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Expression of Two Self-enhancing Antifreeze Proteins from the Beetle *Dendroides canadensis* in *Arabidopsis thaliana*

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Abstract Antifreeze proteins depress the non-equilibrium freezing point of aqueous solutions, but only have a small effect on the equilibrium melting point. This difference between the freezing and melting points has been termed thermal hysteresis activity (THA). THA identifies the presence and relative activity of antifreeze proteins. Two antifreeze protein cDNAs, *dafp-1* and *dafp-4*, encoding two self-enhancing (have a synergistic effect on THA) antifreeze proteins (DAFPs) from the beetle *Dendroides canadensis*, were introduced into the genome of *Arabidopsis thaliana* via *Agrobacterium*-mediated floral dip transformation. Southern blot analysis indicated multiple insertions of transgenes. Both DAFP-1 and/or DAFP-4 were expressed in transgenic *A. thaliana* as shown by RT-PCR and Western blot. Apoplastic fluid from T_3 DAFP-1+DAFP-4-producing transgenic *A. thaliana* exhibited THA in the range of 1.2–1.35°C (using the capillary method to determine THA), demonstrating the presence of functioning antifreeze proteins (with signal peptides for extracellular secretion). The freezing temperature of DAFP-1+DAFP-4-producing transgenic *A. thaliana* was lowered by approximately 2–3°C compared with the wild type.

Keywords Antifreeze protein · *Arabidopsis thaliana* · Cold tolerance · *Dendroides canadensis* · Multi-gene transformation · Transgenic

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Introduction

Antifreeze proteins (AFPs) play an important role in the cold tolerance of certain fish, terrestrial arthropods, plants, and microorganisms (for reviews, see DeVries 1983, 2005; Duman 2001; Davies et al. 2002; Griffith and Yaish 2004; Duman et al. 2010). Although the equilibrium freezing and melting points of aqueous solutions are typically identical and the terms are used interchangeably, in the presence of AFPs this is not the case. AFPs depress the non-equilibrium (hysteretic) freezing point, in the presence of ice, but only have a small effect on the melting point. The difference between the hysteretic freezing and equilibrium melting points is termed thermal hysteresis (DeVries 1986). AFPs are thought to lower the hysteretic freezing temperature by "adsorption–inhibition", first described by Raymond and DeVries (1977). They proposed that AFPs bind to the surface of the growing ice crystal, so the surface of the ice crystal formed between AFPs is highly curved (high surface free energy). Due to the Kelvin effect, the growth of the ice crystal is delayed until the temperature is lowered to the hysteretic freezing point. Several types of binding interactions between ice and AFPs have been suggested. While hydrogen bonding is thought to be the most common form, hydrophobic interactions and van der Waals forces have also been reported as possible interactions, depending on the type of AFP (Davies et al. 2002; Jia and Davies 2002; Jorov et al. 2004).

In addition to thermal hysteresis activity (THA), changes in specific ice crystal morphology (e.g., hexagonal and bipyramidal shapes), and recrystallization inhibition are also indications of the presence of AFPs. Low concentrations of AFPs, or AFPs with low specific activity, typically produce very low THA, if any at all, and usually this situation results only in changed ice crystal morphology and/or recrystallization inhibition. The term ice-active proteins (IAPs) is now properly

used (Wharton et al. 2005) to include proteins that function (1) as true AFPs (those IAPs with greater THA) that actually function as antifreezes, as well as IAPs that only produce (2) low THA (a few tenths of a °C), (3) changes in ice crystal morphology, and (4) recrystallization inhibition. Antifreeze proteins are typically found in freeze-avoiding organisms (those that die if frozen) while the other IAPs are present in many freeze-tolerant species (those that survive freezing, usually only of the extracellular water). Among the AFPs and other IAPs isolated from various organisms, those from freeze-avoiding insects have the highest THA. In contrast, the THA of endogenous IAPs in plants is relatively low (generally less than 0.2°C, but on occasion a bit higher) compared with fish (0.4–1.6°C) and freeze-avoiding insects (2–13°C) (Duman 2001; Duman et al. 2004; Bennett et al. 2005). All of the plants currently known to have IAPs are freeze tolerant.

Overwintering larvae of the beetle *Dendroides canadensis* produce a family of antifreeze proteins which are differentially distributed in the hemolymph, gut fluid, epidermal tissue, and Malpighian tubule fluid (Duman et al. 2002, 2010). *D. canadensis* AFPs (DAFPs) demonstrate high thermal hysteresis activity, 3–9°C, (Wu et al. 1991) compared with AFPs from other animals or plants. Using a yeast two-hybrid screen, group I DAFPs (1, 2, 4, and 6) were found to interact with one another (2 and 4 with 1 and 6 with 4). The interaction between DAFPs enhances their thermal hysteresis activity in a synergistic way when tested at a 1:1 ratio in vitro, producing self-enhancement of THA of 5.5°C at a combined concentration of DAFPs-1 and DAFPs-4 of just 1 mg/ml (Wang and Duman 2005).

In freeze-avoiding insects, such as *D. canadensis*, AFPs function to prevent freezing by blocking inoculative freezing across the cuticle initiated by ice in the environment (Olsen et al. 1998) and by inhibiting ice nucleators in the hemolymph and gut of the insect, thereby promoting supercooling (Olsen and Duman 1997a, b; Duman 2002). Several freeze-tolerant plants (those able to survive freezing) produce IAPs (Urrutia et al. 1992; Duman and Olsen 1993; Duman 1994; Hon et al. 1994; Worrall et al. 1998; Meyer et al. 1999; Smallwood et al. 1999; Huang and Duman 2002; Pudney et al. 2003; Wang and Wei 2003; Griffith and Yaish 2004; Simpson et al. 2005; Kawahara et al. 2009). As noted earlier, the thermal hysteresis activity in freeze-tolerant organisms, including plants, is low and the function is different than that in freeze-avoiding species. Some IAPs in winter rye (*Secale cereale*) are secreted into the surrounding cell walls and may inhibit lethal ice propagation from the apoplast into the symplast (Pihakaski-Maunsbach et al. 2001). Additionally, IAPs may alleviate the injury caused by larger ice crystals formed during recrystallization (Urrutia et al. 1992; Griffith et al. 2005). IAPs from bittersweet nightshade (*Solanum dulcamarum*), peach trees (*Prunus persica*), and winter rye (*Secale cereale*) do not contain signal peptides, which suggests that they may bind to intracellular ice nucleators thereby inhibiting intracellular ice nucleation (Wisniewski et al. 1999; Huang and Duman 2002; Griffith and Yaish 2004). Regardless of the specific functional role, it does not appear that the primary activity of the naturally occurring plant IAPs identified to date is to promote freeze avoidance as none have been identified in freeze sensitive plants.

Higher crop yields could be achieved by both improving freeze tolerance of overwintering crop plants and increasing the freeze avoidance capacity of freezing-sensitive plants during light frosts. Introduction of the hyperactive antifreeze proteins of insects into the genome of plants has the potential to achieve the latter. AFPs and/or IAPs from fish, insects, and plants have been successfully expressed in tomato, tobacco, potato, and *Arabidopsis thaliana* (Hightower et al. 1991; Kenward et al. 1993; Wallis et al. 1997; Kenward et al. 1999; Meyer et al. 1999; Holmberg et al. 2001; Huang et al. 2002; Wang et al. 2008; Zhu et al. 2010). In some systems, AFP expression in transgenic plants produced plants whose extracts exhibited low levels of thermal hysteresis (0.37°C/mg apoplastic protein by nanoliter osmometer, Holmberg et al. 2001) and recrystallization inhibition in vitro (Worrall et al. 1998), as well as reduced electrolyte leakage in leaves after freezing (Wallis et al. 1997; Zhu et al. 2010). However, no other cold tolerance improvement was reported. In contrast, when the *dafp-1* gene from the beetle *D. canadensis* was expressed in *A. thaliana* (Huang et al. 2002), these transgenics had greater thermal hysteresis activity than previously generated transgenic plants (ranging from 0.2°C to 0.8°C, by the capillary method, depending on lines, generations, etc.), and the freezing temperature of the whole plant without roots was lowered by 1–3°C depending on the freezing treatment method used. In a recent study, Wang et al. (2008) expressed a beetle (*Microdera puntipennis dzungarica*) antifreeze protein in tobacco and showed decreased ion leakage, lower malondialdehyde content in the leaves, and improved cold tolerance at −1°C for 3 days.

The objective of the present study was to increase subzero temperature tolerance in *A. thaliana* by expressing two *D. canadensis* antifreeze proteins, DAFP-1 and DAFP-4, which have been demonstrated to have a synergistic, self-enhancing, effect on promoting THA through their interaction. The expression of the transgene(s) at the RNA and protein levels, thermal hysteresis activity, and the effect of these two transgenes on the cold tolerance of transgenic *A. thaliana* were evaluated.

Materials and Methods

To generate transgenic *A. thaliana* expressing both DAFP-1 and DAFP-4, co-transformation of multiple binary vectors

containing different genes (Francois et al. 2002; Goderis et al. 2002) was performed. A Takara PCR kit (Takara Bio, USA) was used to amplify the *D. canadensis* antifreeze protein gene *dafp-4*. PCR primers were purchased from Integrated DNA Technologies. All restriction enzymes were purchased from New England BioLabs. The TOPO TA Cloning kit (Invitrogen) was used for all cloning steps. DNA sequencing was performed using an ABI 3,700 sequencer.

Vector Construction

The *D. canadensis* antifreeze protein gene *dafp-4* (Duman et al. 2002) with signal peptide sequence was amplified from its cDNA by PCR using primers 5′-ACTAG TATGGTTTGGGTTTGCAAAAGT-3′ (Forward) and 5′-GAGCTCTCATGGACATCCCGTAGA-3′ (Reverse). Sequences were designed to include a SpeI site at the 5′, and a SacI site at the 3′. By digestion with SpeI and SacI, *dafp-4* was ligated into the multiple cloning site of plasmid pE1913 (purchased from Purdue University), which was linearized previously with the same enzymes. The resulting plasmid, which contains a mannopine synthase (*mas*) promoter and activator, and three tandem repeats of octopine synthase (*ocs*) activator, tobacco etch viral 5′-translational leader, *dafp-4* coding sequence and agropine synthase (*ags*) terminator, was designated as pE1913-4 (Fig. 1). The marker gene in this vector is the *bar* gene, which is Basta (glufosinate ammonium) resistant. PCR and DNA sequencing were performed to ensure that the conjunction and the gene sequences were correct.

A binary vector, pCIB10g-1 (Huang et al. 2002, vector pCIB10g-340 was renamed pCIB10g-1), was used for DAFP-1 transformation. It contains cauliflower mosaic virus (CaMV) 35S promoter, *dafp-1* (NCBI accession number U79777) coding sequence together with its signal peptide sequence, and CaMV 35S terminator. This vector grants plants kanamycin resistance.

Transformation of Vectors Carrying *dafp-1* and/or *dafp-4* into *Agrobacterium*

Vectors pE1913-4 and pCIB10g-1 were introduced into *Agrobacterium* strain GV3101 via electroporation using a BIO-RAD Bacterial Electroporator. The transformed *Agrobacteria* were grown on LB medium with gentamycin (50 mg/L) and kanamycin (50 mg/L). Positive clones were confirmed by PCR using gene specific primers.

Agrobacterium-mediated Floral Dip Transformation of *A. thaliana*

The floral dip method has been demonstrated to be an efficient transformation approach in different plants (Clough and Bent 1998; Verma et al. 2008; Xu and Shi 2008; Yasmeen et al. 2009; Cheng et al. 2010). Seedlings of the *A. thaliana* ecotype Columbia (Col-0) were transformed using the floral dip method. Soil was autoclaved, and distributed in pots, where *A. thaliana* Col-0 seeds were dispensed. Then the pots were covered with plastic wrap and placed at 4°C for 2 days. They were then moved to a growth chamber at 22°C with continuous incandescent light at approximately 100 μmol m^{-2} s^{-1}. The inflorescence was clipped after most plants had formed primary bolts to obtain more synchronized secondary bolts. Plants were then ready to be transformed when most secondary bolts were about 1–10 cm tall (4–8 days after clipping).

Agrobacterium strain, GV3101, which carries a specific vector (pE1913-4 or pCIB10g-1), was incubated in 5 ml YEP medium containing gentamycin (50 mg/L) and kanamycin (50 mg/L) at 30°C with shaking overnight. A 500-ml culture started from this 5-ml culture was grown under the same conditions until OD_{600} was 2.0 the next day. *Agrobacterium* pellets were resuspended in 500 ml ARA-BIDIP solution (5% Sucrose, 0.05% Silwet-L77).

A. thaliana Col-0 plants, watered well the day before transformation, were dipped into the ARABIDIP solution containing the construct(s) of *dafp-1* and/or *dafp-4*. Then the pots were laid on their sides in a tray, and covered with plastic wrap. The next day, the cover was removed, and the pots were set upright. The growth conditions were the same as previously described, and seeds were collected later from individual transformed plants.

Preliminary Screening of the Transformed *A. thaliana*

Seeds from the transformed *A. thaliana* were screened by kanamycin resistance (marker of the DAFP-1 vector) and/or Basta (glufosinate ammonium) resistance (marker of the

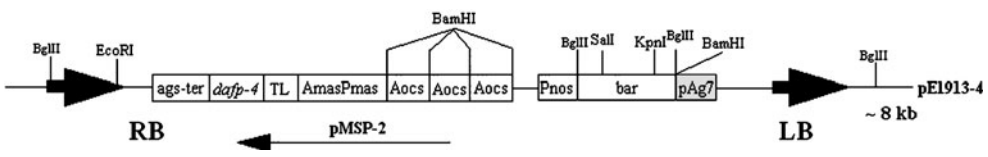

Fig. 1 Diagram of *dafp-4* in vector pE1913. The vector contains a bar gene, which confers Basta (glufosinate ammonium) resistance. Chimeric promoters of the transgene consist of mannopine synthase promoter (*Pmas*) and activator (*Amas*), and three tandem repeats of octopine synthase activator (*Aocs*). *TL* tobacco etch viral 5′-translational leader

DAFP-4 vector). For kanamycin screening, seeds from plants that were transformed with the *dafp-1* construct were sterilized in 1-ml bleach solution (30% bleach, and 5% Triton X-100) for 10 min, rinsed three times in sterile distilled water, resuspended in 2-ml sterile 0.1% Phytagar, and distributed on sterile Gamborg's B5 medium containing kanamycin (50 mg/L) and timentin (300 mg/L), which was used to eliminate *Agrobacterium*. The plates were placed at 4°C for 2 days and moved to the growth chamber at 22°C under constant light. After 12 to 14 days, the dark green seedlings with true leaves (kanamycin resistant) could be discriminated from white dying seedlings (kanamycin sensitive). These kanamycin resistant seedlings were moved to soil pots and grown under conditions described earlier. As for Basta screening, 5-day T_1 seedlings from the *dafp-4* transformation were sprayed with 300 μM Basta at 3-day intervals to eliminate non-transformed seedlings. Seeds from plants that were transformed with both *dafp-1* and *dafp-4* plasmids were selected on the kanamycin plates first, and then were moved to soil pots, after which Basta solution was applied as described above.

THA Screening and Analysis

A nanoliter osmometer (Clifton Technical Physics, Hartford, New York) was used for initial THA screening and the capillary method (Duman 2001) was used for THA analysis of T_3 transgenic plants. The nanoliter osmometer is more sensitive for detection of low THA because it uses a much smaller ice crystal compared with the capillary method (Duman 2001). It also uses less volume of sample. Another advantage of using a nanoliter osmometer compared with the capillary method is that ice crystal shapes, such as the hexagonal shape that indicates the presence of a low concentration of AFP, are more readily visible (Griffith and Yaish 2004). Sixty to seventy individual plants from each transformation that were confirmed as transgene positive by preliminary screening were pierced by a capillary needle and a few nanoliters of fluid were removed by capillary action. The sample was then checked with the nanoliter osmometer. Plants with the highest THA were chosen for further analysis. To be consistent with the previous study (Huang et al. 2002), the capillary method was used for THA assay of T_3 transgenic plants. THAs of total soluble protein (50 mg/ml) and apoplastic protein from each line were tested using the capillary method. Three pools of total protein or apoplastic protein were tested. Measurements were taken in triplicate for each individual sample.

Genomic DNA Analysis by Southern Blot

Genomic DNA from the leaves and stems of T_1 transgenic *A. thaliana* and untransformed control plants were isolated using a Qiagen DNeasy Plant Maxi Prep kit. Twenty micrograms of genomic DNA were digested with EcoRI, separated on a 0.7% agarose gel, and blotted on Hybond N^+ nylon membrane (Amersham Biosciences) in 2× saline-sodium citrate (SSC) using Turboblotter™ Rapid Downward Transfer Systems (Schleicher & Schuell, Keene, NH) as per manufacturer's instructions. ^{32}P-labeled *dafp-4* or *dafp-1* probes were synthesized using random primers DNA labeling system (Invitrogen) and purified using CentriSep columns (Princeton Separations). The blot was prehybridized at 60°C overnight, and hybridized with the ^{32}P-labeled *dafp-1* or *dafp-4* probe at 60°C overnight. After washing in ×2 SSC twice at room temperature (5 min×2), in 2×SSC/1% SDS twice at 60°C (15 min×2), and in 0.1×SSC/0.1% SDS twice at 60°C (15 min×2) the next day, the blot was wrapped and exposed to an X-ray film for 2 days if using the *dafp-4* probe, or 4 to 5 days if using *dafp-1* as a probe.

RNA Analysis by RT-PCR

Total RNA from transgenic *A. thaliana* and untransformed control plants was isolated using TRIzol reagent (Invitrogen) and Phase-Lock-Gel (Eppendorf). RNA was treated with DNase I prior to the synthesis of the first-strand cDNA. One microgram of RNA was used for the first-strand cDNA synthesis (Promega Reverse Transcription System) and an equivalent volume of cDNA was added to PCR (SYBR Green PCR Master Kit, Applied Biosystems). The standard reaction was done at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. This was followed by extension at 72°C for 10 min. The primers were designed to discriminate the two homologous genes (*dafp-1* and *dafp-4*). The primers were: 5′-TCCAAATGCACGTACAGCATGTACTC-3′ (Forward) and 5′-GTAGCTTCGTA-ACAGTTGGTTGATC-3′ (Reverse) for *dafp-1*, and 5′-CCCAAATGCACGTACA-GCATGTACAG-3′ (Forward) and 5′-CCGTAGAATCGGTA CAGGTCTTG-3′ (Reverse) for *dafp-4*. PCR products were separated on a 1.2% agarose gel.

Protein Extraction

To extract total soluble protein, leaves and stems from transgenic and untransformed *A. thaliana* were frozen in liquid nitrogen and homogenized in Tris buffer (50 mM, pH 8.0) that contained 0.1 M KCl, 5% *v*/*v* glycerol, 10 mM EDTA, cysteine proteinase inhibitors (leupeptin and anti-pain, 1 μg/ml), aspartate proteinase inhibitor (pepstatin, 1 μg/ml), and serine proteinase inhibitors (1 mM phenyl-methylsulfonyl fluoride and 1 mM benzamide) (Duman 1994). The homogenate was centrifuged in a microcentri-fuge at 12,000×*g* for 30 min at 4°C, and the supernatant

was dialyzed in deionized water at 4°C for 2 days. Samples were freeze-dried to concentrate the protein. The protein concentration of the samples was determined using the Bio-Rad DC protein assay kit as per manufacturer's instructions.

Apoplastic fluid was isolated from leaves and stems of *A. thaliana* as described by Huang et al. (2002). Leaves and stems were cut into 1-cm lengths, inserted into a 10-ml syringe which was placed on the top of a 1.5-ml centrifuge tube. The apoplastic extract was recovered from the bottom of the tube after centrifugation at 830×*g* for 20 min.

Western Blot

Fifty micrograms of total soluble protein or 5 μl (15 μl for DAFP-4 line, #2) of apoplastic fluid from wild-type and transgenic plants were added to SDS loading buffer (0.125 M Tris, pH 6.8, 20% glycerol, 4% SDS, 10% beta-mercaptoethanol, and 0.004% bromphenol blue) and boiled for 3 min before loading. The mixture was eletrophoresed on a 10–20% (*w*/*v*) gradient polyacrylamide gel (Bio-Rad) at 20 mA until the blue marker dye ran to the bottom of the gel, and then transferred to a nitrocellulose membrane in Towbin's transfer buffer (Towbin et al. 1979) using a semi-dry transfer cell (Bio-Rad) for 25 min at 15 V. After that, the blot was blocked in 5% milk in Tris-buffered saline–Tween 20 (TBS-Tw20) for 3 h and incubated with IgG-purified rabbit anti-DAFP primary polyclonal antibody overnight at 4°C. After two washes (5 min×2) in 5% milk/TBS-Tw20 the next day, the blot was incubated with goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad) at a dilution of 1:3,000 for 3 h and washed three times (10 min×3) in TBS-Tw20. Detection using the Pierce SuperSignal West Femto kit followed for 5 min, and an X-ray film was exposed and developed. For the total soluble protein, two SDS gels loaded with the same amount of samples were run at the same time. One was used for a Western blot, and the other was stained using Coomassie brilliant blue to visualize the loading control (light chain of RubisCO, 13 kDa).

Freezing Temperature Analysis

The freezing temperature of 3–4 weeks old individual T_4 plants without roots (n=15–21) and of individual leaves (one leaf from each plant, n=14–21) were tested using an infrared camera (Wisniewski et al. 1997; Wisniewski et al. 1999) (Inframetrics Model 760 LW, Inframetrics, Billerica, MA). After roots were removed, vacuum grease was applied to the cut area to prevent ice initiation through the cut end of the stem and to stabilize the seedling. Seedlings were then placed in Petri dishes and the Petri dishes were placed into an environmental chamber (Lunaire Limited/Tenney, Model T20S, White Deer, PA) under the detection of the infrared camera. This camera detects a change of 0.1°C on the object surface by displaying different colors for different temperatures. Therefore, when each individual plant freezes, the heat it emits results in a temperature change of several tenths of a degree Celsius. The change of color, identifying the freezing temperature of the plant, could be visualized on the screen. A videotape was made for later analysis.

Quantitative Real-time PCR

Total RNA from *A. thaliana* leaves of each transgenic line was purified using TRIzol reagent (Invitrogen). The RNA was then treated with DNase I (Invitrogen). The concentration of the RNA was determined by reading OD_{260} using a spectrophotometer (Hitachi, U-2000). An equivalent amount of RNA was used for the first-strand cDNA synthesis (Promega Reverse Transcription System). The cDNA samples were treated with RNase H to digest the RNA hybrid strand. The 25 μl reaction included ABI SYBR green master mix, primers at optimized concentration (300 nM), and cDNA derived from 25 ng total RNA (or a series of dilutions for the standard curve). The linearized and purified plasmid (TOPO-*dafp-1* and TOPO-*dafp-4*) standard curves in the range of 10 to 10^7 copies were used to compare the mRNA abundance of *dafp-1* and *dafp-4* genes in the DAFP-1+DAFP-4 lines. Relative quantification was carried out to compare the mRNA abundance of the same gene between different *A. thaliana* lines. Three pools of RNA from each line were used for cDNA synthesis and six replicates of real-time PCR reactions were run for each cDNA sample. The actin-2 gene was used as an internal control for relative quantification. The primers for all genes are shown in Table 1.

The reaction started with one cycle at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 59°C for 30 s, and 72°C for 30 s. Amplification, data acquisition, and the analysis were performed using an ABI PRISM 7700 Sequence Detection System.

Table 1 Primers used in real-time PCR

Gene	Sequence (5′-3′)
actin-2	F: 5′-TCTTGCTTCCCTCAGCACATTCCA-3′
	R: 5′-TTCCTGGACCTGCCTCATCATACT-3′
dafp-1	F: 5′-TCCAAATGCACGTACAGCATGTACTC-3′
	R: 5′-GTCGTAGCTTCGTAACAGTTGGTTGATC-3′
dafp-4	F: 5′-CCCAAATGCACGTACA-GCATGTACAG-3′
	R: 5′-TCCCGTAGAATCGGTACAGGTCTTG-3′

F forward primer, *R* reverse primer

Statistics

The Tukey's multiple comparisons test was used to analyze the data for statistical significance.

Results

Identification of Transgenic Plants

DAFP-1 transformants were screened by kanamycin resistance, and DAFP-4 transformants were screened by Basta resistance. Transgenic plants with both *dafp-1* and *dafp-4* genes were selected using both kanamycin and Basta resistance. The transformation rate of one-gene transformants was 4% and that of two-gene transformants was 1.3%.

Thermal hysteresis activities of T_1 generation plants from all lines were analyzed using the nanoliter osmometer. Approximately 20% of the true transformants confirmed by kanamycin and/or Basta resistance showed THA. Table 2 shows the different activities of each line. DAFP-4 lines are not shown here because THA was not detected, however, hexagonal ice crystals, indicative of a low concentration of DAFP, were found in their total protein extracts. The highest activity among DAFP-1 lines (1.75°C) was obtained from #10, and among DAFP-(1+4) lines from #1 and #2, 3.96°C and 3.85°C, respectively. These lines (DAFP-1, #10; DAFP-1+4, #1 and #2; and DAFP-4, #2 were chosen for further analysis.

Table 2 THA (°C) of T_1 individual transgenic plant

Construct	Line	THA (°C)
DAFP-1	1	1.49
	2	0.13
	3	0.24
	4	0.19
	5	0.58
	6	1.26
	7	0.54
	8	0.66
	9	0.35
	10	*1.75*
	11	1.71
DAFP-(1+4)	*1*	*3.96*
	2	*2.85*
	3	1.12
	4	0.67
	5	1.67
	6	1.32
	7	0.63
	8	1.86
	9	2.16
	10	1.34
	11	1.67
	12	1.30
	13	0.84
	14	1.54

Line numbers and THA values in italics identify the transgenic lines that were selected for further study because they exhibited the highest THA

Genomic DNA Analysis in Transgenic *A. thaliana*

Southern blot analysis showed successful insertions of transgenes (*dafp-1* and/or *dafp-4*) (Fig. 2) and confirmed that all three types of transgenic plants containing DAFP-1, DAFP-4, and DAFP-(1+4) had multiple copies of transgenes in their genome. Either a *dafp-1* or *dafp-4* probe was

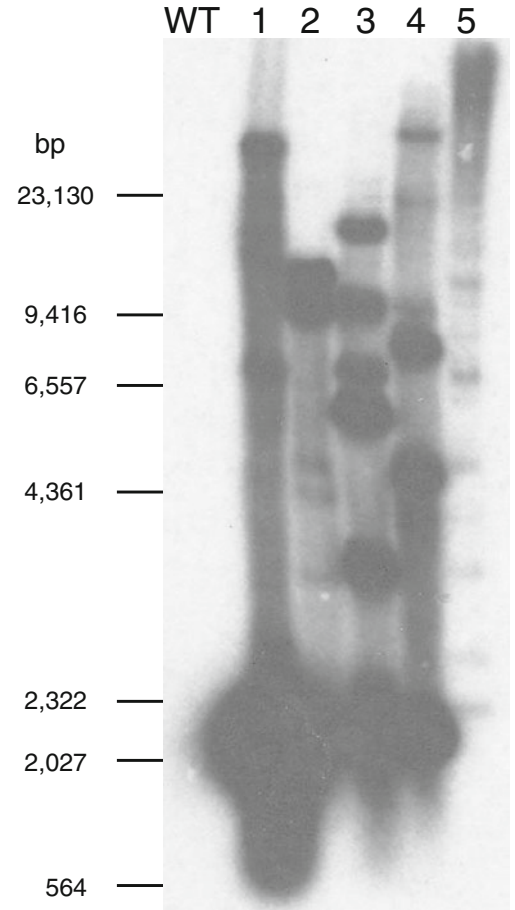

Fig. 2 Southern blot analysis of representative transgenic lines identifying DAFP genes. *WT* the wild-type *Arabidopsis thaliana*, which did not show any bands. *Lane 1*, DAFP-4-producing transgenic *A. thaliana* #2; *lane 2*, is DAFP-1-producing transgenic *A. thaliana* #10; *lane 3*, DAFP-(1+4)-producing transgenic *A. thaliana* #1, *lane 4*, DAFP-(1+4)-producing transgenic *A. thaliana* #2, *lane 5*, is *Dendroides canadensis* genomic DNA as a positive control. The *dafp-1* probe was used in this blot

used for the Southern blots, but both showed the same results. This implied cross hybridization between *dafp-1* and *dafp-4* genes due to a high degree of sequence homology.

mRNA Profiles of DAFP(s) in Transgenic *A. thaliana* by RT-PCR (T_1 Generation)

Reverse transcription polymerase chain reaction (RT-PCR) using *dafp-1* or *dafp-4* specific primers showed the expression of transgenes at the RNA level in the transgenic plants (Fig. 3). As expected, all three types of transgenic *A. thaliana* showed transcription of the corresponding transgenes. No bands were present in the wild-type *A. thaliana* control. Cross reactions were not detected between *dafp-1* primers and *dafp-4* template DNA, and vice versa.

Expression Profiles of DAFP(s) in Transgenic *A. thaliana* by Western Blot

Total soluble protein extracted from *A. thaliana* leaves and stems of the T_3 generation was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting. IgG-purified rabbit polyclonal antibody to DAFPs, which reacts with both DAFP-1 and DAFP-4 with the same affinity, was used as the primary antibody. As demonstrated in Fig. 4, unlike the wild type, all transgenic plants exhibited positive results. Although the predicted mass of DAFP-1 is 8,710 Da, and that of DAFP-4 is 7,325 Da, they both migrated at approximately 14 kDa on the SDS-PAGE. This migration pattern on SDS-PAGE is typical for DAFPs in that they often appear larger than they actually are (Duman et al. 1998). The faster bands are believed to be degraded proteins. Plants that expressed DAFP-1 or both DAFPs-1 and DAFPs -4 demonstrated higher expression of the *D. canadensis* antifreeze proteins than did the DAFP-4-producing plants.

Apoplastic proteins from *A. thaliana* leaves of the T_3 generation also showed DAFP expression on Western blots

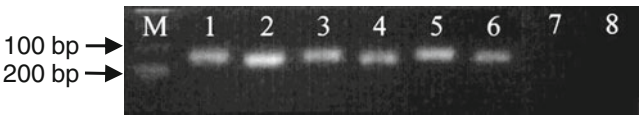

Fig. 3 RT-PCR of transgenic *Arabidopsis thaliana* (T_1 generation). *Lane M*, the DNA ladder. *Lane 1*, DAFP-1-producing transgenic plants, #10; *lane 2*, DAFP-4-producing transgenic plants, #2; *lanes 3* and *4*, DAFPs-(1+4) line, #1; *lanes 5* and *6*, DAFPs-(1+4) line, #2; *lanes 7* and *8*, wild-type plants. Primers for *lanes 1, 3, 5* and *7* are *dafp-1*-specific primers; primers for *lanes 2, 4, 6* and *8* are *dafp-4*-specific primers. The no-Reverse-transcriptase control did not show any bands using either primer (data not shown here). Note the product size is 160 bp for *dafp-1* primers and 144 bp for *dafp-4* primers

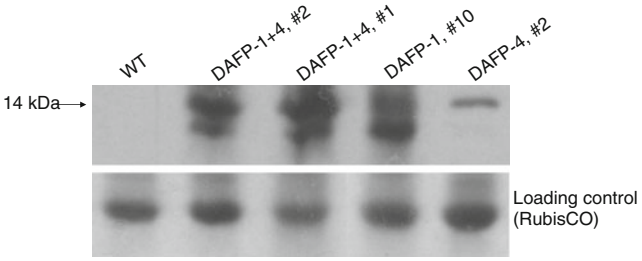

Fig. 4 Western blot analysis of total soluble protein from transgenic *Arabidopsis thaliana* (T_3 generation). Each lane contains 50 μg total soluble proteins, extracted from leaves and stems. IgG-purified rabbit polyclonal antibody to DAFPs, which reacts with both DAFP-1 and DAFP-4 with the same affinity, was used as the primary antibody. The band of RubisCO (light chain, 13 kDa) on the gel was used as the loading control. *WT* the wild-type *A. thaliana*

(Fig. 5). When five microliters of apoplastic fluid were loaded, the DAFP-1 and DAFP-(1+4) lines exhibited positive results, but the DAFP-4 line did not. However, when fifteen microliters of apoplastic fluid from the DAFP-4 line were loaded, a faint band appeared, indicating low expression of DAFP-4. As expected, the wild type did not demonstrate any DAFP expression.

THA Analysis

Antifreeze protein activity (THA) was confirmed in the total soluble protein (50 mg/ml) of all of the transgenic *A. thaliana* lines using the capillary method. As shown in Fig. 6, the two DAFP-(1+4) lines (#1 and #2) demonstrated the highest activity, and the DAFP-1 line showed moderate activity. The DAFP-4 line did not exhibit THA; however, hexagonal ice crystals were detected, indicating a low concentration of antifreeze protein.

The THAs of the apoplastic fluids were also checked (Fig. 6). The trend in relative thermal hysteresis activity was the same as that in the total protein extracts: The DAFP-(1+4) lines showed the highest apoplast activity; the DAFP-1 line showed relatively lower activity; the DAFP-4 line did not show any THA. In contrast to the soluble protein extract, the apoplastic fluid of the DAFP-4 line did not show hexagonal ice crystals, indicating the absence of even slight activity.

The Effect of DAFP(s) on the Freezing Temperature of Transgenic *A. thaliana*

The freezing temperatures of rootless plants of all three types of transgenic *A. thaliana*, compared with the wild type, are shown in Fig. 7a. Both the DAFP-1 plants and the DAFP-(1+4) plants froze at significantly lower temperatures than did the wild type ($p<0.05$). Although the average freezing temperature of the DAFP-4 plants was slightly lower than the wild type, the difference was not

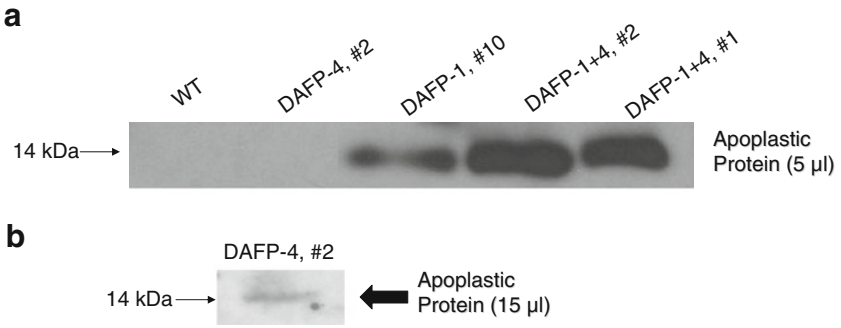

Fig. 5 Western blot analysis of apoplastic protein from transgenic *Arabidopsis thaliana* (T_3 generation). IgG-purified rabbit polyclonal antibody to DAFPs, which reacts with both DAFP-1 and DAFP-4 with the same affinity, was used as the primary antibody. Bands of RubisCO were stained using Coomassie brilliant blue. **a** Western blot of all transgenic lines. Each lane contains 5 µl apoplastic fluid, extracted from leaves and stems. **b** Western blot of DAFP-4-producing *A. thaliana*. Fifteen microliters of apoplastic fluid were loaded

statistically significant. Among transgenic plants, the DAFP-(1+4) lines froze at significantly lower temperatures than both the DAFP-1 and the DAFP-4 lines ($p<0.05$) while the DAFP-1-producing plants froze at lower temperatures than the DAFP-4-producing plants. The DAFP-1 line froze at −15.5±0.2°C, 1.8°C lower than did the wild type. The DAFP-(1+4) line #2 displayed the lowest mean freezing temperature (−16.6±0.2°C), which was 2.9°C lower than the wild type.

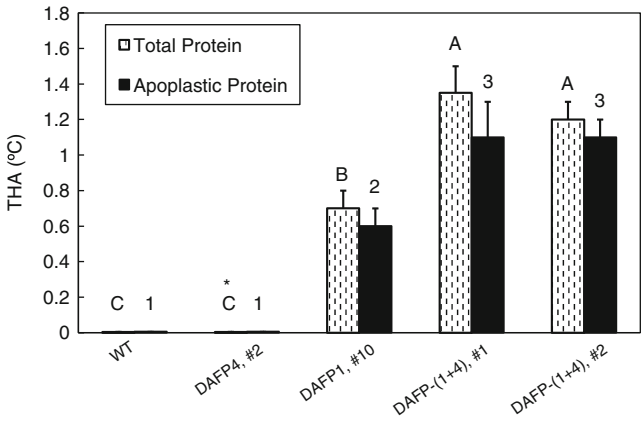

Fig. 6 THA analysis of transgenic *Arabidopsis thaliana*, by the capillary method. Total soluble protein was tested at 50 mg/ml, and the apoplastic fluid was tested at the native concentration. Values are means±standard deviations. Three pooled samples were assayed for each line, and three readings of each sample were recorded. *Numbers and letters above the error bars* indicate statistical significance ($p<0.05$) using the Tukey's multiple comparisons test. Mean values having the same Tukey's ranks (same number or letter) are not statistically different from one another, but are statistically different from those with different ranks (numbers or letters). *Capital letters above the error bars* denote the statistically significant difference, or lack thereof, between total protein samples; numbers indicate the statistically significant difference, or lack thereof, between apoplastic protein samples ($p<0.05$). *Asterisk*, the total protein from DAFP-4 (#2) plant showed hexagonal ice crystals, although no THA was detected

The freezing temperatures of individual leaves from different lines were also recorded (Fig. 7b). The trend was the same as in the whole plants; however, the variation within each line was greater. Leaves from both the DAFP-1 and DAFP-(1+4) lines froze at significantly lower temperatures than did the wild type, and the DAFP-(1+4) lines demonstrated significantly lower freezing temperatures than the DAFP-4 plants ($P<0.05$). Additionally, leaves from only one line of the DAFP-(1+4) plants (#2) froze at a significantly lower temperature than those of the DAFP-1 plants ($p<0.05$). The mean freezing temperatures of the leaves of DAFP-1 line and the DAFP-(1+4) #2 line were 1.6°C and 2.8°C lower than that of the wild type, respectively. Note that the mean freezing temperatures of whole plants were higher in all lines than the freezing temperatures of the single leaves from each line. This was expected because the chance of having an active ice nucleator which initiates freezing at a certain temperature increases with tissue mass.

Profiles of *dafps* in Transgenic *A. thaliana* by Quantitative Real-time PCR

In order to determine the relative expression of *dafp-1* and *dafp-4* in the transgenic *A. thaliana* quantitative real-time PCR was performed. As shown in Fig. 8, the expression of *dafp-4* was 4 fold greater in the 1+4 line, #2 than in the DAFP-4 line, #2; the 1+4 line, #1 was slightly higher than that of the DAFP-4 line, #2 (2-fold greater). The expression of *dafp-1* was higher in the 1+4 lines than in the DAFP-1 line, #10 (3–4-fold).

Absolute quantification was also carried out to compare the expression of *dafp-1* and *dafp-4* in the DAFP-(1+4) lines (Table 3). The expression of *dafp-1* was much higher than *dafp-4* in both of the DAFP-(1+4) lines (approximately 116-fold greater in line #1, and 53 fold greater in line #2). When the THA of solutions of purified DAFP-1 and DAFP-4 was tested with ratios of DAFP-1/DAFP-4 of 53/1 or 116/1,

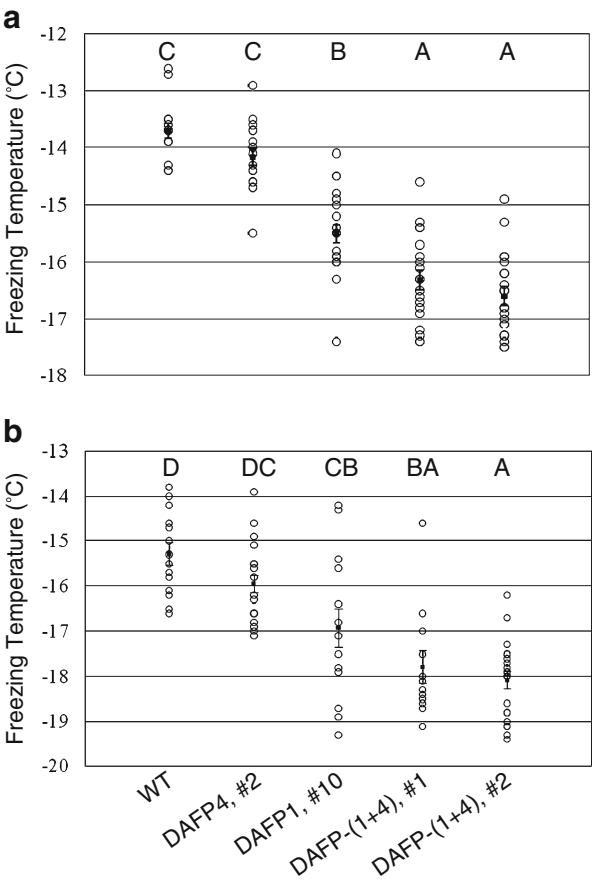

Fig. 7 Freezing temperatures of transgenic *Arabidopsis thaliana* compared with wild type. The freezing temperature of individual plants and leaves were tested using an infrared camera. Each *blue circle* denotes the freezing temperature of an individual plant (**a**) or an individual leaf (**b**). The *black squares* indicate the mean freezing temperature of each line, and the standard errors were shown as *error bars*. *Letters above the error bars* indicate statistical significance ($p<0.05$), or lack thereof, using the Tukey's multiple comparisons test. Mean values having the same Tukey's ranks (same letter) are not statistically different from one another, but are statistically different from those with different ranks (letter). **a** The freezing temperatures of whole plants without roots ($n=15–21$). **b** The freezing temperatures of individual leaves (one leaf from each plant, $n=14–21$)

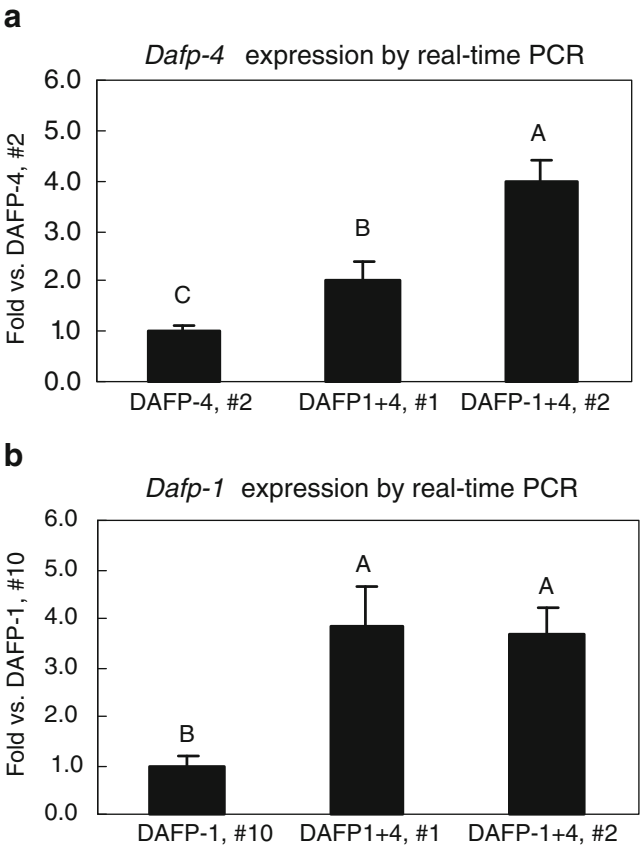

Fig. 8 Transcript level comparison of DAFP-1 and DAFP-4 in transgenic *Arabidopsis thaliana* by real-time PCR. Relative quantification ($\Delta\Delta$Ct method) was carried out for the comparison of the same gene in different lines. Three pools of RNA from each line were used for cDNA synthesis and six replicates of real-time PCR reaction were run for each cDNA sample. The actin-2 gene was used for internal control. Letters above the error bars indicate statistical significance ($p<0.05$), or lack thereof, using the Tukey's multiple comparisons test. Mean values having the same Tukey's ranks (same letter) are not statistically different from one another but are statistically different from those with different ranks (letter). **a** *dafp-4* expression by real-time PCR. **b** *dafp-1* expression by real-time PCR

no THA enhancement was detected. (THA was the same as with DAFP-1 alone.)

Discussion

When cold adapted, many freeze-tolerant plants such as winter rye (*S. cereale* L.), bittersweet nightshade (*Solanum dulcamara*), peach (*P. persica*), and carrot (*Daucus carota*) produce endogenous IAPs but the THA in these plants is very low, typically not more than a few tenths of a degree Celsius or less (Duman 1994; Hon et al. 1994; Smallwood et al. 1999; Wisniewski et al. 1999; Huang and Duman 2002; Griffith and Yaish 2004; Kawahara et al. 2009). IAPs in the apoplast may protect these freeze-tolerant plants by inhibiting recrystallization and/or they may prevent lethal, intracellular ice propagation into the cytoplasm (Pihakaski-Maunsbach et al. 2001; Griffith et al. 2005). In contrast to plants, most IAP-producing animals are freeze avoiding, meaning they die if frozen, and the IAPs which

Table 3 Fold difference of transcript in DAFP-(1+4)-producing plants (lines #1 and #2) by real-time PCR

Line	Transgene	Fold vs. (1+4) #1, *dafp-4*
(1+4) #1	dafp-4	1
	dafp-1	115.9±7.6
(1+4) #2	dafp-4	2.2±0.6
	dafp-1	116.0±8.3

they produce typically exhibit more THA than those of plants, functioning to actually prevent freezing. No freeze-avoiding plants have been reported to contain endogenous AFPs or to demonstrate THA. However, a non-secreted dehydrin with minor THA is present in peach xylem parenchyma cells that avoid freezing and supercool (Wisniewski et al. 1999). As mentioned in the Introduction, several previous attempts have been made to introduce fish or insect AFP genes into transgenic plants, but the freezing temperatures of the transgenics were not lowered, with one exception. This previous study characterized the expression of an AFP gene, with and without its signal peptide, from *D. canadensis* (DAFP-1) in transgenic *A. thaliana* (Huang et al. 2002). THA was detected in total soluble protein extracts from transgenic plants containing either form (with and without a signal peptide sequence) of the *dafp-1* gene. However, THA was only present in the apoplastic fluid of transgenic plants containing DAFP-1 with the signal peptide, indicating that the *D. canadensis* signal peptide of DAFP-1 functions properly in *A. thaliana*. A significant effect of DAFP-1 on the freezing temperature of the transgenic plants was also observed in the Huang et al. (2002) study, with transgenic plants freezing at temperatures 1–3°C lower than the wild type.

The study described here was designed to increase the thermal hysteresis activity of DAFPs in transgenic *A. thaliana* by utilizing the self-enhancing interactions between DAFP-1 and DAFP-4, and to determine if these transgenic plants demonstrated improved capacity to prevent freezing. By constructing two different binary vectors, each having a transgene, we successfully transferred both *dafp-1* and *dafp-4* cDNAs into the genome of *A. thaliana* (Fig. 2). This is, to our knowledge, the first time that two different antifreeze proteins have been expressed in plants. Multiple copies of each gene, including their signal peptides were inserted in *A. thaliana* (Fig. 2), and the expression of both transgenes at both the RNA and protein levels was confirmed by RT-PCR (Fig. 3) and Western blot (Figs. 4 and 5) analyses. The apoplastic fluid of two lines (#1 and #2) of transgenic *A. thaliana* that express both DAFP-1 and DAFP-4 exhibited THAs of 1.35°C (for #1) and 1.20°C (for #2) by the capillary method. These are the highest THAs among the published AFP-producing transgenic plant studies. In addition, the mean freezing temperatures of the two lines were lowered by 2.6–2.9°C compared with the wild type.

Transgenic *A. thaliana* expressing either DAFP-1 or DAFP-4 were also generated for comparison. However, these are not optimal controls for comparison with the transgenic *A. thaliana* expressing both DAFP-1 and DAFP-4 because each line undergoes its own transgenic events and every copy of a transgene inserts randomly into a plant genome. Therefore, the specific position will be different for each copy of the transgene. Position effects can be advantageous if the transgene inserts in an active region that has an enhancer. In contrast, if the transgene resides in the heterochromatin, it is not transcribed. In addition, there are other cases where transgenes are silenced (for a review, see Fagard and Vaucheret 2000). Therefore, multiple copies of a transgene inserted into a genome do not necessarily mean more mRNA or protein will be expressed.

DAFP-1 and DAFP-4 proteins have been found to interact with each other and the interaction enhances the THA of the two proteins in a synergistic way when tested at a 1:1 ratio in vitro (Wang and Duman 2005). Similar synergistic interaction between DAFP-1 and DAFP-4 was also found in transgenic *Drosophila melanogaster*, where *D. melanogaster* containing both DAFP-1 and DAFP-4 showed higher THA compared with *D. melanogaster* containing either DAFP-1 or DAFP-4 alone (Lin et al. 2010). In the present study, the highest THA exhibited in the apoplastic fluids among all the transgenic *A. thaliana* generated was found in plants harboring both *dafp-1* and *dafp-4* genes, suggesting that these two proteins may have interacted to enhance one another in the plants. However, the abundance of *dafp-1* relative to *dafp-4* in the DAFP-1+DAFP-4 lines was 53/1 in DAFP-(1+4) line, #2 and 116/1 in the DAFP- (1+4) line, #1 (Table 3). Moreover, transcript levels of *dafp-1* in the DAFP-1+DAFP-4 lines were 3- to 4-fold greater than that in the DAFP-1 line (Fig. 8). These results make it problematic to determine whether or not the higher activity in the dual DAFP-1/DAFP-4 lines results from the interaction between DAFP-1 and DAFP-4 or from the higher expression of DAFP-1 alone, especially as the ratios for the proteins may be different than those of the transcripts. However, no THA enhancement was detected *in vitro* at DAFP-1/DAFP-4 ratios of 53/1 or 116/1.

It is worth noting that in general the expression of transgenes in the present study at the mRNA level is consistent with the resulting level of protein and these levels correspond with the THA and plant freezing temperature data as well. For instance, based on the quantification of both RNA transcripts and protein, DAFP-4 was expressed at a low level (Table 3; Figs. 4 and 5). Correspondingly, the DAFP-4 line did not show THA but only the induction of hexagonal ice crystals, and this line did not freeze at a significantly lower temperature than the control (Figs. 6 and 7). On the other hand, DAFP-1 was expressed at high levels in all lines that contain *dafp-1*. The THA data and the plant freezing temperature data with regard to DAFP-1 correspond very well for the DAFP-1 line and DAFP-1+DAFP-4 lines.

In terrestrial plants such as *A. thaliana*, studies have found that the translation consensus sequence is A(A/G)(A/C)AAUGGC, where the underlined AUG is the start codon (Lütcke et al. 1987; Rangan et al. 2008). Many researchers

believe that the translation consensus sequence is important in the initiation of translation. In the present study, GAUCCAUGG was used in the *dafp-1* construct and CUAGUAUGG was used for the *dafp-4* construct. Although neither sequence was the same as the consensus sequence, DAFP-1 was expressed at a higher level compared with DAFP-4. Because the quantitative PCR results showed that the mRNA level of DAFP-4 was also much lower than DAFP-1 (Table 3), the difference between the expression of DAFP-1 and DAFP-4 in transgenic *A. thaliana* can possibly be attributed to the promoter of *dafp-4* used. The promoter in vector pE1913-4 did not function well in *A. thaliana* even though it used a superpromoter containing *mas* promoter and activator, and three tandem repeats of *ocs* activator. Consequently, it may be best to use the translation consensus sequence in future studies to enhance translation efficiency.

Despite the higher apoplastic THA obtained in this study (1.35°C in the DAFP-1+DAFP-4 line #1, and 1.2°C in the DAFP-1+DAFP-4 line #2, by capillary method), the temperatures at which these plants froze were only 2.6–2.9°C lower than the wild type. Consequently, although the plant freezing temperature was significantly lower than wild type, it was not lower than the freezing temperature of the transgenic plants expressing only DAFP-1 generated in the earlier DAFP-1 study (Huang et al. 2002). The transgenic *A. thaliana*, in the earlier study showed similarly lower freezing temperatures (1–3°C lower) than the wild type, although the apoplastic THA was only 0.8°C or less in the plants. This implies that even higher THA may be needed to obtain more dramatic effects on the freezing temperature of plants, and thereby more effectively protect sensitive plants from frosts. Another possibility is that more complete expression of the family of DAFPs present in the *D. canadensis* beetles may be required. In the beetles the DAFPs are present as a family of several similar isomers with tissue specific expression (Duman et al. 2002, 2010). However, even the approximately 3°C of protection provided by the DAFPs in these transgenic studies is significant as many late spring or early autumn frosts produce temperatures only slightly below freezing.

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