GENETIC TRANSFORMATION AND HYBRIDIZATION

Expression of insect (*Microdera puntipennis dzungarica*) antifreeze protein MpAFP149 confers the cold tolerance to transgenic tobacco

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Abstract To elucidate the function of antifreeze protein from *Microdera puntipennis dzhungarica* for freezing stress tolerance in plant, the construct of Mp*AFP*149 gene with the signal peptide sequence responsible for secreting the native MpAFP149 into the apoplast space under control of a cauliflower mosaic virus 35S promoter was introduced into tobacco by *Agrobacterium tumefaciens*-mediated transformation. The observation of immunogold localization by TEM (transmission electron microscope) showed that the heterologous MpAFP149 protein was mainly distributed on the cell wall in apoplast of the transgenic tobacco plant. T1 generation transgenic tobacco plants displayed a more frost resistant phenotype and kept the lower ion leakage ratio and MDA (malondialdehyde) content in the leaves compared with wild-type ones at $-1°C$ for 3 days. The results showed that MpAFP149 provided protection and conferred cold tolerance to transgenic tobacco plant during freezing stress.

Keywords *Microdera puntipennis dzungarica* · Antifreeze protein · Transgenic tobacco · TEM · Cold treatment

Abbreviations

AFP	Antifreeze protein
THPs	Thermal hysteresis proteins
THA	Thermal hysteresis activity
GFP	Green fluorescence protein
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
TEM	Transmission electron microscope
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
MDA	Malondialdehyde
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

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Introduction

Antifreeze proteins (AFPs) have been isolated from a variety of organisms ranging from fish (DeVries 1986; Marshall et al. 2004a), insects (Duman et al. 2004; Stefen and Brian 2004), plants (Urrutia et al. 1992; Lin et al. 2004), to bacteria (Sun et al. 1995; Kuwabara et al. 2002; Jack et al. 2004), and they bind to ice crystals inhibiting their continued growth (Raymond and DeVries 1977). AFPs play an important role in modifying the shape of ice crystal, the inhibition of ice growth, and the repression of recrystallization. By adsorption to the ice surface AFP causes the freezing point of a solution to be lowered without influence on the melting point in a noncolligative manner. The difference between the melting point and the nonequilibrium freezing point can be determined and is termed thermal hysteresis activity (THA). THA is widely used as an indicator of AFPs activity, so AFPs are often referred to as thermal hysteresis proteins (THPs). The THAs among species are quite different: insect AFPs are comparatively high ($3–6°C$), as compared to fishes ($0.7–1.5°C$), and especially plants ($0.2–0.5°C$) (Jia and

Davies 2002). Most AFP-producing insects are freeze-avoiding and cannot survive freezing of their body fluids. The AFPs in these insects lower the freezing point of the hemolymph and gut fluid (Olsen and Duman 1997a, b) and prevent inoculative freezing from external ice across the body surface (Olsen et al. 1998), thereby extending the supercooling capabilities of the insects.

Low temperature is one of the major limiting environment factors in the growth, productivity and distribution of plants. Higher yields could be achieved either by improving the freezing tolerance of an overwintering crop, or by increasing the survival of freezing sensitive plants following light frosts (Griffith and Yaish 2004). Henceforth, several attempts have been made to utilize modern plant biotechnology to express AFPs in frost-susceptible crops to increase their freezing tolerance (Holmberg and Bülow 1998). Due to plant-freezing characteristics controlled by expression rate, localization, stability, and activity of heterologous AFPs in plants, success on genetic improvement in freezing resistance for plants are limited (Hightower et al. 1991).

Fish AFPs have been transformed into tomato (Hightower et al. 1991), tobacco (Kenward et al. 1993; Kenward et al. 1999), potato (Wallis et al. 1997) and spring wheat (Khanna and Daggard 2006). However, most of the transgenic plants expressing fish AFPs demonstrated lower freezing temperature. Some of the most potent AFPs are found in insects like moths (Tyshenko et al. 1997) and beetles (Graham et al. 1997) function as hyperactive antifreezes. They produce freezing point depressions of 4–5°C at millimolar concentrations and have specific activities 10–100 times greater than most AFPs from other organisms at comparable concentrations (Graham et al. 1997; Tyshenko et al. 1997; Li et al. 1998). Arabidopsis (Holmberg et al. 2001) and tobacco (Huang et al. 2002) transformed with insect AFPs genes were tested to have thermal hysteresis in apoplasts, and the whole plants freezing temperature was lowed in the presence of ice nucleator.

MpAFP149 gene was isolated from Microdera puntipennis dzungarica, a local beetle in Xinjiang desert region. It was 363 bp with a signal peptide sequence. The deduced transcript encoding 98 amino acids of mature peptide shares 68.37% homology with the published AFP from Tm (Tenebrio molitor) (Marshall et al. 2002). The mature protein fused with GST was expressed successfully in Escherichia coli BL21 (DE3), displaying very high activity in protecting bacteria survival at low temperature with as low as 10 μg ml^{-1} concentration. The cryoprotective effect was linearly correlated with the AFP concentrations (Zhao et al. 2005). Thus, it may be more efficient in improving plants cold tolerance, which encouraged us to transform this AFP gene to plant.

The aim of this report is to evaluate the potential of MpAFP149 to protect transgenic tobacco plants from cold or freezing damage.

Materials and methods

Signal peptide prediction and transient expression of MpAFP149: GFP fusion protein

The first 50 amino acid residues of the MpAFP149 were predicted for the signal peptide cleavage site using the net-based software (http://www.cbs.dtu.dk/services/SignalP) developed by Nielsen et al. (1997).The MpAFP149 gene was inserted before the green fluorescent protein (GFP) gene in-frame in pCAMBIA1302-GFP plasmid to express MpAFP149-GFP fusion protein. The fusion (35S: MpAFP149-GFP) and a control plasmid (pCAMBIA1302-GFP) were introduced into onion (Allium cepa) epidermal cells by particle bombardment (Takeuchi et al. 1992). The onion was cultured in dark for 18 h and the cells were viewed by confocal microscopy (Nikon, JPN).

Plasmid construction

The Microdera puntipennis dzungarica AFP gene (MpAFP149) (GenBank accession number AY821792), encoding MpAFP149 with its signal peptide sequence (363 bp), was obtained by PCR with forward primer 5′-ATGGCTTTGACAACAAAATGG-3′ and reverse primer 5′-TTAACCTTTATTTGGACATCC-3′. The cloning sequence was created with the BamHI and SstI inserting sites at the 5′ and 3′ ends of MpAFP149 gene, respectively. The full sized MpAFP149 fragments were constructed into the pBI121 by replacing the intron-GUS region. Then the HindIII-EcoRI fragment of CaMV35S-MpAFP149-Nos was subcloned into the pCAMBIA1302 to form expression vector pCAMBIA1302-MpAFP149 (Fig. 3). The insert was re-sequenced to confirm the correct maintenance of the open reading frame.

Plant transformation

The expression vector pCAMBIA1302-MpAFP149 was transferred into the competent cells of Agrobacterium EHA105 strain by the liquid nitrogen freeze thaw method (An 1987). DNA was extracted from several transformed kanamycin-resistant A. tumefaciens colonies and presence of the MpAFP149 transgene was confirmed by PCR using primers described above under standard condition. The wild-type tobaccos (Nicotiana tabacum L.) var. Wisconsin 38 were grown on half-strength Murashige and Skoog medium (1/2 MS) for 55–60 days (Murashige and Skoog

1962) under condition of 16 h photoperiod, 25°C temperature. Young leaves were used for gene transformation experiments. Tobacco leaf discs (1–2 in. length) were infected with EHA105 containing pCAMBIA1302-Mp*AFP*149 (Horsch et al. 1985). After 2 days of co-cultivation in the dark at 28°C, the leaf discs were transferred to the generation medium supplemented with 20 mg l^{-1} hygromycin. Presumptive transgenic shoots were performed for two cycles selection, and then induced to root with the same hygromycin concentration. The T0 plants were allowed to flower and set seeds in a growth chamber with a 16 h light/8 h dark photoperiod at 25°C. The plants were grown for 15 weeks in the green house before the ripe seed capsules were harvested.

Germination and growth of transgenic tobacco

Seeds of the T0, along with wild-type tobacco, were sterilized by soaking in 1:9 (v/v) of 30% bleach:ethanol for 10 min. The seeds were then rinsed five times with ethanol and set at the sterile hood overnight for volatilizing the ethanol. Transgenic seedlings carrying the HPTII gene were selected by germinating T0 seeds on 1/2 MS plates containing hygromycin (20 mg ml^{-1}) and then transplanted into pots to full growth at 25°C and 16 h light/8 h dark cycle in the culture room. Presence of the transgene in hygromycin resistant seedlings was verified by experiments as described below.

Identification of transgenic plants

Genomic DNA from leaves was extracted according to Michaels's method (Michaels et al. 1994). These genomic DNA samples were used as template for PCR with the primers described above to identify the gene Mp*AFP*149. Total RNA was isolated from plant leaves using Triozol reagent (Tiangen, China) according to the supplier's instructions. Reverse transcription was carried out using a MLV-kit (TaKaRa, JPN), and the RT-PCR products were analyzed by agarose gel electrophoresis to check the transcription of Mp*AFP*149.

Immunolocalization of expressed AFP

Leaves of wild-type and transgenic tobacco plants were sectioned freehand with double-edged razor blades and fixed overnight in 3% glutaraldehyde at 4°C. To facilitate the infiltration effect of the fixatives, vacuum pumping was used for 0.5–1 h by syringe. After fixation, the samples were rinsed three times with 0.1 M phosphate-buffered saline (PBS, pH 7.2) and then dehydrated gradually in a series of increasing concentrations of ethanol at 10, 30, 50, 70, 90, and 95% for 10 min in each step, and then two steps in 100% ethanol for 60 min. Samples were infiltrated with increasing ratios of acetone:ethanol at 1:1 and 4:1 for 30 min in each step, followed by acetone without ethanol for 30 min at room temperature. Acetone in the samples was substituted with epikote. Samples were then infiltrated with increasing ratios of epikote:acetone at 1:1 and 4:1 for 1 and 3 h, respectively, followed by epikote without acetone overnight at room temperature. Samples were transferred into capsules containing fresh epikote to embed at 37, 45 and 60°C for 1 day, respectively.

Ultra-thin sections (nm) were prepared with an RMC MT-7 ultramicrotome and collected onto 100-mesh copper grids. All procedures were performed by floating the grids, section-side down, on drops of the appropriate solution at room temperature as described by Karlson et al. (2002) with a slight modification. Sections were incubated in 0.2 M PBS (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, pH 7.2) buffer containing 10 mg of bovine serum albumin (BSA) per ml for 30 min to block nonspecific sites. Excess solution was removed. Sections were incubated for 1 h with a dilution (1:50) of anti-Mp*AFP*149 polyclonal antibody (in 0.2 M PBS) and subsequently rinsed five times by transferring grids of PBS droplets for 5 min. Goat anti-mouse immunoglobulin G conjugated to 15 nm diameter gold particles labeled secondary antibodies (Biosynthesis, China) were diluted 1:50 (in 0.2 M PBS) and incubated with specimens for 1 h at 37°C. After washing five times with 0.2 M PBS and five times with double-distilled water according to procedure above, the sections on grids were air dried and post-stained with saturated uranyl acetate for 15 min and briefly with lead citrate for 15 min. The sections were examined and photographed under H-600 transmission electron microscope (Hitachi, JPN).

Immunoblots

Apoplastic proteins were extracted from leaves using vacuum infiltration in a buffer consisting of ascorbic acid buffer (20 mM, pH 3) and calcium chloride (20 mM) (Hon et al. 1994). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% acrylamide separating gel and a 5% acrylamide stacking gel by electrophoresis (Bio-Rad, USA). The separated polypeptides were transferred onto a nitrocellulose membrane by electroblotting (80 V, 3 h) in electrotransfer buffer (48 mM Tris-base, 38.6 mM glycine, 0.375% SDS) using BioRad blotting apparatus. The nitrocellulose membrane blots were blocked with 1% BSA in 0.2 M PBS buffer (pH 7.0) for 2 h and then incubated at room temperature with primary antibody (1:50) against MpAFP149 for 1 h. Following three washes with PBS (pH 7.0) each for 10 min, the blots were incubated for 1 h with alkaline phosphatase-conjugated goat anti-rat antibody

(1:200; Biosynthesis, China) and finally washed as described above. The alkaline phosphatase signal was developed using 3, 3′-diaminobenzidine 4HCl (Amreso, USA) to detect the protein bands on the immunoblots.

Cold-resistance analysis and ion leakage assay

Sixty T0 seeds each from two transgenic lines (T0-5 and T0-39) were germinated on 1/2 MS medium containing hygromycin (20 mg l^{-1}) and the survival rate of seedlings for both lines was about 75%. The plantlets of same age (60-day-old) were transferred to pots and grown in chamber for 1 month before exposed to cold treatment. When transgenic and wild-type tobacco plants achieved similar growth states, three plants from each were chosen at random to subject cold treatment and measured for electrolyte leakage and MDA content. The percentage of electrolyte leakage was measured to evaluate the degree of cold injury in tobacco seedlings. The temperature in the freezing chamber was set to −1°C. Both wild-type and transgenic plants were subjected to −1°C for various times (0, 24, 48, 72 h), phenotypes were observed. Meantime, leaf samples for each group were washed with deionized water and then immersed in 10 ml of deionized water. After vacuum infiltration, the electric conductivity of the supernatant (S1) was detected using a DDS-11A detector (Shanghai, Dapu Instrument, China).The samples were then lethally boiled to detect the final conductivity (S2, maximum conductivity of tissues). The relativity leakage degree was calculated by the ratio S1/S2 (Cui 1995). For statistical analysis, the mean value of three tested plants of each line and for each treatment was calculated and used for comparing with the wild-type plants.

MDA content

The comparative rate of lipid peroxidation was assayed from seedling leaves by determining the level of MDA at −1°C, which was determined using a TBA (trichloroacetic thiobarbituric acid) reaction (Fryer et al. 1998). Seedlings (0.3 g FW) were homogenized in 3 ml of 10% TCA (thiobarbituric acid) and centrifuged at 12,000×g for 5 min at 4°C. The 2 ml supernatant was mixed with 2 ml of 0.6% TBA which was prepared by 10% TCA. Mixture was boiled for 15 min, cooled to room temperature and centrifuged at 12,000×g for 5 min. Supernatant was subjected to analysis with spectrophotometer. MDA content was calculated with multiplying the difference between absorbance at 535 and 600 nm by the extinction coefficient of 6.45 μM l^{-1} and then subtracted the absorbance at 435 nm multiplied by the extinction coefficient of 0.56 μM l^{-1} from the data. Statistical analysis was same as above.

Results

Characteristics of MpAFP149 protein

The cDNA of Mp*AFP*149 encoded a polypeptide of 120 amino acid residues with calculated molecular mass of 12.7 kDa. The deduced MpAFP149 protein, based on the full-length cDNA sequence, contained a signal peptide at 2–23aa (ALTTKWFLIAVVVMCLCSEYYC) which would likely be cleaved off (Fig. 1) and was predicted to express outside cell by soft TMHMM 2.0 protein prediction. The mature MpAFP149 polypeptide (minus the signal peptide) was 98 residues with calculated molecular mass of 10.2 kDa (Fig. 1) and comprised of tandem repeats of 12-aa sequence (TCTxSxxCxxAx) with regularly spaced Cys similar to Tm 4–9 from *T. molitor* (Marshall et al. 2002). The second and sixth amino acid is Cys and first and third amino is Thr, fifth is Ser and eleventh is alanine in every unit. The two Cys within each repeat form a disulfide bond with the exception of repeats 1 and 2, which are linked by an additional disulfide bond. The putative mature peptide of MpAFP149 shared 68.37% similarity to that of Tm 4–9 and contained eight TCT repeats which is one more than Tm 4–9. Removal of the additional repeat element resulted in an increase to 77.19% amino acid identity.

Transient expression of MpAFP149 in onion epidermal cells

To confirm the expression of Mp*AFP*149 gene in plant cells and visualize the sub-cellular localization of
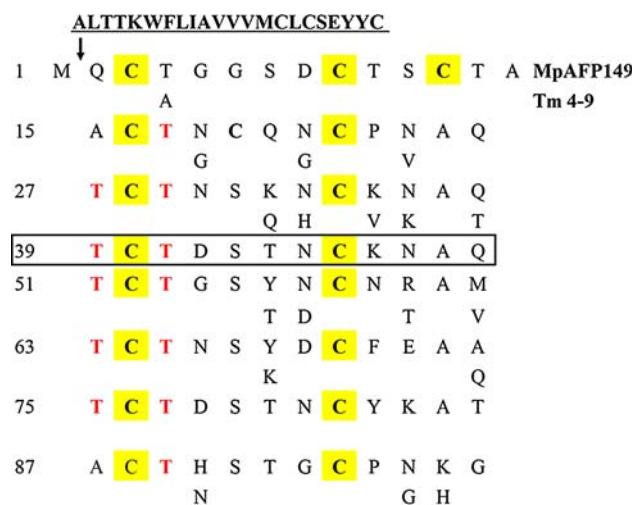

Fig. 1 Sequence alignment of the MpAFP149 and Tm 4–9. The sequence of MpAFP149 is shown in the *top line*. At the 18th positions that vary between these two AFPs, the residue found in Tm 4–9 is shown below. Residues that are highly repetitive are *bold*, with Cys residues highlighted in *yellow* and the Thr residues of the Thr array in *red*. The *underlined* represents the signal peptide and 12 aa in the pane stands for one more repeat unit than that of Tm 4–9
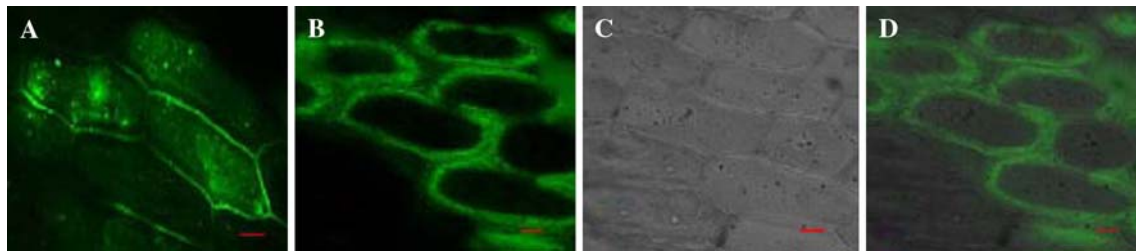
Fig. 2 Subcellular localization of MpAFP149: GFP fusion protein when transiently expressed in onion epidermal cells. **a** The control empty plasmid (*GFP*) viewed under fluorescent filter; **b**, **c** MpAFP149: GFP transformed cells viewed under fluorescent filter (**b**) to show the location of GFP protein and in bright field (**c**) to show the cell shape; **d** merge of **b** and **c**. *Bar* 50 μm
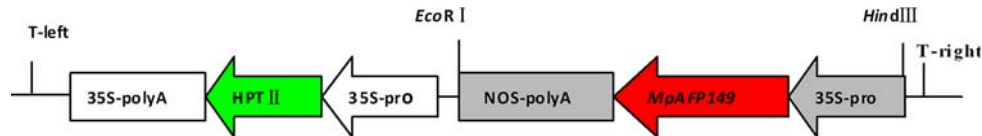

Fig. 3 Gene construct for expression of Mp*AFP*149 gene in tobacco. Expression is controlled by Cauliflower mosaic virus 35S promoter and nopaline synthase polyA terminator. *HPTII* gene encoded hygromycine for screening the transgenic plants

MpAFP149, the full-length Mp*AFP*149 cDNA was fused in-frame with a *GFP*. The resulted Mp*AFP*149: *GFP* plasmid was transiently expressed in onion epidermal cells by the view of fluorescent signal of MpAFP149: GFP around cells (Fig. 2b–d). However, for the control (pCAMBIA1302-GFP transformed) the fluorescence was seen in whole cells without specific expression region (Fig. 2a). It suggested that the MpAFP149 was a secreted protein and the signal peptide could be cut correctly in plant cells, which was consistent with theoretical prediction.

Identification of transgenic plants

The Mp*AFP*149 gene was placed downstream of the constitutive CaMV 35S promoter and upstream of the NOS terminator in a binary plant integration vector (Fig. 3). The resulting vector was transformed into tobacco W38 via *Agrobacterium*-mediated gene transfer to validate the function of Mp*AFP*149 gene during cold treatment. After screened by hygromycin, putative transgenic tobaccos were tested for the presence of the binary vector by PCR with primers that amplified a 363 bp fragment of Mp*AFP*149. Fifty-five out of 66 T0 transformed plants showed the expected band in the agarose gel (data not shown). Among of them, T0-5 and T0-39 showed higher transcript level by RT-PCR analysis (data not shown), which suggested that more AFP protein would be expressed in these two lines. So these two transgenic lines were chosen for detailed analysis in following works. The cDNAs from the transgenic lines of T1-5 and T1-39 gave rise to amplification products of the correct size which indicated that the Mp*AFP*149 gene was transcribed (Fig. 4). No bands were
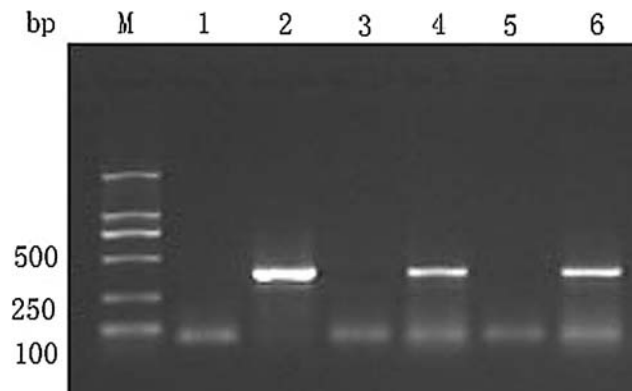

Fig. 4 RT-PCR analysis on T1 transgenic tobacco plants. *Lanes*: *1* negative control (wild-type plant), *2* positive control (pCAM-BIA1302-Mp*AFP*149 as template), *3* T1-5 without RT, *4* T1-5, *5* T1-39 without RT, *6* T1-39, *M* DL 2000 marker (Tiangen)

visible from wild-type plants and RNAs with no-RT. These RT-PCR results showed genetic stability of the integrated genes to tobaccos in the primary stage.

Immunolocalization of expressed AFP

To determine whether heterologous MpAFP149 protein was expressed and where it localized in transgenic tobacco plants, immunogold labeling approach was employed. A polyclonal antibody was raised in mouse against MpAFP149 protein and used for subsequent immunolocalization. Subcellular immunogold localization showed that MpAFP149 protein uniformly accumulated in the outer layers of cell wall of transgenic plant (Fig. 5b), which was absent in the control tobacco plants (Fig. 5a). At the same time, SDS-PAGE and Western blots for apoplastic

Fig. 5 Immunolocalization of heterologous protein MpAFP149 in tobacco leaves by TEM. **a** No gold particles are detected in wild-type tobacco, **b** dense gold particles are presented on the cell wall of transgenic tobacco plant. *Arrowheads* indicate the position of gold particles. Apoplast (*apo*), cell wall (*cw*), bars 1 μm
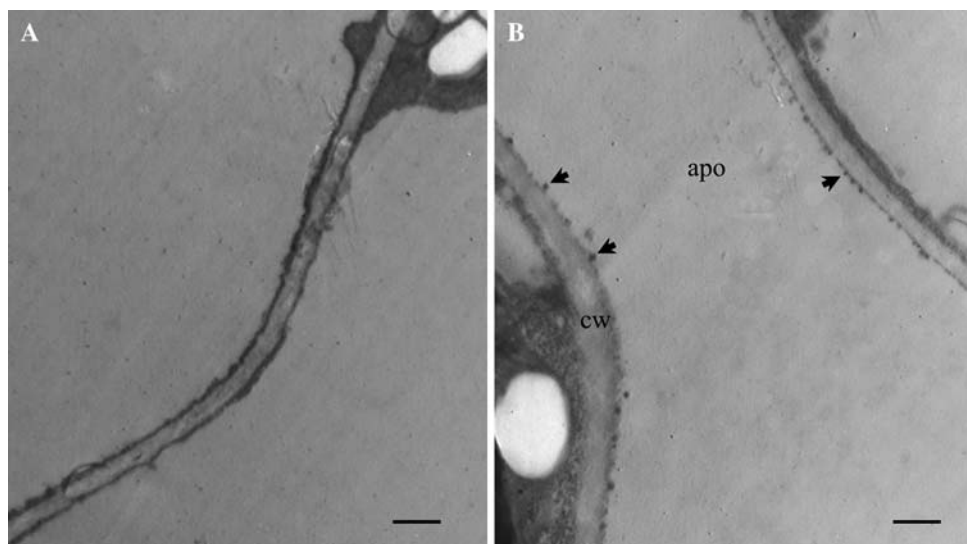
protein from transgenic tobacco lines showed the expected protein band of 10.2 kDa (Fig. 6), which indicated that mature peptide protein MpAFP149 was synthesized in transgenic tobaccos.

Cold resistance analysis of transgenic tobaccos

Transgenic and wild-type tobacco seedlings reached similar states of growth and displaying no visible phenotypic differences were chosen for cold treatment (Fig. 7a). When exposed to −1°C for 1 day, both transgenic and wild-type
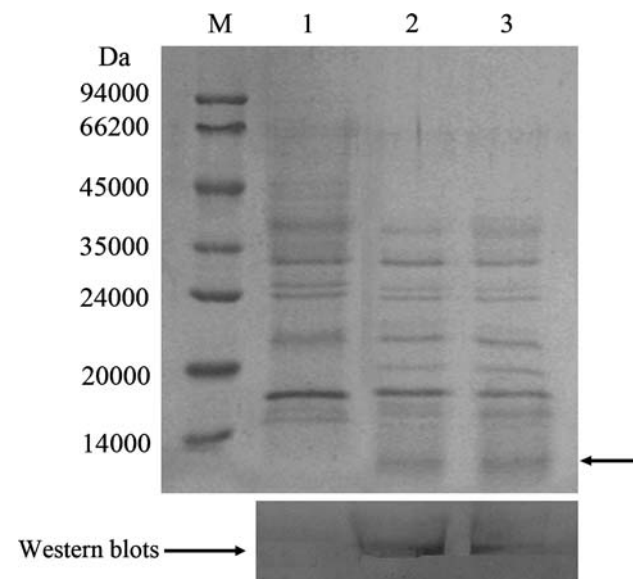

Fig. 6 SDS-PAGE and Western blots analysis for leaf tissue apoplastic extracts from T1 transformed tobaccos. Total soluble protein extracted from leaves was separated by 12% SDS-PAGE gel and immunoblotted with antibody against MpAFP149. *Lanes: 1* WT, *2* T1-5, *3* T1-39. *Arrowheads* indicate that the predicted size of the mature MpAFP149 protein is about 10.2 kDa

tobacco plants only showed moderate dehydration (Fig. 7b). When treated for 2 and 3 days, most leaves of wild-type tobacco were frozen and looked vitrification except the top leaves. However, the transgenic tobacco only exhibited dehydration of several old leaves grown near the base of the plants (Fig. 7c, d). The differences between wild-type tobaccos and MpAFP149 expressing lines were also manifested during stress recovery. During the first day after returned to room temperature, MpAFP149 expressing plants overcame dehydration and achieved complete recovery (Fig. 7e). However, the wild-type tobaccos displayed severe chlorosis and wilting and suffered some level of irreversible damage. These results indicated that transgenic lines carrying Mp*AFP*149 gene showed improved cold tolerance and enhanced recovery from cold treatment.

Ion leakage and MDA content

The semi-permeability of tobacco cytomembranes was disrupted at low temperatures and the effusion of electrolytes resulted in increased electric conductivity in those tissues. Transgenic and control tobacco plants were treated for 0, 1, 2 and 3 days at −1°C. Measurement of the resulting ion leakage (based on three lines per treatment) displayed significantly increasing differences between the control and transgenic plants over time (Fig. 8a). At an initial stage of cold treatment, ion outflow was all little increased and no difference between transgenic and wild-type tobacco plants on 1 day. There was a clear difference in ion leakage rate after 2 days exposure to −1°C: the electrolyte leakage reached 65% for wild-type tobacco, 28% for T1-5 and 27% for T1-39. On day three, the ion outflow was increased in similar tendency as day two, with 71, 28, 36% for wild-type tobacco plants, T1-5 and T1-39 lines, respectively.

Fig. 7 Cold treatment experiment on wild-type tobacco (*left*) and transgenic tobacco (*right*) at −1°C. **a** Before cold treatment (25–28°C), **b** cold treatment for 1 day, **c** cold treatment for 2 days, **d** cold treatment for 3 days, **e** recovery for 5 days at 25–28°C

The increase of malondialdehyde (MDA) paralleled the increase in relative conductivity (rather than one causing the other). MDA concentration in wild-type and transgenic tobacco plants was not significantly different before the cold treatment (Fig. 8b), but it increased more than three times for wild-type tobaccos and two times for T1 transgenic plants after 3 days cold treatment. This result suggested that wild-type plants suffered higher oxidative lipid injury than transgenic tobacco ones, paralleling increases in relative amounts of both ion leakage and MDA clearly with prolonged times of cold treatment. A significant and high correlation ($R^2 = 0.9132$) was observed between MDA content and permeability of cell membranes to ions (Fig. 8c), which suggested that permeability of cold damaged cell membrane increased with the increasing peroxidation of fatty acids.

Discussion

Beetle AFP genes are relatively conserved, though they are a complex family (Graham et al. 2007). The gene MpAFP149, cloned in this study from *Microdera puntipennis dzungarica*, adds another isoform with 68.37% similarity in amino acid to TmAFP 4–9 (Marshall et al. 2002). TmAFPs have been studied in some detail and are composed primarily of a variable number of copies of the 12-amino acid repeat TCTxSxxCxxAx (Graham et al. 1997; Liou et al. 1999) with key Thr residues in alignment on one side of the peptide. The derived MpAFP149 also contained the same repeats units (Fig. 1), so we supposed that the interreaction mechanism between MpAFP149 and ice was similar with TmAFP. Beetle AFPs are more potent when the number of repeats is increased (Leinala et al. 2002; Marshall et al. 2004b). There has one more repeat unit in MpAFP149 than in Tm 4–9, thus MpAFP149 was presumed to produce similar or higher THA than in Tm 4–9. It is natural to introduce MpAFP149 gene into plant to try to improve its cold tolerance.

For plants, ice forms preferentially in the apoplast where the solute concentration is the lowest (Pearce 1988; Steponkus and Webb 1992) and usually results in cellular dehydration and disruption of cell integrity (Levitt 1980) as intracellular water is lost to the growing extracellular ice
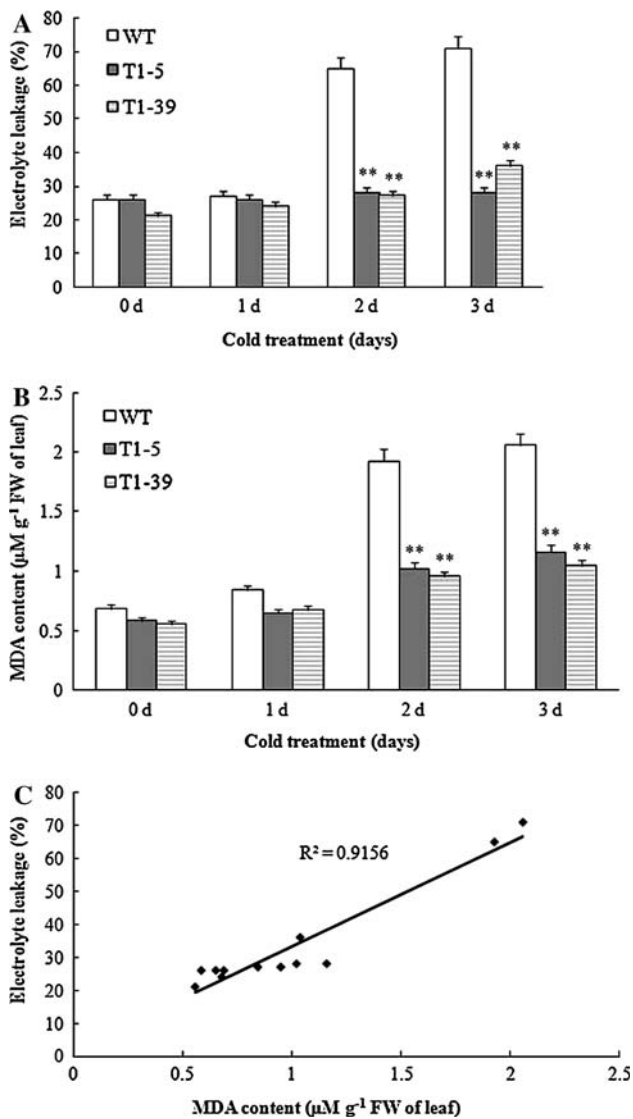
Fig. 8 Effects of cold treatment on permeability of cell membranes (**a**) and MDA content (**b**) of tobacco seedling leaves at −1°C; linear relationship between MDA content and ion leakage (**c**). Data represents the mean ± SD in three independent experiments. **$P < 0.01$ indicates the significant difference between wild-type and transgenic plants. *WT* wild-type tobacco, T1-5 and T1-39 are transgenic tobacco lines

and denaturation of proteins (Pearce 2001). Thus to transfer antifreeze protection to a plant, it is essential that AFPs is targeted to the apoplast space to confer an optimal antifreeze effect (Meyer et al. 1999). Transgenic plants with apoplast-targeted AFP exhibited the higher levels of AFP and are provided the protection at low temperature than those without the signal peptide sequence (Wallis et al. 1997; Kenward et al. 1999; Holmberg et al. 2001; Huang et al. 2002; Khanna et al. 2006). In this research, immunolocalization was the first to be employed for localizing the heterologous insect AFP in transgenic plant. The presence of plant-produced MpAFP149 indicated that the

plant recognized the signal peptide from insect inherent sequence and correctly targeted it to the apoplast (Fig. 5). This was consistent with the result of transient expression of MpAFP149 gene in onion cells (Fig. 2).

Codon usage is generally considered as one of the critical factors that limit the expression rate of heterologous proteins in plants (Kusnadi et al. 1997). In order to achieve high expression levels in plants, spruce budworm AFP gene is optimized and finally improved the thermal hysteresis (Holmberg et al. 2001). However, in this report, although MpAFP149 gene did not be optimized according to tobacco codon bias, heterologous protein was also expressed and targeted in the apoplast of transgenic tobaccos (Figs. 5, 6). It requires for MpAFP149 to be in a very specific native conformation to bind to the ice lattice. The phenotype of cold tolerance and decrease in ion leakage and MDA content from transgenic tobacco plants suggested that at least some of the expressed MpAFP149 were in the native conformation. This implicated that not only was the plant capable of cleaving the signal peptide from the expressed MpAFP149, but it could also remove most or all of the pro-region of insect AFP.

Two factors speculated as contributing to the function of MpAFP149 under cold treatment. The first was the stability of MpAFP149 in the apoplast of transgenic tobacco. If the heterologous AFP produced without signal peptide accumulated in the cytosol of transgenic plant, it is likely that the protein would be degraded very rapidly (Kenward et al. 1999). The relative concentration of protein may be increased due to few proteins especially enzymes in the apoplast. Secondly, the MpAFP149 expressed in the apoplast possessed THA. The THA of apoplast in expressed MpAFP149 transgenic tobacco plants measured by Osmometer (μ.OSMETTE™, USA) was two times that of wild-type ones (data not shown). In addition, recrystallization will be taken place spontaneously when plants are frozen for prolonged periods (Knight et al. 1995). The MpAFP149 with THA in the apoplast of transgenic tobacco plants may inhibit ice growth and recrystalization, hence, it prevents the plant cells from cold damage.

Due to complexity of the cold-tolerance mechanism in plants and the partial participation by the AFP, we selected phenotype experiments, ion leakage assays and MDA content to evaluate the function of the MpAFP149 gene in the transgenic tobacco plants. Duration of exposure to stress determines the degree of injury (Rajashekar et al. 1983). The extent of cold injury in plants with prolonged time was indicated by the increments in tissue conductivity. When treated at −1°C for 2 and 3 days for T1-5 and T1-39, the electrolyte leakage showed less change, while for the wild-type tobacco, it increased by two times, suggesting that the membrane for the wild-type plant was greatly damaged during the cold stress (Fig. 8a). Further,

MDA content is a direct indicator for the situation of the cellular membranes during cold stress, similar results as in the measures for conductivity was obtained. The MDA content for the wild-type plants was dramatically increased almost three times after treated on day two, but the transgenic lines kept a relatively stable MDA level. This result was in agreement with the ion leakage assay. Actually, the high correlation was found between MDA content and membrane permeability to ions ($R^2 = 0.9156$, Fig. 8c). Meantime, freezing phenotype of transgenic and wild-type plants supported the assays in the ion leakage and MDA content. The transgenic tobaccos maintained greater stamina than wild-type ones during cold treatment and was able to recover and continue to grow under normal conditions. In terms of phenotype experiments, ion leakage and MDA content, a relationship between the expression of MpAFP149 and an improvement in cold resistance in transgenic plants was inferred.

Based on the results of this study, transgenic tobacco plants which encoded MpAFP149 with the native signal peptide responsible for secreting the native MpAFP149 into the apoplast space showed the improved cold tolerance by conferring low-temperature protection to membrane system in the cell. The results demonstrated that the MpAFP149 gene may be used as the candidate gene for the improvement of frost resistance of commercially important crops. It would be that levels of protection could be increased further by improving promoter constructs.

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