* L.qinghai, *Roegneria thoroldiana* and *Elymus nutans*. The major reason for this is that the stress decreaseds the stability of the chloroplast and then destroyed them (Yan et al., 2007). Generally, the degree of decreasing chlorophyll content correlates with the degree of damage to the forage crop plants, so that the chlorophyll level can be used as a guide to stress tolerance in these plants.

Under a variety stresses, the respiration rate of forage plants becomes unstable. For example, the respiration rate dramatically decrease after suffering from freezing, heat, high-salinity or flooding. While after drought or chilling, the respiration rate increase initially, then sharply decreases (Sheng, 2010). The respiratory metabolism pathway also alters under stress. The Pentose Phosphate (PPP) pathway increases under drought and mechanical damage conditions in forage crop plants (Sheng, 2010).

3.1 Osmotic adjustment in response to abiotic stress

Different types of stress often produce interrelated effects and induce similar cellular damage. A general phenomenon is cell dehydration. Under the water deficit condition, plants sustain normal physiological processes through osmotic adjustment (OA). Osmotic adjustment is a major trait associated with maintenance of high cell turgor potential and water retention in response to dehydration stress (Hare et al., 1998; Ingram & Bartels, 1996). Osmotic adjustment can result in turgor maintenance, thereby sustaining cell elongation and leaf expansion as water deficits develop. Osmotic adjustment has been correlated with drought and salt tolerance in various forage and turfgrass species, including tall fescue (White et al., 1992), bermudagrass, buffalograss [Bouteloua dactyloides (Nutt.) Columbus] (Qian & Fry, 1997), *Cenchrus ciliaris* (Wilson & Ludlow, 1983), *Andropogon gayanus* var.

bisquamulatus (Geerts *et al*., 1998). Osmotic adjustment measurements can be used to select drought-tolerant cultivars (Morgan,1983). The extent of osmotic adjustment was higher in buffalograss and zoysiagrass (*Zoysia japonica* Steud) with a better drought tolerance than tall fescure (*Festuca arundinacea* Shreb.) (Qian & Fry, 1997).
Osmotically active solutes include amino acids (proline), sugars (e.g.,sucrose, fructans), polyols (e.g., mannitol), and organic ions(e.g.,potassium, sodium) (Chaves et al., 2003). Those solutes are associated not only with turgor maintenance, but also with the maintenance of membrane and protein structures and protection against oxidative damage (Crowe et al., 1992; Hoekstra et al., 2001). OA for creeping bentgrass and velvet bentgrass is associated with the accumulation of water soluble carbohydrates during the early period of drought and increases in proline content following prolonged a period; however, inorganic ions were not found to relate OA in these species (DaCosta & Huang, 2006). In alfalfa (*Medicago sativa* L.), salt stress induces a large increase in the amino acid and carbohydrate pools. Amongst the amino acids, proline shows the largest increase in roots, cytosol, and bbacteroides. Its accumulation is reflected in an osmoregulatory mechanism not only in roots but also in nodule tissue. The concentration of the carbohydrate pinitol is also increased significantly (Fougere et al., 1991). In many other forage and turfgrasses, glycinebetaine and proline makes a significant contribution to OA under abiotic stress (Girousse *et al*., 1996; Marcum, 1994).

3.2 Dormancy is another countermeasure to survive from stresses

Dormancy also is a mechanism by which forage crop plants become quiescent during prolonged environmental stress, especially drought. Grasses temporarily slow the growth of meristem to avoid drought damage and to allow survival (McWilliam, 1968). Poaceae forage plants, such as *Poa scabrella* (Laude, 1953), *Poa bullbosa* (Volaire *et al*., 2001), and some populations of forage grasses such as *Dactylis glomerata* 'Kasbah' (Norton *et al*., 2006), all exhibit summer dormancy.

4. The molecular and genetic response to stress

The plant responses to abiotic stress involves many genes and molecular mechanisms and stress-associated genes, proteins and metabolites from a complex regulatory network.
A large number of genes have been found to be associated with abiotic stress (Jin et al., 2010; Kang et al., 2010; Shinozaki and Yamaguchi-Shinozaki 2000; Thomashow 1999). Genes induced during stress conditions function not only in protecting cells from stress by producing important metabolic proteins, but also in regulating signal transduction in the stress response (Yamaguchi-Shinozaki & Shinozaki, 2006). These gene products are divided into two groups (Shinozaki et al., 2003; Yamaguchi-Shinozaki & Shinozaki, 2006). The first group functions in the direct protection of the plant against stress and includes key enzymes for osmolyte biosynthesis, LEA (late embryogenesis abundant) proteins, detoxification enzymes and enzymes involved in many metabolic processes. The second group contains protein factors involved in further regulation of signal transduction, including various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (Yamaguchi-Shinozaki & Shinozaki , 2006).

4.1 Transcriptional factors

Transcription factor genes play important roles in stress survival by serving as master regulators of sets of downstream stress-responsive genes. Transcription factors regulate

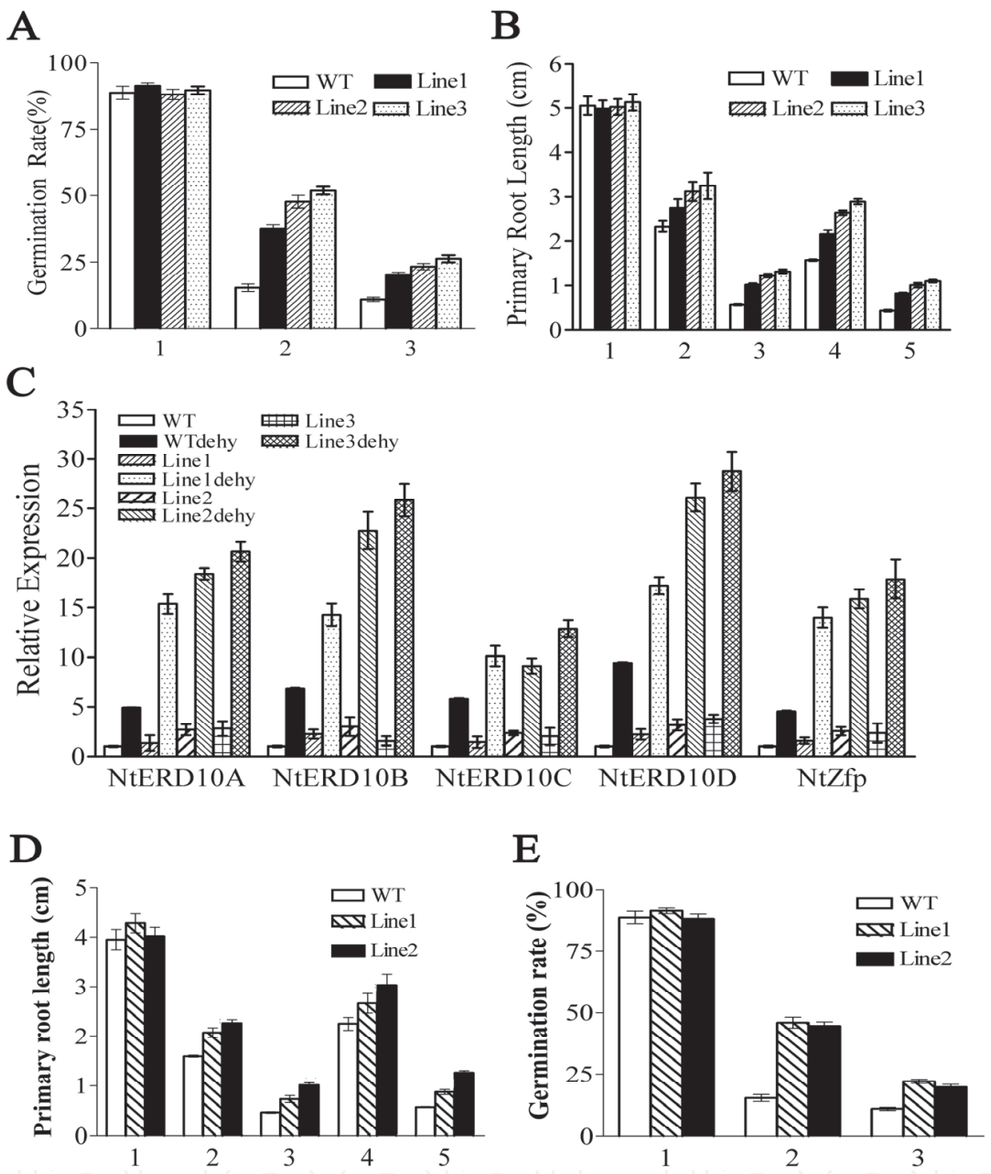
downstream gene expression via binding to specific elements (cis-elements) in target genes and consequently, enhance stress tolerance in plants (Chen & Zhu, 2004; Yamaguchi-Shinozaki & Shinozaki, 2006).

Transcriptional control of the expression of stress-responsive genes is a crucial part of the plant response to a range of abiotic stresses (Singh et al., 2002). Several hundred types of transcription factors have been isolated from higher plants. Important families of stress-responsive transcription factors include AP2/EFR, basic-domain leucine-zipper (bZIP), MYC/B, WRKY, Zinc finger, MADS, NAC (Liu et al., 1999; Yamaguchi-Shinozaki & Shinozaki ,2006).

In the Arabidopsis genome, 145 DREB/ERF related proteins are classified into five groups—the AP-2 subfamily, RAV subfamily, DREB subfamily, ERF subfamily, and others (Sakuma et al., 2002). Many AP2/EREBP transcription factor genes have been isolated from a variety forage crop plants (Chen et al., 2009; Niu et al., 2010; Wang et al., 2010; Wang et al., 2011; Xiong & Fei, 2006). In *Medicago falcate*, MfDREB1 and MfDREB1s encode an AP2/EREBP type transcription factor and are multi-copy genes. Also they are induced by low temperature stress, although hardly induced at all under salt and drought conditions (Niu et al., 2010). *LpCBF3*, encoding the transcription factor DREB1/CBF, is isolated form perennial ryegrass. *LpCBF3* is induced by cold stress, but not by abscisic acid (ABA), drought and salinity. Over-expression of *LpCBF3* in Arabidopsis was found to induce CBF3 target genes and enhance freezing tolerance by measuring electrolyte leakage (Xiong & Fei, 2006). *LpCBF3* can induce downstream gene expression in cold-tolerant perennial ryegrass accessions without cold treatment, but cannot be activated in cold-sensitive perennial ryegrass accessions. Also cold treatment can induce the downstream genes of CBF3 expression in these accessions. Over-expression of *LpCBF3* with a 35S promoter resulted in dwarf-like plants, later flowering and greater freezing tolerance (Zhao & Bughrara, 2008). *HsDREB1A* is isolated from xeric, wild barley in bahiagrass. *HsDREB1A* introduced into bahiagrass under the *HVA1s* promoter from barley, enhanced tolerance under severe salt stress and severe dehydration stress (James et al., 2008). The WXP1 gene from *Medicago truncatula* containing a AP2 domain was induced by cold, ABA and drought treatment mainly in shoot tissues. Over-expression of WXP1 in alfalfa not only induced a number of wax-related genes, but also significantly increased wax accumulation.Transgenic lines were found to enhance drought tolerance and quick recovery after re-watering (Zhang et al., 2005).

In our previous studies, we isolated and identified the AP2/EREBP genes from several forage crops, including *Caragana korshinskii*, *Galegae orientalis* (Chen et al., 2009; Wang et al., 2010; Wang et al., 2011) and *Ceratoides arborescens* (unpublished data).

CkDBF, a DREB like gene isolated from *C. korshinskii*, was confirmed as a transcription factor by one-hybrid experiments and located to the nucleus. CkDBF is induced by high salt, dehydration, low temperature and abscisic acid (ABA). Over-expression of *CkDBF* in transgenic tobacco induces the expression of downstream stress-responsive genes and increases tolerance under high salinity and osmotic stress (Wang et al., 2010) (figure 1). CkDREB, which contains a conserved AP2/ERF domain, is also isolated from *C. korshinskii*. It was located in the nucleus, and had a DRE element-binding activity and transcriptional activation ability. The expression of *CkDREB* is induced by a variety abiotic stress types including high salt, dehydration and low temperature. The over-expression of CkDREB in tobacco was found to enhance the tolerance for high salinity and mannitol stress by measuring the germination rate of seeds and primary root lengths (figure 1). The over-expression of *CkDREB* induces abiotic stress-response genes containing a DRE element in their promoters. These results show that CkDREB is involved in the regulation of stress-response signals (Wang et al., 2011).



a Germination rates of WT (control) and transgenic lines (CkDBF) grown on MS medium(1), or MS medium supplemented with 200mM NaCl (2) or 250mM mannitol (3) (n=100, each experiment was repeated three times). b Primary root growth of WT and transgenic lines tobacco seedlings (CkDBF) under normal condition (1), treated with150mM mannitol (2), 250mMmannitol (3), 100mM NaCl (4), or 200mMNaCl (5)(n=20). c Expression analysis of downstream genes NtERD10A, NtERD10B, NtERD10C, NtERD10D and NtZfp in transgenic tobacco (CkDBF) by using real-time PCR. Genes were amplified with specific primers. The ACTIN gene was used to normalize samples. Experiments were repeated three times. d Primary root growth of transgenic lines (CkDREB) and WT tobacco seedlings under normal condition(1), treated with 100 mM NaCl (2), 200 mM NaCl(3), 150 mM mannitol (4) or 250 mM mannitol (5) (n = 20). e Germination rate of WT(control) and transgenic lines seeded on MS media (1), or MS media supplemented with200 mM NaCl (2) or 250mM mannitol (3). Each experiment was repeated three times.

Fig. 1. Enhanced stress tolerance of transgenic tobacco carrying DREB transcription factor genes from *Caragana korshinskii*.

Previous studies with DREB transcriptional factor genes focused on DREB-1 and 2 types, which generally play important regulation roles. However, as an A-6 type DREB gene, *CkDBF* responds to a variety abiotic stress types , and the finding that over-expression could enhance the multiple stress-tolerance in transgenic plants indicates that the A-6 type factor also plays an essential role in transcriptional regulation, especially in forage crops.

In addition to DREB-type genes, other AP2/EREBP genes have also been investigated in forage crop plants. GoRAV, which was isolated from *Galegae orientalis*, belongs to the RAV family and has two AP2 and B3-like distinct DNA-binding domains. GoRAV is induced by cold, drought, high salinity and ABA (Chen et al., 2009).

Much researchs has been carried out with the aim of improving the stress-tolerance of forage crops through transgenic modification. Thus, introducing the Arabidopsis DREB1A/CBF3 gene into *Lolium perenne* can increase drpught and freezing stress tolerance, the increased stress tolerance being associated with increased activities of antioxidant enzymes (Li et al., 2011). In another experiment, GmDREB1 isolated from soybean, was introduced into alfalfa under the control of Arabidopsis Rd29A promoter and the transgenic lines induced by salt and showed high tolerance (Jin et al., 2010). Also the Arabidopsis *HARDY* gene, belonging to the stress-related AP2/ERF super family of transcription factors, was transformed into *Trifolium alexandrinum* L. By measuring fresh and dry weight, transpiration and sodium uptake in the transgenic lines and wild type, it was found that over-expression of *HARDY* improves drought and salt tolerance in transgenic plants (Abogadallah et al., 2011).

The zinc-finger motifs, which are classified based on the arrangement of Zinc-binding amino acids, are present in many transcription factors and play critical roles in interactions with other molecules (Chao et al., 2009; Sun et al., 2010). A number of zinc-finger transcription factors have been implicated in important biological processes and stress-tolerance regulation.

MsZFN, encoding a zinc-finger protein, was isolated from alfalfa. *MsZFN* is located in the nucleus, and is significantly induced by salt and reach a maximum level at 30 min (Chao et al. 2009). The Alfin1 gene from alfalfa which encodes a putative Zinc finger motif is specifically expressed in roots (Bastola et al., 1998; Winicov, 1993). The Alfin1 protein binds DNA in a sequence-specific manner in vitro, and can also bind to fragments of MsPRP2, which is considered to be root-specific and accumulates in alfalfa roots under a salt environment (Bastola et al., 1998). Over-expression of Alfin1 under the 35S promoter enhanced expression of the endogenous MsPRP2 gene in alfalfa and improved salinity tolerance (Winicov & Bastola, 1999). Alfin1 has been proposed as a root growth regulator, and transgene lines have increased in root growth under normal and saline conditions in alfalfa (Winicov, 2000).

MtSAP1 (*Medicago truncatula* stress-associated protein1) encodes a zinc-finger domains, and It's expression is increased embryos during desiccation, and decreased significantly during the first hours of imbibing. MtSAP1 protein accumulated in the embryo axis under cold, hypoxia, ABA and desiccation related stress, but its expression is not notably changed under mild drought stress. RNAi studies showed that MtSAP1 storage proteins are very important for the success of germination (Gimeno-Gilles et al., 2011).

Other transcription factors also had been investigated. *AtHDG11* encodes a protein classified as a homeodomain-leucine zipper transcription factor number. Over-expression *AtHDG11* in tall fescue, with four 35S enhancers significantly enhance tolerance to drought and salt stress. The enhanced stress tolerance is associated with a more extensive root system, a lower level of malondialdehyde, a higher level of proline and superoxide dismutase (SOD) and catalase (CAT) (Cao et al., 2009).

In *Medicago truncatula*, the HD-Zip 1 transcription factor HB1 is expressed in primary and lateral root meristerms and is induced by salt stress and constitutive expression of HB1 in *M. truncatula* roots alters their architecture (Ariel et al., 2010).
These studies taken together demonstrate that transcription factors play an important role in the acquisition of stress tolerance in forage crops.

4.2 Osmoregulatory genes

In stress-tolerant plants, many genes are involved in the synthesis of osmoprotectants. Osmoregulation is believed to be the best strategy for abiotic stress tolerance, especially if an osmoregulatory gene can be triggered in response to drought, salinity or high temperature (Bhatnagar-Mathur et al., 2008). Osmotically active solutes include amino acids (e.g., proline, glycine betaine), sugars (e.g., sucrose, fructans), polyols (e.g.,mannitol), and organic ions(e.g., potassium, sodium) (Chaves et al.,2003). These active solutes accumulate in a large number of plant species under environmental stress conditions (Ashraf & Foolad, 2007; Chen & Murate, 2011; Mansour, 1998).
Proline plays a vital role in plants, especially in abiotic stress conditions (Ashraf & Foolad, 2007; Aubert et al., 1999; Schat et al., 1997). Many genes are involved in the synthesis and degradation of proline under a variety of stress conditions such as salt, drought and metal toxicity etc. A role for P5CS (Δ^1-pyrroline-5-carboxylate synthase) in the proline biosynthetic pathway under stress conditions, has been emphasized in the last two decades (Kishor et al., 1995; Zhu et al., 1998). In alfalfa, the expression levels of MtP5CS1 in the different plant organs closely correlated with proline levels but transcript abundance was not affected under osmotic stress condition; was seen to significantly accumulate although only in shoots under osmotic stress conditions (Armengaud et al., 2004). BADH (betaine aldehyde dehydrogenase) is a key enzyme in the biosynthesis of glycine betaine and a BADH gene, which originated from *Atriplex hortensis,* was transformed into alfalfa and was found to increase the salt tolerance of the transgenic plants (Liu et al., 2011).
Sugars also play an essential role in osmotic adjustment. Trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2) from yeast, driven by the rd29A promoter, were transformed into alfalfa. The transgenic lines led to increased plant biomass and tolerance under drought, freezing, salt and heat stress conditions (Suárez & Iturriaga, 2009). In alfalfa leaves, sucrose phosphate synthase (SPS) and sucrose synthase (SS) were examined to explore sucrose metabolism under cold (5°C) and heat stress conditions and it was found that(33°C) genes expression was significantly changed in the cold but not the heat condition (Mo et al., 2011).
Putrescine aminopropyltransferase (PAPT) enzyme, highly specific for putrescine as the initial substrate, can yield multiple polyamine products which are associated with osmotic stress. PAPT activity from alfalfa was found to be mainly located in the meristematic shoot tip and floral bud tisseues. A scheme was proposed to comprehensively illustrate the role of PAPT in biosynthesis of several common and unusual polyamines in alfalfa (Bagga et al., 1997).

4.3 Detoxifying genes

In most of the aerobic organisms, elimination of ROS (reactive oxygen species) is needed under environment stress conditions. In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system that includes various enzymes and non-enzymatic metabolites (Vranova et al., 2002). Key

enzymes for detoxifying ROS in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (Yang et al., 2006).

Superoxide dismutases (SODs) are important antioxidant enzymes that occur in virtually all oxygen-respiring organisms (Halliwell & Gutteridge, 1999; Scandalios, 1997). Until now, four types of SODs have been identified. Copper-zinc SOD is the most importance one, which is closely related to resistance to stress in plants (Feng et al., 2005; Song et al., 2006; Wang et al., 2005). PS-CuZn SOD from *Polygonum sibiricum* , which encodes a copper-zinc SOD, is a constitutively expressed gene and has different expression modes in different organs under salinity-alkalinity stress conditions (Qu et al., 2010). Our team isolated a copper-zinc SOD from *Galega orientails*. Expression of this gene, induced by drought and salt stress, indicated that the copper-zinc gene is involved in stress signaling (unpublished). Copper-zinc SOD can be divided into two forms, one is cytosolic and the other is associated with chloroplast isoenzymes (Sheri et al., 1996). Subcellular analysis showed that Cu-Zn SOD of *G.orientails* locates in chloroplast (unpublished).

Transgenic plants with over-expression of the SOD gene in alfalfa were found to be able to resist, and to possess markedly enhanced antioxidant capacities (Bryan et al., 1993; Bryan et al., 2000). The Mn-SOD gene from *Nicotiana plumbaginifolia* is introduced into alfalfa using two different plasmid vectors for targeting to mitochondria or chloroplast. The transgenic lines had enhanced SOD activity and increased re-growth after freezing stress (McKersie et al., 1993). Another study showed that the introduction of Mn-SOD improved survival, vigor and yield over three years in a natural field environment (McKersie et al., 1996). Further observations with many different transgenic plants in both laboratory and field evaluations show may that over-expression of Mn-SOD improve the winter survival and dry-matter yield, although some lines showed the converse. Although many of the transgenic plants had higher winter survival rates and herbage yield, there was no apparent difference in primary freezing tolerance of the cells in the taproot or crown of transgenic alfalfa (McKersie et al., 1999). In another study, a Fe-SOD gene from Arabidopsis, with a chloroplast transit peptide, was over-expressed in alfalfa under the cauliflower mosaic virus 35S promoter. The transgenic alfalfa show a higher superoxide-scavenging capacity and winter survival. The Fe-SOD activity is found to have a close relationship with winter survival, but not with the oxidative stress tolerance and shoot dry matter production in a 2 year trial. The higher winter survival may stem from reducing secondary injury symptoms and enhancing recovery after stress (McKersie, et al. 2000). In a further investigation of transgenic MnSOD in mitochondria of leaves and nodules, MnSOD in the Chloroplasts and FeSOD in the Chloroplasts; it was shown that transgenic lines had a 20% higher photosynthetic activity than the parental line under mild water stress conditions, however there were no major differences between the untransformed and the transformed alfalfa for most parameters examined under a water stress environment (Rubio et al., 2002). To explore two SOD transgenes influencing the SOD stress-tolerance mechanisms, the F1 progeny was generated through a sexual cross of a hemizygous Mit-MnSOD alfalfa and a hemizygous Chl-MnSOD alfalfa. The results showed that the F1 progeny with the two genes inserted had increased total SOD activity and significantly higher storage organ biomass compared with the non-transgene siblings, but had a lower biomass production compared to siblings having only one transgene (Samis et al., 2002).

Catalase is a unique hydrogen peroxide-scavenging enzyme. A catalase gene *Facat1* is isolated from *Festuca arundinacea* Schreb. Facat1 is up-regulated in cold and salt stress treated leaves, and reached an expression peak at 2 and 4 h, respectively. However, under ABA and drought treatment conditions, the expression was down-regulated (Yang et al., 2006).

Targeting the detoxification pathway is an appropriate approach for producing plants with multiple stress-tolerance traits (Bartels et al., 2001). It is expected that with increased understanding of this pathway, a breeding forage crop will be produced with multiple stress-tolerance.

4.4 Signal transduction

Plant cells sense stress through signaling pathways and transmit the signal to cellular machinery activating an adaptive response essential for plant survival. Molecular and biochemical studies suggest that abiotic stress signaling in plants involves receptor-coupled phosphorylation, phosphoinositol-induced Ca^{2+} changes, mitogen-activated protein kinase cascades and transcriptional activation of stress-responsive genes. In addition, protein post-translational modifications and adapter or scaffold-mediated protein-protein interactions are also important in abiotic stress signal transduction (Xiong & Zhu, 2001).

In alfalfa, $P44^{MMK4}$ kinase was specifically activated under drought and cold treatment conditions, but not induced by high salt concentrations or heat shock. Under ABA treatment, MMK4 transcription levels and $p44^{MMK4}$ kinase were not increased or activated (Jonak et al., 1996). In another study SIMK, an alfalfa mitogen-activated protein kinase (MAPK), was found to be highly regulated by salt stress, in terms of its levels and subcellular localization in roots (Baln ka et al., 2000). SIMK is a member of the family of MAPKs(mitogen-activated protein kinases) which are involved in transducing a variety of extracellular signals. It is transiently activated by NaCl, KCl, sorbitol, and in alfalfa reaches maximal activity between 8 and 16 min before a slow inactivation. Unlike other MAPKs in most mammalian and yeast cells, SIMK has a constituive nuclear localization and the activation is not correlated with nucleo-cytoplasmic translocation (Munnik et al., 1999).

The Msapk1 gene, harbouring a unique ankyrin repeat, can be detected in almost every organ of alfalfa. It is found to be induced in the roots of alfalfa upon osmotic stress (Chinchilla et al., 2003). Also MsCPK3, a calmodulin-like domain protein kinase (CPK), was identified in alfalfa. The expression of MsCPK3 is activated by 2,4-Dichlorophenoxyacetic acid (2,4-D), ABA and NaCl but not by kinetin, ABA or salt treatment. Measurement of the recombinant protein activity showed that MsCPK3 involved in auxin and stress related Ca^{2+} signalling pathways (Davletova et al., 2001).

The AnnMs2 gene, an annexin-like protein from alfalfa, is expressed in various tissues especially in buds, flowers and roots. It is activated in cells or tissues under osmotic stress or by ABA. The recombinant AnnMs2 protein is able to bind to phospholipids in the presence of Ca^{2+} and immunofluorescence studies showed that it is mainly localized in the nuclear (Kovács et al., 1998).

Another signaling element active oxygen species (AOS), can act as ubiquitous signal molecule in plants. It is a central component in the stress response and it's level determines the type of response (Vranova et al. , 2002).

The gene *Srl*k, from *M. trnucatula*, a leucine-rich repeat RLK (receptor-like protein kinases) is rapidly induced by salt stress in roots. The gene expression study and a *Srl*k promoter-β-glucuronidase (GUS) fusion location experiment suggested that Srlk is activated in the root epidermis. Through studies using RNAi and Srlk-TILLING mutants, Srlk would appear to be involved in the regulation of the adaptation of *M.truncatula* roots to salt stress (De Lorenzo et al., 2009).

Heme oxygenase is the rate-limiting enzyme in the breakdown of heme changing into carbon monoxide (CO), iron etc (Shekhawat & Verma, 2010) and it plays a vital role in stress

responses. The expression and protein levels of MsHO1 are higher in alfalfa stems and leaves than in germinating seeds and roots and are induced significantly by some pro-oxidant compounds including hemin and nitric oxide donor sodium nitroprusside (Fu et al., 2011).

4.5 Late embryogenesis abundant proteins

LEA proteins, which are suggested to act as desiccation protectants during seed desiccation and in water-stressed seedlings, can be induced by ABA and various types of water-related stress (Espelund et al., 1992). MtPM5, identified as an atypical hydrophobic LEA protein, during stress, is able to stabilize proteins, but is unable to protect cell membranes. MtPM25 is able to rapidly dissolve aggregates in a non-specific manner and sorption isotherms show that when it is unstructured, it absorbs up to threefold more water than MtEM6 (Boucher et al., 2010). In proteomic analysis of the germination of *M. truncatula* seeds associated to desiccation tolerance (DT), 11 polypeptides were identified as late embryogenesis abundant proteins. The abundance changes of MtEm6 and MtPM25 show the two proteins were related to DT (Boudet et al., 2006).

4.6 Transporter genes

Ion transporters selectively transport ions and maintain them at physiologically relevant concentrations. Sodium transporters in plant cells have been extensively studied. Sodium is compartmentalized into the vacuole, through the operation of the vacuolar Na^+/H^+ antiporter, down an electrochemical proton gradient generated by the vacuolar H^+-ATPase and H^+-Ppase (Blumwald et al., 2000). In both prokaryotic and eukaryotic cells, the Na^+/H^+ exchanger plays a key role in the regulation of cytosolic pH, cell volume and Na^+ homeostasis (Padan et al., 2001; Wiebe et al., 2001). Plant Na^+/H^+ antiporters have been isolated from Arabidopsis (Shi et al., 2000), rice (Fukuda et al., 1999) and forage plants (Li et al., 2009; Tang et al., 2010; Yang et al., 2005). MsNHX1, encoding a vacuolar Na^+/H^+ antiporter, was isolated from alfalfa. The expression of MsNHX1 was significantly up-regulated after treated by NaCl and ABA (Yang et al., 2005). MsNHX1 was located to the vacuolar membrane and can partly complement the NaCl-sensitive phenotypes of a yeast mutant. The expression of MsNHX1 in Arabidopsis enhances the resistance to salt stress (An et al., 2008). To investigate the mechanisms of *Medicago intertexta* and *Melilotus indicus* in salt stress, the expression of four genes coding for NHX-type Na^+/H^+ antiporters were measured. The result show that three genes are expressed in *M. intertexta* leaves and roots, and one gene in *M. indicus* roots. *NHX* gene expression may trigger *M. intertexta* to cope with tissue Na^+ accumulation, while in *M. indicus*, the low Na^+ content and the lack of correlation between growth in the presence of NaCl, Na^+ content and *NHX* gene expression indicates that different mechanisms are involved in coping with salt stress (Zahran et al., 2007). TrNHX1, isolated from *Trifolium repens* L., can complement the *Δnhx1* and *Δena1-4Δnhx1* yeast mutants by suppressing their observed phenotypes. A similar result is observed in the presence of LiCl and KCl. Under 150mM NaCl treatment, the expression level of TrNHX1 in roots, shoots and leaves is 1.7, 2.2 and 4.3 times, respectively, that of the controls. The expression levels and Na^+ content in organs also have a close relationship (Tang et al., 2010). SsNHX1, isolated from the halophyte *Salsola soda*, could significantly enhance salt- tolerance in transgenic alfalfa under the stress-inducible *rd29A* promoter even at 400 mM NaCl (Li et al., 2011). Also *GoNHX1* was induced by salt, drought and ABA treatment in *Galega orientalis* (Li et al., 2009).

When the AVP1 gene, a vacuolar H^+-pyrophosphatase gene from *A. thaliana*, was transformed into alfalfa, over-expression enhanced salt and drought tolerance. Compared to the wild-type, the transgenic plants accumulated more Na^+, K^+ and Ca^{2+} in leaves and roots, retained more solutes and water, maintained a higher root activity, had a protected photosynthetic machinery, and maintained a stable cell membrane under abiotic stress conditions (Bao, et al., 2009). Zhao's team reported that co-expressing *Suaeda salsa* SsNHX1 and AVP1 conferred greater salt tolerance to transgenic plants than did SsNHX1 alone (Zhao et al., 2006). These studies provide a promising way for improving salt and drought tolerance in forage crop plants.

4.7 Cold-acclimation specific gene

Many plants increase freezing tolerance upon exposure to low non-freezing temperatures, a phenomenon known as cold acclimation. Cold acclimation includes the expression of certain cold-induced genes that function to stabilize membranes against freeze-induced injury (Thomashow, 1999). In forage grasses, many genes (Mohapatra et al., 1989; Tamura & Yonemaru, 2010; Tominaga et al., 2001; Zhang et al., 2009) and proteins (Kosmala et al., 2009) are regulated during cold acclimation.

In alfalfa, some CAS (cold-acclimation-specific) genes are specifically expressed during cold-acclimation and their expression level measured by mRNA abundance is positively correlated with the freezing-tolerance of cultivars (Mohapatra et al., 1989). From alfalfa, MsaCIA, cas15 (cold acclimation-specific gene), cas17, and MsaCIC, induced by low temperature have been isolated (Castonguay et al., 1994; Laberge et al., 1993; Monroy et al., 1993; Wolfraim & Dhindsa 1993), although they were not induced by ABA or other stresses (Mohapatra et al., 1989). Encoding a putative nuclear protein, the cas15 transcript level is increased significantly with cold acclimation but is hardly detectable in the absence of cold acclimation. Thus, the accumulation of cas15 and the prolonged cold acclimation have a close relationship (Monroy et al., 1993). In red clover, the homolog of the alfalfa MsaCIA is induced by cold, but the homolog-like MsaCIB, MsaCIC genes were not induced by cold. The expression level of MsaCIA transcripts was 3 times higher in the cold-acclimated, regenerative, F49R (cold tolerant) genotype, compared to the cold-acclimated, non-regenerative, F49M (cold sensitive) genotype. It was also shown that enhanced expression of MsaCIA and the regenerative trait are either linked (Nelke et al., 1999). MsaCIC is similar to bimodular proteins that are developmentally regulated in other plant species (Castonguay et al., 1994). Calcium, a second messenger, can also play an important role in cold acclimation in alfalfa. The influx of extracellular $^{45}Ca^{2+}$ at 4 ^{o}C is 15 times higher than at 25 ^{o}C. The addition of a calcium ionophore or a calcium channel agonist caused an influx of extracellular $^{45}Ca^{2+}$ and induced the expression of *cas* genes (as reporters in low-temperature signal transduction) at 25^{o}C, while the addition of calcium channel blockers inhibited the influx of extracellular $^{45}Ca^{2+}$ as well as the expression of cas genes (Monroy & Dhindsa, 1995). Associated with winter survival, the cold acclimation-responsive gene, such as RootCAR1, may act as a molecular marker for identifying winter hardy plants in semi-dormant or non-dormant alfalfa germplasm in winter of the seed year (Cunningham et al., 2001).

Forage crop plants are the foundation of animal husbandry. In general, forage crops are located in harsh environments, especially in China. This implies that forage crop plants contain essential stress-tolerance gene resources. Over previous decades, the study of stress tolerance mechanisms mainly focused on physiology and morphology, the molecular and

genetic mechanism being less understood. The discovery and use of stress-tolerance-associated genes to confer forage plant stress tolerance, is clearly a promising approach. New studies aimed at revealing the signaling transduction, transcriptional regulation and gene responses in forage plants, will contribute to this end.

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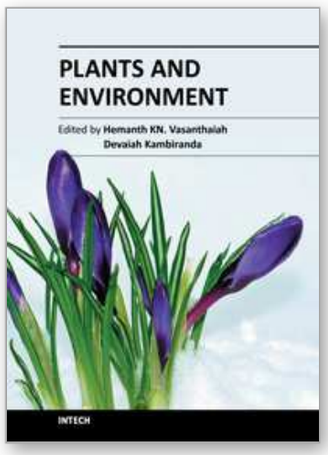

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Changing environmental condition and global population demands understanding the plant responses to hostile environment. Significant progress has been made over the past few decades through amalgamation of molecular breeding with non-conventional breeding. Understanding the cellular and molecular mechanisms to stress tolerance has received considerable scientific scrutiny because of the uniqueness of such processes to plant biology, and also its importance in the campaign â€œFreedom From Hungerâ€. The main intention of this publication is to provide a state-of-the-art and up-to-date knowledge of recent developments in understanding of plant responses to major abiotic stresses, limitations and the current status of crop improvement. A better insight will help in taking a multidisciplinary approach to address the issues affecting plant development and performance under adverse conditions. I trust this book will act as a platform to excel in the field of stress biology.

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