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AGROBACTERIUM-MEDIATED TRANSFORMATION OF PERENNIAL RYEGRASS (_LOLIUM PERENNE_ L.) FOR COLD TOLERANCE

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By

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ABSTRACT

AGROBACTERIUM-MEDIATED TRANSFORMATION OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) FOR COLD TOLERANCE

By

Carmille Joanna C. Bales

Perennial ryegrass (*Lolium perenne* L.) is an agronomically important grass grown in the temperate regions of the world. It is widely used as a forage crop and turfgrass for its high quality and yield. However, its growth is limited due to its inability to survive in extreme winter temperatures. The aim of the present study is to obtain cold tolerant perennial ryegrass by transforming it the cold regulated transcriptional activator *Lp*CBF3 gene using the *Agrobacterium*-mediated gene transfer method and evaluate overexpression of the *Lp*CBF3 gene under the control of the constitutive promoter, cauliflower mosaic virus 35S (CaMV35S). Embryogenic calli of perennial ryegrass cv. 'Inspire' were transformed by an *Agrobacterium* strain EHA105 containing a modified pFGC5941 plasmid.

Results of the study showed that transgenic perennial ryegrass overexpressing the *Lp*CBF3 gene was successfully regenerated. Presence and stable integration of the gene in the genome was confirmed by PCR analysis and Southern hybridization. Glufosinate resistance and electrolyte leakage tests indicated the expression of the gene in the transformants. Cold acclimated transgenic plants showed increased tolerance to freezing temperature. In this study, 35S:*Lp*CBF3 transgenic plants did not show growth retardation but had reduced tillering ability.

"I have fought the good fight, I have finished the race, I have kept the faith."
2 Timothy 4:7

To God be the glory...

This piece of work is humbly dedicated to
Papsy, Mamsy, Dodong and Joseph

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CHAPTER I

LITERATURE REVIEW

Importance of perennial ryegrass

Perennial ryegrass (*Lolium perenne* L.) is a native of Europe, Asia, and South Africa and is an agronomically important grass in the temperate regions of the world. It is widely used as a forage crop and as turfgrass for its high quality and yield. The United States produced over 50,000 certified and 70,000 tons of uncertified perennial ryegrass seeds in 2006-2007 (Forage and Turf Crop Statistics, 2006). In a 2002 Michigan Turfgrass survey, an estimated total of 17,030 acres of perennial ryegrass is used in golf courses, parks, schools, and cemeteries. For pastures, less than 10,000 is the estimated acreage of perennial ryegrass in Michigan (Leep, 2007).

Perennial ryegrass is used for forage predominantly in the coastal Northwest, the Midwest, the Northeast, and irrigated intermountain valleys of the West. Its palatability and digestibility make this species highly valued for dairy and sheep forage systems. Also, the use of perennial ryegrass for turf has increased in recent years with selection for more dense-growing and persistent turf types. It is also considered one of the most versatile turfgrass species. For turf, perennial ryegrass is used alone or in combination with other grasses. Disease susceptibility and limited cold and heat tolerance, however, limit its persistence and zone of adaptation (Hannaway et al., 1999).

Michigan producers prefer perennial ryegrass for its high quality and yield, but it has low winter hardiness (ability of a plant to survive winter). Humphreys and Eagles

(1988) reported that the cold tolerance (ability of plants to tolerate stresses when exposed to temperatures below 0°C) of perennial ryegrass needed to be improved before the species could be used in the United Kingdom and northern continental climates. Of all the perennial cool season grasses, ryegrass is the least winter hardy, so that survival can be risky in areas with cold winters with no snow cover (Kaye, 2007). Frame (1989) compared herbage productivity of several grass species, including perennial ryegrass, in the United Kingdom and determined that perennial ryegrass performed poorly compared to the other grass species mainly because production was affected by winter damage.

Perennial ryegrass is a cool-season grass belonging to the Poaceae family and is either diploid (2n=2x=14) or tetraploid (4x=28) with a two-locus self-incompatibility system, which ensures a high degree of genetic variation in populations (Bolaric et al., 2005). Diploid types are used in permanent pastures. They tiller more, resulting in very solid, durable, and long lasting pastures. They also have higher dry matter content (Kaye, 2007). Tetraploid types tend to be taller and less dense than diploid types, even in early stages of regrowth (Cosgrove et al., 1999). Past research results indicate that diploid cultivars of perennial ryegrass have a higher cold tolerance than tetraploid cultivars (Sugiyama, 1998; Warnock et al., 2005).

Improving cold tolerance is one of the most important breeding objectives for perennial ryegrass. However, conventional breeding methods have so far been unsuccessful in achieving this objective because of the quantitative characteristic of the cold tolerance trait (Skinner et al., 2006; Skøt et al., 2002). Modern biotechnology provides an opportunity to improve cold tolerance and winter hardiness in perennial ryegrass, as well as other plant species by manipulating genes related to cold tolerance

and their expression (Kosmala et al., 2006; Zhang et al., 2004). Forage and turfgrass managers in Northern temperate climates could greatly benefit from new high-quality, cold-tolerant perennial ryegrass cultivars.

Cold tolerance mechanisms in plants

Cold stress, caused by chilling (<20^{0}C) or freezing (<0^{0}C) temperatures, adversely affects the growth and development of plants. It prevents the expression of the full genetic potential of plants because it directly inhibits metabolic reactions and, indirectly results in cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses (Chinnusamy et al., 2007; Ouellet, 2007).

Sudden exposure to temperatures near 0^{0}C, called cold shock, greatly increases the chances of injury (Taiz and Zeiger, 2006). As temperatures drop below 0^{0}C, ice formation is generally initiated in the intercellular spaces due, in part, to the extracellular fluid having a higher freezing point than the intracellular fluid (Thomashow, 1999). When plants are exposed to freezing temperatures for an extended period, the growth of extracellular ice crystals results in the movement of liquid water from the protoplast to the extracellular ice, causing excessive dehydration (Browse and Xin, 2001; Taiz and Zeiger, 2006).

In many plants, freezing tolerance increases in response to low non-freezing temperatures, a phenomenon called cold acclimation (Chinnusamy et al., 2007; Thomashow, 1999). A key function of cold acclimation is to stabilize membranes against freezing injury. Chilling damage can be minimized if exposure is slow and gradual. It has

been shown that cold acclimation is associated with changes in gene expression (Guy, 1990; Shinozaki & Yamaguchi-Shinozaki, 2000; Thomashow, 1999). This observation led to the hypothesis that changes in gene expression cause some of the biochemical and physiological changes that occurred in response to low temperature and were likely to contribute to an increase in freezing tolerance (Buskirk & Thomashow, 2006). Expression of certain genes and synthesis of specific proteins are common to both heat and cold stress, but their response pathways differ (Thomashow, 2001). At least two pathways govern the changes after environmental stresses, the abscissic acid (ABA) or ABA-independent pathways (Knight et al., 2004; Liu et al., 1998). In the ABA-independent process, cold temperatures trigger the transcription of the CRT-binding factor (CBF) family.

CBF genes

More than 100 genes are up-regulated by cold stress in many crop species (reviewed in Thomashow, 1998; Yamaguchi-Shinozaki and Shinozaki, 2006; Agarwal et al., 2006). Because cold stress is clearly related to ABA responses and to osmotic stresses, not all genes up-regulated by cold stress necessarily need to be associated with cold tolerance, but many of them are. In Arabidopsis, cold acclimation involves action of the CBF cold-response pathway (Thomashow, 2001). Within 15 min of exposing plants to low temperature, transcripts accumulate for a family of genes that are transcriptional activators called C-repeat binding factors, CBF1, CBF2, and CBF3 (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Medina et al., 1999; Thomashow, 2001) also called DREB1b, DREB1c, and DREB1a (Liu et al., 1998; Shinozaki & Yamaguchi-Shinozaki,

2000), respectively. The CBF/DREB1 proteins bind to CRT/DRE elements (C-repeat/ dehydration responsive, ABA-independent sequence elements) present in the promoters of COR (cold-regulated) and other cold-responsive genes to stimulate their transcription (Stockinger et al., 1997; Yamaguchi-Shinozaki & Shinozaki, 1994). CBF proteins are involved in the transcriptional response of the CBF regulon, a collection of numerous cold and osmotic stress-regulated genes whose expression is regulated by the CBF proteins (Fowler et al., 2005).

Gilmour et al. (1998) proposed that a transcription factor already present in the cell at normal growth temperatures recognizes the CBF promoters and induces expression upon exposure to cold stress. They named the unknown activator (s) "ICE" (inducer of CBF expression). ICE presumably recognizes a cold-regulatory element present in the promoters of each CBF gene. At warm temperatures, ICE is suggested to be in an "inactive" state but upon exposure of the plant to low temperature, a signal transduction pathway is activated that results in modification of either ICE or an associated protein which allows ICE to induce CBF gene expression (Thomashow, 1999).

Chinussamy et al. (2003) identified ICE1, which encodes a MYC-like bHLH transcriptional factor that regulates the transcription of CBF genes in the cold. The authors reported that CBF1, 2, and 3 are not regulated in an identical fashion. Overexpression of ICE1 enhances the expression of the CBF regulon in response to low temperature, resulting in plants that are more freezing tolerant than wild-type plants following cold acclimation. ICE1 functions as an upstream regulatory protein that positively controls the transcription of CBF3. However, it does not appear to be involved in the cold induction of CBF1 or CBF2. The authors stated that MYC-like proteins other

than ICE1 may contribute to cold-induced expression of CBF1 and CBF2. Gilmour et al. (2004) compared the effects of the overexpression of each CBF gene on Arabidopsis growth and development, freezing tolerance, and gene expression, and found that each of the three CBF transcription factors is differentially regulated and may not have the same functional activities.

Novillo et al. (2004) proposed that CBF2 acts as a negative regulator of CBF1 and CBF3. This suggestion was based on their finding that a T-DNA insertion in the CBF2 gene resulted in constitutive expression of CBF1 and CBF3. The *cbf2* mutants constitutively expressed CBF target genes and were more freezing tolerant than the wild-type plants. The authors proposed a model in which CBF1 and CBF3 are quickly induced in response to low temperature followed closely by the induction of CBF2 which, in turn, leads to the suppression of CBF1 and CBF3 expression in Arabidopsis. This ensures that expression is transient and tightly controlled.

Novillo et al. (2007) showed that the three CBFs do not have overlapping functions in Arabidopsis. CBF1 and CBF3 have different roles than CBF2 in both constitutive freezing tolerance and cold acclimation. CBF1 and CBF3 seem to transactivate the same targets but both factors have an additive effect and are required to induce the whole CBF regulon.

These findings indicate a complex regulation of the CBF/DREB1 gene expression (Figure 1).

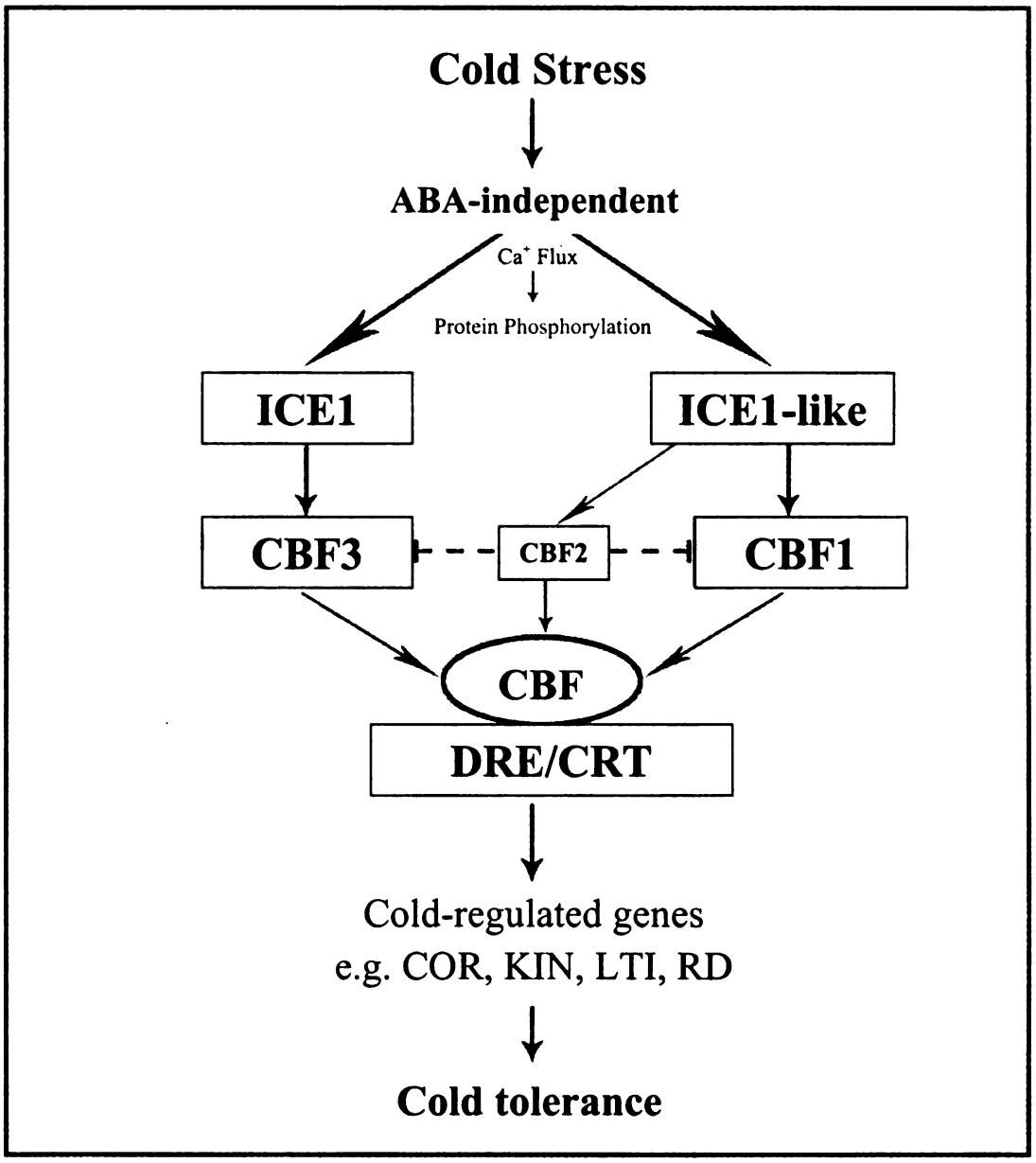

Figure 1. Diagram of the cold-responsive pathway in Arabidopsis. Solid arrows show positive downstream regulation while dotted lines show negative feedback regulation. (adapted from Chinnusamy et al., 2007; Novillo et al., 2004; Yamaguchi-Shinozaki & Shinozaki, 2006; Zhu et al., 2007).

Genetic approaches to cold tolerance improvement

Homologous genes of CBF/DREB1 have been isolated in many species such as wheat (Miller et al., 2006; Shen et al., 2003b; Vágújfalvi et al., 2003); *B. napus* (Gao et al., 2002; Jaglo et al., 2001); rice (Dubouzet et al., 2003; Oh et al., 2005; Wang et al., 2008); barley (Choi et al., 2002; Skinner et al., 2005; Xue, 2003); maize (Qin et al., 2004); oat (Brautigam et al., 2005); perennial ryegrass (Xiong & Fei, 2006; Zhao & Bughrara, 2008); soybean (Li et al., 2005); strawberry (Iezzoni et al., 2002) and sweet cherry (Kitashiba et al., 2004) among others. Overexpression of the Arabidopsis CBF/DREB1 genes in transgenic B. napus (Jaglo et al., 2001), tobacco (Kasuga et al., 2004), peanut (Bhatnagar-Mathur et al., 2007), maize (Al-Abed et al., 2007), and potato (Behnam et al., 2007) induced expression of homologs of Arabidopsis CBF/DREB1-targeted genes and increased not only freezing but also drought and salt stress tolerance of transgenic plants.

Initial experiments done on Arabidopsis (Gilmour et al., 2000; Liu et al., 1998) and rice (Kasuga et al., 1999) showed that overexpression of CBF3/DREB1A resulted in strong expression of target stress-inducible genes, and transgenic plants acquired higher tolerance to drought, salinity and freezing. In grass species, the Arabidopsis CBF3/DREB1A improved drought tolerance of tall fescue, an important perennial cool-season grass (Zhao et al., 2007). With the use of *RD29A* (Responsive to Desiccation 29A; also known as *COR78*), the authors found that transgenic plants accumulated high levels of proline and increased expression of *AtP5cs2*, a known downstream target gene of CBF/DREB1 in Arabidopsis. Recently, *Zm*CBF3 was isolated from maize containing a

nuclear localization signal (NLS) and a conserved CRT-binding activator protein (AP2) domain consistent with the previously identified CBFs from Arabidopsis, rice and other plant species. Expression of *Zm*CBF3 was induced by cold stress and ABA, suggesting that it is involved in the cold acclimation pathway in maize (Wang et al., 2008).

For Chinese lawngrass (*Zoysia sinica* Hance), CBF1/DREB1b from Arabidopsis driven by CaMV 35S promoter was introduced and of the three independent transgenic lines obtained, two exhibited stunted growth and decreased tillering. However, the transgenic lines had significantly strong chilling tolerance as compared to the wild type ones (Li et al., 2006).

In perennial ryegrass, a CBF3/DREB1A homolog was isolated by Xiong and Fei (2006) from the cultivar 'Caddyshack' and was designated as *Lp*CBF3. Expression of this gene was induced by cold stress, similar to the other CBF3/DREB1A homologs, but not by ABA, drought or salinity. Overexpression of *Lp*CBF3 in Arabidopsis revealed induced expression of two Arabidopsis CBF3/DREB1A target genes, *COR15A* and *RD29A*.

Another CBF3 homolog from a freeze-tolerant perennial ryegrass accession (PI598441) was isolated, sequenced and characterized recently by Zhao and Bughrara (2008). Their study was based on the results of a cold tolerance evaluation of 300 perennial ryegrass accessions conducted in the field (Warnock et al., 2005). The 30 most cold-tolerant accessions were chosen and further evaluated for freezing tolerance in the laboratory to determine the temperature at which 50% of the plants were killed (LT_{50}). Among the 30 accessions, PI598441 was considered to be the most freezing-tolerant with

LT_{50}= -11^{0}C. This accession was originally collected from Switzerland at 860 m elevation. Analysis of *Lp*CBF3 gene expression indicated the presence of three homologs of *Lp*CBF3 in the PI598441 genome and only one amino acid variation in the *Lp*CBF3 protein compared to the cold-sensitive accessions. When compared with the *Lp*CBF3 gene isolated by Xiong and Fei (2006), more than 74% of the amino acids were identical and considerable differences were observed in the non-AP2 regions. In both studies, *Lp*CBF3 was overexpressed in Arabidopsis to determine its function. The transgenic plants containing the *Lp*CBF3 driven by the 35S promoter resulted in dwarf plants that flowered late and showed increased freezing tolerance. These phenotypic characteristics were also observed in a previous study by Gilmour et al. (2000) in Arabidopsis overexpressing *At*CBF3. The dwarfism trait is desirable in turfgrass breeding since it may reduce mowing frequency in turf grasses.

Drought-tolerant perennial ryegrass was obtained by introducing *Lp*CBF3 (Xiong & Fei, 2006) through the particle bombardment method (Liebao et al., 2007). Transgenic plants recovered after drought exposure, as manifested by increased membrane stability and chlorophyll content compared to control plants. The gene was constitutively expressed in both non-stressed and stressed conditions but with higher transcript levels under stress. This study shows the potential of *Lp*CBF3 not only for cold tolerance but also for other environmental stress tolerance as previously shown in rice (Kasuga et al., 1999) and tall fescue (Zhao et al., 2007).

Interestingly, CBF3 driven by the maize ubiquitin (UBI) promoter did not show any growth inhibition or visible phenotypic alterations in perennial ryegrass. The authors

discussed the possibility that lower levels or fewer target genes were activated by CBF3, as also observed by Oh et al. (2005) in rice. This promoter minimizes the effects of CBF3 on the plant growth in rice and perennial ryegrass. Unlike in dicots including Arabidopsis, the UBI promoter does not induce dwarfism in rice and perennial ryegrass.

Agrobacterium-mediated transformation of turfgrass

One of the most important breakthroughs in plant biotechnology was the ability to insert foreign DNA into plant cell genome and regenerate whole plants expressing the foreign genes (Smith, 2001). The generation of transgenic plants with improved characteristics such as resistance or tolerance to herbicides, diseases, insects, drought, salinity, and temperature has revolutionized agriculture where they have been used.

Three major techniques have been used to insert foreign genes into plant cells. The first method involves the use of enzymes that digest the plant cell wall resulting in a plant protoplast that allows the foreign gene to be inserted into the cell. Protoplasts can take up foreign genes either by chemical treatment or passing an electrical current through the solution containing the protoplasts and the foreign genes. Protoplasts are then cultured to reform a cell wall and regenerate entire plants in cell culture (Smith, 2001)

Another method is microprojectile bombardment or biolistics. Gold or tungsten microparticles are coated with foreign DNA (genes) and shot into cultured plant cells. If a particle enters an individual cell, it can deliver the DNA into the cell, which is then cultured into a transgenic plant.

The last major method is the use of the soil-borne bacterium called *Agrobacterium tumefaciens*. This microorganism is a pathogen that infects plants cells

and transfers a piece of its own DNA into the cell's genome. The use of *A. tumefaciens* in biotechnology progressed so rapidly that today it is relatively straightforward to insert multiple transgenes into well over 100 different species and genotypes of plants (Christou and Capell, 2007).

In monocots, the protoplast transformation method has had limited success because the process is tedious and regeneration problems are common. It was soon replaced by the biolistic method, which has proven effective for most crops, but multiple copies of the transgene are integrated into the genome, possibly leading to silencing of gene expression. Among the methods, the *Agrobacterium* gene transfer system has been routinely used to transform dicots and has several advantages over the biolistic method, such as the transfer of relatively large segments of DNA with little rearrangement and the integration of low copy number of T-DNA into active regions of chromosomes (Hiei & Komari, 2006). For monocots, however, early studies suggested that they are recalcitrant to *Agrobacterium* infection since most monocots are not among its natural hosts. A study by Hiei et al. (1994) conclusively proved that *Agrobacterium* could successfully transform a monocot such as rice with relatively high frequencies. Today, many monocots are efficiently transformed by *Agrobacterium* and studies have been done to improve this method.

In forage and turfgrasses, protocols for efficient genetic transformation using *Agrobacterium* have been reported for switchgrass (Somleva et al., 2002), creeping bentgrass (Aswath et al., 2005; Luo et al., 2004; Yu et al., 2000), colonial bentgrass (Chai et al., 2004), tall fescue (Dong & Qu, 2005), bermudagrass (Li et al., 2005), orchardgrass (Lee et al., 2006), lawngrass (Li et al., 2006), bromegrass (Nakamura and Ishikawa,

2006), zoysiagrass (Toyama et al., 2003), Kentucky bluegrass (Gao et al., 2006) and darnel ryegrass (Ge et al., 2007). This transformation system is a valuable tool for functionality tests of candidate genes in forage and turfgrass species (Ge et al., 2007).

For perennial ryegrass, the first genetic transformations were achieved with the microprojectile bombardment method. The earliest reported work was done by Hensgens et al. (1993) studying the transient and stable expression of GUS under the control of the CaMV 35S promoter in perennial ryegrass as well as rice and barley. Stable transformants by the bombardment method were obtained by transforming non-embryogenic cell suspension culture, using the *hpt* and *gusA* gene (van der Maas et al., 1994). In this study, the constitutive promoter of the rice gene, GOS2, was used to regulate the *gusA* reporter gene. Transgenic forage-type perennial ryegrass containing the hygromycin phosphotransferase (*hpt*) gene construct driven by the rice *Act1 5'* regulatory sequences were transformed by bombardment of embryogenic suspension cells (Spangenberg et al., 1995). Protoplasts of perennial ryegrass were bombarded to express GUS gene (*uidA*) and neomycin phosphotransferase II gene (*npt II*). This study revealed the presence of albino regenerants in the antibiotic-resistant clones which is higher than in the non-transformed clones from control experiments (Wang et al., 1997). Dalton et al. (1999) regenerated perennial ryegrass transgenics from cell suspension colonies expressing the hygromycin resistance (*hyg*) gene under the CaMV35S promoter and the β-glucuronidase (*gus*) gene under the control of a truncated rice *actin1* promoter and first intron, or a maize ubiquitin promoter and first intron.

The same method was used to obtain turf-type perennial ryegrass containing the neomycin phosphotransferase II (*nptII*) gene driven by the maize ubiquitin promoter

(ubi), and the majority of the transgenic lines showed integration of two to six transgene copies (Altpeter et al., 2000). The use of the gene transfer method for the potential of RNA-mediated virus resistance of perennial ryegrass was also explored (Xu et al., 2001).

Hisano et al. (2004) reported the overexpression of wheat fructosyltransferase genes, *wft1* and *wft2*, in transgenic perennial ryegrass plants under the control of CaMV 35S promoter which resulted in an increased level of fructan and consequently tolerance to freezing at the cellular level. This was the first attempt to improve cold tolerance of perennial ryegrass using the gene transfer method.

The utility of the *Agrobacterium*-mediated transformation system in perennial ryegrass was first studied by Wu et al. (2005) to obtain salt-tolerant perennial ryegrass by inserting a rice vacuolar membrane Na+/H+ antireporter (*OsNHX1*) gene. The resulting transgenic plants had improved salt-tolerance, suggesting that these plants can be planted in saline soil. The authors also showed that the addition of acetosyringone during the incubation of bacteria and co-cultivation process resulted in an increase in resistant green shoot frequency, confirming acetosyringone as a key factor in *Agrobacterium*-mediated cereal crop transformation.

Forage-type transgenic perennial ryegrass plants contained at least one intact gus gene, but GUS expression was not observed. It was discussed that the possibility of silencing of gus gene in the transgenics appear to suppress GUS activity by co-transformation of multiple *gus* and *hpt* genes under the control of the CaMV 35S promoter (Sato and Takamizo, 2006). A previous co-transformation study obtained from the bombardment method (Dalton et al., 1999) did not report silencing of GUS activity even if four copies of the GUS transgene was observed.

The development of a high throughput *Agrobacterium* genetic transformation procedure from embryogenic callus lines (derived from meristematic regions of the vegetative tillers) enabled generation of a large number of independently transformed lines of perennial ryegrass (Bajaj et al., 2006). This study reported an average transformation frequency of 7% (number of transgenic plants/number of calli used). Wu et al. (2007) reported an efficient regeneration and transformation procedure obtaining 22 independent transgenic lines from 229 calli infected (9% efficiency) of perennial ryegrass expressing the phosphinothricin acetyltransferase gene (*bar*).

Additionally, the improvement of the nutritional value of perennial ryegrass was explored by increasing its fructan content through the integration of heterologous fructan biosynthetic genes: sucrose 1-fructosyltransferase and fructan 6G-fructosyltransferase genes from onion. The obtained transgenic perennial ryegrass showed a three-fold increase in fructan content (Gadegaard et al., 2008).

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CHAPTER II

AGROBACTERIUM-MEDIATED TRANSFORMATION OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) FOR COLD TOLERANCE

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is widely used as a forage crop and as turfgrass for its high quality and yield. Its utility for turf has increased over the years and is now considered as one of the most versatile turfgrass species. For turf, perennial ryegrass is used alone or in combination with other grasses. Disease susceptibility and limited cold and heat tolerance, however, limit its persistence and zone of adaptation (Hannaway et al., 1999).

Of all the perennial cool season grasses, ryegrass is the least winter hardy, so that survival can be risky in areas with cold winters with no snow cover (Kaye, 2007). Improving cold tolerance is one of the most important breeding objectives for perennial ryegrass. However, conventional breeding methods have so far been unsuccessful in achieving this objective because of the quantitative characteristic of the cold tolerance trait (Skinner et al., 2006; Skøt et al., 2002). Modern biotechnology provides an opportunity to improve cold tolerance and winter hardiness in perennial ryegrass, as well as other plant species, by manipulating genes related to cold tolerance and their expression (Kosmala et al., 2006; Zhang et al., 2004). Forage and turfgrass managers in Northern temperate climates could greatly benefit from new high-quality, cold-tolerant perennial ryegrass cultivars.

In forage and turfgrasses, protocols for efficient genetic transformation using *Agrobacterium* have been reported for switchgrass (Somleva et al., 2002), creeping bentgrass (Aswath et al., 2005; Luo et al., 2004; Yu et al., 2000), colonial bentgrass (Chai et al., 2004), tall fescue (Dong & Qu, 2005), bermudagrass (Li et al., 2005), orchardgrass (Lee et al., 2006), lawngrass (Li et al., 2006), bromegrass (Nakamura and Ishikawa, 2006), zoysiagrass (Toyama et al. 2003), Kentucky bluegrass (Gao et al., 2006) and Darnel ryegrass (Ge et al., 2007). Transformation is a valuable tool for functionality tests of candidate genes in forage and turfgrass species (Ge et al., 2007).

In many plants, freezing tolerance increases in response to low non-freezing temperatures, a phenomenon called cold acclimation (Thomashow, 1999; Chinnusamy et al., 2007). Freezing damage can be minimized if exposure is slow and gradual. It has been shown that cold acclimation is associated with changes in gene expression (Guy, 1990; Thomashow, 1999; Shinozaki & Yamaguchi-Shinozaki, 2000). This observation led to the hypothesis that changes in gene expression lead to some of the biochemical and physiological changes that occurred in response to low temperature and were likely to contribute to an increase in freezing tolerance (Buskirk & Thomashow, 2006).

More than 100 genes are up-regulated by cold stress in many crop species (reviewed in (Thomashow, 1998; Yamaguchi-Shinozaki & Shinozaki, 2006; Agarwal et al., 2006). Because cold stress is clearly related to ABA responses and to osmotic stresses, not all genes up-regulated by cold stress necessarily need to be associated with cold tolerance, but many of them are. In Arabidopsis, cold acclimation involves action of the CBF cold-response pathway (Thomashow, 2001). Within 15 min of exposing plants to low temperature, transcripts accumulate for a family of genes that are transcriptional

activators called C-repeat binding factors, CBF1, CBF2, and CBF3 (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Medina et al., 1999; Thomashow, 2001) also called DREB1b, DREB1c, and DREB1a (Liu et al., 1998; Shinozaki & Yamaguchi-Shinozaki, 2000), respectively. The CBF/DREB1 proteins bind to CRT/DRE elements (C-repeat/ dehydration responsive, ABA-independent sequence elements) present in the promoters of *COR* (cold-regulated) and other cold-responsive genes to stimulate their transcription (Yamaguchi-Shinozaki & Shinozaki, 1994; Stockinger et al., 1997). CBF proteins are involved in the transcriptional response of the CBF regulon, a collection of numerous cold and osmotic stress-regulated genes whose expression is regulated by the CBF proteins (Fowler et al., 2005).

Homologous genes of CBF/DREB1 have been isolated in many species such as wheat (Miller et al., 2006; Shen et al., 2003b; Vágújfalvi et al., 2003); *B. napus* (Gao et al., 2002; Jaglo et al., 2001); rice (Dubouzet et al., 2003; Oh et al., 2005; Wang et al., 2008); barley (Choi et al., 2002; Skinner et al., 2005; Xue, 2003); maize (Qin et al. 2004); oat (Brautigam et al. 2005); perennial ryegrass (Xiong & Fei, 2006b; Zhao & Bughrara, 2008); soybean (Li et al., 2005) and sweet cherry (Kitashiba et al., 2004) among others. Overexpression of the Arabidopsis CBF/DREB1 genes in transgenic *B. napus* (Jaglo et al., 2001), tobacco (Kasuga et al., 2004), peanut (Bhatnagar-Mathur et al. 2007), maize (Al-Abed et al., 2007), and potato (Behnam et al. 2007) induced expression of homologs of Arabidopsis CBF/DREB1-targeted genes and increased not only freezing but also drought and salt stress tolerance of transgenic plants.

In perennial ryegrass, a CBF3/DREB1A homolog was isolated by Xiong and Fei (2006) from the cultivar 'Caddyshack' and was designated as *Lp*CBF3. Expression of this gene was induced by cold stress, similar to the other CBF3/DREB1A homologs, but not by ABA, drought or salinity. Overexpression of *Lp*CBF3 in Arabidopsis revealed induced expression of two Arabidopsis CBF3/DREB1A target genes, *COR15A* and *RD29A*.

Another CBF3 homolog from a freeze-tolerant perennial ryegrass accession (PI598441) was isolated, sequenced and characterized by Zhao and Bughrara (2008) and was designated as *Lp*CBF3. Their study was based on the results of a cold tolerance evaluation of 300 perennial ryegrass accessions conducted in the field (Warnock et al., 2005). The 30 most cold-tolerant accessions were chosen and further evaluated for freeze tolerance in the laboratory to determine the temperature at which 50% of the plants were killed (LT_{50}). Among the 30 accessions, PI598441 was considered to be the most freeze-tolerant with LT_{50}= -11^{0}C. This accession was originally collected from Switzerland at 860 m elevation. Analysis of *Lp*CBF3 gene expression indicated the presence of three homologs of *Lp*CBF3 in the PI598441 genome and only one amino acid variation in the *Lp*CBF3 protein compared to the cold-sensitive accessions. When compared with the *Lp*CBF3 gene isolated by Xiong and Fei (2006), more than 74% of the amino acids were identical and considerable differences were observed in the non-Activator Protein (non-AP2) regions. In both studies, *Lp*CBF3 was overexpressed in Arabidopsis to determine its function. The transgenic plants containing the *Lp*CBF3 driven by the 35S promoter resulted in dwarf plants that flowered late and showed increased freezing tolerance. These phenotypic characteristics were also observed in a previous study by Gilmour et al.

(2000) in Arabidopsis overexpressing *At*CBF3. The dwarfism trait is desirable in turfgrass breeding since it may reduce mowing frequency in turf grasses. To date, drought-tolerant improvement of perennial ryegrass using *Lp*CBF3 (Liebao et al., 2007) has been explored but the generation of cold tolerant from top performing but cold-sensitive cultivars has not been reported.

Objectives of the study

(1) Obtain cold tolerant perennial ryegrass by genetic transformation with the cold-regulated transcriptional activator *Lp*CBF3 gene using *Agrobacterium*-mediated gene transfer.

(2) Evaluate the expression of *Lp*CBF3 gene under the control of a constitutive promoter, cauliflower mosaic virus 35S (CaMV35S).

MATERIALS AND METHODS

Plant material and explants

Five top performing perennial ryegrass cultivars, "Citation Fore," "Inspire," "Silver Dollar," "Transformers," and "UNO," were obtained from the Scotts Company (Marysville, Ohio) and screened for callus induction performance and regeneration. Of the five cultivars tested, "Inspire" and "Transformer" showed high induction and regeneration capacity and were used for subsequent transformation experiments. Mature seeds were surface sterilized as described previously by Liu et al. (2006).

Culture medium

Sterilized seeds were placed in callus induction medium containing MS basal salts (Murashige and Skoog, 1962) supplemented with 5 mg l^{-1} 2,4-D, 0.2 mg l^{-1} benzylaminopurine (BAP), 1g l^{-1} casein hydrolysate and 30 g l^{-1} sucrose (Sato and Takamizo, 2006), solidified with 3g l^{-1} Phytagel and induced in the dark at 24 ± 2^0C. White shoots and roots were removed after 2 wk. Viable calli tissues were selected and transferred to subculture medium with MS containing 3 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BAP, 1g l^{-1} casein hydrolysate and 30 g l^{-1} sucrose, solidified with 3 g l^{-1} Phytagel. Microscopically selected embryogenic calli were subcultured for 8 wk at 2-wk intervals. Actively growing embryogenic calli were again microscopically selected, separated into small pieces and used for transformation experiments.

The regeneration medium used contained MS basal salts supplemented with 3 mg l^{-1} BAP, 1 mg l^{-1} NAA, 1 mg l^{-1} kinetin, 1g l^{-1} casein hydrolysate and 30 g l^{-1} sucrose,

solidified with 3 g l^{-1} Phytagel. Rooting media consisted of half-strength MS supplemented with 0.5 mg l^{-1} NAA, 0.5 g l^{-1} casein hydrolysate and 15 g l^{-1} sucrose, solidified with 3 g l^{-1} Phytagel (Liu et al., 2006). All media were adjusted to pH 5.7-5.8 before the addition of Phytagel and autoclaved at 121^0C for 25 min.

Vector construction

The T-DNA expression cassette containing the *Lp*CBF3 gene (Zhao and Bughrara, 2008) constructed for the experiment was driven by cauliflower mosaic virus 35S (CaMV35S) promoter contained in the binary expression vector pFGC5941 with octopine synthase 3′ (OCS 3') at the terminator end (Figure 2). The vector's *Chalcone synthase A* (CshA) intron region was replaced with *Lp*CBF3 using the unique enzymes AscI (5') and XbaI (3'). The pFGC5941 vector also contains the *bar* gene, a selectable marker gene conferring glufosinate (Basta) resistance, which is under the control of *mannopine synthase* promoter (pMAS) with MAS 3′ at the terminator end. With these, the modified recombinant binary vector pFGC5941 was inserted into *Agrobacterium tumefaciens* by electroporation.

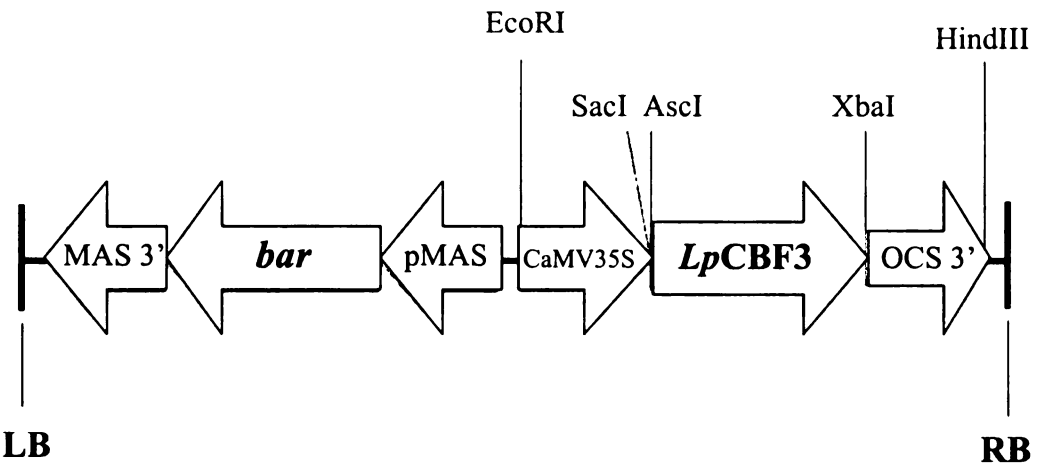

Figure 2. Linear plasmid map of the T-DNA of the pFGC 5941 binary vector used for transformation containing *Lp*CBF3 gene driven by cauliflower mosaic virus 35S (CaMV35S) promoter.

Agrobacterium transformation

Agrobacterium tumefaciens strain EHA 105 harboring the modified binary plasmid pFGC5941 was used for transformation. Single colonies containing the plasmid construct were selected and transferred to YEP liquid medium supplemented with 50 mg l^{-1} kanamycin, 50 mg l^{-1} rifamycin and 200μM acetosyringone and grown overnight at 28^0C with shaking (175 rpm). After achieving an optical density (OD) of 600 nm at 0.8-1.0, *A. tumefaciens* cells were pelleted by centrifugation at 5000 rpm for 10 min and resuspended in MS-B5 medium supplemented with 30 g l^{-1} sucrose and 200 mg l^{-1} acetosyringone. The final OD_{600} was adjusted to 0.2 for infection.

Pre-cultured mature seed-derived embryogenic calli were prepared by transferring microscopically identified embryogenic sectors to subculture medium on Whatman #1

paper filters. Embryogenic sectors ranged in size from 2 to 3 mm and approximately 20 pieces weighed 0.1 g per plate. Plates were prepared immediately before inoculation. Ten to 15 ml final inoculum of *A. tumefaciens* was added to each plate containing filters of callus tissue, making sure to cover calli completely. Calli were loosened from the filter paper using forceps and plates were intermittently shaken manually for 10 min. The inoculum was aspirated using a sterile pipette and calli were transferred to another sterile Petri dish containing a sterile 85-mm Whatman #1 filter. Approximately 20 pieces of inoculated calli were arranged in a 4-cm diameter circle, and 200 µl sterile water was added to the center of the circle. Plates were placed in the dark at 24 ± 2^{0}C for 2 d. Calli were transferred directly to subculture medium containing 250 mg l^{-1} cefotaxime and cultured for 3 d under the same conditions.

Selection and regeneration of transgenics

After co-cultivation, calli were transferred to selective subculture medium containing 20 mg l^{-1} DL-phosphinothricin (PPT) and 250 mg l^{-1} cefotaxime. Plates were subcultured for 6 wk at 2-wk intervals and maintained in the dark at 24 ± 2^{0}C. Embryogenic sectors were transferred to selective regeneration medium containing 10 mg l^{-1} PPT and 100 mg l^{-1} cefotaxime. They were kept for 1 wk in the dark and then moved to illumination (16h:8h L/D), covered with cheesecloth the first week. Regenerated shoots were transferred to selective rooting medium supplemented with 100 mg l^{-1} cefotaxime in a Magenta box for induction of roots. After 4 wk, rooted plantlets were

acclimated by opening the lid of the box for 3 d before transplanting into potting soil in the greenhouse at 26 ± 2^0C.

Leaf painting assay for glufosinate resistance

Fully emerged leaves of two-week old perennial ryegrass 'Inspire' transgenic lines were used. A line was drawn across half of the leaf blade using a black permanent marker. Above the line and toward the leaf tip, a 1-inch long strip of herbicide solution (25 mg l^{-1} solution of glufosinate (PPT) containing 0.01% Tween 20 wetting agent) was applied using a cotton bud applicator. Plants were scored for susceptibility or resistance to the herbicide 3 to 7 d after application (Peña, 2005).

PCR analysis

Genomic DNA was extracted from young leaf tissues of non-transformed and putatively transformed greenhouse-grown plants as previously described by James et al. (2008). Extracted DNA (100 ng) was used in 25µl PCR reactions with Taq DNA polymerase (Promega, WI, USA) supplemented with 3% DMSO. The 883-bp fragment of *Lp*CBF3 was amplified using the primer set, 5'-ACTGAGGTAGCGCTAGCTCCTATT-3' (forward) and 5'- ACAATCACATTACCAGAAACTGC- 3' (reverse). The PCR reaction parameter began with an initial hot start at 98 °C for 3 min, then 30 cycles of denaturation (94 °C; 30 s), annealing (62 °C; 20 s) and extension (72 °C; 20 s), followed by a final extension of 7 min at 72 °C. Amplified products were analyzed by electrophoresis in 1% (w/v) agarose/ethidium bromide gels.

Southern hybridization

For Southern blot analysis, 20 μg of genomic DNA extracted from leaf tissues of each transgenic line and non-transgenic plants were digested with *SacI* and subjected to electrophoresis in a 1% agarose gel. DNA was transferred to Hybond-N+ membranes (Amersham Biosciences, Buckinghamshire, UK). Digoxigenin (DIG) probe labeling of a 381-bp partial length of *Lp*CBF3 gene was done using the PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany) using the primers 5'-ATTCCTGCCTCAACTTCGCTGACT- 3' (forward) and 5'-TCTGGTAACTCCACAGTGCCACAT- 3' (reverse). Pre-hybridization, hybridization and detection was followed according to the manual of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany).

Whole plant freezing test

Four-week-old transgenic and non-transgenic plants grown in the greenhouse (26 ± 2^0C) were transferred to 4, 0, and -4^0C under 100 ± 20 μmol m^{-2} s^{-1} light conditions for 24h and were then returned to 26^0C for 2 wk. Survival rate was defined as the number of surviving replicates from each transgenic lines divided by the total number plants exposed to the freezing test from each transgenic lines and non-transgenic lines. Three replicate tests were done for each transgenic line.

Northern blot analysis

Total RNA from leaves of transgenic and non-transgenic plants was extracted at 0, 15 and 30 min, and 1, 2, 4 and 24 h at 4^0C cold treatment by using Plant RNA

Reagent (Invitrogen Concert ™) following manufacturer's instructions. Five micrograms of total RNA was loaded into a 1.2% formaldehyde agarose gel and transferred onto Hybond-N+ membranes (Amersham Biosciences) according to a protocol modified from Sambrook et al. (1989). The probe was prepared from DNA plasmid containing *Lp*CBF3 gene and labeled using the PCR DIG probe synthesis kit (Roche Applied Science, Mannheim, Germany). Pre-hybridization, hybridization and detection was performed according to the manual of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany).

Electrolyte leakage test for cold tolerance

Leaves of two month-old transgenic and non-transgenic plants were cut into 1-cm segments for the electrolyte leakage freeze test. After washing with ion-free distilled water (Millipore Milli-Q System, Bedford, MA), three leaves were placed in test tubes (16 x 125 mm) and incubated in ice prior to the freeze tests. Samples were then placed in a low temperature bath set at -2°C in a Completely Randomized Design. After 1 h, ice formation was effected by introducing a small piece of ice into the test tubes (Uemura et al., 1995). Each tube was capped with foam plugs and incubated a further 1 h at -2°C. The bath temperature was then lowered 1^0C every 20 min. Tubes were removed at each temperature and incubated an additional hour at that same temperature in a separate bath. Tubes were placed on ice after removal from the bath until all tubes have been removed. These modifications were described by Gilmour et al. (2000) using five glycol baths. The samples were thawed overnight on ice in a 4^0C cold room.

The next day, distilled water (3 ml) was added to each tube, and the samples were shaken gently for 3 h. Conductivity of the resulting solution was measured using a conductance meter (YSI model 35). A value for 100% leakage was obtained by freezing each sample at -80°C for 1 h and re-extracting with the original solution. The percentage of electrolyte leakage from leaves was determined by the ratio of electrolyte leakage to 100% electrolyte leakage. A plot of temperature versus percent electrolyte leakage was used to determine the value for 50% electrolyte leakage which was defined as the LT_{50} (Gilmour et al., 1988).

Statistical analysis

The data were analyzed by t-test at P=0.05 to compare the percent electrolyte leakage of transgenic and non-transgenic plants for each treatment, using the SAS statistical analysis software, Version 9.1 (SAS Institute, Cary, NC).

RESULTS

Perennial ryegrass tissue culture and establishment

Mature seeds from five perennial ryegrass cultivars were initially tested for calli induction using MS medium with hormone combinations from different published sources (Bajaj et al., 2006; Liu et al., 2006; Sato & Takamizo, 2006; Wu et al., 2007). Of the five cultivars, Inspire and Transformer were identified to have relatively high calli induction frequency (data not shown). However, after several subcultures, Transformer calli were lost due to contamination, thus the use of this cultivar was discontinued as a source of calli for future transformation experiments.

Among the tested calli induction media, those containing 2,4-D and casein hydrolysate in the MS basal medium were effective in producing embryogenic calli. The combination of 5 mg l^{-1} 2,4-D, 0.2 mg l^{-1} BAP, and 1g l^{-1} casein hydrolysate (Sato and Takamizo, 2006) was proven to have more than 67.5% calli induced from seeds. After 2 weeks of culture in the induction medium, calli developed at the peripheral portion of perennial ryegrass seeds (Figure 3a). These calli were picked and separated from growing shoots and were subcultured for three to six times to produce compact and friable calli (Figure 3b).

After 6-8 weeks of subculture, embryogenic sectors of calli were microscopically identified and transferred to regeneration medium. Embryogenic calli derived from a single seed which generated green shoots after 3-4 wk in regeneration medium were identified to be potential source of calli for the transformation experiments (Fig 3c and 3d). Maximum regeneration ability of the tested calli lines ranged from 4 to 6 months.

Regenerated plantlets (Fig 3e) were transferred into the greenhouse after 2 months from regeneration (Fig 3f). All in all, it took a minimum of 6 months for perennial ryegrass to be propagated *in vitro* from seeds to greenhouse establishment.

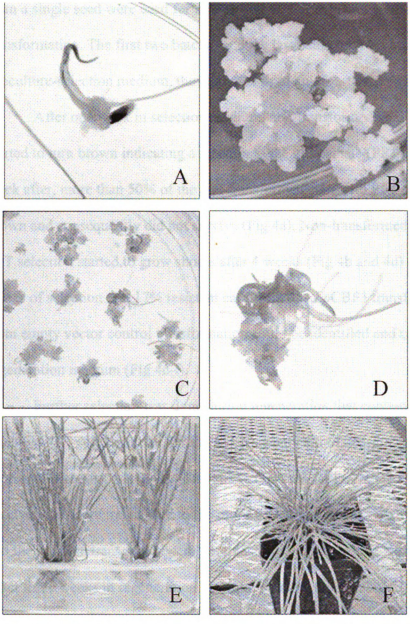

Figure 3. Regeneration of perennial ryegrass cv. Inspire under *in vitro* conditions. a) callus induction from peripheral portion of mature seed with growing shoot; b) embryogenic callus; c, d) green shoots regenerating from callus e) rooting of regenerated shoots; f) regenerated perennial ryegrass plant established in the greenhouse.

Perennial ryegrass transformation

Five batches of calli were co-cultivated with *Agrobacterium* EHA105 for a total of 3,683 embryogenic calli transformation explants (Table 1). Embryogenic calli derived from a single seed were used for inoculation of all treatments in one batch of transformation. The first two batches and the last batch were all contaminated at the subculture-selection medium, thus only the third and fourth batches were continued.

After one week in selection medium, non-transformed calli in selective medium started to turn brown indicating a reaction to the glufosinate (PPT) in the medium. A week after, more than 50% of the non-transformed calli with PPT selection turned dark brown and consequently did not survive (Fig 4a). Non-transformed calli controls without PPT selection started to grow shoots after 4 weeks (Fig 4b and 4d). It was only after 6 weeks of selection that 17% resistant calli from 35s:*Lp*CBF3 transformation and 22% from empty vector control transformation could be identified and transferred to the regeneration medium (Fig 4c-f).

Further selection was done during regeneration that contained only half of the selective PPT concentration. Of the 211 resistant calli of 35s:*Lp*CBF3, only 7 (1%) regenerated shoots. Also, only 7 from 151 resistant calli of pFGC 5941 transformation survived and were transferred to the rooting medium. Regenerated shoots of the controls and the putative transgenics were not transferred to the rooting medium at the same time. After three weeks in regeneration medium, putative transgenic plants did not grow as fast compared to the non-transformed plants (Figure 4g). Thus, they had to remain at the regeneration medium for three weeks more before they were transferred for rooting.

Table 1. Frequency of surviving perennial ryegrass transgenics from PPT selection and leaf painting herbicide assay.

	Calli Infected						Resistant Calli					Total (%)[b]	Resistant Shoots					Total (%)[c]	Rooted Plantlets (%)[d]	Herbi-cide resistant
	Batch					Total	Batch						Batch							
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5			
35S:*Lp*CBF3	246	-	230	238	227	941	con[a]	-	135	76	con	211 (22.42)	-	-	3	4	-	7 (1.5)	**2 (0.4)**	2
p5941 vector control	-	212	218	221	243	894	-	con	91	60	con	151 (16.89)	-	-	2	5	-	7 (1.6)	**2 (0.4)**	2
Control (with PPT)	107	113	102	92	136	550	con	con	0	0	con	0	-	-	0	0	-	0	**0**	0
Control (without PPT)	97	103	120	109	155	584	con	con	101	97	con	198 (33.90)	-	-	24	24	-	48	**48 (20.9)**	0
Total						3683														

[a] Contaminated cultures

[b] Number in parenthesis indicates relative frequency of resistant calli (% = number of resistant calli/total number of calli infected with *Agrobacterium*)

[c] Number in parenthesis indicates regeneration frequency (% = number of shoots/total number of calli infected with *Agrobacterium* from two batches)

[d] Number in parenthesis indicates relative frequency of rooted plantlets (% = number of rooted plantlets/total number of calli infected with *Agrobacterium* from two batches)

Since the previous controls were already established at the greenhouse by the time the putative transgenics were ready, new controls were transferred to the rooting medium together with the transgenic batch.

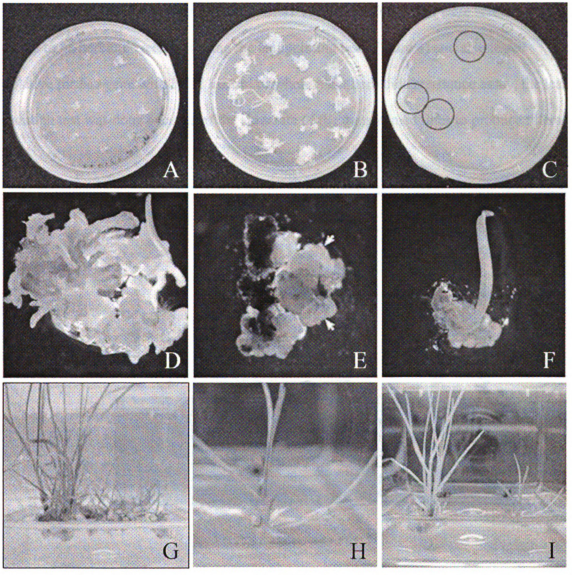

Figure 4. Transgenic perennial ryegrass cv. 'Inspire' obtained after *Agrobacterium*-mediated transformation. a) Non-transformed calli in selective medium (supplement with 20 mg l^{-1} PPT); b) non-transformed calli in medium without PPT; c) three putative transgenic shoots from transformed calli in selective medium; d) shoot emerging from non-transformed calli without PPT; e) emerging green shoots from calli in selective medium; f) resistant shoot in selective medium; g-h) shoots in rooting medium; i) albino shoot in regeneration medium.

Only two rooted plantlets from the 35s:*Lp*CBF3 transformation were transferred to the greenhouse and were positive for herbicide tolerance. PCR analysis showed that these two lines were positive for both *Lp*CBF3 (883 bp) and *bar* (445 bp) genes (Figure 5). The two rooted plants from the pFGC 5941 vector transformation were screened, and found to be herbicide resistant using the leaf painting test (Figure 6) and also tested positive for *bar* gene with PCR. Instead of whole plant herbicide resistance assay, the leaf painting test was done because of the slow rate of the transformed lines to produce tillers.

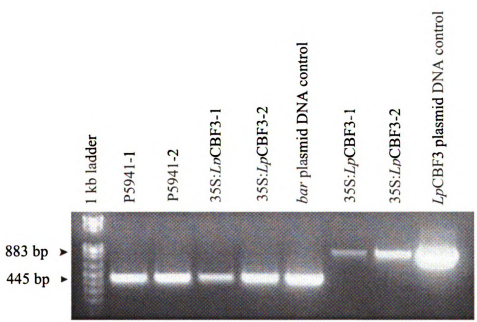

Figure 5. Polymerase Chain Reaction (PCR) results of two 35s:*Lp*CBF3 transgenic lines positive for *Lp*CBF3 (883bp) and *bar* gene (445bp) and two empty vector (pFGC5941) transgenic controls containing the *bar* gene.

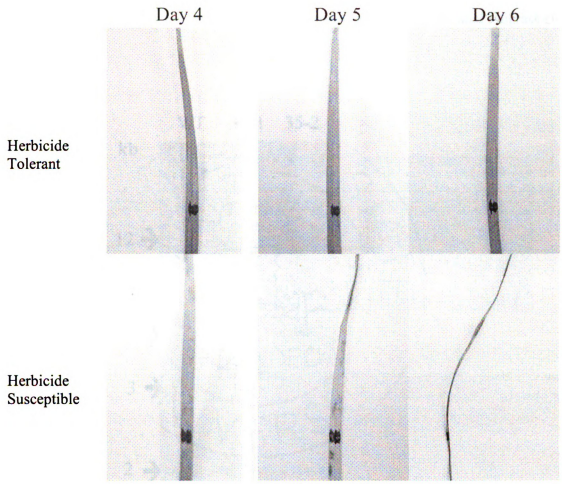

Figure 6. Visual observation of leaf painting test during the 4th, 5th and 6th days after application of 25 mg l^{-1} PPT on perennial ryegrass cv. 'Inspire' leaves.

Molecular characterization

Results of the Southern blot analysis (Figure 7) showed that the number of inserted *Lp*CBF3 copies in two transgenic lines were 3 and 4, respectively. This confirmed the integration of the *Lp*CBF3 gene into the genome of perennial ryegrass cv. 'Inspire'.

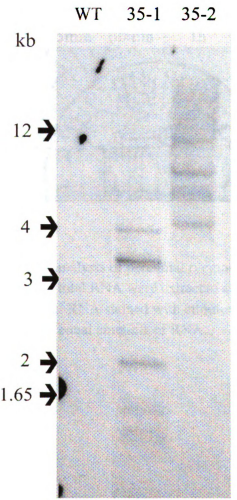

Figure 7. Southern blot analysis of perennial ryegrass 35S:*Lp*CBF3 transgenic lines (designated as 35-1 and 35-2). DNA was digested with *SacI* and probed with the *Lp*CBF3 gene.

Northern blotting results (Figure 8) showed that the *Lp*CBF3 gene was expressed in the transgenic plants. The exposure at 4^0C acclimation temperature at earlier time points did not immediately affect the level of expression of the *Lp*CBF3 transcripts. It was only after 2 hours of cold exposure that transcript levels increased until 24 hours.

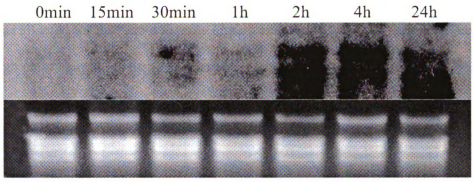

Figure 8. Northern blot analysis of perennial ryegrass transgenic line exposed at 4^0C cold temperature and total RNA were extracted at 0, 15 and 30 min, and 1, 2, 4 and 24 hours. Ribosomal RNA stained with ethidium bromide is used as an internal control to verify equal amounts of RNA.

Plant height and whole plant freeze tolerance tests

Transgenic plants in the greenhouse did not tiller well as compared to the control (wild-type) lines (Figure 9 and 10) thus it was difficult to get enough tillers for replications for plant height measurements (Figure 11) and for whole plant freeze tests. Growth of all transgenic perennial ryegrass had the same trend regardless of the presence of the *Lp*CBF3 gene and were relatively slower or limited than the control plants.

After the initial exposure of the transgenic plants at 4^0C, a period of at least 2 to 3 weeks was allowed for the plants to grow more leaves for more tissue samples. There was not enough plant material to do all the desired freezing tests.

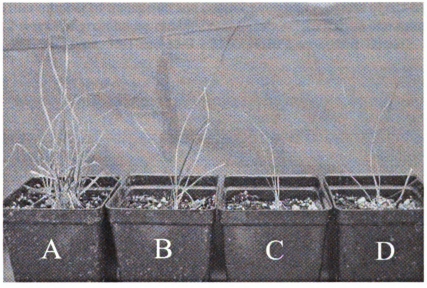

Figure 9. Two-month old transgenic plants established in the greenhouse. a) Wild-type control potted 3 weeks early; b) wild-type control potted with transgenics; c) 35S:*Lp*CBF3 transgenic; d) empty vector transgenic control.

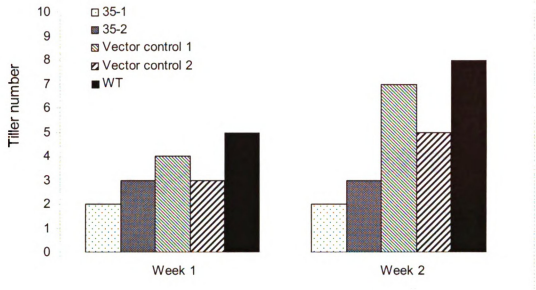

Figure 10. Tiller number of transgenic and control perennial ryegrass plants measured at first 2 weeks of greenhouse establishment. Data is taken from one sample of each line. WT-wild type; 35-1 and 35-2 – 35S:*Lp*CBF3 transgenic lines.

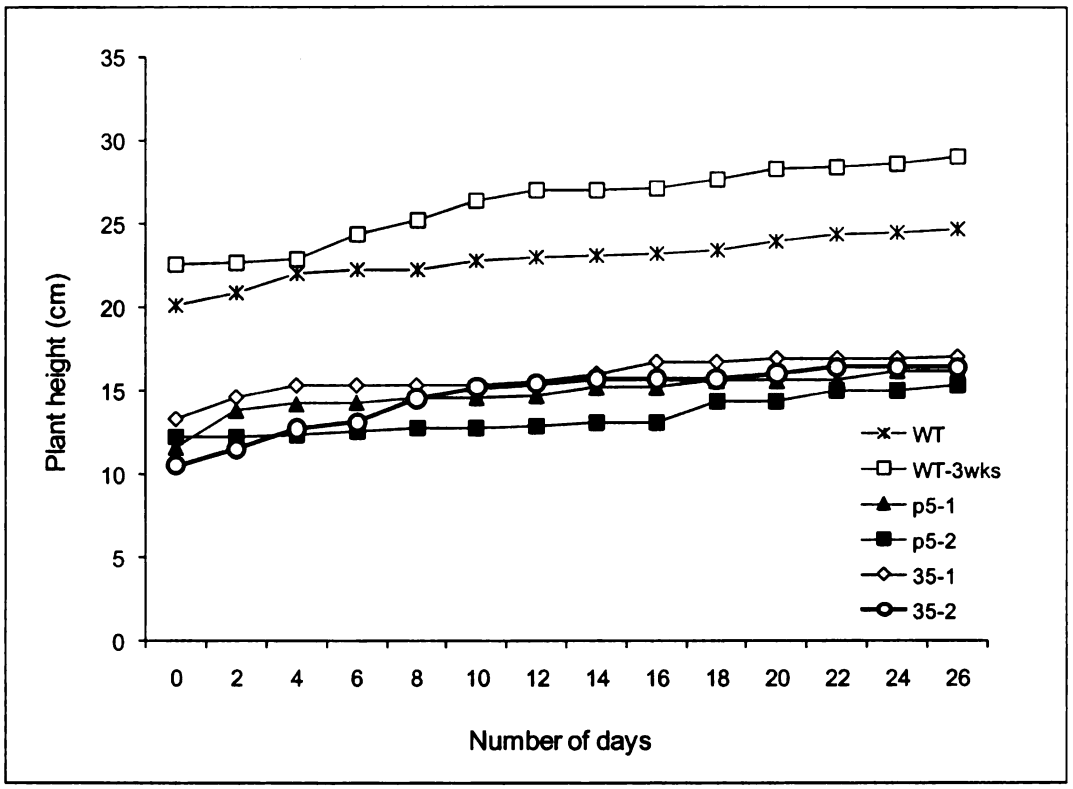

Figure 11. Plant height (cm) of transgenic and control perennial ryegrass plants measured at 2-day intervals for 3 weeks. WT-3wks control has been established in the greenhouse 3 weeks earlier. Plant height data is taken from the base of the plant to the longest blade of one sample from each line. WT-wild type; p5-1 and p5-2 – transgenic vector control lines; 35-1 and 35-2 – 35S:*Lp*CBF3 transgenic lines.

Electrolyte leakage test

The plot of the percent electrolyte leakage (Figure 12) from detached leaves of the perennial ryegrass transgenics and controls revealed that acclimated 35S:*Lp*CBF3 had a lower percentage of ion leakage at low temperature treatment as compared to the wild type (non-transformed) and empty vector controls.

Table 2 shows the temperature by which 50% of the plants were killed (LT_{50}) for each line at low temperature exposure. Acclimated 35S:*Lp*CBF3 line had the lowest LT_{50} of -5.5^{0}C while nonacclimated 35S:*Lp*CBF3 had a LT_{50} value similar to the acclimated vector control at -4^{0}C. Non-transgenic controls had the highest LT_{50} at -3^{0}C.

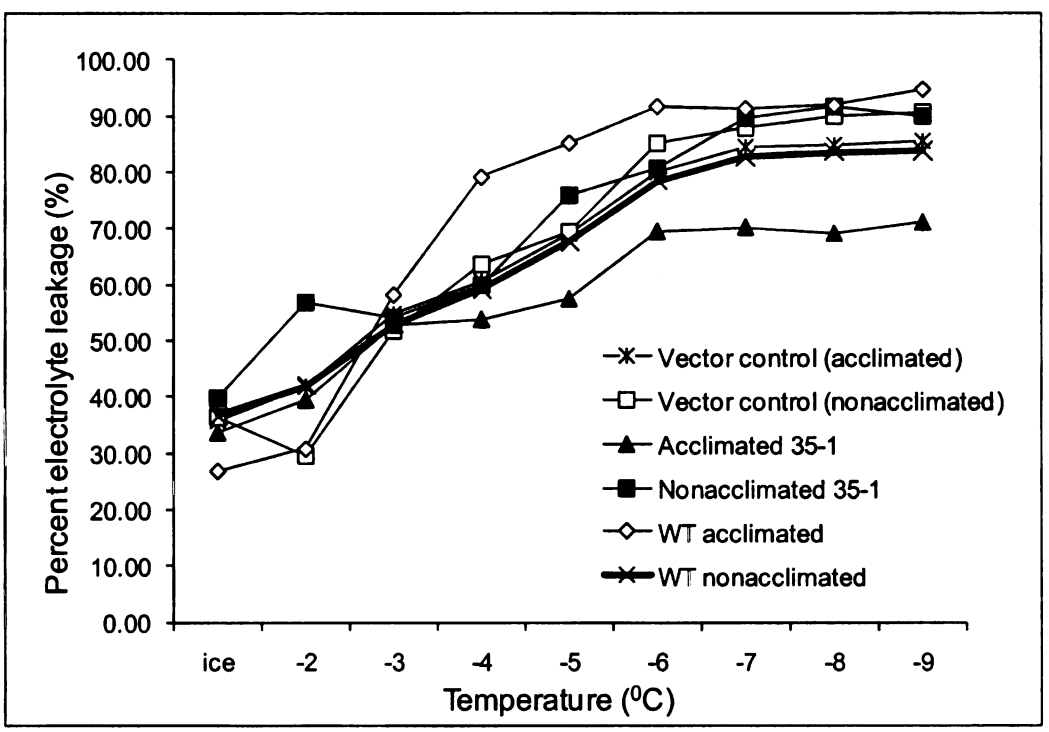

Figure 12. Percent electrolyte leakage of perennial ryegrass transgenic lines and controls. WT – wild type; 35-1 - 35S:*Lp*CBF3 transgenic lines.

Table 2. Freezing tolerance of perennial ryegrass cv. Inspire. Plants were placed at low temperature for the times indicated, and LT_{50} values were estimated using the electrolyte leakage test plot (Fig 10). LT_{50} value was determined as the midpoint between the minimum and maximum percent leakage of each line.

Lines	LT_{50} (^{0}C)	
	Acclimated	Nonacclimated
Vector control	-4	-3.5
35S:*Lp*CBF3	-5.5	-4
Non-transgenic (WT) control	-3	-3

Table 3 shows statistical differences of the treated lines at different temperatures. In all low temperature treatments, there was a highly significant difference among all lines tested except at non-frozen (ice) control and at -3^{0}C temperatures. At temperatures -7^{0}C to -9^{0}C, acclimated 35S:*Lp*CBF3 line had a significantly lower electrolyte leakage than the rest of the lines. At temperatures -4^{0}C and -6^{0}C, electrolyte leakage of acclimated and non-acclimated 35S:*Lp*CBF3 were not significantly different but differed significantly at -5^{0}C (Figure 13). At -2^{0}C, nonacclimated 35S:*Lp*CBF3 had a higher electrolyte leakage compared to the rest of the tested lines but did not differ significantly with the wild-type controls starting at -5^{0}C temperature and lower (Figure 14). At -4^{0}C, acclimated and non-acclimated 35S:*Lp*CBF3 did not differ significantly with the transgenic vector controls (Figure 15). The electrolyte leakage test reveals the expression of *Lp*CBF3 gene on acclimated and nonacclimated perennial ryegrass when exposed to low temperature levels.

Table 3. Mean percent electrolyte leakage from detached leaf segments of transgenic perennial ryegrass plants and controls exposed to different cold temperature settings for 1 hour with three replications.

Treatments	Temperature (^{0}C)								
	Ice control^{ns}	-2***	-3^{ns}	-4**	-5***	-6**	-7**	-8**	-9**
Acclimated Vector control	35.50	41.78 a	54.89	60.63 ab	69.01 ab	79.85 ab	84.85 b	84.69 b	85.49 b
Nonacclimated Vector control	36.41	29.67 a	51.79	63.79 ab	69.32 ab	85.05 b	88.00 b	89.94 b	90.50 b
Acclimated 35S:*Lp*CBF3	33.65	39.45 a	52.71	53.72 a	57.38 a	69.36 a	70.06 a	69.02 a	70.96 a
Nonacclimated 35S:*Lp*CBF3	39.61	56.75 b	54.05	60.00 a	75.98 bc	80.70 ab	89.66 b	91.49 b	89.80 b
Acclimated non-transgenic control	26.93	30.81 a	58.24	79.18 c	85.22 c	91.68 b	91.29 b	91.86 b	94.75 b
Nonacclimated non-transgenic control	25.89	32.60 a	54.57	72.68 bc	87.29 c	89.96 b	91.42 b	90.73 b	93.53 b
P-value (t-test)	0.1863	0.0002	0.9368	0.0010	<0.0001	0.0072	0.0069	0.0022	0.0027

* significant at <0.05 ** significant at <0.01 *** significant at <0.001 ^{ns} not significant

Means within columns followed by the same letter are not significantly different as indicated by the P-value.

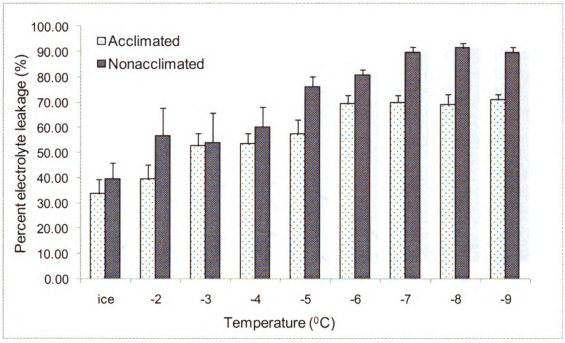

Figure 13. Comparison of the percent electrolyte leakage of 35S:*Lp*CBF3 transgenic lines at acclimated and nonacclimated conditions. Error bars represent standard deviation.

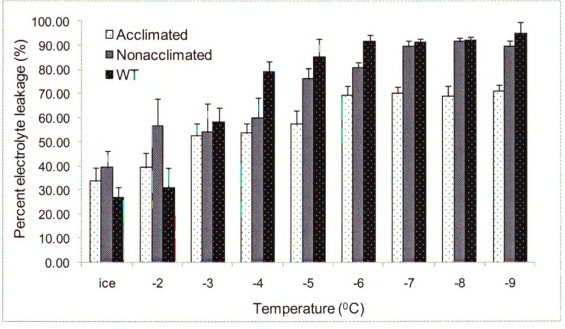

Figure 14. Comparison of the percent electrolyte leakage of 35S:*Lp*CBF3 transgenic lines and control. Error bars represent standard deviation

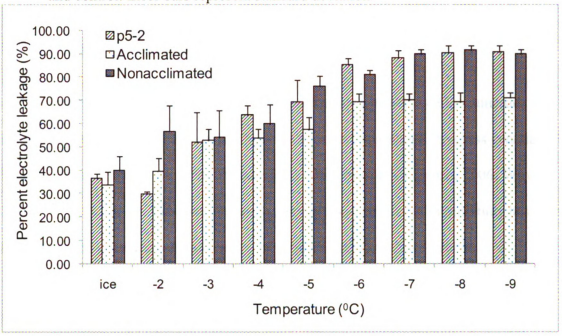

Figure 15. Comparison of the percent electrolyte leakage of 35S:*Lp*CBF3 transgenic lines and transgenic empty vector (p5-2) control. Error bars represent standard deviation.

DISCUSSION

Tissue culture and transformation of perennial ryegrass

In vitro propagation of perennial ryegrass has been established by numerous studies and the use of calli culture has been widely used in perennial ryegrass genetic transformation (Bajaj et al., 2006; Hisano et al., 2004; Sato & Takamizo, 2006; Wu et al., 2007; Wu et al., 2005). The tissue culture regeneration system used in this study has been efficient in the generation of sufficient embryogenic calli for use in genetic transformation. The adaption of a concentration of 5 mg l^{-1} of 2,4-D in the callus induction medium as reported to be optimal in perennial ryegrass (Liu et al., 2006; Sato & Takamizo, 2006) worked well in the regeneration frequency of the embryogenic calli. Contamination, however, has always been a problem in the tissue culture production of plants. This was one of the reasons to terminate the transformation of perennial ryegrass Transformer cultivar.

Plant genotype, source and age of explants and culture medium could greatly affect the response of perennial ryegrass in tissue culture. The production of resistant calli from cv. Inspire was relatively high but possibly because of the stringent selection in PPT, the production of rooted shoots was reduced. A concentration of 20 mg l^{-1} PPT was used in the selective subculture medium and was decreased to 10 mg l^{-1} during the regeneration but this was still too stringent for the perennial ryegrass transformants. Wu et al (2007) using bar as the selectable marker only supplemented 5-10 mg l^{-1} PPT in the selective media.

Additionally, it was also observed that a low frequency of albino shoots were obtained from the regeneration of perennial ryegrass in PPT selection (Liebao et al. 2007). These albino shoots did not produce roots in the rooting medium and eventually died. This was also observed by Liebao et al (2007) in particle-bombarded perennial ryegrass regenerants.

Agrobacterium-mediated transformation is often the preferred approach for grass transformation because it often yields plants with low number of transgene copies and does not require expensive equipment (Li et al., 2005). In this study, transformation efficiency (0.4%) was very low compared to previous reports on the use *Agrobacterium* for the transformation of perennial ryegrass. Bajaj et al. (2006) reported an average of 7% transformation efficiency of the method while Wu et al. (2007) had an efficiency of 9.6%. Li et al. (2006) who recovered only 3 transgenic plants from Chinese lawngrass transformation explained that low transformation efficiency could be a result of the effect of cefotaxime in the media on shoot regeneration.

Molecular analysis

All putative transgenics tested positive for the *bar* and *Lp*CBF3 gene by PCR analysis. This confirms the presence of the genes in the transgenic plants. Further analysis by Southern hybridization showed 3 and 4 insertions in the two 35S:*Lp*CBF3 transgenics indicating the successful insertion of the *Lp*CBF3 gene into the perennial ryegrass genome. Wu et al. (2007) confirmed 1–3 band pattern for perennial ryegrass transgenic using *bar* gene as the probe. Bajaj et al. (2006) had perennial ryegrass *hptII*

transgenic plants with insertions ranging from one to three with the exception of one plant with four copies. Sato and Takamizo (2006) observed at least five copies of the transgenes in the leaf tissue of their transgenic perennial ryegrass. It is not unusual to have more than one copy of the transgene in *Agrobacterium* transformation (Li et al., 2005).

Northern analysis at 4^0C low temperature treatment of 35S:*Lp*CBF3 transgenic plant detected *Lp*CBF3 transcripts in all time points. It was at 2 hour cold exposure that transcripts increased which does not corroborate with Zhao and Bughrara's (2008) findings with *Lp*CBF3 overexpressed in Arabidopsis plants. In their study, transcript levels of *Lp*CBF3 increased after 15 min of plant exposure in the cold. It could be hypothesized that transcript expression levels of this gene differ among plant systems. In this case, increase in *Lp*CBF3 transcripts takes a longer time in perennial ryegrass than in Arabidopsis.

Effect on the growth

Most reports on the overexpression of the *At*CBF3/DREB1A showed stunted growth of the Arabidopsis transgenic lines (Gilmour et al., 2004; Kasuga et al., 1999; Liu et al., 1998). Similarly, overexpression of rice *Os*DREB1A in Arabidopsis showed growth inhibition (Dubouzet et al., 2003). Also, studies done by Xiong and Fei (2006a) and Zhao and Bughrara (2008) overexpressing the *Lp*CBF3 in Arabidopsis had generated stunted plants. In the present study however, 35S:*Lp*CBF3 transgenic plants did not show growth retardation but had reduced tillering ability instead. Chinese lawngrass transformed with CBF1 also exhibited decreased tillering (Li et al., 2006). However, it

was also observed that vector control transgenics also exhibited the same growth pattern as the 35S:*Lp*CBF3 transgenic plants. It could be suggested that the insertion of the transgene by *Agrobacterium* in a random manner in the genome can potentially have an effect on some genes that influence growth of plants.

Liebao et al. (2007) observed that their *UBI:CBF3* transgenics did not exhibit growth inhibition nor visible phenotypic alterations. No differences in morphology or growth were also observed by Al-abed et al. (2007) on maize overexpressing *At*CBF3. This was also observed by Oh et al. (2005) in rice and claimed that this may be because lower levels of target genes are activated by CBF3 in rice than in Arabidopsis. Thus, the effect on the growth of rice and perennial ryegrass may be minimal. The difference of gene targets and expression level also confirms the Northern analysis findings of this study.

Electrolyte leakage test

Freezing tolerance of the transgenic plants was evaluated by electrolyte leakage test. The relative amount of ions released from the detached leaves were measured at specific low temperature treatments and at 100% ion leakage by severely freezing it. Essentially, the fewer ions released at a low temperature, the more cold tolerant the plant. Perennial ryegrass Inspire has been known to be cold susceptible and unable to cold acclimate. Results of the leakage test has confirmed that the presence of the *Lp*CBF3 gene in the genome of Inspire has enabled it to cold acclimate thereby releasing relatively fewer ions at lower temperatures. Although its LT_{50} is still higher than the native PI598441 (LT_{50}= -11^{0}C), LT_{50}=-5^{0}C is still significantly lower than the non-acclimated

and acclimated wild type control. This proves that we have successfully generated a cold tolerant perennial ryegrass cultivar from Inspire. Zhang et al. (2009) has shown that perennial ryegrass cv. 'Caddyshack' can cold acclimate and has exposed the plant at 4^0C for up to two weeks. It would be interesting to see if these *Lp*CBF3 Inspire transgenics could increase cold tolerance at longer periods of cold acclimation.

CONCLUSION

We have successfully regenerated transgenic perennial ryegrass overexpressing the *Lp*CBF3 gene. Presence of the gene in the genome and stable integration was confirmed by PCR analysis and Southern hybridization. Leaf painting assay for glufosinate resistance and electrolyte leakage test indicated the expression of the gene in the transformants. Cold acclimated transgenic plants may suggest increased tolerance to freezing temperature. In this study, 35S:*Lp*CBF3 transgenic plants did not show growth retardation but had reduced tillering ability.

Further studies could be done on the evaluation of expression of the transcript levels of *Lp*CBF3 gene at different low temperatures and at longer lengths of cold acclimation exposure of transgenic 35S:*Lp*CBF3 perennial ryegrass. Further evaluation of downstream cold-responsive genes would help confirm the activation of these genes with the induction of *Lp*CBF3. Studying the progeny of the transgenics would determine stable inheritance of gene expression as shown in T_0 transgenics.

This study has established evidence that cold susceptible top performing perennial ryegrass could be made more tolerant to cold temperatures. Forage and turfgrass managers in temperate climates could greatly benefit from new high-quality, cold-tolerant perennial ryegrass cultivars.

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