Acta Physiol Plant
DOI 10.1007/s11738-013-1383-x

ORIGINAL PAPER

Carrot antifreeze protein enhances chilling tolerance in transgenic tomato

Sarma Rajeev Kumar · Rajamani Kiruba · Srinivasan Balamurugan · Hélia G. Cardoso · Arnholdt-Schmitt Birgit · Ahmed Zakwan · Ramalingam Sathishkumar

Received: 28 March 2013 / Revised: 25 August 2013 / Accepted: 30 August 2013

Abstract
Key message **The expression of carrot antifreeze protein enhanced chilling tolerance in heterologous host system tomato and AFP can be a potential gene candidate for producing chilling tolerant crop plants.**
Abstract In an attempt to improve chilling tolerance, the carrot gene encoding the antifreeze protein (AFP) was cloned under the control of constitutive CaMV35S promoter and genetically transformed the tomato var. PKM1 using *Agrobacterium*-mediated genetic transformation. Putative transgenic plants were confirmed by PCR using *AFP*-specific primers and grown to maturity. The integration of *AFP* transgene in the tomato genome was confirmed by Southern blot analysis. The *AFP* gene expression in transgenic plants was determined using semi-quantitative reverse transcription PCR. Upon exposure to chilling stress (4 °C), a significant decrease in membrane injury index was observed in AFP transgenic tomato lines without any phenotypic aberrations when compared with WT plants. Hence, this study clearly proves that the development of chilling tolerant tomato plants will soon become a reality.

Communicated by P. Sowinski.

S. Rajeev Kumar · R. Kiruba · S. Balamurugan · R. Sathishkumar (✉)
Plant Genetic Engineering Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore 641046, India
e-mail: rsathish@buc.edu.in

H. G. Cardoso · A.-S. Birgit
EU Marie Curie Chair, ICAAM, University of Evora, Evora, Portugal

A. Zakwan
Defence Institute of Bio-Energy Research, Haldwani 263139, India

Keywords Antifreeze protein · *Lycopersicon esculentum* · Chilling tolerance · Genetic transformation · Membrane injury index

Abbreviations

AFP	Antifreeze protein
CTAB	Cetyl trimethyl ammonium bromide
MS	Murashige and skoog
sq RT-PCR	Semi-quantitative reverse transcriptase PCR
MII	Membrane injury index

Introduction

Abiotic stresses like cold, drought, soil salinity and heat are the major environmental factors that adversely affect the growth, productivity and quality of crop plants (Movahedi et al. 2012). Cold, drought and salinity stress have been proved to be interconnected and affect the water relations in cellular, tissue, organ and whole plant leading to a series of morphological, physiological, biochemical and molecular changes (Beck et al. 2007). They are the primary cause of crop loss worldwide, reducing productivity and pose a threat to the sustainability of the whole agricultural industry. Among the abiotic stresses, low temperature (mainly 'chilling and freezing stresses') is a major one that affects the plants primarily by dehydration. Chilling stress is injurious to sensitive plant tissues. It cannot increase the fluidity of bio-membranes and result in membrane leakiness leading to cell death. During low-temperature exposure, membrane conductivities are altered, viscosity of water increases and the closure of stomata is blocked resulting in dehydration. It retards metabolism, delays

Published online: 17 September 2013

energy dissipation and induces formation of free radicals resulting in oxidative stress (Beck et al. 2007). The adaptability of plants during stress is through altered gene expression (Kumar et al. 2012). The cold-responsive genes encode proteins that are involved in membrane integrity, several stress-protective proteins (e.g. dehydrins and anti-freeze proteins) and low molecular mass compounds (e.g. sugars, proline and mannitol), which act as osmolytes (Zhu et al. 2012).

Cold tolerance is regulated by multiple genes and many of these candidate genes have been exploited for enhancing the cold tolerance (Park et al. 2004). The genetic modification for enhancing stress tolerance is based on the manipulation of genes that maintain the function and structure of different cellular components. Transgenic technology has emerged as a promising approach for crop improvement.

Antifreeze proteins (AFPs) are a family of proteins capable of protecting organisms from damage caused by freezing conditions by lowering freezing point non-colligatively, whilst leaving the melting point unchanged by a process termed as thermal hysteresis (TH). In the last 40 years, AFPs have been reported from many different kingdoms including fishes (De Vries 1971), bacteria, fungi (Duman and Olsen 1993) and plants (Griffith et al. 1992; Smallwood et al. 1999; Huang and Duman 2002). The two main properties of AFPs are thermal hysteresis (TH) and ice recrystallization inhibition (IRI). The main characteristic feature of AFPs is thermal hysteresis activity (THA) that is widely used as an indicator of AFP activity. The second hallmark feature of AFP is "recrystallization inhibition" because it binds to the surface of ice nuclei and inhibits ice crystal growth (it inhibits the growth of small ice crystals into large ones). This binding is an irreversible process, as any off rate of binding would lead to further growth of ice crystals (Knight and DeVries 1994). A large surface area involves multiple bindings and interactions, all of which would need to be broken simultaneously to release the ligand. In addition to the structural match, binding of a protein to any one of the planes of ice results in irreversible binding that will stop the growth of ice crystals. On the other hand, it has been proposed that the interaction between hydrophobic aminoacids in AFP and membrane lipids contributes to membrane stability during thermotropic phase transition at chilling temperature (Hays et al. 1996). (Tomczak et al. 2001, 2002a, b) proposed a role for the AFP in the stabilization of membranes at low temperature and suggested that AFPs alter the molecular packing of the acyl chains in membrane lipids.

A novel cold-induced AFP was identified from carrot (Smallwood et al. 1999). The theoretical 3D model of carrot AFP showed 10-loop β-helix, 24 tandem repeats of amino acids (PXXXXXLXXLXXLXLSXNXLXGXI) with leucine rich repeats (LRR). The site-directed mutagenesis in carrot AFP ice-binding motif revealed the importance of conserved asparagines in the β-loop and its THA was directly correlated to the length of its asparagine binding site and the size of ice-binding face (Zhang et al. 2004a).

Tomato is one of the major vegetable crops that is grown and consumed in almost all regions of the world. Tomato (*Solanum lycopersicum* formerly *Lycopersicon esculentum*) is a pre-eminent model system for genetic transformation studies, and it is the second *Solanaceous* species that is intensively studied due to its simple diploid genetics, short generation time and availability of rich genetic and genomic resources. The plant genome size is of 950 Mb, encoding ~35,000 genes. Tomato is rich in vitamins A, C, tocopherols, fibre and also lycopene (~20–50 mg/100 g), which is one of the most powerful antioxidants protecting the cells from free radicals. It has become increasingly evident that environmental factors such as drought, salinity, extremes of temperature, light and biotic stresses have profound effects on tomato production. Hence, we have selected a chilling-sensitive plant, tomato, to study the effect of carrot AFP in enhancing chilling tolerance.

The first report of *Agrobacterium*-mediated genetic transformation in tomato was in 1986 (Mc Cormick et al. 1986). Zhang et al. (2004b) showed that tomato plants could not cold acclimate even though they possess CBF genes, but the same has been proved to enhance the stress tolerance in *Arabidopsis*. A number of studies incorporating abiotic stress-tolerant genes in tomato have been reported by different groups (Park et al. 2004; Roy et al. 2006; Khare et al. 2010; Singh et al. 2011; Yarra et al. 2012; Lyu et al. 2012). Nevertheless, any alteration towards the enhanced survival of tomato plants during chilling conditions will represent a major breakthrough in high-altitude farming.

Materials and methods

Gene isolation and cloning in plant expression vector

Seeds of *Daucus carota* var. Kuroda were germinated in commercial soil mix (Keltech Energies, Karnataka, India), regularly nourished with 1/10 strength sterile MS salt solution, maintained at 25 °C under 16 h photoperiod using cool white fluorescent lamps (~50 μmol m^{-2} s^{-1}) and 80–85 % humidity. Genomic DNA (gDNA) was isolated from young leaves of 1-month-old carrot plants following the protocol of Doyle and Doyle (1990). 100 ng of gDNA was used as template for PCR amplification of the full-length *AFP* gene (GenBank Accession AJ131340, *AFP* is a gene lacking introns, showing an ORF with 1.1 kb) using

the gene-specific primers encompassing restriction sites for cloning (Fw: 5′-CAA CCC GGG ATG GTA ATA TTG AAT CA-3′ and Rev: 5′-AGT AGT GAG CTC CTA GCA TTC TGG CAA TGG-3′). PCR was carried out for 35 cycles, each cycle having 60 s at 94 °C, 45 s at 50 °C and 60 s at 72 °C. An initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 10 min were included. The PCR product was purified and cloned first in pDRIVE TA vector (Qiagen PCR cloning kit, Qiagen, CA, USA). The ligated product was then transformed to *Escherichia coli* DH5α and the *AFP* sequence were confirmed by sequencing. The gene was then subcloned under CaMV35S promoter in pBI121 binary vector by replacing *GUS* gene at the *Xma*I and *Sac*I site and pBI121::AFP was then moved to *E. coli* DH5α. Finally, the pBI121::AFP was moved to *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method, which was then used for stable plant genetic transformation.

Agrobacterium-mediated genetic transformation

A single colony of *A. tumefaciens* EHA105 strain harbouring pBI121::AFP vector was inoculated into liquid Luria–Bertani (LB) medium containing 60 mg/l rifampicin and 75 mg/l kanamycin and incubated overnight at 28 °C at 180 rpm. A bacterial solution with OD 0.3–0.4 was used for explants infection.

Seeds of *Lycopersicon esculentum* cv. PKM1 were in vitro germinated in half-strength MS media and 7 days old cotyledonary leaves were used as explants. Cotyledonary leaves around 2 cm length were aseptically excised and inoculated into petri dishes (6 cm diameter) containing solid MS medium (30 g/L sucrose and 8 g/L agar) supplemented with 0.1 mg l^{-1} IAA and 2 mg l^{-1} Zeatin to induce callogenesis (callogenesis medium). Explants (30–40) were inoculated per petri dish in a total of 25 petri dishes (three repetitions were performed). Three days after inoculation, the explants were immersed in the bacterial solution for 10 min, followed by drying in a sterile filter paper for 10 min, transferred to a fresh medium with the same composition and incubated in dark for 3 days. After the co-cultivation period, the explants were briefly washed with sterile water having 300 mg l^{-1} carbenicillin, followed by inoculation in fresh media with the same hormone combination containing 75 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin (selection medium). These cultures were incubated at 22–24 °C with 16 h photoperiod. After 30 days, the developed calli were excised and transferred to the regeneration medium (MS with Gamborg's B5 vitamins supplemented with 0.1 mg l^{-1} IAA, 2 mg l^{-1} Zeatin solidified with 8 g/l agar) containing the selection medium. The cultures were then incubated for 8–10 weeks under same conditions and plants were transferred to fresh media every 15 days. Regenerated shoots were then transferred to shoot elongation medium (same as callus induction media). After 2 months, elongated shoots with 4–6 cm length, were transferred to rooting media (1/2 strength MS media with 0.1 mg l^{-1} IAA solidified with 0.2 % gelrite) devoid of antibiotics and incubated under the same conditions as described above. The well rooted plants were transferred to a sterile mixture of vermiculite and soil (1:1) nourished MS salt solution and subsequently acclimatized to the ex vitro conditions. The hardened putative transgenic lines were subsequently transferred to the containment facility for further growth. Observations were recorded to calculate the percentage of plantlet regeneration.

The genetic transformation experiment was repeated in three independent experiments.

Screening of tomato transgenic plants

gDNA was isolated from young leaves of both the putative transformants and WT tomato plants and *AFP*-specific primers were used for PCR screening. Southern blot was performed for the PCR-positive transgenic lines (AFP 1 and AFP 3) and WT plant as control using 40 µg of gDNA, which was digested with *Xba*I and *Sac*I at 37 °C overnight. The digested samples were resolved in 1 % agarose gel and blotted on positively charged nitrocellulose membrane (BioTrace NT, Pall, USA) by capillary transfer. Hybridization and detection was done using biotin labelling and detection kit (Fermantas, USA). The labelling of probes, hybridization and washing of the membrane were performed as per the manufacturer's instruction. The biotin-labelled *AFP* PCR products were used as probe and detection was done using streptavidin alkaline phosphatase conjugate.

Evaluation of AFP during chilling stress

Plant material

To evaluate the involvement of the *AFP* transcript in tomato plants under chilling treatment, transgenic lines (AFP1, AFP 3 and AFP 3*) and WT tomato plants were exposed to 4 °C for 48 h under a 16 h photoperiod. For RNA extraction, young leaf samples located at the second and third node were harvested and immediately frozen in liquid nitrogen.

Gene expression analysis by reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasy plant mini kit (Qiagen, CA, USA) by following the manufacturer's instructions and on column DNase digestion was

performed using RNase-free DNase (Qiagen Inc, Valencia, CA, USA). First-strand cDNA was synthesized using 2 μg of RNA as per the manufacturer's instruction using RETROscript® First Strand Synthesis kit (Ambion INC, Austin, Texas, USA). One μl of cDNA (diluted 1:10) was used as template for PCR using Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). AFP-specific primers were used to amplify an 80 bp amplicon (Fw: 5′-CGA CAA GCA AGC TTT ACT CCA A-3′ and Rev: 5′-CGT CTG ACA CCC ATG AGT CTG T-3′). β-tubulin was used as reference for gene expression analysis (Fw: 5′-TTA ATT CCC TTC CCA CGT CTT C-3′ and Rev: 5′-TGC TGT GAT CCA CGA GAG GTT-3′ was used to amplify 79 bp). PCR was carried out for 30 cycles (Bio-Rad, Hercules, CA, USA). Each cycle consisted of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. An initial denaturation step at 95 °C for 5 min and a final extension step at 72 °C for 10 min were included. The products were resolved in 2 % agarose gel and stained using ethidium bromide solution (80 ng/ml).

Membrane injury index (MII)

The MII was assayed by quantifying the electrolyte leakage using a conductometer to measure the membrane injury due to alterations in their semipermeable properties. Electrolytic leakage was performed as per Xu et al. (2005). Five leaf discs/plant were collected at 0, 24 and 48 h from leaves located at the two to three nodal regions of the plant. The discs were equilibrated in deionized water for 4 h and initial conductivity was measured. The samples were autoclaved to get all the solutes out from the cells and final conductivity was measured. The conductivity of the solution was measured as follows: EL (%) = initial conductivity/final conductivity × 100. Three replicates were performed per plant and the data obtained were the mean ± SD.

Phenotypic analysis

Phenotypic analysis, which consisted in the evaluation of plant growth, plant height and morphology of flowers and fruits, was performed in transgenic lines and WT plants during and after chilling stress treatment.

Results

Tomato genetic transformation

There is no universal method for plantlet regeneration and genetic transformation, as the responses vary even between the genotypes of the same species. In our study, cotyledonary leaves as a source of explants resulted in efficient transformation and regeneration of the transgenic plantlets, which started to regenerate in 45 days after the co-cultivation period (Fig. 1a–c). About 50–60 % of rhizogenesis was observed after 30 days in rooting media (Fig. 1d). The plants were then transferred to containment facility for further studies (Fig. 1e, f).

Screening of putative transgenic tomato plants by PCR and DNA blot analysis

After kanamycin selection, putative transgenic tomato plants were tested for the presence of transgene. The *AFP* fragment was amplified from the putative transformants by PCR for initial confirmation (data not shown). From the transgenic lines identified by PCR, two lines, AFP 1 and AFP 3, were analysed by Southern blot hybridization using

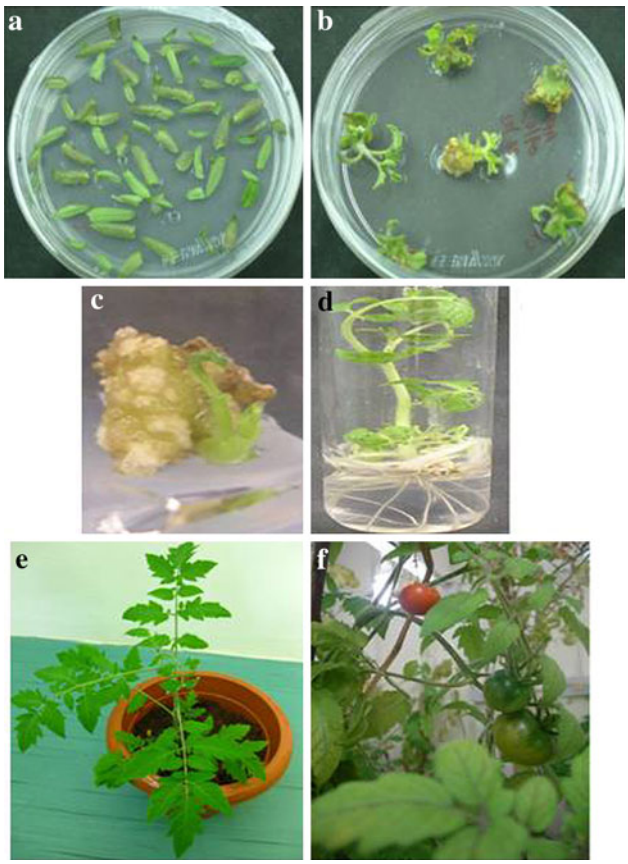

Fig. 1 *Agrobacterium tumefaciens*-mediated genetic transformation in *Lycopersicon esculentum* cv PKM1 using cotyledonary leaves as explant. **a** Preculture of explants in MS with Gamborg's B5 vitamins supplemented with 0.1 mg l^{-1} IAA and 2 mg l^{-1} Zeatin. **b**, **c** Regeneration of putative transgenics in selection media (MS with Gamborg's B5 vitamins supplemented with 0.1 mg l^{-1} IAA, 2 mg l^{-1} Zeatin + 75 mg/l kanamycin and 300 mg/l carbenicillin). **d** Induction of roots in 1/2 MS. **e** Putative transgenic plant in the containment facility. **f** Fruiting in transgenic plants

the *AFP* full-length probe. The results confirmed the stable integration of transgene *AFP* in the transgenic tomato lines analysed (Fig. 2a).

Analysis of *AFP* gene expression by SQ RT-PCR

Three transgenic T_1 lines were selected for gene expression studies. The transgenic lines AFP1, AFP 3 and AFP 3* showed transcript accumulation, which indicated that the AFP was efficiently transcribed in the heterogeneous host system. There was no transcript accumulation in WT plants (Fig. 2b). The *AFP* expression level was stable, with no changes between 0 and 48 h after chilling treatment; however expression level varied between the transgenic lines. These RT-PCR results indicate the genetic stability of the integrated gene in tomato lines.

AFP and MII

In this study, MII in WT and transgenic lines did not show any significant difference at normal condition. However, when the temperature was reduced to 4 °C, the leakage was higher in WT plants and further increased to 60 % after 48 h of stress, whereas in transgenic lines it was maintained at the level of 30 % as a maximum (Fig. 3).

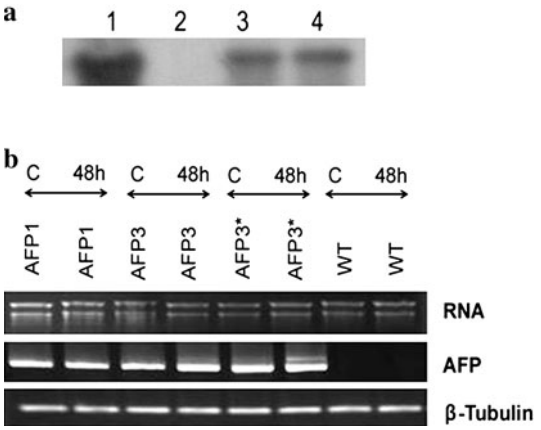

Fig. 2 **a** Southern blot analysis of genomic DNA from transgenic and WT tomato plants. The DNA was digested with *Xba*I and *Sac*I to release the fragment of AFP and the blot was probed with biotin labelled with full length AFP PCR product. *Lane 1* positive control (AFP PCR product), *lane 2* WT DNA, *lane 3* and *4* DNA from transgenic tomato lines AFP 1 and AFP3. **b** Gene expression analysis of *AFP* in transgenic tomato by semi-quantitative RT-PCR. WT and transgenic plants were given chilling stress at 4 °C for 48 h and total RNA was isolated from all the samples. cDNA was synthesized using 2 µg of RNA. β-Tubulin was used as the reference gene. The sqRT-PCR was repeated twice to verify the results. *C* Control sample, *48 h s* sample after 48 h stress

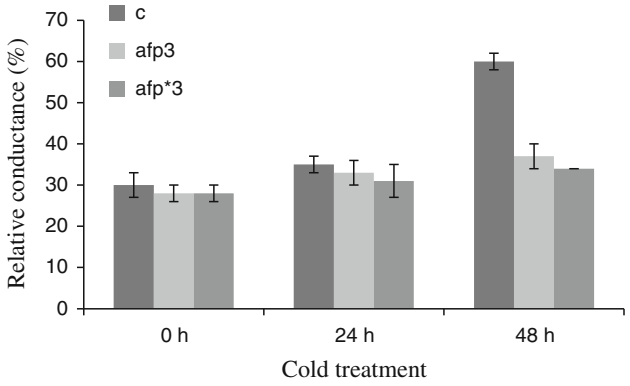

Fig. 3 Effects of low-temperature stress on the electrolyte leakage assay in the transgenic and WT plants exposed to 4 °C for 48 h. Data are the mean ± SD of three replicates

Phenotypic analysis of transgenic lines

The transgenic tomato plants did not show any significant variations in morphological and agronomical characters such as plant growth, plant height, fruit size and shape and flowering period.

Discussion

Development of a highly efficient transformation and regeneration system is a pre-requisite for the genetic improvement of crop plants (Gupta et al. 2012). The source and age of the explants play an important role in inducing in vitro regeneration and genetic transformation (Davis et al. 1991). Cotyledonary leaves were reported to be the best explant for the genetic transformation of tomato (Kaur and Bansal 2010; Khare et al. 2010; Singh et al. 2011). *A. tumefaciens* culture with optical density of 0.2–0.6 at 600 nm was found to be optimal for co-cultivation. Our study corroborated with other reports that the optical density of 0.1–1.0 was essential for efficient transformation in tomato (Mc Cormick et al. 1986; Raj et al. 2005; Singh et al. 2011). Co-cultivation period for 3 days was found to be optimal for effective transformation, while more than 3 days led to overgrowth of bacteria. The aminoglycoside derivative kanamycin (interferes with the protein synthesis) was used for selection of transformants. On selection medium, no transformed shoots showed the symptoms of chlorosis; however, it was observed in non-transgenic shoots 15 days after selection, which resulted in total bleaching due to lack of chlorophyll synthesis and growth inhibition by 30 days. A preliminary study was performed to select the most appropriate concentration of kanamycin and it was found to be 75 mg/l where absolute arrest of growth was observed. The genetic transformation efficiency was found to be 31.2 % (48 plants showing the

amplification of the transgene in a total of 150 plants analysed). This is similar to the report in the var. Pusa Ruby where tomato leaf curl virus coat protein gene was expressed. Sharma et al. (2009) reported the maximum transformation efficiency of 41.4 % and the lowest rate of 14.2 % was reported by Raj et al. (2005).

Xu et al. (2005) reported that carrot AFP driven under CaMV35S resulted in growth retardation in transgenic tobacco plants; however in our study, there were no phenotypic anomalies among the tomato transgenic plants expressing AFP lines during chilling stress. The overall morphology of transgenic plants including the number of flowers and fruits was similar to the WT plants. We also observed that the AFP expressing transgenic lines continued to proliferate with new tissues during the recovery period (after low-temperature stress), whereas the WT plants could not survive.

The RT-PCR results confirmed that AFP gene was expressed in transgenic line forming functional AFP proteins, which protected membranes during chilling stress as indicated by electrolyte leakage assay. The mode of AFP action relies probably on its interaction with membrane lipids, where it alters molecular packing of the acyl chains, as previously proposed (Tomczak et al. 2001, 2002a, b). There is also a report that multiple forms of membrane damage can induce cellular dehydration, and alteration in membrane fluidity will help to prevent dehydration (Steponkus 1984). Tomczak et al. (2002b) AFP inhibits leakage across chloroplast membrane when they are chilled, which is a result of alteration in the acyl chains in the hydrophobic bilayer core (due to AFP lipid interaction). The AFP from different fish species was found to maintain mammalian oocytes and their membranes after exposing to 4 °C. Thus, the AFP possesses the ability to protect cells and membranes during low-temperature stress by interacting with the membrane system (Rubinsky et al. 1991).

To conclude, this is first report of expression of a functional carrot AFP in tomato transgenic plants. From this study, we could confirm that carrot AFP led to chilling tolerance in transgenic tomato plants. Our results clearly prove that carrot antifreeze protein can protect tomato plants at chilling temperature, the effect being probably through the stabilization of membrane system in transgenic plants.

This finding will open up new avenues to investigate whether carrot AFP can confer low-temperature tolerance in other chilling-sensitive economically important crops. Hence, engineered cold-tolerant crops could soon become a reality.

Author contribution RS conceived and planned the work. RKS, RK and SB carried out all the benchwork. RKS prepared the manuscript and RS revised it. HCG and ASB helped RKS to carry out the SQ RT-PCR work. AZ had overseen the work. All authors read and approved the final manuscript. The authors declare that no conflict of interest exists.

Acknowledgments The authors thank Defence Institute of Bio-Energy Research, India for the financial support through a research grant to RS and for a fellowship support to RKS.

References

Beck EH, Fetitig S, Knake C, Hartig K, Bhattarai T (2007) Specific and unspecific responses of plants to cold and drought stress. J Biosci 32:501–510

Davis ME, Lineberger RD, Miller AR (1991) Effects of tomato cultivar, leaf age, and bacterial strain on transformation by *Agrobacterium tumefaciens*. Plant Cell Tis Org Cult 24:115–121

De Vries AL (1971) Glycoproteins as biological antifreeze agents in antarctic fishes. Science 172:1152–1155

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Duman JG, Olsen MT (1993) Thermal hysteresis protein activity in bacteria, fungi and phylogenetically diverse plants. Cryobiology 30:322–328

Griffith M, Ala P, Yang DS, Hon WC, Moffatt BA (1992) Antifreeze protein produced endogenously in winter rye leaves. Plant Physiol 100:593–596

Gupta N, Rathore M, Goyary D, Khare N, Anandhan S, Pande V, Ahmed Z (2012) Marker-free transgenic cucumber expressing *Arabidopsis* CBF1 gene confers chilling stress tolerance. Biol Plant 56:57–63

Hays LM, Feeney RE, Crowe LM, Crowe JH, Oliver AE (1996) Antifreeze glycoproteins inhibit leakage from liposomes during thermotropic phase transitions. Proc Natl Acad Sci USA 93:6835–6840

Huang T, Duman JG (2002) Cloning and characterization of a thermal hysteresis (antifreeze) protein with DNA-binding activity from winter bittersweet nightshade, *Solanum dulcamara*. Plant Mol Biol 48:339–350

Kaur P, Bansal KC (2010) Efficient production of transgenic tomatoes via *Agrobacterium*-mediated transformation. Biol Plant 54:344–348

Khare N, Goyary D, Singh NK, Shah P, Rathore M, Anandhan S, Sharma D, Arif M, Ahmed Z (2010) Transgenic tomato cv. Pusa uphar expressing a bacterial mannitol-1-phosphate dehydrogenase gene confers abiotic stress tolerance. Plant Cell Tis Org Cult 103:267–277

Knight CA, DeVries AL (1994) Effects of polymeric, non-equilibrium "antifreeze" upon ice growth from water. J Crys Grow 143:301–310

Kumar SR, Anandhan S, Dhivya S, Zakwan A, Sathishkumar R (2012) Isolation and characterization of cold inducible genes in carrot by suppression subtractive hybridization. Biol Plant 57:97–104

Lyu JL, Min SR, Lee JH, Lim YH, Kim JK, Bae CH, Liu JR (2012) Overexpression of a trehalose-6-phosphate synthase/phosphatise fusion gene enhances tolerance and photosynthesis during drought and salt stress without growth aberrations in tomato. Plant Cell Tis Org Cult. doi:10.1007/s11240-012-0225-7

Mc Cormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*Lycopersicon esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84

Movahedi S, Sayed Tabatabaei BE, Alizade H, Ghobadi C, Yamchi A, Khaksar G (2012) Constitutive expression of Arabidopsis DREB1B in transgenic potato enhances drought and freezing tolerance. Biol Plant 56:37–42

Park EJ, Jeknic Z, Sakamoto A, Denoma J, Yuwansiri R, Murata N, Chen TH (2004) Genetic engineering of glycine-betaine synthesis in tomato protects seeds, plants and flowers from chilling damage. Plant J 40:474–487

Raj SK, Singh R, Pandey SK, Singh BP (2005) *Agrobacterium* mediated tomato transformation and regeneration of transgenic lines expressing tomato leaf curl virus coat protein gene for resistance against TLCV infection. Curr Sci 88:1674–1679

Roy R, Purty RS, Agrawal V, Gupta SC (2006) Transformation of tomato cultivar Pusa ruby with *bspA* gene from *Populus tremula* for drought tolerance. Plant Cell Tis Org Cult 84:55–67

Rubinsky B, Arav A, Fletcher GL (1991) Hypothermic protection—a fundamental property of "antifreeze" proteins. Biochem Biophys Res Comm 180:566–571

Sharma MK, Solanke AU, Jani D, Singh Y, Sharma AK (2009) A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. J Biosci 34:423–433

Singh S, Rathore M, Goyary D, Singh RK, Anandhan S, Sharma DK, Ahmed Z (2011) Induced ectopic expression of At-CBF1 in marker-free transgenic tomatoes confers enhanced chilling tolerance. Plant Cell Rep. doi:10.1007/s00299-011-1007-0

Smallwood M, Worrall D, Byass L, Elias L, Ashford D, Doucet CJ, Holt C, Telford J, Lillford P, Bowles DJ (1999) Isolation and characterization of a novel antifreeze protein from carrot (*Daucus carota*). Biochem J 340:385–391

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Ann Rev Plant Physiol 35:543–584

Tomczak MM, Hincha DK, Estrada SD, Feeney RE, Crowe JH (2001) Antifreeze proteins differentially affect model membranes during freezing. Biochim Biophys Acta 1511:255–263

Tomczak MM, Hincha DK, Estrada SD, Wolkers WF, Crowe LM, Feeney RE, Tablin F, Crowe JH (2002a) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. Biophys J 82:874–881

Tomczak MM, Vigh L, Meyer JD, Manning MC, Hincha DK, Crowe JH (2002b) Lipid unsaturation determines the interaction of AFP type I with model membranes during thermotropic phase transitions. Cryobiology 45:135–142

Xu W, Liu M, Shen X, Lu C (2005) Expression of a carrot 36 kD antifreeze protein gene improves cold stress tolerance in transgenic tobacco. For Stud China 7:11–15

Yarra R, He SJ, Abbagani S, Ma B, Bulle M, Zhang WK (2012) Overexpression of a wheat Na+/H+ antiporter gene (TaNHX2) enhances tolerance to salt stress in transgenic tomato plants (*Solanum lycopersicum* L.). Plant Cell Tis Org Cult 111:49–57

Zhang DQ, Liu B, Feng DR, He YM, Wang SQ, Wang HB, Wang JF (2004a) Significance of conservative asparagines in the thermal hysteresis activity of carrot antifreeze protein. Biochem J 377:589–895

Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, Thomashow MF (2004b) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. Plant J 39:905–915

Zhu B, Peng RH, Xiong AS, Xu J, Fu XY, Zhao W, Jin XF, Meng XR, Gao JJ, Cai R, Yao QH (2012) Transformation with a gene for myo-inositol *O*-methyltransferase enhances the cold tolerance of *Arabidopsis thaliana*. Biol Plant 56:135–141