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Transgenic Tobacco Expressing a Ring Domain-Containing Protein of *Capsicum annuum* Confers Improved Cold Tolerance

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Zinc finger proteins function in plant tolerances to stresses from cold, dehydration, and salt. To determine the mechanisms for those underlying defenses, we previously used cDNA microarrays and northern blot analysis to identify a gene for the ring zinc finger protein (RDCP1) from hot pepper (*Capsicum annuum*). In that study, we showed that the *RDCP1* gene was strongly induced by cold stress and, to a lesser degree, by ABA and high salt. Here, we have used a Ti-plasmid and *Agrobacterium*-mediated transformation to engineer *RDCP1* under the control of the CaMV35S promoter for constitutive expression in tobacco. The resultant RDCP1 transgenic plants exhibit significantly increased tolerance to low temperatures. Moreover, some of those transgenics have greater drought tolerance. In addition, none of the RDCP1 transgenic plants show any visible alterations from the wild phenotype. These current results demonstrate the biological role of RDCP1 in conferring stress tolerance.

Keywords: *Capsicum annuum*, cold tolerance, ring domain-containing protein, transgenic tobacco

Plants are frequently subjected to abiotic environmental stresses, such as low temperatures, drought, and high salinity. These conditions have significant detrimental effects on growth and crop yields. Protective mechanisms can include a range of strategies, e.g., physiological and biochemical processes that normally are mediated via the upregulation of expression for stress-regulated genes (Thomashow, 1999; Zhu 2001a, b; Hasegawa et al., 2002; Xiong and Zhu, 2002; Xiong et al., 2002). Molecular studies using DNA microarrays have identified genes regulated by such stresses (Schenk et al., 2000; Bohnert et al., 2001; Seki et al., 2001; Kreps et al., 2002). Many of these genes are controlled at the transcriptional level (Shinozaki and Yamaguchi-Shinozaki, 1997; Cheong et al., 2002; Shinozaki et al., 2003; Zhu and Provart, 2003).

The protein products of these stress genes are grouped according to their associated responses. Class 1 includes proteins involved in direct protection, while Class 2 comprises those that are active in the stress signal-transduction pathway and the control of expression by stress-tolerance genes (Qiang et al., 2000; Schenk et al., 2000; Bohnert et al., 2001; Seki et al., 2001, 2002; Kreps et al., 2002). Among these, focus has been placed on genes encoding for factors that are components of the abiotic stress signal-transduction pathway because of their capacity to regulate several target genes.

Researchers are attempting to engineer abiotic stress-tolerant crop plants via the overexpression of stress-regulated genes whose products confer direct or indirect protection (Holmberg and Bulow, 1998). The primary genes in those studies have been transcription factors.

Zinc finger proteins are associated with plant responses to cold, dehydration, and salt. For example, SCOF-1 from soybeans can confer enhanced cold tolerance in transgenic tobacco (Kim et al., 2001). Another zinc-finger protein, STZ of *Arabidopsis*, is associated with drought tolerance in transgenic plants, although their growth is retarded (Sakamoto et al., 2004). Other zinc finger proteins, including ZTP2 of petunia (Sugano et al., 2003), OSISAP1 of rice (Mukhopadhyay et al., 2004), and ZFP245 of rice (Huang et al., 2005), are involved in abiotic stress tolerance in transgenic plants.

One interesting zinc finger transcription factor is the ring zinc finger domain protein, whose domain differs from the zinc finger in that four pairs of zinc ligands bind to two zinc ions within the ring zinc finger domain, while two pairs of zinc ligands bind to one zinc ion in the zinc finger proteins. This ring zinc finger protein has been implicated in a variety of functions, including cell differentiation, cell cycle regulation, signal transduction, and stress responses (Hershko and Ciechanover, 1998). Sahin-Cevik and Moore (2006) have demonstrated that an H2-type zinc ring finger protein from *Citrus* is induced by cold and drought stresses.

To characterize the cold-defense mechanisms in plants, we previously used a combination of cDNA microarray and northern blot analyses to evaluate altered levels of cold-regulated transcripts in hot pepper (*Capsicum annuum*) (Hwang et al., 2005). Through our analysis of the 3.1 K red pepper cDNA microarray, we were able to identify 317 cold-inducible genes. Using sequencing analysis, we selected 46 up-regulated genes and 3 that were down-regulated by chilling. Among the 49 genes that appeared to be up-regulated in response to cold, 19 of them also seemed to be simultaneously regulated by salt stress. Many transcription factors were included, such as a family of four ethylene-responsive element binding proteins (CaEREBP-C1 to C4), the bZIP protein (CaBZ1), RAV1, the zinc finger proteins, a heat shock factor (HSF1), and the WRKY (CaWRKY1) protein (Hwang et al., 2005). Among these, one gene encoding for the C3HC4-type ring zinc finger protein transcription factor, RDCP1, was identified as being induced by cold stress.

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In that study, we demonstrated that the RDCP1 gene was strongly induced by cold stress, and, to a lesser degree, by ABA and salt treatments (Hwang et al., 2005).

Here, we describe our efforts to determine the involvement of the ring zinc finger domain-containing protein in the signal-transduction pathway of cold stress, and to generate abiotic stress-resistant plants. In doing so, we introduced the cold stress-inducible ring zinc finger domain-containing protein (RDCP1) gene into tobacco, then assessed the resultant transgenic plants with regard to several stress tolerances.

MATERIALS AND METHODS

Plant, Bacteria, and Culture Conditions

Tobacco plants (*Nicotiana tabacum* var. Xanthi) were maintained on a medium containing MS salts, vitamin mix, 3% sucrose, and 0.7% phytagar (pH 5.6 to 5.7). They were grown in a temperature-controlled culture room at 23°C under constant light. The binary vector, pB7WG2D, under the control of the CaMV 35S promoter, was used for producing pB7WG2D/35S-RDCP1. This construct was transferred into *Agrobacterium tumefaciens* strain, LBA4404, which was grown on a YEP medium of 1% yeast extract, 1% peptone, 0.5% NaCl, and 1.5% agar (pH 7.2) for approximately 48 h at 28°C.

Construction of the Binary Vector pB7WG2D/35S-RDCP1

To construct the plant overexpression vector pB7WG2D/35S-RDCP1, we utilized Gateway™ Cloning Technology (Invitrogen, USA). Briefly, two homologous recombination reaction steps were conducted, first with BP, then with LR. We initially used PCR to generate the RDCP1 gene, flanked by the attB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) and attB2 (5′-GGGGACAAGTTTGTACAAGAAAGCTG-GGT-3′) sequences at each end. PCR was conducted with the following primers: RDCP1-*att*B1 (5′-AAAAAGCAG-GCTCGCTTTGCTTTTCTCCTTCTAAAGACTC-3′) and RDCP1-*att*B2 (5′-AGAAAGCTGGGTCAGTATGAGTCTCTATGCAC-CATAATGG-3′). The BP reaction was performed with purified *att*B-PCR products and a donor vector, the 4470-bp pDONR™201 (Invitrogen, USA), that harbored a kanamycin selection marker. A 10 μL BP reaction mixture was then made using 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 the BP Clonase™ enzyme mix, and was adjusted with water to the desired final volume. This reaction was run for 4 h at 25°C. The BP reaction product was then transferred into *Escherichia coli* DH5α cells via heat-shock, and the reaction mixture was placed on an LB plate containing 50 μg mL^{-1} kanamycin for 16 h at 37°C. The transformants were then verified via PCR. Afterward, to transfer *RDCP1* in the entry clone, pDONR201/35S-RDCP1, we performed an identical homologous recombination reaction (LR reaction) between that entry clone and the binary overexpression vector, pB7WG2D, in order to generate the final plant overexpression vector pB7WG2D/35S-RDCP1. This LR reaction facilitated the recombination of an *att*L in pDONR201/35S-RDCP1 with an *att*R in pB7WG2D (Fig. 1). The overexpression vector used here was the 12542-bp pB7WG2D™201 (Invitrogen), harboring streptomycin and spectinomycin selection markers. A 10 μL LR reaction mixture was prepared with 4 μL (150 ng) of the entry clone, 1 μL (150 ng) of the destination vector (pB7WG2D™), 2 μL of 5X BP Clonase™ reaction buffer, and 2 μL of LR Clonase™ enzyme mix. This was added to a final volume with deionized water, then held for 2 h at 25°C. The LR reaction product was then transferred into *E. coli* DH5α cells via heat-shock for propagation and maintenance because this strain is sensitive to the effects of *ccd*B. The LR reaction mixture was placed on an LB medium plate containing 50 μg mL^{-1} kanamycin for 16 h at 37°C. Transformants were verified by PCR.

Generation of Transgenic Tobacco Plants

The overexpression vector, pB7WG2D/35S-RDCP1, was transferred into *Agrobacterium* via the freeze-thaw method. Tobacco transformation was conducted according to the procedures of Kwon et al. (1994). The regenerated plants were then transferred to a soil mixture for further analysis.

RDCP1	44	CLDCVHEPVITLCGHLYCWPCIYKWIY--FQSVSSENSDQQQPQ---CPVC	90
Ring protein	33	CFDFAHEPVVTLCGHLYCWPCIYKWLH--VQ-SASLASDEH-PQ---CPVC	76
Ring ZF protein	59	CLDTAHDPVVTLCGHLFCWPCIYKWLH--VQ-LSSVSVDQHQNN---CPVC	103
Zn binding protein*1	47	CLDTAHDPVVTLCGHLFCWPCIYKWLH--VQ-LSSVSVDQHQNN---CPVC	91
RMA1	51	CLDSVQEPVVTLCGHLFCWPCIHKWLD--VQSFSTSDEYQRHRQ---CPVC	96
Zn binding protein*2	24	CLDQVRDPVVTLCGHLFCWPCIHKWTYASNNSRQRVDQYDHKREPPKCPVC	74
Os04g0530500	42	CLDFAAEPVVTLCGHLYCWPCIYEWLCPGVGSTASNNSSLARRQ---CPVC	89
Zn binding protein*3	26	CLDLAQDPIVTLCGHLFCWPCLYKWLH--LH--------SQSKD---CPVC	63
Os04g0766200	111	CLELAQDPVVTLCGHLFCWPCLYEWLH--VH--------AHSRE---CPVC	148
Zn binding protein*4	30	CLELAQDPVVTLCGHLFCWPCLYEWLH--VH--------AHSRE---CPVC	67
Putative protein	134	CLDLSKEPVLTCCGHLYCWPCLYQWLQIS----------DAKE---CPVC	170
Zn binding protein	144	CLDLSKDPVVTNCGHLYCWSCLYQWLQVS----------EAKE---CPVC	180
consensus	1	CLDla.EPVITLCGHLyCWPCIYkWLh..v.....CPVC	51

Figure 1. Comparisons among amino acid sequences for DNA-binding ring zinc finger domain of RDCP1 and other ring zinc finger proteins. Numbers indicate positions. RDCP1 (Accession number AAR99376, *C. annuum*), Ring protein (AAN05420, *Populus* x *canescens*), RING ZF protein (CAB43879, *Arabidopsis thaliana*), Zn binding protein*1 (protein binding/ubiquitin-protein ligase/zinc ion binding protein, NP_194477, *A. thaliana*), RMA1 (NP_192260, *A. thaliana*), Zn binding protein*2 (protein binding/ubiquitin-protein ligase/zinc ion binding protein, NP_194556, *A. thaliana*), Os04g0530500 (NP_001053390, *Oryza sativa*), Zn binding protein *3 (protein binding/ubiquitin-protein ligase/zinc ion binding protein, NP_564078, *A. thaliana*), Os01g0766200 (NP_001044354, *O. sativa*), Ring finger protein (BAD53343, *O. sativa*), Zn binding protein*4 (protein binding/ubiquitin-protein ligase/zinc ion binding protein, NP_191362, *A. thaliana*), Putative protein (CAB67631, *A. thaliana*), Zn binding protein*5 (protein binding/ubiquitin-protein ligase/zinc ion binding protein, NP_181733, *A. thaliana*).

Genomic DNA Extraction and Polymerase Chain Reaction Analysis

Genomic DNA was extracted from tobacco plants by using a G-spin genomic DNA extraction kit (iNtRON, Korea) according to the manufacturer's instructions. PCR was conducted in a Peltier Thermal Cycler PTC-100 (MJ Research, USA) with Ex-Taq polymerase (Takara, Japan), with the following cycling parameters: 95°C for 5 min; then 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 10 min. For PCR-identification of the RDCP1 transgenic tobacco plants, we used two primers: RDCP1-SO (5′-GTTCACAAACGTTCTTGATAC-3′) and RDCP1-ASO (5′-GAACTTCAAACCTCATTTCTTAG-3′). PCR products were separated via 1% agarose gel electrophoresis.

RNA Preparation and Northern Blot Analysis

To characterize the expression of *RDCP1* in our transgenic tobacco plants, we extracted total RNA with Tri Reagent (MRC, USA; Roh et al., 2006) from the leaves of 28 transgenic lines of tobacco grown on MS media for 3 to 4 weeks. For northern blot analysis, total RNA was electrophoresed on a 1.2% denaturing formaldehyde/MOPS agarose gel. The blot was prepared on a Hybond-N^+ membrane (Amersham, USA) via capillary transfer, with 20× SSC as the transfer buffer. Pre-hybridization was conducted for 15 min at 65°C in hybridization solution (1% BSA, 1mM EDTA, 0.5M $NaHPO_4$, and 7% SDS). Afterward, hybridization solution containing radio-labeled cDNA probes was added, followed by overnight hybridization at 65°C in a Hybaid oven (Hybaid, USA). These probes were labeled via random oligonucleotide priming (Amersham). Stringent membrane washing was conducted with 0.2× SSC/0.1% SDS at 65°C before the washed membrane was exposed to X-ray film at -70°C.

Cold- and Drought-Stress Treatments

For all treatments, plants were obtained either by germinating seeds in a soil mixture (control) or by rooting plantlets on an MS selection medium containing phosphinothricin (transgenics). The plants were then grown for 3 weeks in soil or Jiffy pots before stress was induced. For the cold treatment, control and transgenic plants were chilled for 24 h at -6°C. Drought was initiated by withholding water from soil-grown control and transgenic plants for 14 d. Moisture contents were measured from detached, air-dried leaves at various time points.

RESULTS

Isolation of a Cold-Related Ring Zinc Finger Protein cDNA

Using a combination of cDNA microarray and northern blot analyses, we previously identified a cold-inducible gene encoding for a ring zinc finger protein from *C. annuum* (Hwang et al., 2005). Sequence analysis indicated that the RDCP1 protein shares strong homology with DNA-binding ring zinc finger proteins in other plant systems. All ring zinc finger proteins harbor a cysteine-rich domain -- defined by the consensus sequence, $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}$ $CX_2CX_{(4-48)}CX_2C$ – that is involved in DNA interactions (Kosarev et al., 2002). In the current study, the conserved ring zinc finger domain of RDCP1 was aligned with other ring zinc finger proteins, including *Arabidopsis* ring zinc finger protein and rice Os04g0530500 (Fig. 1).

Construction of Plant Overexpression Vector pB7WG2D/35S-RDCP1

Two homologous recombination steps were necessary for preparing the plant overexpression vector, pB7WG2D/35S-RDCP1. The first step involved introducing the RDCP1 gene into a donor vector, pDONR201, to generate an entry clone, pDONR201/35S-RDCP1. The second step entailed moving *RDCP1* in the donor vector into the plant overexpression vector, pB7WG2D, in order to construct pB7WG2D/35S-RDCP1.

First-round PCR was conducted with a set of RDCP1-specific primers, and the full-length RDCP1 cDNA fragment was used as template. Second-round PCR with the same attB1 and attB2 primer set resulted in a PCR product that featured *RDCP1* flanked by attB1 and attB2 sequences at each end (Fig. 2).

A homologous recombination reaction between the specific attachment (*att*) sites "*attB*" and "*attP*" on the DNA molecule (BP reaction) facilitated the transfer of the gene of interest into the *attB* expression clone or the *attB*-PCR product into an *attP*-harboring donor vector, thus creating an entry clone. Then, to transfer the RDCP1 gene in that entry clone, pDONR201/35S-RDCP1, into the overexpression vector, pB7WG2D, we conducted an identical homologous recombination reaction (LR reaction) between attL in the entry clone and attR in the overexpression vector. This generated the final overexpression vector, pB7WG2D/35S-RDCP1 (Fig. 2).

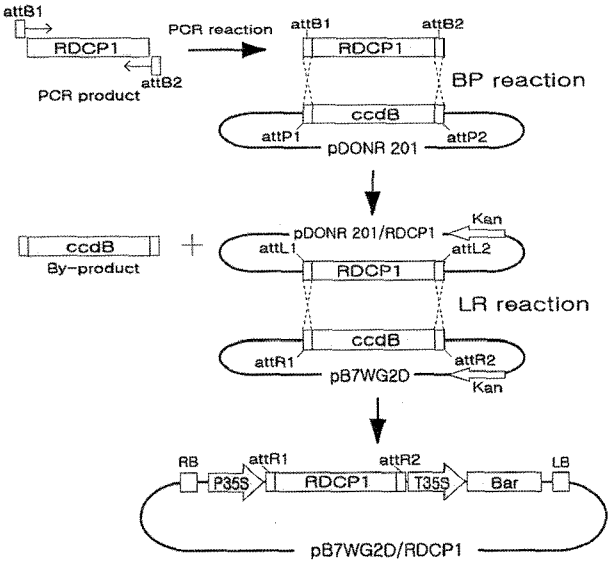

Figure 2. Plasmid construction of binary vector, pB7WG2D/35S-RDCP1, to introduce *RDCP1* into tobacco. PCR primer set was designed to generate full-length *RDCP1* cDNA flanked by attB1 and attB2 sequences at each end. P35S and T35S are CaMV35S promoter and terminus, respectively. Bar is Basta resistance gene. attB1, attB2, attP1, attP2, attL1, attL2, attR1, and attR2 are sequences used for recombination.

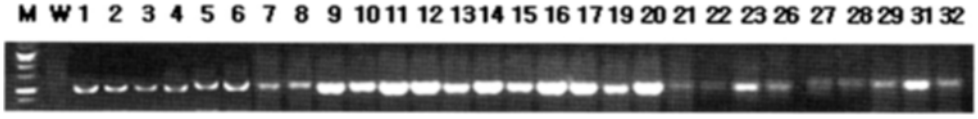

Figure 3. Genomic PCR analysis using RDCP1-SO and RDCP1-ASO primer set. M, molecular marker; W, PCR product from wild-type genome. Lanes 1-17, 19-23, 26-29, 31, and 32 are transgenic tobacco plants. PCR product (1138 bp) was obtained with RDCP1-SO and RDCP1-ASO primers.

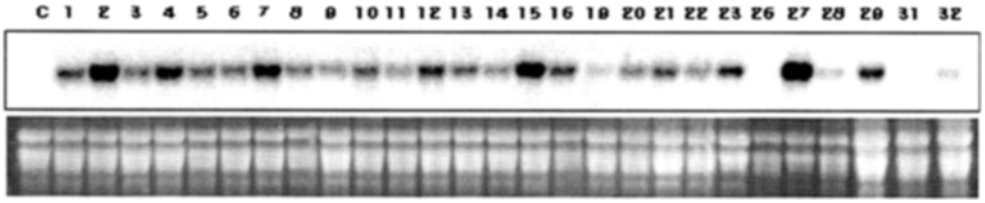

Figure 4. Northern blot analysis for mRNA expression of RDCP1 gene. RNA transferred onto membrane was hybridized with [α-^{32}P]dCTP-labeled RDCP1 cDNA (upper panel). Total RNA was isolated from transgenic tobacco lines and electrophoresed (lower panel). C, wild-type control. Lanes 1-16, 19-23, 26-29, 31, and 32 are transgenic lines.

Generation and Selection of RDCP1 Transgenic Tobacco Plants

RDCP1 was introduced into tobacco via *Agrobacterium*-mediated transformation, and was constitutively expressed under the control of the CaMV35S promoter. To verify this integration, phosphinothricin-resistant transgenic plants were PCR-analyzed. The resultant PCR products showed the expected band patterns; in the transgenics (Fig. 3, Lanes 1-17, 19-23, 26-29, 31, and 32), 1138-bp PCR fragments were acquired, but no bands were detected in the wild type (Lanes 18, 24, 25, and 30) when either the RDCP1-SO or RDCP1-ASO primer sets were used.

We selected 28 transgenic lines harboring the *RDCP1* transgene to monitor their expression in 3- to 4-week-old plants. None of these transgenics had any visible alterations from the wild-type morphology.

RDCP1 Gene Expression in Transgenic Tobacco Plants

RDCP1 expression was analyzed with northern blots. Whereas the wild-type control plants showed no *RDCP1* mRNA expression, various amounts of transcript were detected from each transgenic line, with the highest levels being measured from Lines, 1, 2, 4, 7, 12, 15, 16, 22, 24, and 26. Another 15 lines (3, 5, 6, 8-11, 13, 14, 19-21, and 28) had intermediate levels of expression. Finally, although Lines 17, 23, and 27 had been verified as harboring the *RDCP1* gene insertion via genomic PCR, no *RDCP1* mRNA expression was observed in our northern blot analysis (Fig. 4).

Cold and Drought Treatment of RDCP1 Transgenic Tobacco Plants

Biofunctional analysis was conducted by applying cold treatment to soil-grown wild-type and five RDCP1 transgenic lines. These plants were exposed to 24 h of chilling at -6°C. The degree of cold resistance differed significantly between the two genotypes, with all transgenic tobacco plants, except those from Line 12, showing enhanced tolerance and the wild types being extremely sensitive to this stress (Fig. 5). We also assessed the effect of drought conditions by withholding water from our test plants, and found

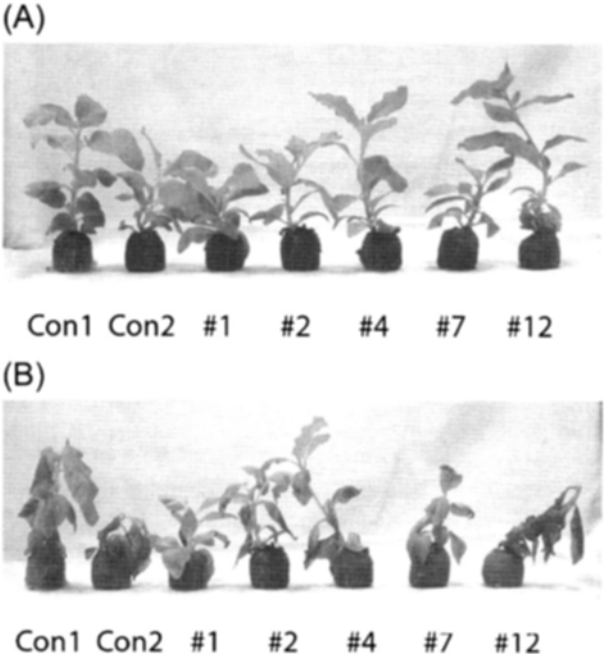

Figure 5. Effect of cold stress on RDCP1-transgenic and control (wild-type) tobacco plants grown in soil or Jiffy-pots for 3 weeks. Before (**A**) and after (**B**) 24 h of treatment. Con, control plants; transgenic Lines #1, 2, 3, 4, 7, and 12 were transformed with *RDCP1*.

that those from transgenic Lines #3, #4, and #7 were most tolerant (Fig. 6A). In addition, when the leaves were air-dried, plants of those same transgenic lines showed better capacity for retaining water than did the wild-type plants (Fig. 6B). However, not all of the cold-tolerant plants were also drought-tolerant. Again, no differences in phenotype were apparent between the transgenics and wild-type plants when grown in culture tubes or soil.

DISCUSSION

We previously used microarrays and northern blots to iso-

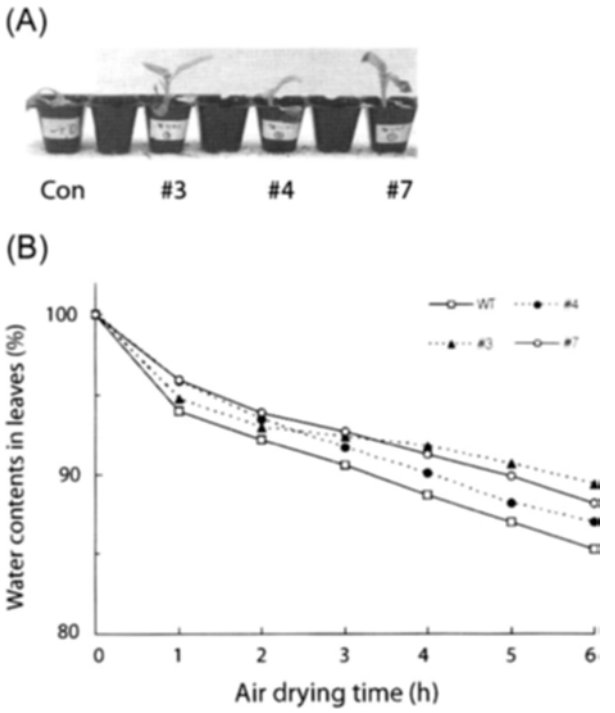

Figure 6. Effect of drought stress on RDCP1-transgenic and control tobacco plants grown in soil or Jiffy-pots for 3 weeks. (**A**) Drought was induced by withholding water for 2 weeks, then re-watering for 1 week. (**B**) Water-retention capacity was measured by air-drying detached leaves from transgenic and control plants. Con, control; #3, 4, and 7, lines transformed with *RDCP1*.

late several cold-regulated transcription factors in *C. annuum* (Hwang et al., 2005). Expressional changes in transcript levels were investigated there, and one gene identified as cold-inducible in that study was selected for the research presented here. This gene, *RDCP1*, also could be induced to a slight degree by ABA and salt treatments. It had originally been submitted to the Genbank database as a drought stress-inducible hot pepper zinc finger protein (Genbank accession # AY513612; Cho and Kim, 2004). However, in our previous study, this *RDCP1* gene was induced only very weakly by drought, with expression levels being just slightly enhanced when compared with our control hot pepper plants (Hwang et al., 2005).

Here, we demonstrated that transgenic tobacco plants over-expressing RDCP1 exhibited enhanced cold tolerance (Fig. 4). Likewise, these transgenics showed slightly improved tolerance to drought stress, but had no change in their response to salt treatment (data not shown). These observations are somewhat consistent with the patterns of RDCP1 gene expression in hot pepper plants. However, not all plants evidencing cold tolerance also manifested tolerance under drought conditions (Fig. 6).

The ring zinc finger proteins harbor a cysteine-rich domain defined by the consensus sequence, $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}CX_2CX_{(4-48)}CX_2C$, in which C, H, and X are cysteine, histidine, and any amino acid, respectively (Kosarev et al., 2002). RDCP1 contains the ring domain of $CX_2CXHX_6CXHX_2CX_2CX_{21}CX_2C$ between 44 and 90 amino acid residues. Ring domain zinc finger proteins have been classified into two groups, Ring-HC and Ring H2, based on the presence of cysteine and histidine at the fifth co-ordination site, respectively (Joazeiro and Weissman, 2000; Kosarev et al., 2002). Our sequence analysis of RDCP1 indicated that its proteins also are members of the Ring-HC group.

When the zinc finger protein, STZ, is over-expressed in *Arabidopsis*, growth is inhibited in the resultant transgenic plants. Moreover, that degree of retardation may be correlated with STZ expression levels (Sakamoto et al., 2004). However, none of our RDCP1 transgenic tobacco plants, including those from Line #2 (with the highest level of mRNA expression), showed any phenotypic alterations from the morphology observed in the wild type.

The degree of cold tolerance was only minimally correlated with the level of transgene expression (Fig. 4, 5). In fact, some plants with high transgene expression did not appear to be at all tolerant of cold or drought. Others showed pronounced tolerance to both types of stress.

We are now conducting studies that utilize a yeast two-hybrid system to isolate genes that interact with RDCP1. Other research has been designed to facilitate the characterization of RDCP1, using RNAi transgenic plants. Our goal is to elucidate the RDCP1-involved cold signal-transduction pathway.

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