Review

Advances in the molecular breeding of forage crops for abiotic stress tolerance

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Abstract Forages are the backbone of sustainable agriculture. They includes a wide variety of plant species ranging from grasses, such as tall fescue and bermudagrass, to herbaceous legumes, such as alfalfa and white clover. Abiotic stresses, especially salinity, drought, temperature extremes, high photon irradiance, and levels of inorganic solutes, are the limiting factors in the growth and productivity of major cultivated forage crops. Given the great complexity of forage species and the associated difficulties encountered in traditional breeding methods, the potential from molecular breeding in improving forage crops has been recognized. Plant engineering strategies for abiotic stress tolerance largely rely on the gene expression for enzymes involved in pathways leading to the synthesis of functional and structural metabolites, proteins that confer stress tolerance, or proteins in signaling and regulatory pathways. Genetic engineering allows researchers to control timing, tissue-specificity, and expression level for optimal function of the introduced genes. Thus, the use of either a constitutive or stress-inducible promoter may be useful in certain cases. In this review, we summarize the recent progress made towards the development of transgenic forage plants with improved tolerance to abiotic stresses.

Keywords Forage, transgenic, abiotic stress, biomass, molecular breeding, genetically modified organisms

Introduction

Forages are the vegetative portions of plants that are consumed by animals, including the cultivated plants cut for fodder (hay, silage and rotation pasture), permanent pastures and rangelands, as well as crop residue. Forage crops and grassland play a major role in the agricultural development for most parts of the world. They contribute to our food supply through animals. Forages provide ground cover and a habitat for wild life. Thus, forages are very important for soil and wildlife conservation. Forages are the most widely grown, but probably least appreciated, commodity. Compared to cash crops, research and development in this agricultural sector has been neglected to a large extent.

Forage crops include mainly grasses and legume species, although a variety of forbs and browse plants also provide forage for livestock in rangeland areas. Difficulties associated with conventional breeding arise from the diversity in pollination of the different species, irregularities in the fertilization and seed setting, the perennial nature of most forage species as well as differences in the evaluation and maintenance of new lines. Small floral parts make hybridization tedious in many species. Therefore, the methods for forage breeding programs are as varied as the species upon which they are based.

The potential for ruminant livestock to increase the world food supplies is substantial. Thus, the production of an increased amount of forage with limited resources is essential. Forages are frequently grown on marginal lands. Abiotic stresses, such as temperature extremes, high photon irradiance, as well as supplies of water and inorganic solutes, are the limiting factors for the growth and productivity of major cultivated forage crops (Seligman and Sinclair 1995). More than one type of stress can coexist, and one stress can decrease a plant's ability to resist a second one. Drought is considered the most common abiotic stress that limits crop growth and

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productivity. However, other abiotic stresses, notably salinity and acidity, are increasingly significant in limiting the growth of both forage grasses and cereals. Globally, low temperature is also a major limitation for plant growth, and this has a major impact on grasses via, for example, vernalization and low temperature damage during anthesis (Tester and Bacic 2005). In addition, future agricultural production, including forage cultivation and management, will encounter multifaceted challenges from global climate change. Carbon dioxide (CO_2) and other greenhouse gases are accumulating in the atmosphere at unprecedented rates, causing increased radiative forcing (Le Quéré et al. 2009; Shindell et al. 2009). Increased emissions of greenhouse gases will increase annual temperatures. Growing season temperatures are expected to warm more than the annual averages, with reduced precipitation expected to accompany higher temperatures in some regions. Additionally, heat waves are expected to increase in frequency, intensity, and duration (Christensen et al. 2007; Tebaldi et al. 2006). Therefore, traits associated with tolerance to abiotic stresses are the major targets for biotechnological improvement of forage crops.

Conventional approaches to breeding crop plants with improved abiotic stress tolerances have so far met limited success, which is especially true for forage species of complex breeding systems. Conventional breeding methods are disadvantageous because thousands of genes are transferred in each cross including genes which are not necessarily useful, in addition to the desired traits for the target species. Other major limitations in conventional breeding include the barriers for gene transfer from incompatibility and species differences. Instead of relying on sexual recombination to thoroughly mix the parental genes, genetic engineering preserves the integrity of the parental genotype, inserting only a small additional piece of information that controls a specific trait. This is done by splicing a well-characterized piece of foreign DNA, containing a known gene, into a chromosome of the host species using restriction enzymes. This kind of engineering enables the host organism to recognize the new information and use it at the appropriate time as well as cellular location and to the proper extent. Therefore, overcoming problems of sexual incompatibility and species barriers between organisms, this technology helps the breeders and molecular biologists introduce only the gene of

interest with more selective modification, representing a significant advance. Indeed, conventional and molecular breeding methods are different but complementary ways of improving crops, and either method or their combination can be appropriate or inappropriate for particular cases, depending on the breeding objectives.

Molecular breeding continues to be the most rapidly adopted technology in agricultural history due to its social and economic benefits. The estimated global area of transgenic crops for 2009 was 134 million hectares, which is more than 7% over the previous year (Marshall 2010). A sustained rate of annual growth of more than 10% per year has been achieved since their introduction in 1996. On a worldwide scale, transgenic soybean, corn, cotton and canola are cultivated in significant production areas. By contrast, transgenic research in forages has been lagging behind that of major cash crops. Forage breeders have succeeded to some extent in producing stress-tolerant lines/cultivars of certain crops through conventional breeding (Ashraf 1990; Ashraf et al. 1986; Carrow and Duncan 2003; Cattivelli et al. 2008; Reed 1996; Zhou et al. 2009). However, the magnitude of genetic variation in the gene pools of many important forage crops is low. Modern molecular techniques involve the identification and use of molecular markers that can enhance breeding programs. Increased abiotic stress tolerance may be introgressed into commercial lines from tolerant landraces using marker-assisted breeding approaches, which depend on a large amount of sequence information.

Unfortunately, most dedicated forage species are poorly represented in the nucleotide databases. The sequencing of the rice (Yu et al. 2002), soybean (Schmutz et al. 2010), and *Brachipodium* (2010) genomes provides an invaluable resource for exploiting syntenic alignment with other grasses and legumes, which facilitates fine mapping in the unsequenced genomes of many other species. Molecular and physiological information on the processes of abiotic stress tolerance in grasses is being developed, and it is clear that a significantly greater effort is required to both complement and guide breeding and genetic manipulation programs (Tester and Bacic 2005).

Discovery of the molecular mechanisms and key genes behind common stresses open up the possibility for increasing tolerance. However, application of this information to the production of transgenic forage crops

with demonstrably improved abiotic stress tolerance has been slow. There have been a number of reviews published discussing the progress and potential of developing stress-tolerant forages (Kim 2009; Lee 1996; Wang and Ge 2006; Yamada et al. 2005; Yamada et al. 2009; Zhang et al. 2006). In this paper, we will summarize recent developments in utilizing transgenic approaches to develop stress-tolerant forage crops. The uses of transgenic technology with various forage plants are listed in Table 1.

All abiotic stresses alter the expression of a number of genes. Plants' response to environmental stress is controlled by intricate molecular network cascades. These cascades activate stress response mechanisms that re-establish homeostasis as well as protect and repair damaged proteins and membranes (Wang et al. 2003). Some responses are common to all stresses, while some gene expression events are specific to a particular stress. Therefore, plant engineering strategies for abiotic stress tolerance rely on the expression of either genes that are involved in signaling and regulatory pathways (Seki et al. 2003; Shinozaki et al. 2003), genes that encode proteins conferring stress tolerance (Wang et al. 2004), or enzymes present in the pathways that lead to the synthesis of functional and structural metabolites (Apse and Blumwald 2002; Park et

Table 1 Genetic engineering of major cultivated forage crops for abiotic stress tolerance

Gene (source):: Promotor	Gene product/function	Transgenic host	Performance of transgenic plants	Reference
::CaMV35s*WXP1* (*Medicago truncatula*)	AP2 domain-containing putative transcription factor	Alfalfa	Enhanced drought tolerance demonstrated by delayed wilting and quicker and better recovery when the dehydrated plants were re-watered	Zhang et al. 2005
::CaMV35s *CER6WXP1* (*Medicago truncatula*)	AP2 domain-containing putative transcription factor	Alfalfa	Transgenic lines showed higher net photosynthetic rate, transpiration rate, and stomatal conductance and higher efficiency of photosystem II, quantum yield of photosystem II, coefficient of photochemical quenching, and apparent electron transport rate under water stress and after re-watering	Jiang et al. 2010
::CaMV35s*Mn-SOD* (*Nicotiana plumbaginifolia*)	Superoxide Dismutase	Alfalfa	Enhances tolerance to diphenyl ether herbicide, acifluorfen, and freezing; increased regrowth after freezing stress of stress	McKersie et al. 1993
Mn-SOD (Nicotiana plumbaginifolia)::CaMV35s	Superoxide Dismutase	Alfalfa	A3-year field trial suggests enhanced water-deficit tolerance, determined by chlorophyll fluorescence, electrolyte leakage, and re-growth from crowns	McKersie et al. 1996
Mn-SOD (Nicotiana plumbaginifolia)::CaMV35s	Superoxide Dismutase	Alfalfa	Improved the winter survival and subsequent herbage yield in some independent transgenics; winter survival and subsequent yield actually lessened	McKersie et al. 1999
Mit-Mn-SOD and Chl-Mn-SOD (Nicotiana plumbaginifolia):: CaMV35s	Superoxide Dismutase	Alfalfa	F1 generation of a sexual cross between hemizygous Mit-Mn-SOD and Chl-Mn-SOD transgenics was evaluated. Both transgene containing F1 had a lower shoot and storage organ biomass compare to the single gene-transgenics	Samis et al. 2002
Fe-SOD (Arabidopsis thaliana)::CaMV35s	Superoxide Dismutase	Alfalfa	Increased winter survival was observed over 2 years, but does not show resistance to methyl viologen-induced oxidative stress. Total shoot dry matter production over 2 harvest years was not associated with Fe-SOD activity	McKersie et al. 2000
AVP1 (Arabidopsis thaliana)::CaMV35s	Vacuolar H+-pyrophosphatase	Alfalfa	Enhanced salt and drought tolerance. Increased solute accumulation and water retention, and steady intracellular ion homeostasis, higher photosynthesis capacity and the lesser cell membrane damage during salt or water-deficit stress	Bao et al. 2009
AVP1 (Arabidopsis thaliana)::CaMV35s	Vacuolar H+-pyrophosphatase	Creeping bentgrass	Transgenic plants were much tolerant under 100 mM NaCl, and also exhibited faster recovery from damages from exposure to 200 and 300 mM NaCl	Li et al. 2010

Gene (source):: Promotor	Gene product/function	Transgenic host	Performance of transgenic plants	Reference
OsNHX1 (Ooryza sativa)::CaMV35s	vacuolar Na+/H+ antiporter	Perennial ryegrass	Transgenic plants survived a 350 mM NaCl for 10 weeks, while wild-type plants did not. The leaves of transgenic plants accumulated higher concentrations of Na^+, K^+ and proline than those of the control plants.	Wu et al. 2005
AtNHX1 (Arabidopsis thaliana)::CaMV 35s	vacuolar Na+/H+ antiporter	Tall fescue	Transgenic plants grow well up to 200 mM NaCl, whereas untransformed plants exhibited growth retardation at 100 mM	Tian et al. 2006
AtNHX1 (Arabidopsis thaliana)::CaMV 35s	vacuolar Na+/H+ antiporter	Tall fescue	T1 and T2 lines showed no phenotypic changes or yield reduction and were more resistant to a 200 mM NaCl solution than control plants. Biomass accumulation was higher in transgenic plants under saline condition compared to control	Zhao et al. 2006
SsNHX1 (Salsola soda)::rd29A	vacuolar Na+/H+ antiporter	Alfalfa	Transgenic plants showed a strong salt-tolerant ability and could grow in conditions with up to 400 mM NaCl treatment over 50 days	Li et al. 2010
TPS1-TPS2 (Yeast)::rd29A	trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase	Alfalfa	Increased accumulation of trehalose in transgenic plants under stress conditions. Transgenic plants showed a significant increase in drought, freezing, salt, and heat tolerance	Suárez et al. 2009
P5CS (Vigna aconitifolia)::CaMV35s	Δ^1-pyrroline-5-carboxylate synthetase	Barrel medic	Transgenic plants accumulates high levels of proline and display enhanced osmotolerance. Nitrogen fixation was significantly less affected by salt treatment compared to wild-type plants	Verdoy et al. 2006
BetA (Escherichia coli)::CaMV35s	Choline dehydrogenase	Kentucky bluegrass	Transgenic phenotypes were not assessed for stress tolerance	Zhang et al. 2010
ZBD1 (Zoysia tenuifolia)::rice Act1	betaine aldehyde dehydrogenase	Italian ryegrass	Transgenic plants showed better re-rooting ability in vitro in the presence of 300 mM NaCl. Chlorophyll fluorescence of the transgenic was significantly less affected under salt stress	Takahashi et al. 2010
GmDREB1 (Glycine max)::rd29A	Dehydration responsive element transcription factor	Alfalfa	Transgenic had increased free proline and soluble sugars and were more tolerant to salt stress	Jin et al. 2010
HsDREB1A (Hordeum spontaneum)::HVA1s and Dhn8	Transcription factor	Paspalum notatum	T0 and their apomictic progeny survived 200 mM salt stress and repeated cycles of severe dehydration stress under controlled environment conditions	James et al. 2008
DREB1A/CBF3 (Arabidopsis thaliana) ::rd29A promoter	Transcription factor	Tall fescue	Transgenic plants showed an increased expression of *AtP5CS2*, accumulated high level of proline and increased resistance to drought	Zhao et al. 2007
CBF1 (Arabidopsis thaliana)::CaMV35s	Transcription factor	Tall fescue	Tolerance against high salinity and osmosis stresses. Growth of transgenic plants was also inhibited under normal environmental conditions	Wu et al. 2006
AtHDG11 (Arabidopsis thaliana)::CaMV35s	Transcription factor	Tall fescue	Transgenic plant had a more extensive root system, a lower level of malondialdehyde, a nearly normal Na+/K+ ratio, a higher level of proline and a kinetically accelerated induction of SOD and CAT activities and tolerance against drought and salt stress	Cao et al. 2009
hva1 (Hordium vulgare)::ABA2 or Ubi-1	LEA3 protein	Creeping bentgrass	Transgenic lines maintained high water content in leaves and showed significantly less extent of leaf wilting under water-stressed condition. Stress-inducible *hva1* expression is the better choice for water stress tolerance	Fu et al. 2007

Promoter::gene (source)	Gene product/function	Transgenic host	Performance of transgenic plants	Reference
wft1 and wft2 (Triticum aestivum)::CaMV35s	Sucrose–fructan 6-fructosyltransferase and sucrose–sucrose 1-fructosyltransferase, respectively	Perennial ryegrass	A freezing tolerance test using the electrical conductivity method indicated that transgenic plants that accumulated a greater amount of fructan than non-transgenic plants and have increased tolerance on a cellular level to freezing	Hisano et al. 2004
ipt (Arabidopsis thaliana)::SAG12	adenine isopentenyl phosphotransferase	Creeping bentgrass	Root isopentenyl adenine (iPA) content increased 2.5-3.5 timesin transgenics while decreased by 20% in wild type under heat stress. iPA accumulation stimulated tiller formation and root production, and delayed leaf senescence under heat stress	Xu et al. 2009
ipt (Arabidopsis thaliana)::SAG12 -or HSP18.2 promoter	adenine isopentenyl phosphotransferase	Creeping bentgrass	Transgenic lines exhibited higher CK content and a higher CK-to-ABA ratio, higher turf quality, photochemical efficiency, chlorophyll content, leaf relative water content, and root: shoot ratio under drought stress	Merewitz et al. 2010

al. 2004; Rontein et al. 2002). Based on these knowledge, recent efforts to improve plant stress tolerance by genetic transformation have resulted in several important achievements. A number of gene constructs have been used to engineer forage crops to provide stress tolerance. Here, we summarize the information from a recent survey of the literature and provide a framework to demonstrate the range of materials and the methods presently under production and testing.

Transgene expression for enhanced antioxidant metabolism

Stress-induced production of reactive oxygen species (ROS) is an important aspect of environmental stress response in plants. ROS form in the cell as a natural byproduct of normal oxygen metabolism in both unstressed and stressed cells. Physiological concentrations of ROS in aerobic organisms are beneficial and involve cell signaling pathways as well as survival from invading pathogens. However, during times of environmental stress, such as drought, salinity, metal toxicity, high and low temperature, wounding and pathogenic infection, ROS levels can increase dramatically and cause oxidative stress. The presence of unpaired valence shell electrons in ROS result in significant damage to cellular components. Plants respond to oxidative stress with increased enzymatic and non-enzymatic antioxidant processes (Alscher and Hess 1993). Alleviation of oxidative damage by using different antioxidants and ROS scavengers can enhance plant

resistance to various abiotic stresses, such as salt, drought and chilling. Among the antioxidative enzymes, superoxide dismutase (SOD) and ascorbate peroxidase (APX) provide the first line of defense against ROS. SOD, the first enzyme in the detoxifying process, converts superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2), and APX reduces H_2O_2 to water using ascorbic acid as a specific electron donor (Asada 1992; Asada 1999; Foyer et al. 1994). ROS are generated from the reaction of chloroplast O_2 and electrons that escape from the photosynthetic electron transfer system (Foyer et al. 1994). O_2^- can be produced in various cellular compartments where an electron transport chain is present, such as the mitochondria, chloroplast microsome, glyoxysome, peroxisome, apoplast and cytosol. However, chloroplasts, mitochondria and peroxisomes are the biggest contributors of cellular Ros (Fridovich 1986). As phospholipid membranes are impermeable to charged O_2^-, the local scavenging tools for ROS are crucial. Therefore, plants have localized SOD with distinct metal co-factors. For example, Fe-SOD is located in chloroplasts; Mn-SOD in mitochondria and peroxisomes; and Cu-Zn-SOD in chloroplasts, the cytosol and possibly the extracellular space. Such diversity in SODs with different metal requirements is possibly linked to the differential availability of soluble transition metal compounds from the biosphere in relation to the O_2 content of the atmosphere in different geographical areas (Bannister et al. 1991).

Reduced water availability is one of the limiting factors in forage production. During drought stress, over-reduction of the photosynthetic electron transport chain forces the

ensuing excitation energy to dissipate via non-photochemical quenching, which leads to the production of superoxide due to a drought-impaired electron transport system (Alam et al. 2010b; Price et al. 1989). Chloroplasts are highly sensitive to damage by ROS, therefore, overexpression of SOD could be a good strategy to clean up ROS and decrease plant damage from drought. Overexpression of a *Mn-SOD* cDNA from *Nicotiana plumbaginifolia* in transgenic alfalfa (*Medicago sativa*) led to reduced injury from water-deficit stress, as determined by chlorophyll fluorescence, electrolyte leakage, and re-growth from crowns (McKersie et al. 1996). A 3-year field trial of these transgenic plants indicated that their yield and survival were significantly improved, which suggests that tolerance against oxidative stress could be an important strategy for the forage plants to adapt to field conditions.

Increased production of activated oxygen species has been associated with the development of injury symptoms from chilling (Einset et al. 2007; Lee et al. 2007a), freezing (Kendall and McKersie 1989; Liang et al. 2008), and ice encasement (McKersie et al. 1982). Tolerance to these environmental stresses correlates with an increased capacity to either scavenge or detoxify activated oxygen species (Kendall and McKersie 1989 ; Malan et al. 1990). McKersie et al. (1993) overexpressed a *Mn-SOD* cDNA from *Nicotiana plumbaginifolia* into alfalfa using *Agrobacterium*-mediated transformation. The *Mn-SOD* construct contained a transit peptide for targeting to either the mitochondria or chloroplasts and exhibited tolerance against freezing and paraquat, which is an ROS-generating herbicide. The transgenic plants showed an increased rate of re-growth after freezing injury. Enhanced Mn-SOD activity may limit the membrane injury to a few cells and prevent development of a secondary injury. The observation that differences among the plants were greatest during the second cycle of re-growth, not the first supports the idea. Similarly, an *Arabidopsis Fe-SOD* cDNA was also overexpressed in alfalfa (McKersie et al. 2000). The transgenic plants showed increased Fe-SOD activity and winter survival over 2 years in field trials; however, they did not exhibit oxidative stress tolerance, as measured by resistance of leaves to methyl viologen. There was no detectable difference in the growth pattern, carbohydrate accumulation and primary freezing injury between the control and transgenic alfalfa plants.

Therefore, the improvement in winter survival neither appeared to be a consequence of improved oxidative stress tolerance associated with photosynthesis, nor was it a consequence of a change in primary freezing injury. Thus, it appears that *Fe-SOD* overexpression reduced secondary injury symptoms and, thereby, enhanced recovery from stresses experienced during winter. Another candidate, *2-Cys-Prx*, has been shown to possess an important antioxidant function. Peroxiredoxins (Prx) are ubiquitous thiol-based peroxidases that detoxify various peroxide substrates via their catalytic cysteine residues by using thiol-containing proteins as reductants (Wood et al. 2003). Prx enzymes are involved in multiple cellular processes such as antioxidant defense (Neumann et al. 2003), H_2O_2-mediated cellular signaling (Choi et al. 2005) and molecular chaperone-based folding (Jang et al. 2004). Based on the number of conserved Cys residues that participate in the catalytic cycle, Prxs are largely divided into two groups, 1-Cys Prxs and 2-Cys Prxs. Having two functions, 2-Cys Prx acts as a peroxidase and as a molecular chaperone, alternating between the two under heat shock and oxidative stresses (Kim et al. 2009). We overexpressed an *Arabidopsis* 2-Cys Prx gene into tall fescue under the control of a CaMV 35S promoter (Kim et al. 2010). The transgenic plants showed enhanced tolerance to MV-induced oxidative stress and heat stress. Under heat stress (42C), the transgenic plants were capable of maintaining their chlorophyll fluorescence (Fv/Fm) for 24 hrs, while the control plants lost chlorophyll fluorescence very quickly.

The pyramiding of genes is another way of increasing plant performance under stress conditions. In the same way the plant breeders are continually developing new varieties that contain the most effective combination of existing characteristics, there is now a similar trend with transgenic crops. As overexpression of one enzyme may not alter the function of the entire antioxidant pathway, in certain cases, overexpression of more than one antioxidant enzyme may provide enhanced protection against either oxidative or abiotic stresses (Pitcher et al. 1991; Tepperman and Dunsmuir 1990; Torsethaugen et al. 1997). In furtherance of this goal, a sexual cross was conducted between a hemizygous *Mit-MnSOD* plant and a hemizygous *Chl-MnSOD* transgenic

alfalfa plant, which had previously been screened in field trials for improved persistence. The F_1 population showed significantly higher total SOD activity and improved winter persistence (Samis et al. 2002). However, the F_1 progeny containing both transgenes had a lower shoot and storage organ biomass, compared with the siblings that had only one of the transgenes.

Pyramiding of multiple genes has also been practiced using entirely transgenic approaches in both model and forage plants (Kwon et al. 2002) demonstrated that overexpression of both the CuZnSOD and APX genes in tobacco chloroplasts resulted in enhanced tolerance to methyl viologen (MV)-induced oxidative stress, compared with expression of either of these genes alone. (Lee et al. 2007b) introduced a chimeric gene construct, *SWPA 2::CuZnSOD::APX* (Tang et al. 2006), into tall fescue plants (*Festuca arundinacea*) by *Agrobacterium*-mediated transformation. High levels of transgene expression and subsequent enhanced tolerance was exhibited under a wide range of abiotic stresses, including methyl viologen, H_2O_2, and the heavy metals, copper, cadmium, and arsenic. Such pyramiding of multiple transgenes will hopefully become an increasing feature in new forage cultivars in the near future.

Transgenes expression for ion transporters

Salinity is one of the most widespread agricultural problems in arid and semi-arid regions, making fields unproductive. More than 50% of all arable lands are supposed to experience serious salinization by the year 2050 (Ashraf 1994). Salt stress severely inhibits plant growth for two primary reasons: one, due to either a salinity-based osmotic or water-deficit effect and two, via either a NaCl salt-specific or ion-excess effect, which causes changes in the K^+/Na^+ ratio and increases the concentrations of Na^+ and Cl^- in the cytosol (Munns and Tester 2008). Sodium toxicity primarily occurs because Na^+ and K^+ ions are similar to plant transporters and enzymes. Plant cells typically maintain a high K^+/Na^+ ratio in their cytosol with a relatively high K^+ concentration, on the order of 100-200 mM, and low Na^+ concentration, approximately 1-10 mM (Higinbotham 1973). To prevent salt damage, plants restrict the uptake of environmental

Na^+, increase the efflux of Na^+ from the cell, and sequester Na^+ into the large intracellular vacuole. A number of studies have revealed that exposure to salt up-regulates Na^+/H^+ antiporter activity, suggesting a role for the exchanger in salt tolerance. The activity of this secondary transport system is driven by the proton-motive force, generated by the vacuolar membrane H^+-ATPase and H^+-pyrophosphatase, which also respond to salt levels through transcriptional and posttranscriptional regulation (Maeshima 2001). Thus, the Na^+/H^+ antiporters play a major role in internal pH, cell volume, and Na^+ homeostasis (Padan et al. 2001; Wiebe et al. 2001).

The Na^+/H^+ antiporter system is found in animals, yeast, bacteria and plants cells (Blumwald et al. 2000). Overexpression of the vacuolar Na/H^+ antiporter and H^+-pyrophosphatase pump (H^+-PPase) provides enhanced tolerance to both salinity (Apse et al. 1999; Zhang and Blumwald 2001) and drought stress (Gaxiola et al. 2001; Park et al. 2005) in model plants. These results suggest that enhanced vacuolar H^+-pumping in the transgenic plants provided an additional driving force for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter. Consequently, an effort to engineer forage crops has also been undertaken. A Na^+/H^+ antiporter gene in the rice vacuolar membrane (Fukuda et al. 2004) was transferred to perennial ryegrass (Wu et al. 2005). The leaves of these transgenic plants accumulated higher concentrations of Na^+, K^+ and proline than in the control plants. The transgenic plants survived in 350 mM NaCl after 10 weeks, while wild-type plants did not.

Tall fescue plants have also been successfully transformed by *Agrobacterium* overexpressing *Arabidopsis* AtNHX1, the vacuolar Na^+/H^+ antiporter gene, to embryogenic calli of several tall fescue genotypes and the regeneration of transgenic T_0 plants (Zhao et al. 2005). Genetic analysis of transgenic salt-tolerant phenotypes and the K^+/Na^+ levels in independent transgenic T_0 and T_1 lines suggest that overexpression of the vacuolar Na^+/H^+ antiporter allows the transgenic lines to sequester a large amount of Na^+ into the root vacuoles. This sequestering event likely results in less transport of Na^+ to the leaves (Zhao et al. 2007b). The salt-tolerant transgenic plants showed neither phenotypic abnormalities nor yield reduction under control condition. Under normal conditions, the germination rates for the transgenic progenies were

similar to non-transgenics, whereas under saline conditions the transgenics had dramatically higher rates. Salt stress also had a less deleterious effect on the vegetative growth of transgenic plants compared to the wild-type plants. Wild-type plants showed symptoms of salt toxicity, such as progressive chlorosis, reduced leaf size and growth inhibition, while the transgenic plants were not affected by a NaCl concentration below 200 mM.

Similar to model plants, application of the vacuolar H^+-pyrophosphatase (H^+-PPase) gene has also been reported in forage crops. (Bao et al. 2009) reported on overexpression of the *Arabidopsis* H^+-pyrophosphatase gene, *AVP1*, in alfalfa. Transgenic plants accumulate more Na^+, K^+ and Ca_2^+ in leaves and roots compared with wild-type plants. Transgenic alfalfa showed considerable growth in the presence of 200 mM NaCl and under drought conditions, while wild-type plants exhibited chlorosis, growth inhibition, and death. Increased solute accumulation, water retention, and steady intracellular ion homeostasis might help transgenic plants have higher photosynthesis capacity and the less cell membrane damage during either salt or water-deficit stress. Comparing the salt tolerance between transgenic rice plants expressing the *Suaeda salsa* Na^+/H^+ antiporter, SsNHX1, and co-expressing SsNHX1 with *Arabidopsis* AVP1, it was found that simultaneous expression of *SsNHX1* and *AVP1* conferred more tolerance to the co-expressing plants than those only expressing *SsNHX1* (Zhao et al. 2006). This result suggests that a similar approach would also be helpful for forage crops for enhanced H^+-pumping of the vacuolar solute accumulation.

Engineering for the synthesis of osmoprotectants

Physiologically, salinity imposes an initial water deficit, which results from the relatively high solute concentrations in the soil (Yamaguchi and Blumwald 2005). Cellular ion exclusion cannot provide complete adaptation for plants to high soil salinity, presumably due to the osmotic stress component of salinity stress (Blumwald et al. 2000). Drought, hypoxia and cold stress impose osmotic stress on plants. To overcome osmotic stress, plants tend to accumulate certain low molecular mass osmoprotectants and osmolytes, such as quaternary amines, amino acids

and sugar alcohols. These compounds provide plants the ability to raise the osmotic potential of the cell, thus balancing the osmotic potential of an externally increased osmotic pressure and stabilizing the membranes and/ or macromolecular structure. However, the ability to accumulate such compounds varies greatly among the species. For example, rice, potato and tobacco accumulate limited amounts of the potent osmoprotective compound, glycine betaine. This accumulation makes them excellent targets for introducing osmoprotectant/osmolyte-producing enzyme systems. Manipulation of osmolyte and compatible solute synthesis using transgenic approaches has yielded impressive results for tolerances against salt, cold and drought stress in model plants (Pommerrenig et al. 2007; Tarczynski et al. 1993). A number of forage plants have also been engineered for enhanced accumulation of various compatible solutes to confer tolerance against high salinity, cold and drought.

Proline has received much attention as an osmolyte in transgenic overproduction. Accumulation of proline was reported in many plant species under diverse abiotic stress conditions, especially salt and osmotic stress, but also for cold and frost (Han and Hwang 2003; Hong et al. 2000; Kavi Kishor et al. 2005; Nanjo et al. 1999; Zhu et al. 1998). A number of protective roles have been proposed for proline, such as mediator for osmotic adjustment (Handa et al. 1986); stabilizer for sub-cellular structures (Schobert and Tschesche 1978); free-radical scavenger (Hong et al. 2000; Saradhi et al. 1995); heavy metal detoxifier (Rai 2002; Sharma et al. 1998); energy sink (Alia and Saradhi 1991); and signaling/regulatory molecule that is able to activate multiple responses . A Δ1-pyrroline-5-carboxylate synthetase (*P5CS*) gene, which was cloned from *Vigna aconitifolia*, was transformed to a model legume, *Medicago truncatula*, which accumulated high levels of proline and displayed enhanced osmotolerance (Verdoy et al. 2006). Nitrogen fixation was significantly lower after salt treatment compared with wild-type plants.

Synthesis of glycine betaine (GB) has also received much attention in plant genetic engineering (Sakamoto and Murata 2001). GB is a zwitterionic quaternary ammonium, which is known to function as an extremely efficient compatible solute (Le Rudulier et al. 1984). GB is synthesized in higher plants via the choline-betaine pathway. It has two oxidization steps: choline→betaine

and aldehyde→betaine. The first step is catalyzed by choline monooxygenase and the second by betaine aldehyde dehydrogenase (BADH) (Sakamoto and Murata 2002). Genes encoding enzymes involved in betaine synthesis have been used to produce transgenic plants that show improved tolerance to salinity and other environmental stresses (Holmström et al. 2000; Jia et al. 2002; Kishitani et al. 2000; Li et al. 2003; Quan et al. 2004). The *E. coli betA* gene encodes choline dehydrogenase, which catalyzes the conversion of choline to glycine betaine via the intermediate, betaine aldehyde. Overexpression of *betA* produces salt- and freezing-tolerant phenotypes in tobacco and potato (Holmberg and Bülow 1998; Lilius et al. 1996). The *BADH* gene, (*ZBD1*) was cloned from salt-tolerant zoysiagrass (*Zoysia tenuifolia* Willd. ex Trin.) (Oishi and Ebina 2005) and was transformed into Italian ryegrass (*Lolium multiflorum*) (Takahashi et al. 2010); the plant height, fresh weight, dry weight, and number of tillers were higher in transgenic plants in 300 mM NaCl. The transgenic plants also had better re-rooting ability *in vitro* inthe presence of NaCl. Transgenic plants maintained higher relative Fv/Fm values over the non-transgenic plants, suggesting less damage to photosystem II of the transgenics. Although the authors did not measure the betaine content precisely in the transgenic plants, the results are reasonable because betaine has a pleiotropic function for stress tolerance in addition to its function as an osmoprotectant. Recently, Zhang et al. (2010) introduced the *betA* gene, encoding choline dehydrogenase, and a mutant *als* gene, encoding the enzyme acetolactate synthase, into three Kentucky bluegrass cultivars. However, the genes' subsequent effects on the transgenic plants were not reported.

The *codA* gene encodes choline oxidase, which is the enzyme that converts choline into glycine betaine, and has been cloned from a soil bacterium, *Arthrobacter globiformis* (Deshnium et al. 1995). Transformation of *Arabidopsis thaliana* with the cloned *codA* gene, under the control of the constitutive promoter, enabled the plant to accumulate glycinebetaine and enhanced its tolerance to salt and cold stress (Hayashi et al. 1997). In an effort to obtain the synergistic and protective effects from compatible solutes and antioxidants, Ahmad et al. (2010) pyramided *CuZnSOD*, chloroplasts targeting *APX* and a bacterial choline oxidase (*codA*) gene under the control

of the stress inducible *SWP2* promoter in potato plants using transgenesis. GB accumulation in these transgenic plants helped maintain higher activities of SOD, APX and catalase, following oxidative, salt and drought stress treatments. In our laboratory, we are also developing transgenic tall fescue overexpressing the *codA* gene for increased GB synthesis (unpublished data). The preliminary results indicate better root growth and shoot biomass accumulation in the transgenic plants (Fig. 1).

Trehalose is a nonreducing glucose disaccharide that also functions as a compatible solute in stabilizing biological structures under abiotic stress in bacteria, fungi, and invertebrates (Garg et al. 2002). Overexpression of the *E. coli* trehalose biosynthetic genes (*otsA* and *otsB*) as a fusion gene resulted in 3-10 times higher trehalose accumulation in transgenic rice plants, depending on the growth conditions. Several independent transgenic lines exhibited sustained plant growth, less photo-oxidative damage, and more favorable mineral balance under salt, drought, and low-temperature stress conditions (Garg et al. 2002). A bifunctional, yeast *TPS1-TPS2* (trehalose-6-phosphate synthase) gene, which is another candidate for trehalose biosynthesis, has been expressed in *Arabidopsis* and confers tolerance to several abiotic stresses (Miranda et al. 2007). The same gene was also introduced into alfalfa, using either the constitutive 35S promoter or the stress-regulated *rd29A* promoter (Suárez et al. 2009). Trehalose accumulated in all the different lines at similar levels under stress conditions. However, plants overexpressing this bifunctional enzyme driven by the constitutive promoter had slower growth and reduced
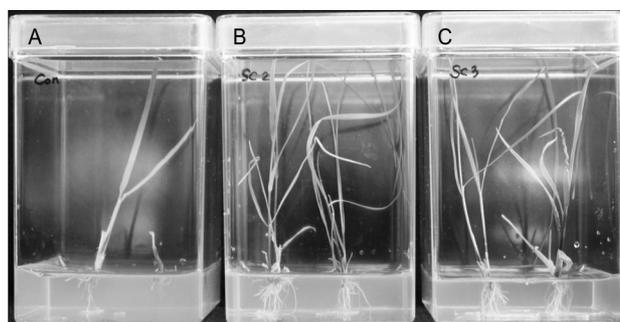
Fig. 1 *In vitro* salt-treatment assay of wild-type and transgenic tall fescue overexpressing *codA* gene driven by a CaMV 35S promoter. Non-transgenic control (A) and two independent transgenic lines (B and C) were presented as an example to show the effect of salt treatment. The photographs were taken after 4 weeks of culture in 200 mM NaCl treatment

biomass, down to approximately 21%. In contrast, when the genes were controlled by a stress-inducible *rd29A* promoter, the transgenics had a significant increase in biomass, up to 62% compared with the wild-type plants under non-stressed conditions. These results indicate that constitutive trehalose formation might divert the carbon skeletons that are normally required for macromolecules synthesis involved in growth. Nevertheless, the transgenic plants displayed a significant increase in drought, freezing, salt, and heat tolerance. Therefore, stress-inducible over-accumulation of trehalose would be a better option in engineering crops. These observations demonstrate the feasibility of engineering forage crops for increased tolerance of abiotic stress, especially salt, drought and cold, as well as enhanced productivity through either tissue-specific or stress-dependent overproduction of various compatible solutes.

Manipulation of signaling and regulatory elements

Microarray and proteomics techniques have allowed researchers to catalog the expression of numerous genes under various adverse environmental conditions for many plant species. Many of the products from these genes are thought to promote stress tolerance and regulate gene expression through various signal transduction pathways (Alam et al. 2010a; Alam et al. 2010b; Shinozaki et al. 2003; Xiong et al. 2002). Using these regulatory proteins, many genes involved in stress response can be simultaneously regulated with a single gene that encodes a stress inducible transcription factor (Kasuga et al. 1999). Thus, this technology offers the possibility of enhancing tolerance towards multiple stresses including drought, salinity, and freezing (Bhatnagar-Mathur et al. 2008). Transcription factors (TFs) are master regulators that control gene clusters. A single TF can control the expression of many target genes through specific binding of the TF to the cis-acting element at the promoters of its respective target genes (Nakashima et al. 2009).

Several major transcriptional regulatory systems have been identified in *Arabidopsis*. For instance, dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factor (CBF) and DREB2 systems function during ABA-independent gene expression. On the other hand,

the ABA-responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) system functions in ABA-dependent gene expression. Engineering the regulatory machinery involving transcription factors has emerged as a new tool for controlling the expression of many stress-responsive genes. (Zhao et al. 2007a) introduced *Arabidopsis DREB1A/CBF3*, driven by an inducible *rd29A* promoter, into the tall fescue genome via *Agrobacterium*-mediated transformation. As expected, the transgenic plants also showed an increased expression of *AtP5CS2*, a downstream target gene of *DREB*, observed in *Arabidopsis*. The transgenic tall fescue lines accumulated high levels of proline and showed an increased resistance to drought stress. Another *DREB1A* transcription factor ortholog was isolated from a wild barley (*Hordeum spontaneum*) and overexpressed in bahiagrass (*Paspalum notatum*) under the control of stress-inducible *HVA1s* and *Dhn8* promoters (James et al. 2008). These transgenic plants survived severe salt stress and repeated cycles of severe dehydration stress under controlled environmental conditions, in contrast to non-transgenic plants. Recently, a soybean *DREB* ortholog, *GmDREB1*, was introduced into alfalfa plants under the control of an *Arabidopsis Rd29A* promoter, which conferred salt tolerance to these transgenic plants (Jin et al. 2010). These transgenic lines had significantly lower ion leakage, higher chlorophyll fluorescence value, and higher free proline and total soluble sugar content under salt stress.

The homeodomain (HD) transcription factor family having Steroidogenic Acute Regulatory related lipid transfer (START) domainsis another important group of transcriptional regulators, also known as the Class IV Homeodomain-Leucine Zipper transcription family (Nakamura et al. 2006). The majority of START-domain containing proteins in plants were found to contain a homeodomain, and these HD-START transcription factors are unique to plants (Schrick et al. 2004). An *Arabidopsis AtHDG11* was overexpressed in transgenic tall fescue under the control of the CaMV 35S promoter with four enhancers (Cao et al. 2009). The constitutive expression of the *AtHDG11* resulted in significantly enhanced tolerance to drought and salt stress. These transgenic plants had a more extensive root system, a lower level of malondialdehyde, a nearly normal Na^+/K^+ ratio, a higher level of proline and a kinetically accelerated induction of SOD and

CAT activities during drought and/or salt stress. These characteristics indicate that the stress tolerance might be associated with the pleiotropic effect of AtHDG11 expression.

Modulation of transcriptional factors may also alter the anatomical features of plants, such as wax formation. Wax present on plant surfaces plays an important role in water conservation and in protecting aerial organs from damage caused by environmental stresses. Because multiple biochemical processes are involved in wax biosynthesis, transgenic expression of regulatory genes could be an effective way to manipulate wax accumulation in plants. A transcriptional regulator, *WXP1* (*WAX PRODUCTION1*), has been characterized as a putative AP2 domain-containing transcription factor gene from the model legume, *M. truncatula*, which activates wax production. Constitutive overexpression of *WXP1* under the control of the CaMV35S promoter led to a significant increase in cuticular wax loading on the leaves of a transgenic alfalfa (Zhang et al. 2005). Transgenic alfalfa plants with increased cuticular waxes showed an enhanced drought tolerance, demonstrated by delayed wilting after watering ceased as well as a quicker and better recovery when the dehydrated plants were re-watered. Recently, *WXP1* has also been transferred to white clover (*Trifolium repens* L.) directed by an *Arabidopsis* CER6 promoter (Jiang et al. 2010). Constitutive expression of the *WXP1* resulted in higher net photosynthetic rates, efficiency of PSII, relative water content and leaf water potential under drought-stressed conditions in the transgenic plants. Therefore, genetic modification of leaf cuticular waxes through transcriptional regulation has great potential for enhancing stress tolerance.

Conclusions

Transcriptomic, proteomic and metabolic analyses have identified and characterized several genes that are induced by abiotic stresses and their associated signaling and regulatory pathways in plants. These knowledge uncover the cross-talk between abiotic stress and signaling pathways, providing potential candidate genes for stress tolerance. Among them, the most promising candidates were transferred to the forage crops. In many cases, the

stress tolerance was concomitant with some abnormalities. Therefore, the tolerance-related mechanism should always be assessed thoroughly with respect to its cross-talk with other stress-related mechanisms. The choice of promoter, which confers constitutive, spatial and/or temporal expression of the transgene, is one of the key determinants to obtaining successful stress tolerance without any undesirable traits. Currently, the performance of stress tolerance in transgenic plants is reported on the basis of either laboratory tests or short-term greenhouse trials. These may not mimic actual field conditions, which are generally associated with multiple, simultaneous stresses. As physiological and molecular responses during short and long exposures to stress could differ, the practical aspects, such as biomass, yield and quality, should be addressed to assess the performance of the transgenic plants. For example, proline accumulation has been reported to confer salt and drought tolerance, although it is controversial as to whether proline is useful in sustaining yield in the field. Diverse types of forage plants may have distinct physiological and molecular responses to stress. Therefore, comprehensive profiles of the stress-associated transcriptome, proteome and metabolome would be useful in dissecting the response specific to forage species and would eventually assist in breeding stress-tolerant cultivars.

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