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Article

Expressing the Tyrosine Phosphatase (CaTPP1) Gene from *Capsicum annuum* in Tobacco Enhances Cold and Drought Tolerances

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As one way to approach to cold defense mechanism in plants, we previously identified the gene for protein-tyrosine phosphatase (CaTPP1) from hot pepper (*Capsicum annuum*) using cDNA microarray analysis coupled with Northern blot analysis. We showed that the *CaTPP1* gene was strongly induced by cold, drought, salt and ABA stresses. The *CaTPP1* gene was engineered under control of CaMV 35S promoter for constitutive expression in transgenic tobacco plants by *Agrobacterium*-mediated transformation. The resulting CaTPP1 transgenic tobacco plants showed significantly increased cold stress resistance. It also appeared that some of the transgenic tobacco plants showed increased drought tolerance. The CaTPP1 transgenic plants showed no visible phenotypic alteration compared to wild type plants. These results showed the involvement of protein tyrosine phosphatase in tolerance of abiotic stresses including cold and drought stress.

Key words: Capsicum annuum, *cold tolerance, drought tolerance, transgenic tobacco, tyrosine phosphatase*

Plants have developed sophisticated protective mechanisms to overcome various abiotic environmental stresses such as low temperature, high salinity and drought [Thomashow, 1999; Hasegawa *et al.*, 2002; Zhu 2001a, b; Xiong *et al.*, 2002; Xiong and Zhu, 2002). Many studies have shown that these protective mechanisms are regulated via an alteration in the expression levels of many stress genes. Molecular studies using DNA microarrays have identified many genes that are regulated by various abiotic stresses [Schenk *et al.*, 2000 Bohnert, 2001; Seki *et al.*, 2001, 2002; Kreps *et al.*, 2002). It is also known that many of these stress genes are regulated at the transcriptional level [Shinozaki and Yamaguchi-Shinozaki, 1997; Cheong *et al.*, 2002; Zhu and Provart, 2003; Shinozaki *et al.*, 2003).

The protein products of these stress genes are classified into two categories according to the function upon various stresses; the first category is the proteins that are involved in direct protect functions, the second category is the proteins that are involved in stress signal transduction pathway and expression controls of stress-tolerant genes [Qiang *et al.*, 2000). Genes induced by these abiotic stresses are thought to function not only in protecting cells by producing important metabolic proteins and cellular protects, but also in regulating genes that are involved in transducing the stress response signal [Shinozaki and Yamaguchi-Shinozaki, 1997, Kasuga *et al.*, 1999, Seki *et al.*, 2002; Xiong *et al.*, 2002). Among these, genes encoding factors that are components of abiotic stress signal transduction pathway drew attention because they control several target genes. These factors include kinases, phosphatases and transcription factors.

There have been many attempts and developments through the years to engineer abiotic stress tolerant crop plants by overexpression of abiotic stress-regulated genes whose products function either directly or indirectly to protect plants against stresses [Holmberg and Bulow, 1998; Bajaj *et al.*, 1999). In particular, transcription factors were the primary genes to introduce into plants.

Many results indicated that plant mitogen-activated protein kinase (MAPK) is rapidly activated when exposed to variety of abiotic and biotic stresses including cold, drought, wounding and during plant-pathogen interactions [Jonak *et al.*, 1996; He *et al.*, 1998; Agarawal *et al.*, 2002; Tu *et al.*, 2002; Cheong *et al.*, 2003; Kim *et al.*, 2003]. Also studies have shown that tyrosine phosphorylation is very important for the activation of MAPK signal

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pathway in higher plants [Zhang and Klessig, 1997; Xu *et al.*, 1998]. The regulation of phosphorylation levels is mediated by the balanced activities of protein tyrosine kinase and phosphatases (PTPases) [Hunter, 1995]. Regarding this, several studies showed that involvement of protein tyrosine phosphatases in stress responses in lower plant, *Chlamydomonas eugametos* (VH-PTP13) [Haring *et al.*, 1995] and higher plant, *Arabidopsis* (AtPTP1) [Xu *et al.*, 1998; Gupta and Luan, 2003].

As one way to approach to cold defense mechanism in plants, we previously investigated changes of expression in cold-regulated transcripts levels in hot pepper plant (*Capsicum annuum*) using cDNA microarray analysis coupled with Northern blot analysis [Hwang *et al.*, 2005]. By analysis of the 3.1 K red pepper cDNA microarray, we identified 317 cold inducible genes. After sequencing analysis, 42 up-regulated genes and three down-regulated genes by cold treatment were selected for further analysis. Among the 42 genes that appeared to be up-regulated by cold, 19 genes appeared to be regulated by salt stress at the same time. Among up-regulated genes by cold-stress, many transcription factors were included: a family of 4 ethylene responsive element binding protein (CaEREBP-C1 to C4), bZIP protein (CaBZ1), RAV1, zinc finger proteins, heat shock factor (HSF1) and WRKY (CaWRKY1) protein [Hwang *et al.*, 2005]. In addition, interestingly enough, one protein tyrosine phosphatase (CaTPP1) was included. We showed that the gene encoding CaTPP1 was induced by cold stress. It was also induced by salt, ABA and drought [Hwang *et al.*, 2005].

In this report, in an effort to study involvement of protein tyrosine phosphatase (CaTPP1) in the signal transduction pathway of cold stress and to generate abiotic stress-resistant plants, we introduced the cold stress inducible *CaTPP1* gene from *C. annuum* into tobacco plants and tested the resulting transgenic plants had various abiotic stress tolerances.

Materials and Methods

Plant, bacteria and culture media. Hot pepper (*Capsicum annuum* cv Bugang) plants for RNA isolation were grown in a temperature-controlled green house at 25°C. Tobacco plants (*Nicotiana tabacum* var. Xanthi) were kept on an MS medium (MS salt, vitamin mix, 3% sucrose, 0.7% phytoagar, pH 5.7) and maintained in a temperature controlled culture room of 23°C under constant light conditions. Binary vector pB7WG2D under control of the CaMV (cauliflower mosaic virus) 35S promoter was used for constructing pB7WG2D/35S-CaTPP1. This construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* was grown on YEP (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$, pH 7.2) at 28°C for approximately 48 hr.

The reactions for construction of binary vector pB7WG2D/35S-CaTPP1. In order to construct the plant overexpression vector, pB7WG2D/35S-CaTPP1, Gateway™ Cloning Technology (Invitrogen Life Technologies) was applied. For this, two steps of homologous recombination reaction were performed. One was BP reaction, the other was LB reaction. First of all, *CaTPP1* gene flanked by attB1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3') and attB2 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3') sequence at each end was generated by PCR. The PCR reaction was performed using CaTPP1-*att*B1 (5'-AAAAAGCAGGCTCCTCCAATTCCACTCAATTCAAAACTAC-3) and CaTPP1-*att*B2 (5'-AGAAAGCTGGGTCGGGGAACTCAACAGCATCATTGAAC-3') primers.

The BP reaction was performed with purified *att*B-PCR products and donor vector, pDONR. The donor vector was a 4,470 bp pDONR™201 (Invitrogen™ Life Technologies) containing a kanamycin selection marker. The 10 μL BP reaction mixture was constructed by 100 ng of *att*B DNA (second PCR product), 150 ng of *att*P DNA (pDONR™ vector), 2 μL of 5X BP Clonase™ reaction buffer, and 2 μL of BP Clonase™ enzyme mix, and water adjust a final volume. The reaction was performed at 25°C for 4 hr.

The BP reaction product was transferred into *E. coli* DH5α cell by heat shock method [Sambrook *et al.*, 2001]. The BP reaction mixture was spread on LB plate containing 50 $\mu g \cdot mL^{-1}$ kanamycin for 16 hr at 37°C. Transformants were confirmed by PCR.

Then in order to transfer *CaTPP1* gene in entry clone, pDONR201/35S-CaTPP1 to binary vector, pB7WG2D, the same homology recombination reaction (LR reaction) between entry clone, pDONR201/35S-CaTPP1 and overexpression vector, pB7WG2D was performed to generate final plant overexpression vector, pB7WG2D/35S-CaTPP1. The LR reaction facilitated recombination of an *att*L in pDONR201/35S-CaTPP1 with an *att*R in pB7WG2D to create the plant overexpression vector, pB7WG2D/35S-CaTPP1 (Fig. 3A).

The overexpression vector was a 12,542 bp pB7WG2D™ (Invitrogen™ Life Technologies) containing a streptomycin and spectinomycin selection marker. The 10 μL LR reaction mixture was constructed by 4 μL (150 ng) of entry clone, 1 μL (150ng) of destination vector (pB7WG2D™ vector), 2 μL of 5X BP Clonase™ reaction buffer, 2 μL of LR Clonase™ enzyme mix and added to a final volume of deionized water for 2 hr at 25°C.

The LR reaction product was transferred into *E. coli* DH5α cell by heat shock method for propagation and maintenance, because the DH5α strain was sensitive to ccdB. The LR reaction mixture was spread on LB medium plate containing 50 $\mu g \cdot mL^{-1}$ kanamycin for 16 hr at 37°C. Transformants were confirmed by PCR. Table 1 showed all the oligonuclecotides that were used as primers for construction of pB7WG2D/35S-CaTPP1.

***Agrobacterium* transformation and generation of transgenic tobacco plant.** The overexpression vector, pB7WG2D/35S-CaTPP1 was transferred into *Agrobacterium* by the freeze-thaw method [Holster *et al.*, 1978]. Tobacco transformation was performed using the procedures of Kwon *et al.*, (1994). Regenerated transgenic plants were transferred to a soil mixture for further analysis.

Genomic DNA extraction and polymerase chain reaction analysis. Genomic DNA was extraction from tobacco plants by the G-spin genomic DNA extraction kit (iNtRON, Korea) by manufacturer's instruction. Polymerase chain reaction (PCR) amplification was followed in Peltier thermal cycle PTC-100 (MJ Research, USA) with Ex-Taq polymerase (Takara, Japan) using the following cycling parameters; 95°C for 5 min, followed by 35 cycle of 95°C for 1 min, 52°C for 1 min, 72°C for 2 min and final extension of 72°C for 10 min. For PCR identification of CaTPP1 transgenic tobacco plants, CaTPP1-attB1 and CaTPP1-attB2 primer set were used. PCR products were separated by 1% agarose gel electrophoresis.

RNA preparation and Northern blot analysis. To test the expression of CaTPP1 transcript in transgenic tobacco plants, total RNA was extracted using Tri-Reagent (MRC, USA). For this, each lines of 13 transgenic tobacco leaves grown on MS medium for approximately 3~4 weeks were used.

For Northern blot analysis, total RNA was electrophoresed on 1.2% denaturing formaldehyde/MOPS agarose gel. The Northern blot was prepared on a Hybond-N^+ membrane (Amersham, USA) by a capillary transfer method. Prehybridization was performed for 15 min at 65°C in hybridization solution (1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, 7% SDS) before the hybridization solution containing radio-labeled cDNA probe was added, followed by hybridization overnight at 65°C in the Hybaid oven (Hybaid, USA). Stringent membrane washing was performed with 0.2× SSC/0.1% SDS at 65°C. The washed membrane was then exposed to X-ray film at −70°C.

Abiotic stress treatments. Cold stress treatment of hot pepper plants for RNA extraction was carried out at 4°C, for various durations. For salt treatment, 500 mM NaCl was applied by the soil-drenching method. Drought conditions were simulated by air-drying for various durations. ABA treatment was conducted via the spraying of 10^{-4} M ABA solution. The cold treatment of CaTPP1 transgenic tobacco plants for the biofunctional assay was conducted by keeping plants at −6°C for 24 hr with control and transgenic plants growing in the soil mixture. For drought treatment, control and transgenic tobacco plants growing in the soil mixture were not watered for two weeks. The tobacco plants used in abiotic tresses treatments were grown for 3~4 weeks in soil mixture or Jiffy pot. These plants were either germinated in soil mix or root-developed in an MS selection medium containing phosphinothricin.

Results

Isolation and expression patterns of a gene encoding CaTPP1. Using cDNA microarray analysis coupled with Northern blot analysis, we previously identified a gene encoding protein tyrosine phosphatase (CaTPP1) as cold inducible gene from hot pepper [Hwang *et al.*, 2005]. The fact that a number of genes are induced by cold, drought and salt simultaneously in various plants mean that there is some cross-talks between the plant defense mechanisms against various abiotic stresses. In addition the involvement of ABA in the plant environment stress responses has long been recognized [Shinozaki *et al.*, 2003]. Therefore, the expression of several genes has been monitored by RNA blot analysis, according to the effects of various stresses, including those associated with cold, salt, ABA and drought. The gene which encodes for CaTPP1 was induced not only by salt, drought and ABA stresses and, to a slight degree, by cold treatment (Fig. 1).

Characterization of the *CaTPP1* cDNA. Sequence analysis showed that the CaTPP1 protein shares high homology with tyrosine phosphatase in plant systems. All protein tyrosine phosphatase contain (I/V)HCXAGXXR(S/T)G as conserved motif which have the catalytic cysteinyl residue involved in the formation of phosphoenzyme reaction intermediate [Guan and Dixon, 1990]. The CaTPP1 was also shown to contain conserved catalytic motif, ^{158}IHCKRGKHRTG^{168} (Fig. 2).

Hydropathy analysis revealed the CaTPP1 does not contain transmembrane domain. Thus, based on sequence homology comparison with other protein tyrosine phosphatases and absence of transmembrane domain, CaTPP1 is thought to be a member of intracellular PTPase family. In Fig. 2, the conserved catalytic domain of CaTPP1 was aligned with other tyrosine specific phosphatases including *Arabidopsis* AtPTP1 and human PTP1B.

Generation and selection of CaTPP1 transgenic tobacco plants. The plant overexpression vector, pB7WG2D/35S-CaTPP1, was constructed using Gateway™ Cloning

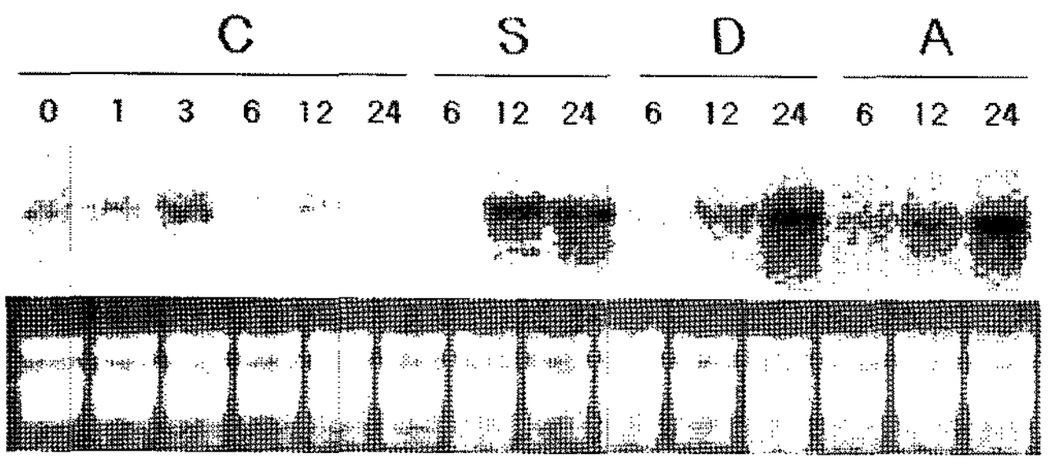

Fig. 1. Expression pattern of the *CaTPP1* gene at cold, salt, drought and exogenous ABA treatment. Fifteen μg of the total RNA was used for the RNA gel blot analysis. N: control, C: cold for 1, 3, 6, 12 and 24 hr, S: salt 500 mM for 6, 12 and 24 hr, D: drought for 6, 12 and 24 hr. A: ABA 10^{-4} M for 6, 12 and 24 hr. Bottom panel indicates ethidium bromide stained RNA gel.

CaTPP1	158	...IHCKRGKHRTG...	168
AtPTP1	263	...VHCSAGTGRTC...	274
PTP	261	...VHCSAGTGRTG...	271
PTP1	264	...VHCSAGTGRTG...	274
PTP1B	214	...VHCSAGTGRSG...	224
AtPTP1	263	...VHCLAGISRSA...	179
PTP	250	...VHCSAGVGRTG...	260
consensus	1	...(I/V)HCXAGXXR(S/T)G...	65

Fig. 2. Comparison of phosphatase catalytic domains of CaTPP1 with those of the other protein tyrosine phosphatase proteins. The numbers are the position of amino acid sequence of catalytic region. CaTPP1 (Accession# AY789640, *Capsicum annuum*), AtPTP1 (Accession# AF055635, *Arabidopsis*), PTP (Accession# CAA06615, *Pisum sativum*), PTP1 (Accession# CAA06975, *Glycine max*), PTP1B (Accession# CAB65732, human), VH-PTP13 (Accession# CAA54910, *Chlamydomonas eugametos*) and PTP1* (Accession# CAA98809, *Saccharomyces cerevisiae*).

Technology (Invitrogen Life Technologies). This technology involved two steps of homologous recombination. The first step was to introduce *CaTPP1* gene into donor vector, pDONR201 to generate entry clone, pDONR201/35S-CaTPP1. The second step was to move the *CaTPP1* gene in donor vector into plant overexpression vector, pB7WG2D to construct pB7WG2D/35S-CaTPP1 (Fig. 3A).

The *CaTPP1* gene of *C. annuum* was introduced into tobacco plants using an *Agrobacterium*-mediated transformation method and expression constitutively under the control of the CaMV 35S promoter. To confirm the integration of the *CaTPP1* gene into the tobacco genome, DL-Phosphinothricin resistant transgenic tobacco plants were analyzed by PCR. Genomic DNA PCR of wild type and transgenic tobacco plants was performed using CaTPP1-attB1 and CaTPP1-attB2 primer set. The resulting PCR products showed the expected band pattern: in

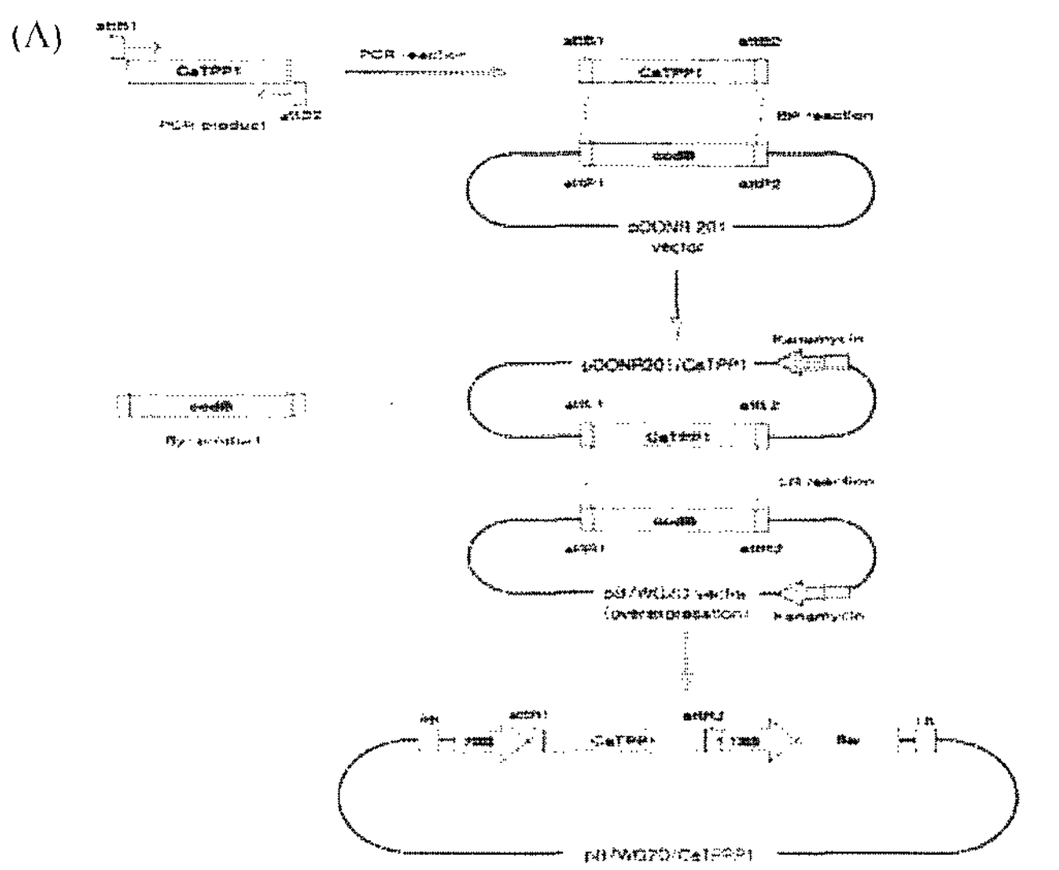

Fig. 3. Plant overexpression vector construction and genomic PCR analysis of CaTPP1 transgenic tobacco plants. (A) Construction of binary vector, pB7WG2D/35S-PIF1, for the introduction of the PIF1 gene into tobacco plants. *att*B1 and *att*B2 primer set used in the PCR was designed for generating full-length PIF1 cDNA flanked by attB1 and attB2 sequence at each end. P35S and T35S are CaMV 35S promoter and terminus, respectively. Bar is Basta resistant gene. attB1, attB2, attP1, attP2, attL1, attL2, attR1 and attR2 are the sequences that were used for recombination. B. Genomic RCR analysis of transgenic tobacco plants using CaTPP1-attB1 and CaTPP1-attB2 primer set. M, molecular marker. C, PCR product from control line. Lanes 1-15, transgenic potato plants. PCR product of 1.2kb was obtained from CaTPP1-attB1 and CaTPP1-attB2 primer set.

CaTPP1 transgenic tobacco plants, 1.2kb PCR fragments were obtained with CaTPP1-attB1 and CaTPP1-attB2, respectively, but no band was found in the wild type with CaTPP1-attB1 and CaTPP1-attB2 primer set. These PCR results confirmed transfer of the *CaTPP1* gene into tobacco genomic DNA (Fig. 3B).

All the transgenic tobacco plants showed no apparent visible phenotype alteration compared to wild type tobacco plants. Thirteen transgenic lines harboring the *CaTPP1* transgene were selected for further analysis. Three- to four-week-old lines of transgenic tobacco plants, rooted in the culture tubes, were transferred to a soil mixture for biofunctional analysis.

***CaTPP1* gene expression in transgenic tobacco plant.** Expression of *CaTPP1* in transgenic tobacco plants was

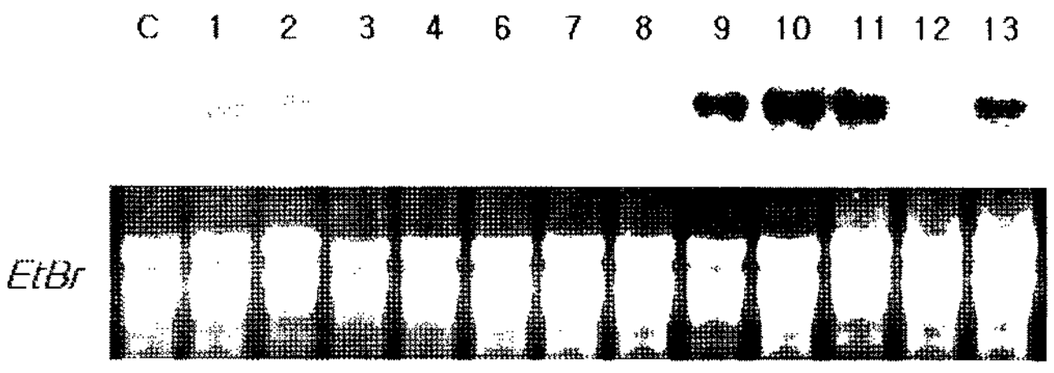

Fig. 4. Expression of the *CaTPP1* gene in the T0 generation of transgenic tobacco plants by Northern blot analysis. Total RNA was isolated transgenic lines and electrophoresed (lower panel). Transferred RNA onto the membrane was hybridized with [α-^{32}P] dCTP-labeled *CaTPP1* cDNA *(EtBr)*. C, wild type control plant. Lanes 1-4, 6-13, transgenic tobacco plant lines.

detected by Northern blot analysis. In wild type control plants, no *CaTPP1* mRNA expression was detected (Fig. 4). Each line of transgenic plants showed various expression levels of the *CaTPP1* gene. Four lines, 10, 11, 12 and 15, showed the strongest *CaTPP1* mRNA expression. The transgenic lines 1, 2, 7, 8, 9 and 13 showed low level expression. However, although the lines 3, 4 and 6 were confirmed to have *CaTPP1* gene insertion by genomic PCR, they had no *CaTPP1* mRNA expression by Northern blot analysis.

Cold and drought treatments of CaTPP1 transgenic tobacco. The biofunctional analysis of CaTPP1 transgenic tobacco plants was carried out by cold treatment. To test enhanced cold tolerance of CaTPP1 transgenic tobacco plants, the wild type, and CaTPP1 transgenic lines grown in the soil mixture were treated at −6°C for 24 h. There was a significant difference in cold resistance between transgenic and nontransgenic control tobacco plants. As shown in Fig. 5A, the transgenic tobacco plants showed enhanced cold tolerance, while wild type plants showed very sensitive to cold stress. In addition to cold stress the tolerance of drought stress was also tested. Especially the transgenic lines #10 and #7 showed tolerance of drought stress (Fig. 5B). However, not all the plants that showed cold tolerance showed drought tolerance. All the transgenic tobacco plants showed no apparent visible phenotype alteration compared to wild type tobacco in the soil mixture.

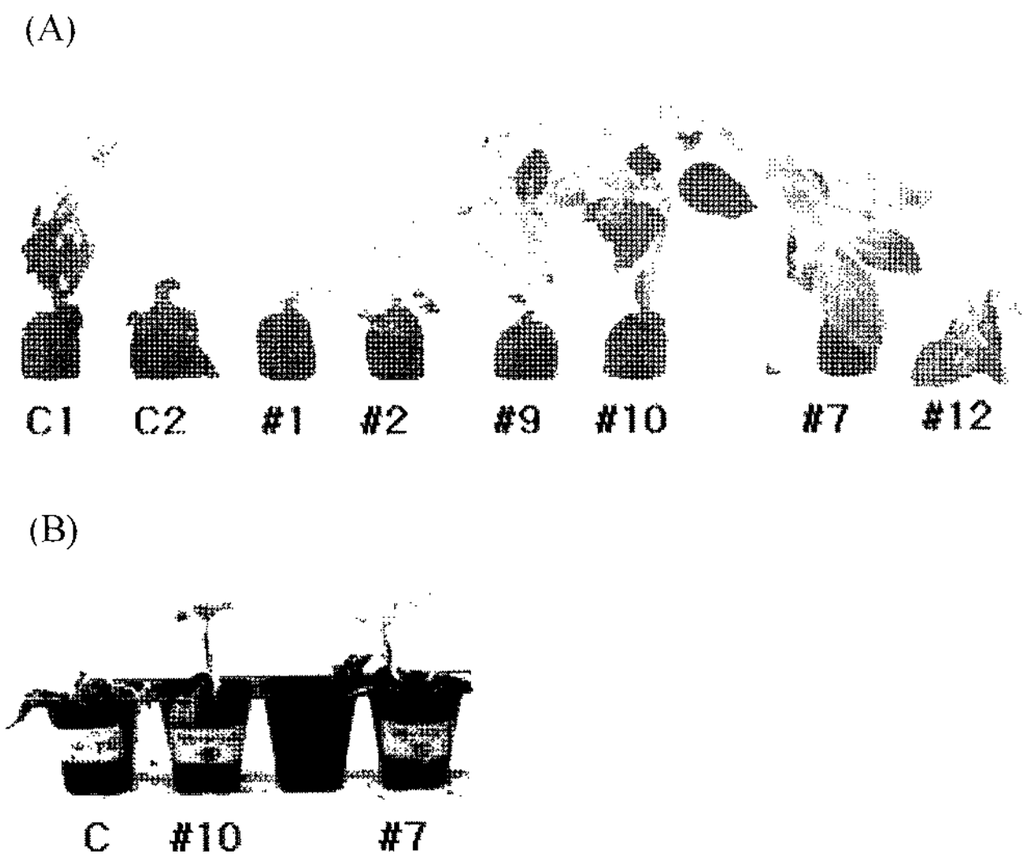

Fig. 5. Cold and drought stress treatment of CaTPP1 transgenic and nontransgenic tobacco plants. Transgenic tobacco plants were grown in soil mixture (Jiffy-pot) for three weeks and treated with cold stress for 24h (A) and drought stress by non-watering for two weeks and re-watered for one week (B). C, C1 and C2 are wild type control plants. #1, 2, 9, 10, 7, and 12 are transgenic lines transformed with *CaTPP1* gene.

Discussion

We previously identified and isolated several cold-regulated genes by investigation of changes of expression in cold-regulated transcripts levels in hot pepper plant (*Capsicum annuum*) using cDNA microarray analysis coupled with Northern blot analysis [Hwang *et al.,* 2005]. The tyrosine specific protein kinase from hot pepper (CaTPP1) gene that was used for this study was one of the genes that were identified as cold inducible. We showed that the gene encoding CaTPP1 was induced not only by cold stress, but also by salt, ABA and drought (Fig. 1).

Mitogen-activated protein kinase (MAPK) cascades are known as one of the major pathways by which the extracellular signals such as growth factors, hormones, and stress stimuli were transduced into intracellular response in yeast and mammalians as well as plants [Cheong *et al.,* 2003; Emerling *et al.,* 2005]. Interestingly, many results indicated that plants MAPK is rapidly activated when exposed to variety of abiotic and biotic stresses including cold, drought, wounding and during plant-pathogen interactions [Jonak *et al.,* 1996; He *et al.,* 1999; Agarawal *et al.,* 2002; Tu *et al.,* 2002; Kim *et al.,* 2003; Jeong *et al.,* 2003]. It is reported that there are at least four subgroups on plant MAPKs based on the phylogenetic analysis of amino acid sequences of all cloned plant MAPKs [Jonak *et al.,* 1999]. Among them, MAPKs in subgroups I and II are mostly involved in signaling of pathogens and abiotic stresses, whereas at least some of subfamily III are involved in cell cycle regulation [Borge *et al.,* 1996; 1999]. In higher plants, tyrosine phosphorylation is important for the activation of MAPKs [Suzuki and Shinshi, 1995; Zhang and Klessig, 1997]. This suggests that protein kinases and phosphatases that are capable of phosphorylating and dephosphorylating tyrosine in protein

substrates play an important role in abiotic stress signal transduction pathway. However, to our knowledge except protein tyrosine phosphatase (AtPTP1) from *Arabidopsis* no other protein tyrosine phosphatase was known to be involved in abiotic stress signal transduction pathway. Here we showed protein tyrosine phosphatase (CaTPP1) is involved in response to abiotic stress including cold.

Xu *et al.,* (1998) showed that AtPTP1 expression was negatively regulated by cold temperature in *Arabidopsis*. However, in our current and previous study, we showed that CaTPP1 expression is positively regulated by cold stress. This finding may implicate the presence of somewhat different mechanisms for plant response to stresses such as cold and drought conditions. Previously, we also showed that the *CaTPP1* gene was induced by ABA treatment as well as cold stress treatment [Hwang *et al*., 2005]. This result may implicate the involvement of ABA in CaTPP1 signal transduction pathway. Regarding this, it was demonstrated that ABA-induced stomatal closure was intermediated by protein tyrosine phosphatase in *Commelina communis* [MacRobbins, 2002].

In this study we demonstrated the transgenic plants overexpressing CaTPP1 showed enhanced cold tolerance (Fig. 5). In addition to tolerance to cold stress, we also tested whether the CaTPP1 transgenic tobacco had tolerance to other stresses. As we may expected from *CaTPP1* gene expression pattern by various stresses, the CaTPP1 transgenic tobacco showed enhanced drought stress tolerance, but no tolerance at all to salt stress, although it showed induced expression by salt stress at transcript level (Fig. 4). However, not all the plants that showed cold tolerance did showed drought stress. This result is not consistent with the *CaTPP1* gene expression pattern in hot pepper plants.

We tested tolerance of abiotic stresses including cold, drought and salt stresses with the CaTPP1 transgenic plants that showed relatively high transgene expression. The level of cold tolerance showed little correlation to the expression level of transgene (Figs. 4, 5). Some plants that showed high transgene expression level appeared to be not resistant to cold or drought stress at all. And some transgenic plants showed strong cold tolerance showed also drought tolerance, but some did not.

Currently, studies are being conducted in order to isolate protein(s) which interact with CaTPP1. Moreover, another study, designed to facilitate the characterization of CaTPP1 using RNAi transgenic plants is underway. Thus, we hope that this study will allow us to elucidate the CaTPP1-involved cold signal transduction pathway.

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