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Transgenic Tobacco Plants Over-expressing Arabidopsis Transcriptional Factor *CBF1* Show Morphological and Biochemical Characteristics Associated with Cold Tolerance

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Abstract: Transcriptional factor *CBFs* (C-repeat/dehydration responsive element binding factors) play important roles in plants' adaptation to low temperature and are prospective to be used in the engineering of cold-tolerant crops. Arabidopsis *CBF1* was expressed in tobacco plants in the present study to investigate the effects of *CBF1* on growth performance and cold tolerance in cold sensitive plants. Tobacco plants over expressing *CBF1* had shorter stature, thicker stems, thicker and glossier leaves, higher chlorophyll content, weaker apical dominance and longer time to flowering than control tobacco plants. Over expression of Arabidopsis *CBF1* in tobacco plants dose-dependently promoted surviving low temperature by increasing photochemical efficiency (Fv/Fm), soluble sugar content and proline content and decreasing malondiadehyde (MDA) content under low temperature stress. These results indicated that Over expression of *CBF1* could conferred elevated tolerance to low temperature upon cold sensitive plants.

Key words: *CBF1*, *Nicotiana*, cold tolerance, growth performance

INTRODUCTION

Low temperature as a key environmental factor represents one of the principle limitation affecting plants species distribution and crop productivity. In the past several decades, extensive attention has been paid to the molecular mechanisms of cold tolerance in plants because of the agricultural demands for improvement in cold tolerance for agronomic plants. Many plants originating in the temperate zone have the ability to sense low temperature and respond to it by activating mechanisms that lead to an increase to freezing temperature, an adaptive process known as cold acclimation (Thomashow, 1999). The most prominent advance in understanding cold acclimation in the past ten years was the discovery of the C-repeat/dehydration -responsive element biding factors(*CBFs*) cold-response pathway (Thomashow, 2001). It has been demonstrated that many water deficit and cold responsive genes contain the C-repeat (CRT) and dehydration responsive element (DRE) in their promoter sequences (Horvath *et al.*, 1993; Baker *et al.*, 1994; Iwasaki *et al.*, 1997). Proteins that bind to the DRE/CRT element and mediate transcription were isolated by the yeast (*Saccharomyces cerevisiae*) one-hybrid system and named DRE-binding proteins (DREBs)/CRT-binding factors (Stockinger *et al.*, 1997; Liu *et al.*, 1998). Arabidopsis encodes a small family of cold-responsive transcriptional activators known either as *CBF1*, *CBF2* and *CBF3* (Medina *et al.*, 1999) or *DREB1b*, *DREB1c* and *DREB1a* (Liu *et al.*, 1998; Kasuga *et al.*, 1999), respectively. In Arabidopsis CBF genes are induced within 15 min of plants being exposed to low temperatures followed at about 2 h by induction of cold-regulated genes contain the CRT/DRE-regulatory element (Gilmour *et al.*, 1998; Liu *et al.*, 1998). Over the next few days at low temperature, the plants increase in freezing tolerance reaching a maximum level within 1 to 2 weeks. Constitutive expressions of the CBF genes in transgenic Arabidopsis and canola have been proved to induce COR (Cold-regulated) genes expression and increase freezing tolerance without a low temperature stimulus (Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000). The homologous of the Arabidopsis *CBFs* have recently been found in many plants, including canola oil seed rape (*Brassica napus*), soybean (*Glycine max*), broccoli (*Brassica oleracea*), tomato (*Lycopersicon esculentum*), alfalfa (*Medicago sativa*), cherry (*Prunus avium*), wheat (*Triticum aestivumfrom*), rye (*Secale cereale*), corn (*Zea mays*), rice (*Oryza sativa*), strawberry (*Fragaria ananassa*) and barley (*Hordeum vulgare*) (Yang *et al.*, 2005). Some of them have been proved to increase cold

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tolerance in Arabidopsis (Jaglo-Ottosen *et al*., 2001; Kitashiba 2004; Dubouzet *et al*., 2003). Since *CBFs* are conditionally expressed and play central roles in plant cold response network (Yang *et al*., 2005), it is important to learn about the effect of *CBFs* on plants growth and development. Over expression of *CBF3* and *CBF1* in Arabidopsis and tomato respectively has been proved to result in dwarfish phenotype, retardation growth habit and lower seed production as well (Gimour *et al*., 2000; Liu *et al*., 1998). We report here that Over expression of Arabidopsis *CBF1* resulted in morphological and biochemical changes associated with cold tolerance in transgenic tobacco plants.

MATERIALS AND METHODS

Plant material: Tobacco (*Nicotiana tobacum*) plants were transformed by T-DNA construct containing *bar*, *cbf1* and *uidA* (Fig. 1-a). *Arabidopsis CBF1* cDNA was attached to the ubiquitin promoter (PUbi) of maize. The exogenous genes were expressed in line TC8 at a high level and in line TC9 at a much lower level (Fig.1-b, Fig. 1-c). T1 generation of line TC8, TC9 and the non-transgenic control tobacco (CK) were used in the present study. Four hundred seeds for each line were sown and incubated in a greenhouse. Seedlings of transgenic lines were selected by Glufosinate-ammonium (Sigma chemical). Plants at the four-leaf stage were transplanted into pots with peat moss and grown under similar conditions in a green house.

Comparison of phenotype between transgenic lines and the control: The Comparison was conducted with TC8, TC9 and CK plants, fifteen uniform plants for each line. The chlorophyll content was measured using the 4th leaf of a plant at seven-leaf stage according to the method described by Wellburn (1984). The diameter of basal stem and leaf thickness (1 cm^2 portion near the main vein in the middle of the 6th leaf) were measured at the early bolting stage. The plant height was measured at the late flowering stage.

Low temperature treatment: To examine the freezing tolerance of the whole plants, transgenic and control plants at seven-leaf stage grown in green house were transferred to the open field. The minimal temperature of the days during experiment (from 28th Oct to 29th Nov. in 2005) declined gradually from 12°C to -5°C. The behaviors of the plants and the air temperature were recorded every day. For low temperature treatment, plants were transferred to a growth chamber operating with continuous light. The Photosynthetic Photon Flux Density (PPFD) was 150 μ mol $m^{-2}s^{-1}$ and the relative humidity was 75%. The temperature was set at 25°C for 2 days, 12°C for 2 days so that plants could adapt to low temperature and 4°C for 5 days. Measurements were carried out before and after low temperature treatment.

Chlorophyll florescence measurements: Chlorophyll fluorescence was recorded using Portable Chlorophyll Fluorometer PAM-2100 (Heinz Walz GmbH, Germany) in the growth chamber at the relevant temperature. After 30 min of dark adaptation the samples excited with actinic radiation of 1800 μ mol m^{-2} s^{-1} at 650 nm. Fluorescence was detected for 40s and the Fv/Fm ratio was calculated.

Lipid peroxidation analysis: For the measurements of lipid peroxidation in leaves, the thiobarbituric acid (TBA) test, which determines MDA as an end product of lipid peroxidation (Heath and parker, 1968), was used. Fresh leaf (0.4-0.5 g) was homogenized in 4 mL of 10% (w/v) TCA solution. The homogenate was centrifuged at 6000 rpm for 15 min and 2 mL of the supernatant was added to 2 mL of 0.67% (w/v) TBA in 10% TCA. The mixture was incubated in boiling water for 30 min and the reaction was stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 6000 rpm for 5 min and the absorbance of the supernatant was measured at 532 nm, subtracting the value for non-specific absorption at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient 155 mm^{-1} cm^{-1}.

Proline measurements: Proline was extracted from leaf material (0.4-0.5g FW) in 4 mL of 3% (W/V) sulfonic salicylic acid. The sample was heated at 100°C for 10 min, after cooling, centrifuged for 10 min at 6000 rpm. The supernatant was analyzed for proline content using the acid ninhydrin method (Troll and Lindsley, 1955).

Total soluble sugar measurements: Total soluble sugars were extracted from leaf material (0.4-0.5 g FW) in 80% (V/V) ethanol (4 mL) at 80°C for 30 min. After cooling, sample was centrifuged for 10 min at 6000 rpm and the supernatant was stored on ice while the pellet was re-extracted twice in 2 mL of 80% ethanol, the three supernatants were combined. The aqueous extract was supplemented with 20-25 mg of powdery activated carbon followed by being heated at 80 for 30 min with shaking at times to remove chlorophyll. 80% ethanol was added to make up to a final volume of 10 mL. The mix was then filtered through filter paper. The filtrate was assayed for sugar content using anthrone reagent (Yem and Willis, 1954).

Statistical analysis: Values are expressed as the mean±SD of three independent replicates of each experiment. In each experiment the chlorophyll fluorescence measurements were carried out 15 times, while the determinations of MDA, proline and soluble sugar were performed with three parallels in all cases. The significance of the differences between control and transgenic plants were statistically evaluated using One-way ANOVA process of SPSS 12.0 for window (SPSS Inc.)

RESULTS

Transgenic plants exhibited dwarf phenotype: *CBF1* expression did not affect seed germination. The first germinations occurred in 8 days and the final germination rates were 86, 82 and 84% for CK, TC9 and TC8, respectively. However, the morphology, phenology, development of the transgenic plant were affected by Over expression of *CBF1*. Line TC8 that expressed high level of *CBF1* exhibited smaller leaves and plant size than line TC9 and control plants (Fig 1-d, e, f), although the leaf number per plant was the same for the three lines. Leaves of line TC8 were much thicker than those of line TC9 and control. Line TC8 leaves were glossiness while the line TC9 and control leaves were glaucousness. The chlorophyll content in leaves of line TC8 was 48% higher than that of control plants. The transgenic tobacco plants had a shorter but thicker stem than control plants (Table 1, Fig. 1-d). TC8 plants exhibited more compact stature than the control plants because of their thicker leaves and shorter internodes. The axillary buds were swelled in transgenic line TC8. Those in the lower part even developed side shoots with 1-3 expanded leaves (Fig. 1-e). These side shoots were very small in size during the whole lifetime. In contrast, the axillary buds in control plants remained small and repressed for the whole

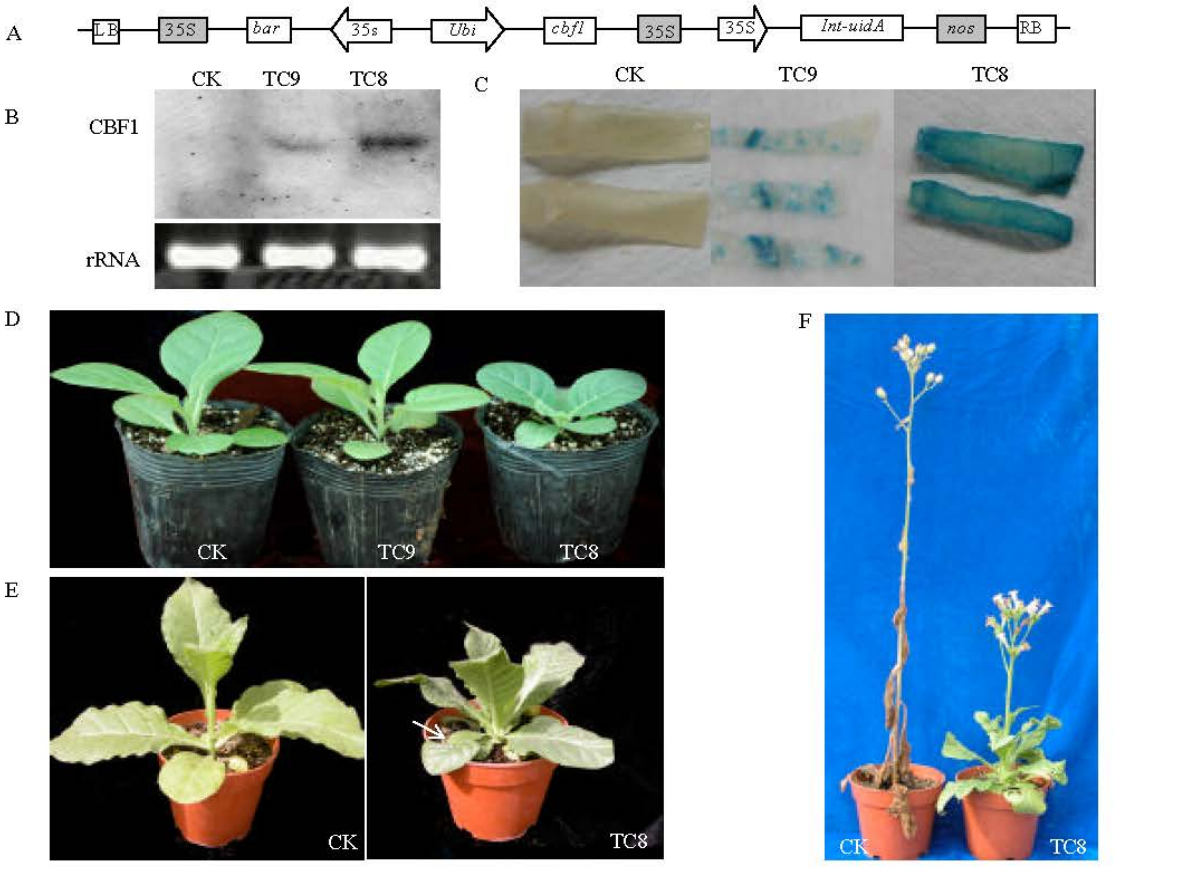

Fig. 1: Growth characteristics of *CBF1*-expressing transgenic tobacco plants. A, transformed T-DNAregion. B, Northern analysis of total RNA (20 μg) prepared from control plant (CK) and fromtransgenic plant TC8, TC9, showing that there was no transcript of *cbf1* in control plant, a lowtranscript level in TC9 and a much higher transcript level in TC8. C, GUS activity in transgenic plants, showing the translation of exogenous genes. D, Non-transformed plant (CK) and transgenic *CBF1*-expressed plant (TC8, TC9) after 35 days growth at greenhouse. E, CK and line TC8 after 50 days growth. The arrow shows the developed side shoot. F, CK and line TC8 after 130 days growth

Fig. 2: Freezing tolerance of *CBF1*-expressing transgenic tobacco plants in the open field. The picture was taken when the minimal air temperature was - 2 for five days

Table 1: The effects of *Arabidopsis CBF1* on the performance of tobacco plants

Item Tobacco line	CK	TC9	TC8
Plant height (cm)	83.6±2.8	76.9±4.3	40.9±3.2
Main stem diameter (mm)	5.6±0.38	6.43±0.20	8.93±0.20
Leaf thickness (μ m)	170±10	195.10±16.4	286.6±50.4
Development of Axillary's buds	Remained small and repressed	Swelled slightly	Swelled or developed 1-3 expended leaves
Leaf number	12±1	12±1	14±1
Leaf chlorophyll Content (μg/g FW)	645.66 ±67.53	809.35 ±53.92	958.83 ±37.96
Leaf glossiness	Glaucousness	Glaucousness	Glossiness
Time to flowering (d)	66.2±4.7	70.4±4.2	95.4±7.3

duration of vegetative growth. The effects of *CBF1* expression were observed also in line TC9 but much weaker than that in line TC8 because of lower transcript level of *CBF1* in line TC9 (Table 1).

Transgenic plants showed delayed flowering time: There was a substantial difference in time to flowering between the control and *CBF1* over expressing plants; i.e. the control plants bolted and formed flowers well before line TC8 did. In one experiment, for instance, the control and TC9 plants began to bolt at 66.2 days and 70.4 days respectively, whereas TC8 plants took 95.4 days to initiate bolting (Table 1). The TC8 plants were at flowering stage when the control plants were at mature stage, (Fig. 1-f). The delay in flowering time observed in the *CBF1*-over expressing plants, significantly, did not "simply" involve a slower overall growth rate, but appeared to involve a developmental delay in flowering. In one experiment, for instance, the control plants produced 12 leaves per plants, whereas TC8 plants produced 14 leaves. The final seed yield was similar between the control and transgenic plants, which indicated that Over expression of *CBF1* did not affect the fecundity of transgenic plants (Fig. 1-f).

Transgenic plants exhibited enhanced tolerance to low temperature stress: Transgenic and control plants at seven-leaf stage were transferred from green house to an open field. The minimal temperature declined gradually from 12°C to -5°C during experiment (from 28th Oct to 29th Nov. in 2005). No visible damage was observed in both transgenic and control plants from day one to day 15 when the minimal air temperature fell from 12°C to 3°C. Cold injury was observed in the control plants but not in transgenic plants during day 16 to 18 when the minimal air temperature decreased to –1°C and the early winter frosts occurred. This fact indicated that transgenic plants were more tolerant to low temperature than control plants. Cold injury occurred in the TC9 plants within day 19 and 20 when the minimal air temperature decreased to –2°C, in the TC8 plants until day 33, the end of this experiment, when the minimal air temperature was lower than -4°C (Fig. 2). It might be deduced that the expression of Arabidopsis *CBF1* could enhance cold tolerance in tobacco and that the degree of enhancement was dependent on the transcript level of *CBF1*.

Changes in the Fv/Fm parameter and MDA contents in transgenic tobacco plants under low temperature stress: To ascertain the beneficial effect of *CBF1* constitutive expression in tobacco cells, the control, TC8, TC9 plants were exposed to 25°C for 2 days, 12°C for 2 days and 4°C for 5 days. Photo inhibition of photosynthesis (the reduction of the Chlorophyll fluorescence parameter (Fv/Fm)) was limited in transgenic plants; and this limitation was more pronounced in TC8 plants than in TC9 plants (Fig.3-a). This fact showed that photochemical efficiency of PSII was less affected in *CBF1*-over expressing transgenic plants.

Low temperature stress induces the accumulation of free radicals, which damage cells by initiating or accelerating the membrane lipid peroxidation (Wise and Naylor, 1987) and yields elevated levels of MDA. There was a slight elevation of MDA level in both control plant and TC9 plants, but not in TC8 plants, at chilling temperature (12°C). When the temperature decreased to 4°C, the increase of MDA content were significant in all plants. What is noticeable is that the MDA level declined again in TC8 plants, but kept increasing in TC9 and control plants after one day of cold treatment (Fig 3-b). The results indicated that Over expression of *CBF1* could alleviate the lipid peroxidation in plant cells under low temperature stress.

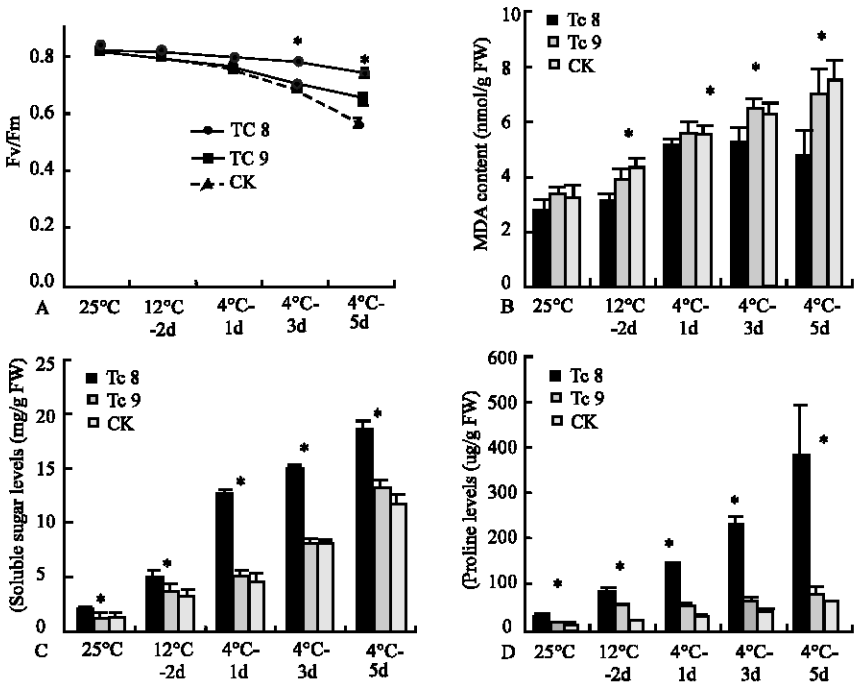

Fig. 3: The influence of low temperature Photo inhibition on the Fv/Fm parameter (A) and formation of MDA (B), changes in levels of soluble sugar (C) and proline (D). Control and transgenic tobacco plants constitutively expressing *CBF1* were transferred to a chamber for low temperature treatment. *Differences significant at $p \leq 0.05$

Effect of constitutive expression of *CBF1* on proline and soluble sugar contents: The accumulation of proline (Nanjo *et al*., 1999) and soluble sugar (Olien and Clark, 1993) are commonly observed biochemical changes in plants during cold acclimation. We found that the constitutive expression of *CBF1* affect the metabolism of these solutes in tobacco plants. The soluble sugar content was dramatically increased in TC8 plants but not significant in TC9 plants in comparison with that found in CK plants under both normal and low temperature stress conditions (Fig 3-c). The difference of proline levels between transgenic plants and control plants was more obvious. At room temperature, the proline content in TC8 plants was approximately 2-fold higher than those in control plants. Under low temperature stress, proline contents were significantly increased in TC8 plants but to a much less extent in TC9 plants in comparison with that found in control plants. After five-day exposure to 4°C, the proline level in line TC8 was up to 381.8 ug g^{-1} FW, about 4-fold of that in line TC9 and 5-fold of that in control plants (Fig.3-d).

DISCUSSION

Though transcripts encoding CBF-like proteins were also found to accumulate rapidly in response to low temperature in tomato, the expression model differs from that in cold-acclimate plants (Gilmour *et al*., 2000). The transcript levels of *CBF1* in tomato appeared to return to those found in warm-grown plants after 24 h of exposure to low temperature and remained at low levels after one week of cold treatment. Heterology expression of Arabidopsis *CBF1* confers elevated tolerance to chilling stress in transgenic tomato, but did not induce the transcript of COR genes homologues (Hsieh *et al*., 2002a). Cold tolerance in the *CBF1*- expressing transgenic rice plants (*Oryza sativa L.*) was not significantly different from that of the wile-type plants, but some cold-responsive genes were up regulated (Lee *et al*., 2004). The results of present study revealed that Over expression of Arabidopsis *CBF1* could confer increased tolerance to low temperature in tobacco. Further more, we found that there was a positive correlation between the transcript level of *CBF1* and the degree of plant tolerance to cold. We speculate that the mechanism of *CBF1* conferring elevated cold tolerance to plants might be conserved but partially activated in cold sensitive plants.

In vivo chlorophyll fluorescence induction provides rapid, non-invasive methods for following changes in the photosynthetic apparatus before any visible symptoms can be observed (Janda *et al*., 1998). A decrease in the Fv/Fm ratio indicates the lower photochemical efficiency

of PSII, the part of the photosynthetic electron transport chain, which is the most susceptible to various stress effects (Krausa and Weis, 1991; Janda, 1994). Under low temperature stress the Fv/Fm parameters of line TC8 was proved to be higher than those of control plants, showing that photosynthetic electron transport was less affected in line TC8. Photo inhibition and low temperature stress would lead to the intense accumulation of active oxygen species in plant cells (Janda, 1994; Hodgson and Raison, 1991). The MDA content reflecting the oxidative damage in leaves of the control and TC9 plants increased continuously under the low temperature stress. By contrast, the MDA content in the leaves of TC8 plants was increased after one day of cold treatment, but decreased afterward. In *CBF1*-expressing transgenic tomato plants the CAT1 gene and catalase activity were highly induced and H_2O_2 content was proved to be lower (Hsieh *et al*., 2002a). These results indicated that expressing of *CBF1* could alleviate Photo inhibition and oxidative damage in cold sensitive plants under low temperature stress.

Increased levels of proline and sugars occur with cold acclimation in a wide variety of plants and are thought to contribute to the enhancement of freezing tolerance, in part, through stabilizing membranes (Guy *et al*., 1992; Prasad *et al*., 1994; Carpenter and Crowe 1998; Nanjo *et al*., 1999). We found that the transgenic tobacco plants showed higher levels of proline and soluble sugar than control plants either at room temperature or at 4°C, indicating that expression of *CBF1* could induce biochemical changes associated with cold tolerance in transgenic tobacco. Similar biochemical changes were also found in *CBF3*- expressing transgenic Arabidopsis. The difference in the ability to tolerate low temperature stress between line TC8 and line TC9 might be due to the difference in *CBF1* transcript level and biochemical changes. Whether COR gene homologues in tobacco were activated by overexpressed *CBF1* remains to be determined.

CBFs are conditionally expressed in cold-acclimate plant of wild-type and play key role to induce biochemical and physiological changes in plants so that to defense stresses of low temperature (Gilmour *et al*., 1998; Jaglo-Ottosen *et al*., 2001). The constitutive Over expression of *CBFs* in plant would result in some unexpected effects. Over expression of *CBF3* in Arabidopsis caused stunted growth and decrease in seed production. Although effects of *CBF1* on plant growth and development was not observed in transgenic Arabidopsis plants (Jaglo-Ottosen *et al*., 1998), the *CBF1*-expressing transgenic tomato plants exhibited an apparent dwarfism along with a reduction in fruit set and seed number per fruit (Hsieh *et al*., 2002b). Here we found that in addition to a dwarfism phenotype the *CBF1*-over expressing transgenic tobacco plants had thicker stems and thicker and glossy leaves. The glossiness might be a favor to reflect light and reduce transpiration. The compact stature of the TC8 plants might be help to withstand abiotic stresses. The extent of morphology changes of transgenic plants was dependent on the level of transcript of *CBF1*. Similar relationship between morphology and transcript levels of *CBF3* was also observed in transgenic Arabidopsis (Liu *et al*., 1998; Gilmour *et al*., 2000).

CBFs appear to be one of "muster switches" that integrate activation of multiple components of the cold acclimation response (Thomashow, 2001). *CBF1* might also affect synthesis or transportation of phytohormones in plants. It has been shown in transgenic tomato plants that Over expression of *CBF1* may be interfering with GA biosynthesis in (Hsieh *et al*., 2002 a, b). A novel gibberellin (GA)-deficient mutant showed dwarfism and late-flowering, but the phenotype was rescued by exogenous GA(3) like known mutants defective in GA biosynthesis. Genetic and molecular analyses revealed that the mutant phenotypes are caused by increased or ectopic expression of a putative AP2 transcription factor that is phylogenetically close to CBF genes (Magome *et al*., 2004). Here we found that over expression of *CBF1* in tobacco could reduce apical dominance to some extent, showing by the appearance of axillary shoots with 1-3 small leaves in the transformants. Axillary buds growth might be triggered either by a redirection of the cytokinin supply from the roots, necessary to initiate axillary bud growth, or by a decline in auxin supply from the apex, enabling the axillary buds to start cytokinin synthesis or by the conversion of an inactive precursor to an active cytokinin (Tamas, 1995). The regulatory effect of *CBFs* on metabolism of phytohormone is worthy of research.

In conclusion, expression of *CBF1* could confer tolerance to low temperature in tobacco, which may be due to the fact that Over expression of *CBF1* caused less reduction in photochemical efficiency, less lipid peroxidation, more osmolytes accumulation, reduced plant size, compact stature and glossiness. The engineering of cold-tolerant crops by incorporating a master switch gene like *CBF1* may be an efficient approach. To avoid possible negative effect of *CBF1* on transgenic plants, it is important to bear in mind that proper promoter should be selected for plants to regulate the expression of *CBF1*. The benefit of using an inducible promoter to drive *DREB1a* has been demonstrated (Kasuga *et al*., 1999). By replacing the cauliflower mosaic virus 35S promoter with inducible promoter such as RD29A in transgenic Arabidopsis, enhanced stress tolerance was achieved

without a penalty on plant growth (Kasuga *et al*., 1999). If the target plants are agronomic crops, stress-inducible promoters should be considered in order to prevent the adverse effects. The growth and development characters exhibiting in TC8 plants suggested that Over expression of *CBF1* promote transverse growth and make plant stronger. If applying GA can reverse the dwarfish phenotype of *CBF1*-over expressing transgenic plants, constitutive promoter might be taken into account for plants used for defending ground or turf establishment. In turfgrass species for example, the growth speed of transgenic plants would be regulated artificially and the retardation and transverse growth would reduce mowing and save labor.

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