

Ectopic overexpression of a salt stress-induced *pathogenesis-related class 10 protein (PR10)* gene from peanut (*Arachis hypogaea* L.) affords broad spectrum abiotic stress tolerance in transgenic tobacco

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Abstract Pathogenesis-related proteins are induced in plants in response to stress, pathogen attack or abiotic stimuli, thus playing a cardinal role in plant defense system. A cDNA containing the full-length ORF, *AhSIPR10* (474 bp, GenBank acc. no. DQ813661), encoding a novel Salinity-Induced PR class 10 protein was isolated from callus cell lines of peanut (*Arachis hypogaea*). Real-time quantitative reverse transcription PCR (qRT-PCR) data showed rapid upregulation of *AhSIPR10* transcription in peanut callus cultures across salinity, heavy metal, cold and mannitol-induced drought stress environments. Likewise, *AhSIPR10* expression was also responsive towards defense/stress signaling molecules salicylic acid (SA), methyl jasmonate, abscisic acid (ABA) and H₂O₂ treatments. Methyl jasmonate or ABA-induced *AhSIPR10* expression was, however, antagonized by SA treatment. A functional role of *AhSIPR10* in alleviation of abiotic stress tolerance was further validated through its over-expression in tobacco.

Analysis of T1 transgenic tobacco plants overexpressing *AhSIPR10* gene showed enhanced tolerance to salt, heavy metal and drought stress through leaf disc senescence, chlorophyll content, seed set and germination assays, thus corroborating a role of salt inducible-PR10 protein in mitigation of abiotic stress-induced damage. Transgenic tobacco lines overexpressing *AhSIPR10* displayed adequate photosynthetic CO₂ assimilation rates under salt, heavy metal and drought stress environments.

Keywords Abiotic stress · Absciscic acid · *Arachis hypogaea* · Jasmonic acid · PR proteins · Ribonuclease · Salicylic acid

Abbreviations

ABA	Absciscic acid
<i>AhSIPR10</i>	<i>Arachis hypogaea</i> salinity-induced PR class 10
JA	Jasmonic acid
MeJA	Methyl jasmonate
MS	Murashige and Skoog medium
PR (10)	Pathogenesis-related (class 10)
SA	Salicylic acid
WT	Wild-type

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Introduction

Pathogenesis-related (PR) proteins are a diverse group of proteins, including chitinases, glucanases, endoproteases and peroxidases, proteinase inhibitors, as well as small proteins such as osmotins, defensins, thionins and lipid transfer proteins (LTPs). Notably, some of the PR proteins are induced de novo upon stress, pathogen attack or abiotic

stimuli, while others are expressed in a tissue- or developmental stage-specific manner and their transgenic over-expression has been reported to give biotic and abiotic stress tolerance (Muthukrishnan et al. 2001; Van Loon et al. 2006; Guan et al. 2010; Chhikara et al. 2011; Subramanyam et al. 2011). Based on their primary structure, serological relationships, and biological activities, PR proteins have been designated to 17 families (Liu and Ekramoddoullah 2006), the largest being the PR10 family with more than 100 members reported across more than 70 plant species and encoded by multigene families (Fernandes et al. 2008; Lebel et al. 2010). Despite significant diversity in the nucleotide and protein sequences, PR10 proteins share several characteristic features such as small size (15–19 kDa), acidic pI, resistance to proteases and cytosolic localization (Markovic-Housely et al. 2003).

Many PR10 proteins share significant amino acid homology with food allergens (Hoffmann-Sommergruber 2002) and several others such as birch Bet v 1, pepper CaPR10, lupin LaPR10, jícama SPE16, peanut AhPR10, pea PR10.1 and maize ZmPR10 exhibit ribonuclease activity (Wu et al. 2003; Park et al. 2004; Chadha and Das 2006; Srivastava et al. 2006; Xie et al. 2010). Involvement of PR10 proteins with cytokinin- and brassinosteroid-mediated signaling cascades suggests a tentative role in regulation of plant architecture and development (Mogensen et al. 2002; Markovic-Housely et al. 2003; Srivastava et al. 2007). Immature flowers of *Vitis vinifera* expressed a large subset of PR10 genes in contrast to stem and intact embryos, suggesting a possible role during sexual reproduction (Lebel et al. 2010). Unusually high affinity of yellow lupine LIPR10.2B for zeatin, suggests that PR10 proteins may act as cellular cytokinin reservoirs in the aqueous cellular environment (Fernandes et al. 2008). Flores et al. (2002) identified a novel PR10 member (ocatin) from *Oxalis tuberosa* accounting for 40–60% of tuber storage proteins, possessing antibacterial and antifungal activities. Several lines of evidence corroborate and implicate a role of PR10 proteins during pathogen infection (Liu et al. 2003, 2006; Chadha and Das 2006; Xie et al. 2010), and under abiotic stress such as drought (Dubos and Plomion 2001), salinity and cold stress (Hashimoto et al. 2004; Kav et al. 2004; Srivastava et al. 2006), extreme temperature (Sule et al. 2004; Bahr-amnejad et al. 2010), ultraviolet radiation (Rakwal et al. 1999), heavy metals (Rakwal et al. 1999; Liu et al. 2006) and herbicides (Castro et al. 2005).

In a previous study on proteome analysis of peanut callus cultures subjected to salt stress, it was noted that several proteins with similarities to the PR10 family members were upregulated (Jain et al. 2006). In this study, we report on successful cloning of a salt stress-inducible cDNA from an *Arachis hypogaea* cell line, *AhSIPR10*, encoding a PR10 protein that is transcriptionally induced

by various abiotic stresses such as salt (NaCl), heavy metals (ZnCl₂), mannitol-induced drought and cold temperature (4°C) in addition to defense related signaling molecules such as, abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA) and H₂O₂. Ectopic expression of *AhSIPR10* in tobacco also augmented tolerance to salt, heavy metal and drought stresses in transgenic plants.

Materials and methods

Cell lines and treatments

Callus lines of *A. hypogaea* were developed from leaf explants as previously described by Jain et al. (2001). Seven-day-old freshly subcultured callus cultures were used for stress treatments and molecular analyses.

Isolation of *AhSIPR10* cDNA clone

Total RNA was extracted using TriPure[®] reagent (Roche, Indianapolis, IN, USA) as per the recommended protocol, and stored at –80°C until further use. Forward and reverse primers for reverse transcription–PCR (RT–PCR) were designed by aligning *PR10* sequences of *Glycine max* (GenBank acc. no. AF529303.1), *Pisum elatius* (GenBank acc. no. U65422.1), *P. sativum* (GenBank acc. no. U65420.1), *Medicago truncatula* (GenBank acc. no. Y08641.1), *Lupinus luteus* (GenBank acc. no. AF170091.1) and *L. albus* (GenBank acc. no. AB070618.1). Five µg total RNA was reverse-transcribed using the AccuScript[®] High Fidelity reverse transcriptase (Stratagene, La Jolla, CA). *AhSIPR10* was amplified using first strand cDNA template and gene specific primers (Table 1). The thermal cycling (MJ Research, Waltham, MA, USA) protocol entailed activation of Platinum[®] *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C, primer annealing at 56°C for 15 s, and extension at 72°C for 30 s each. The amplification reactions were finally extended for 10 min at 72°C and held at 4°C. The PCR product was cloned in pGEM-T Easy cloning vector (Promega, Madison, WI, USA) followed by transformation into *E. coli* DH5α cells (Invitrogen, Carlsbad, CA, USA) and five different clones were sequenced at the DNA sequencing facility (Microsynth, Balgach, Schweiz). *18S rRNA* was included as an internal control in RT–PCR. Multiple sequence alignment analyses were performed using and MegAlign 6.1 suit.

Real-time quantitative RT–PCR (qRT–PCR) analyses

Quantitative RT–PCR was performed using the Brilliant II SYBR[®] Green qPCR mix on a *Mx3000P* platform

Table 1 List of gene-specific primers used for polymerase chain reaction (PCR)

Gene	Forward primer (seq. 5' → 3')	Reverse primer (seq. 5' → 3')
<i>AhSIPRI0</i> ^a	ATTCTAGAATGGGCGTCTTCACTTTCGAG	CGAAGCTTCTAATATTGAGTAGGGTT
<i>AhSIPRI0</i> ^b	TGAAGGACACAACGGAGGATCCA	CCTTGAAGAGAGCTTCACCCCT
<i>AhSIPRI0</i> ^c	ATGTCCA7GGATGGGCGTCTTCACTTTCGAG	GATGACTAGTTAATATTGAGTAGGGTTGG
18S rRNA	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
<i>EF1a</i>	AGTTTGCTGAGCTCCAGACCAAGA	TCCCTCACAGCAAACCTTCCAAGT

^a *AhSIPRI0* primers used for RT–PCR and cDNA cloning

^b Cloning cDNA insert in pCambia vector

^c qRT–PCR

18S rRNA and *EF1a* primers were used for internal controls for RT–PCR and qRT–PCR, respectively

(Stratagene, Agilent Technologies, Santa Clara, CA) and primers as mentioned in Table 1. The PCR reactions were prepared according to the manufacturer's instructions and contained 200 nM of both the forward and reverse gene-specific primers and 2 µl of the fivefold diluted RT reaction in a final volume of 25 µl. The thermal cycling protocol entailed activation of SureStart *Taq* DNA polymerase at 95°C for 15 min. The PCR amplification was carried out for 40 cycles with denaturation at 94°C for 10 s, and primer annealing and extension at 56 and 72°C for 30 s each, respectively. Optical data were acquired following the extension step, and the PCR reactions were subject to melting curve analysis beginning at 55–95°C, at 0.2°C s^{−1}. Elongation factor 1-alpha (*EF1a*) (GenBank acc. no. EZ748096) was used as the reference gene for normalizing the transcript profiles. The real time PCR data were calibrated against the transcript levels in control callus, following the 2^{−ΔΔC_t} method for relative quantification of transcript abundance (Livak and Schmittgen 2001). The data are presented as average ± SD of three independently made RT preparations used for PCR run, each having 3 replicates. Gene specific primers used for qRT–PCR analyses are listed in Table 1. The RT–PCR products were cloned in TOPO[®] 2.1 (Invitrogen, Carlsbad, CA, USA) and sequenced to confirm fidelity of the amplification reaction.

Southern blot analysis

Genomic DNA was isolated following the protocol of Murray and Thompson (1980). Ten µg DNA was digested overnight with restriction enzymes *NcoI* and *SpeI*, and size fractionated on 0.8% agarose gel. The DNA was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) using the alkaline transfer protocol and UV cross-linked (Sambrook et al. 1989). The membrane was pre-hybridized at 58°C for 2 h in 0.5 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, and 7% SDS. [α -³²P]dCTP labeled full-length PCR-amplified *AhSIPRI0* probe was prepared by random priming method using the Amersham

Megaprime[™] DNA Labeling System (Amersham Biosciences, Piscataway, NJ, USA), denatured and added to the fresh pre-hybridization solution for 18 h at 58°C. The membrane was washed sequentially in 3 × SSC and 0.1% SDS, 0.5 × SSC and 0.1% SDS, and 0.1 × SSC and 0.1% SDS for 30 min each. Scanning and recording of images was performed with a phosphorimager (FUJI FLA-5000, FUGIFILM, Tokyo, Japan).

Agrobacterium-mediated transformation of tobacco

The *AhSIPRI0* cDNA was amplified from callus cell lines with primers containing the *NcoI* and *SpeI* restriction sites nested within the forward and reverse primers (Table 1), respectively. The PCR-amplified product was appropriately restricted with *NcoI* and *SpeI*, sequenced and subcloned between respective sites in *pCAMBIA-1302* vector to yield *pCAM-PR10*, containing 35S promoter, hygromycin (*hptII*) and kanamycin, as plant and bacterial selection markers, respectively. Following mobilization of *pCAM-PR10* into *Agrobacterium tumefaciens* strain LBA4404, tobacco (*Nicotiana tabacum* cv. Xanthium) leaf sections were transformed according to Horsch et al. (1985). Putative transgenic plants (T0) were regenerated in the presence of 20 mg l^{−1} hygromycin and further screened by PCR and Southern blot analyses. The seeds from T0 plants were germinated on hygromycin-containing medium to select for the T1 transgenic lines.

Stress tolerance assays for transgenic tobacco lines

To monitor effects of abiotic stress conditions on *AhSIPRI0* overexpressing tobacco plants, 7-day-old tobacco seedlings germinated on basal MS (Murashige and Skoog 1962) medium were transferred to the same with NaCl (200 mM), ZnCl₂ (5 mM) or mannitol (100 mM) amendments for salinity, heavy metal, and drought stress treatments, respectively. Likewise, 7-day-old seedlings were also transplanted in plastic pots (15 × 15 cm in size) containing

agropeat and vermiculite (3:1, v/v) under controlled environmental conditions (28/20°C day/night) and irrigated with 1,000 ml water every day. Leaf disc punches from young fully expanded leaves were floated on liquid MS basal medium containing NaCl (200–600 mM), ZnCl₂ (5–20 mM), and mannitol (200–600 mM). Chlorophyll content in the leaf discs was estimated after 3 days according to the procedure of Arnon (1949). All experiments were repeated at least three times with different transgenic lines and presented as average $\pm$ SD.

Measurement of photosynthetic CO₂ assimilation rates

For photosynthetic CO₂ assimilation measurements, 15-day-old tobacco plants maintained under greenhouse conditions (as described previously) were irrigated with either 400 mM NaCl or 10 mM ZnCl₂ solution, every alternate day. For drought stress treatment, irrigation was restricted to 300 ml water every day, as described by Rivero et al. (2009). Photosynthetic CO₂ assimilation rates were measured with an LI-6400 instrument (Li-Cor, Lincoln, NE), using the following parameters: 28°C leaf temperature, 21% O₂, 1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, 40% relative humidity, and from 0 to 1,200 μbar CO₂ concentration (1 bar = 100 kPa). The data were collected for eighth and ninth fully expanded young leaves per plant ($\approx$ 50 days old), three plants per treatment, and presented as average $\pm$ SD.

Results

Cloning and characterization of *AhSIPR10* cDNA

Salt stress-induced transcriptional upregulation of *AhSIPR10* was observed in callus cell lines cultured on medium amended with 200 mM NaCl (Fig. 1) and the full-length cDNA was cloned in pGEM-T Easy vector. *AhSIPR10* cDNA (GenBank Acc. no. DQ813661) contains a full-length open reading frame (ORF) of 474 bp, encoding a putative protein of 157 amino acid residues. Deduced amino acid sequence for AhSIPR10 predicted a

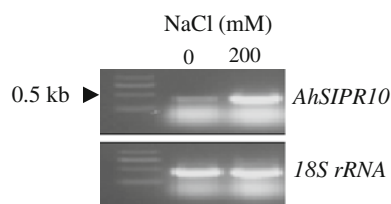


Fig. 1 a RT–PCR amplification of *AhSIPR10* in callus cell line of *A. hypogaea* maintained on MS medium with or without 200 mM NaCl. 18S rRNA was included as internal control

molecular mass of 16.9 kDa with an acidic pI of 5.14. NetPhos (Blom et al. 1999) prediction for putative phosphorylation sites returned four serine, three threonine and two tyrosine residues, implicating its involvement in phosphorylation/dephosphorylation events. A possible nucleic acid binding function is supported by the presence of glycine-rich conserved motif GXGGXG (position 45–50), known as the P-loop (phosphate binding loop) that is frequently found in protein kinases as well as in nucleotide-binding proteins, was discernible (Saraste et al. 1990). A phosphate-binding site may be a likely place for binding of a RNA phosphate group that may be correlated with a ribonucleolytic activity of the protein. Evidence exists to show involvement of the mitogen-activated protein kinase (MAPK) and protein phosphatase in PR10 protein expression (Jwa et al. 2001; Rakwal et al. 2001; Xiong and Yang 2003). Predicted AhSIPR10 sequence also revealed presence of 12 (out of 13) strictly conserved residues among the PR proteins from various species (Jwa et al. 2001), and four conserved Lys53 Glu95, Glu147 and Tyr149 residues required for ribonucleolytic activity (Chadha and Das 2006; Liu et al. 2006) (marked by arrowheads and asterisk, respectively, Fig. 2a). Finally, intracellular and cytosolic localization of the AhSIPR10 protein was confirmed based on absence of any signal peptide or membrane-binding domains in line with other members of PR10 family (Van Loon et al. 2006).

Multiple sequence alignment analyses (LaserGene 6, MegAlign 6.1) showing phylogenetic affiliations of *AhSIPR10* and several other PR10 family members clearly established that most PR10 family members tend to cluster together with the homologues of the same plant family groups (Fig. 2b), probably reflective of multiple and independent gene duplication events in the common ancestors, or presuming a strong concerted evolution of species specific *PR10* loci (Radauer et al. 2008; Lebel et al. 2010).

Abiotic stress-induced expression of *AhSIPR10* gene

Sequence alignment data had revealed no significant sequence similarity to a previously identified PR10 protein from peanut roots (Chadha and Das 2006). However, AhSIPR10 and a peanut allergen Ara h 8 (Mittag et al. 2004) showed 93 and 89% homology at the nucleotide and amino acid level, respectively. Whereas, AhSIPR10 and another isoform variant of Ara h 8 (Riecken et al. 2008) were 96% similar at both nucleotide and amino acid level. Sequence specific primers used for quantitative RT–PCR analyses were carefully designed to span the region of relative diversity between the three genes, and the qRT–PCR products were cloned and sequenced to ascertain the fidelity of PCR reaction. Furthermore, qRT–PCR analyses showed that

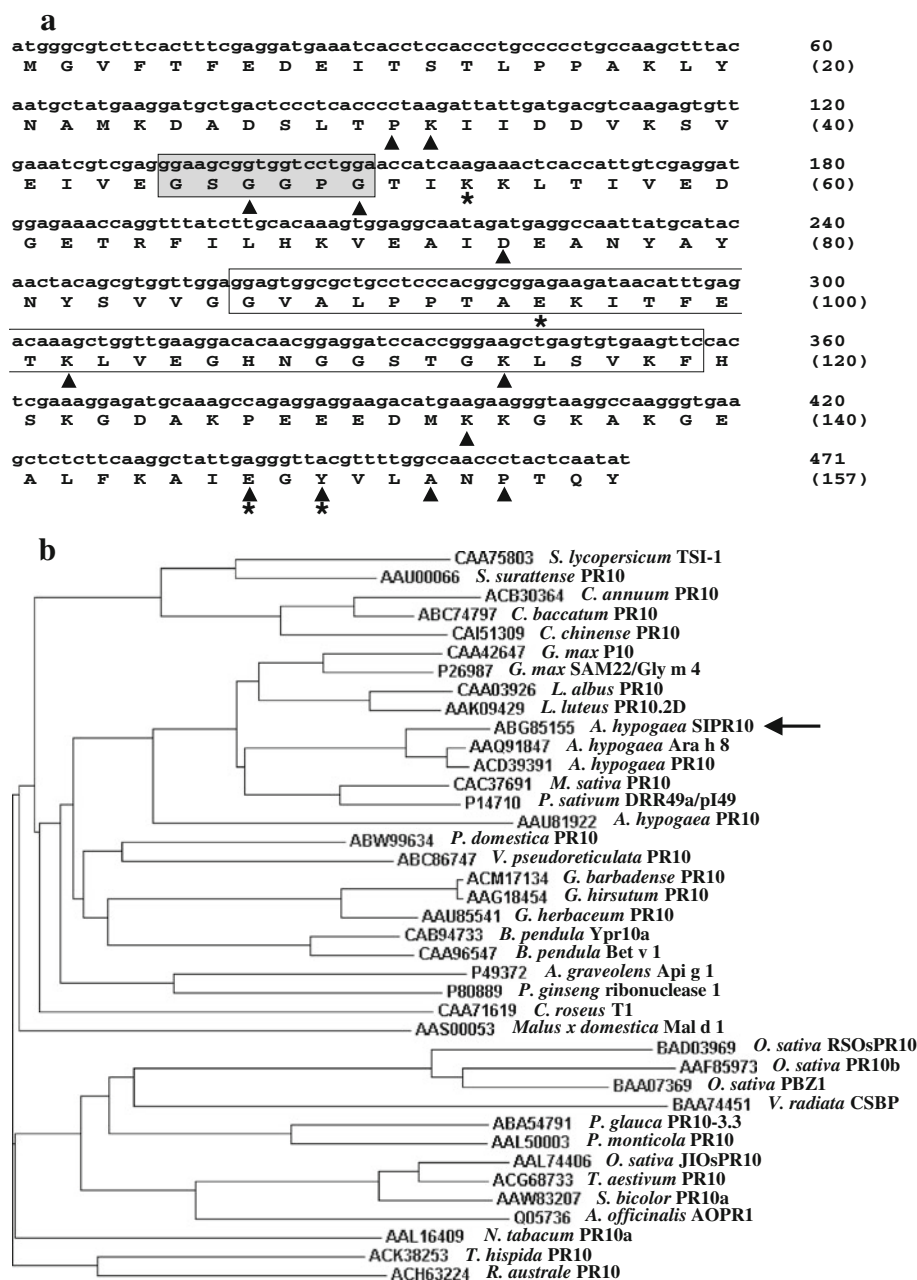


Fig. 2 **a** cDNA and predicted amino acid sequence of *AhSIPR10*. Glycine-rich P-loop motif and the Bet v 1 domain are highlighted (filled and open boxes, respectively). The residues conserved among PR proteins and those essential for ribonuclease activity are marked by arrowheads and asterisk, respectively. **b** A phylogenetic tree showing the respective affiliations of various PR10 proteins from higher plants. The GenBank accession numbers for the PR10 sequences used for phylogenetic analyses are ABG85155, AAQ91847, AAU81922 and ACD39391 (*A. hypogaea*), CAA42647 and P26987 (*G. max*), CAA03926 (*Lupinus albus*) and AAK09429 (*L. luteus*), CAC37691 (*Medicago sativa*), P14710 (*Pisum sativum*), BAA74451 (*Vigna radiata*), ACB30364 (*Capsicum annuum*), ABC74797 (*C. baccatum*)

and CAI51309 (*C. chinense*), ACM17134 (*Gossypium barbadense*), AAU85541 (*G. herbaceum*) and AAG18454 (*G. hirsutum*), CAA75803 (*Solanum lycopersicum*) and AAU00066 (*S. surattense*), AAL16409 (*N. tabacum*), CAA71619 (*Catharanthus roseus*), CAB94733 (*Betula pendula*) and CAA96547 (*B. pendula*), ABW99634 (*Prunus domestica*), AAS00053 (*Malus x domestica*), ABC86747 (*Vitis pseudoreticulata*), ACK38253 (*Tamarix hispida*), ACH63224 (*Rheum australe*), P80889 (*Panax ginseng*), BAD03969, AAL74406 and BAA07369 (*Oryza sativa* Japonica group), and AAF85973 (*O. sativa* Indica Group), ACG68733 (*Triticum aestivum*), AAW83207 (*Sorghum bicolor*), P49372 (*Apium graveolens*), Q05736 (*A. officinalis*), ABA54791 (*Picea glauca*), and AAL50003 (*Pinus monticola*)

salt stress-induced transcriptional upregulation was evident only in case of *AhSIPR0*, as compared to very low basal abundance for either variant of Ara h 8 (data not shown).

Time-course qRT-PCR analyses data is presented to examine the effects of abiotic stress conditions on *AhSIPR10* gene expression. Transcriptional upregulation of

AhSIPR10 gene in callus cell line was evident as early as 24 h in response to 400 mM NaCl, 5 mM ZnCl₂ and 400 mM mannitol-induced water deficit stress (Fig. 3). However, transcriptional upregulation of *AhSIPR10* observed under cold temperature (4°C) treatment at 24 h was transient and only moderately higher transcriptional activity, above that of control callus, was maintained later along 72 h. The stress-induced increase in *AhSIPR10* transcription was not affected by dark incubation (data not shown). Likewise, elevated *AhSIPR10* transcription was discernible following treatment with stress/defense pathway signaling molecules including 100 μM ABA, 100 μM MeJA, 0.5 mM SA and 200 μM H₂O₂ (Fig. 4). Notably, ABA, SA and MeJA-induced increase in *AhSIPR10* level was further enhanced by salt (400 mM NaCl) and drought (400 mM mannitol) treatments. However, no such additive effect of salt or drought treatment was observed on H₂O₂-induced upregulation of *AhSIPR10* transcription. Interestingly, antagonistic effects of ABA and MeJA were observed on SA-induced expression of *AhSIPR10*, both in the presence or absence of 400 mM NaCl (Fig. 5).

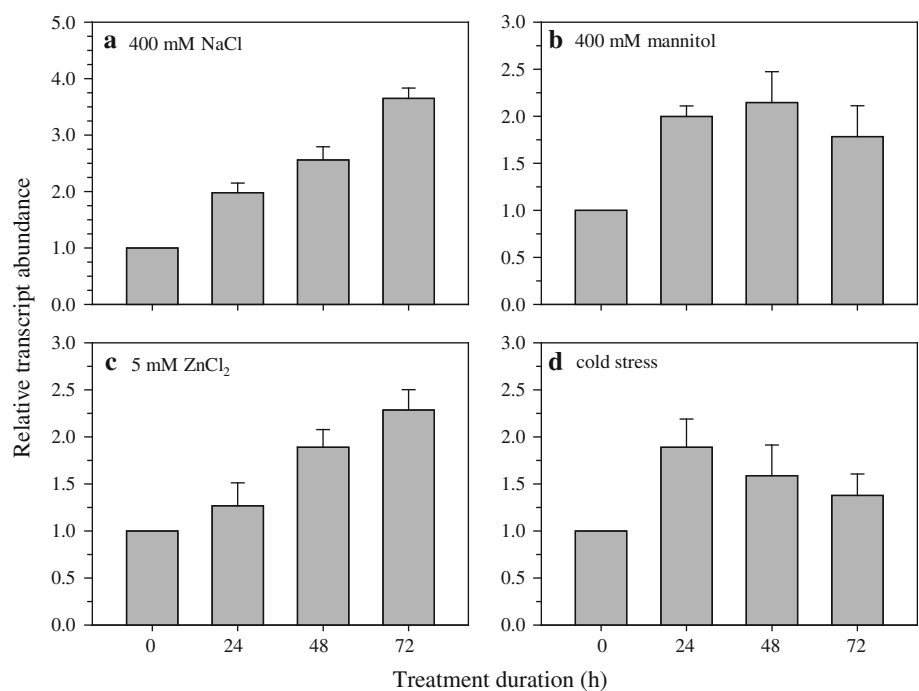
Overexpression of *AhSIPR10* in transgenic tobacco enhances abiotic stress tolerance

A functional role of *AhSIPR10* gene in alleviation of abiotic stress-induced damage was further accredited through overexpression in transgenic tobacco. Following *Agrobacterium*-mediated transformation, putative hygromycin

resistant regenerants were screened for transgene integration by PCR, Southern blot hybridization and RT-PCR in young fully expanded leaves. Finally, 30 independently transformed T0 transgenic lines were identified and grown to reproductive maturity. Transgenic T0 and the T1 lines were similar in morphology and growth characteristics to the wild-type untransformed plants. Molecular evidence for stable transgene integration and expression in a few selected T1 transgenic events is shown in Fig. 6a–c.

Figure 7a illustrates the ameliorative effects of overexpression of *AhSIPR10* on survival of 7-day-old wild-type and the T1 transgenic (line S7) seedlings under salt-stress conditions. Wild-type (WT) and S7 seedlings were morphologically indistinct under ambient growth conditions. However, NaCl affected seedling growth and development in a dose-dependent manner (data not shown). WT seedlings showed stunted growth and chlorosis symptomatic of compromised survival under salinity stress. Figure 7b summarizes the data on survival of T1 transgenic seedlings in presence of 200 mM NaCl. The data on salt stress tolerance were further corroborated by sustained vegetative growth and reproductive fitness as indicated by viable seed set, seed number and seed weight of transgenic lines continuously irrigated with 200 mM NaCl solution (data not shown). Similarly, adequate survival and growth differential of T1 seedlings over WT controls was discernible in the presence of mannitol- or heavy metal-induced stress (Fig. 7c–f). The seedling survival rates for select T1 transgenic events were 34–66, 30–53 and 38–56% higher

Fig. 3 Time-course (0–72 h) qRT-PCR analysis showing transcriptional activation of *AhSIPR10* across **a** 400 mM NaCl, **b** 400 mM mannitol, **c** 5 mM zinc chloride and **d** 4°C cold stress treatments in the cell lines of *A. hypogaea*. *EF1a* was used as the reference gene and the data were calibrated relative to the transcript levels in control callus prior to stress treatment (at 0 h). The data are presented as average ± SD of three independently made qRT preparations used for PCR run, each having 3 replicates



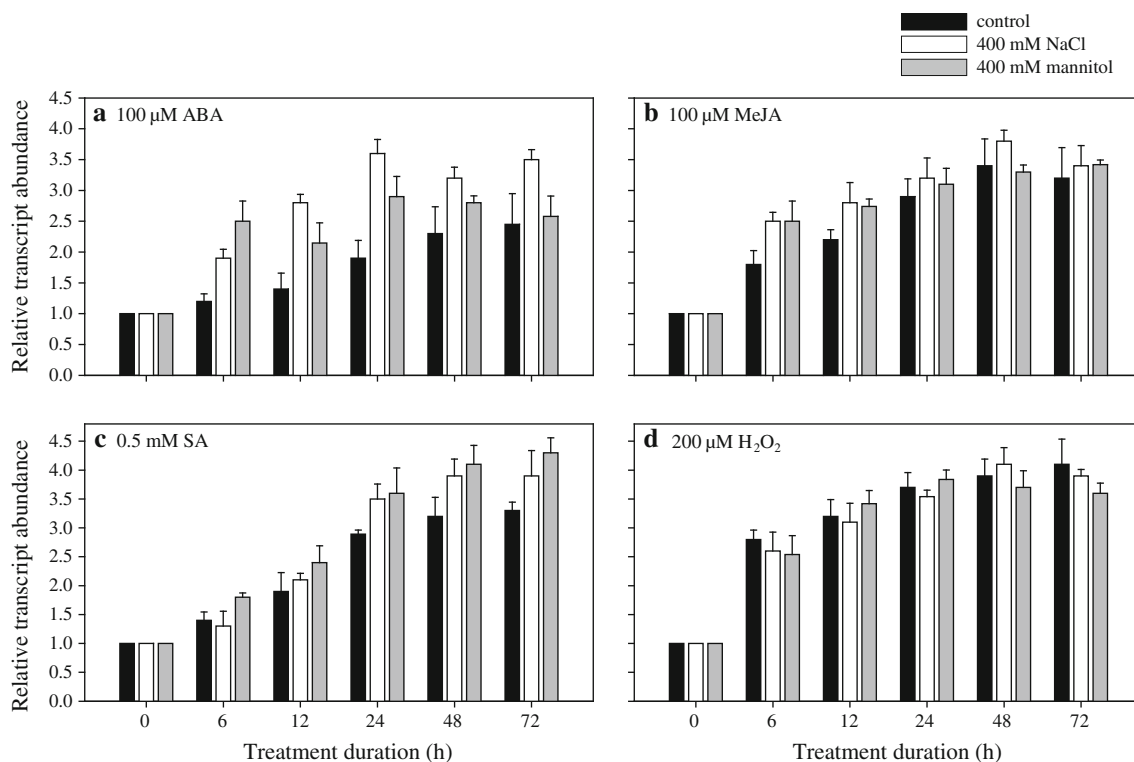


Fig. 4 Time-course (0–72 h) quantitative RT-PCR analysis showing transcriptional activation of *AhSIPR10* in the cell lines of *A. hypogaea* in response to **a** 100 μ M ABA, **b** 100 μ M MeJA, **c** 0.5 mM SA and **d** 200 μ M H_2O_2 treatments. *EF1a* was used as the reference gene and

the data were calibrated relative to the transcript levels prior to elicitor or stress treatment (at 0 h). The data are presented as average $\pm$ SD of three independently made RT preparations used for PCR run, each having 3 replicates

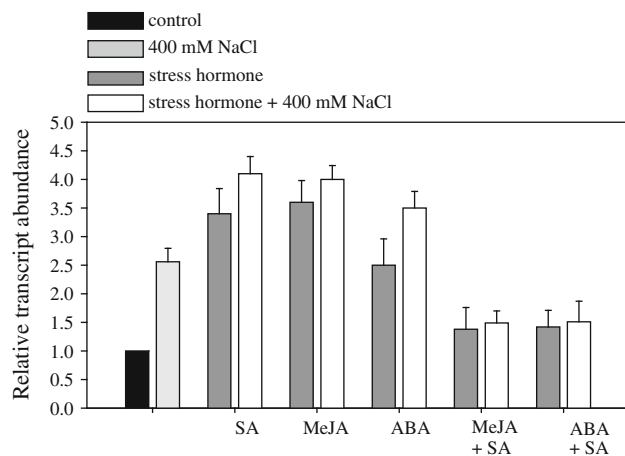


Fig. 5 Quantitative RT-PCR data showing antagonistic affect of ABA and MeJA on SA-induced expression of *AhSIPR10* in the cell lines of *A. hypogaea*. Seven-day-old freshly subcultured callus was transferred to fresh MS medium amended with 400 mM NaCl either alone or in combination with 100 μ M ABA, 100 μ M MeJA or 0.5 mM SA, as indicated. *EF1a* was used as the reference gene and the data were calibrated relative to the transcript levels in control callus (at 48 h). The data are presented as average $\pm$ SD of three independently made RT preparations used for PCR run, each having 3 replicates

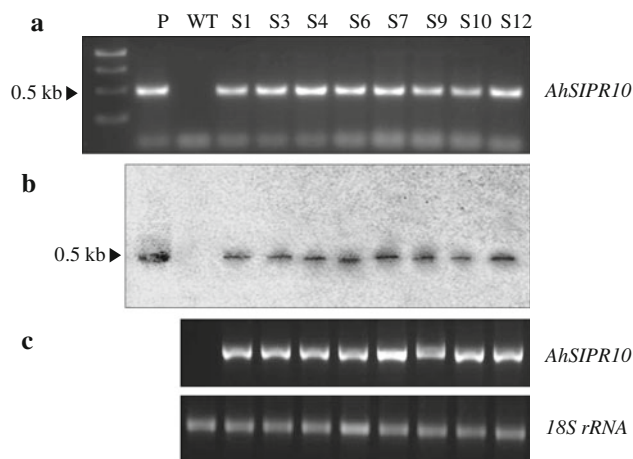


Fig. 6 **a** Genomic PCR, **b** southern blot and **c** RT-PCR analyses confirming stable integration and expression of *AhSIPR10* in young fully expanded leaves of transgenic tobacco plants (P, *pCAM-PR10* plasmid, see “Materials and methods”, WT wild-type untransformed tobacco control, S1–S12, independently transformed T1 transgenic events.)

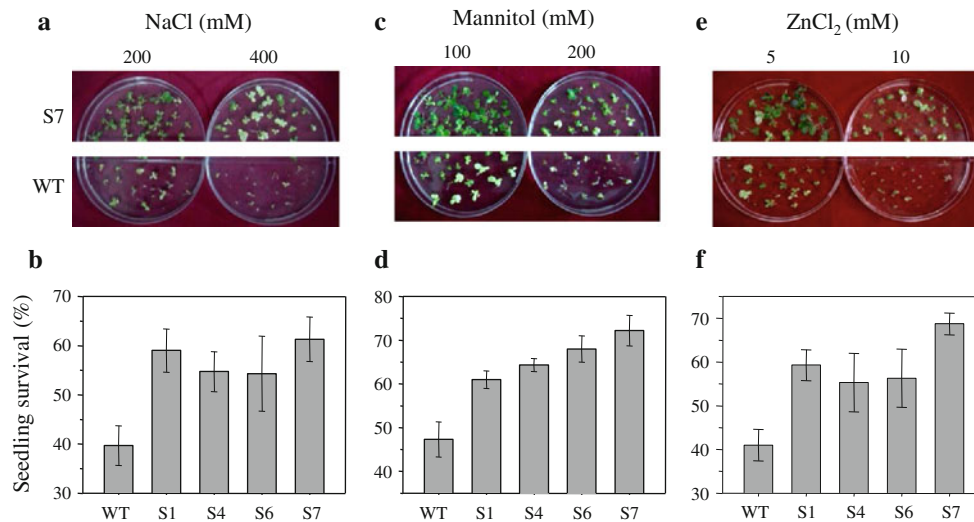


Fig. 7 Survival of 7-day-old tobacco seedlings under abiotic stress conditions imposed by **a** NaCl, **c** mannitol and **e** ZnCl₂. The quantitative data were scored after 15 days of treatment at **b** 200 mM

NaCl, **d** 100 mM mannitol and **f** 5 mM ZnCl₂, and presented as mean $\pm$ SD of three independent experiments (WT wild-type untransformed tobacco control; S1, S4, S6, S7, T1 transgenic line.)

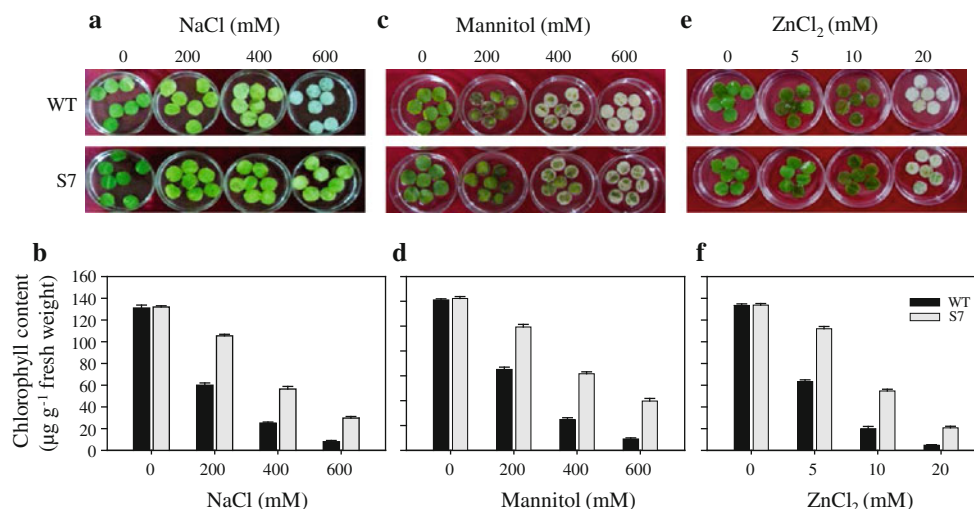


Fig. 8 Tobacco leaf disc senescence assay showing bleaching and loss of chlorophyll under **a, b** 200–600 mM NaCl **c, d** 200–600 mM mannitol and **e, f** 5–20 mM ZnCl₂ stress. The data were scored after

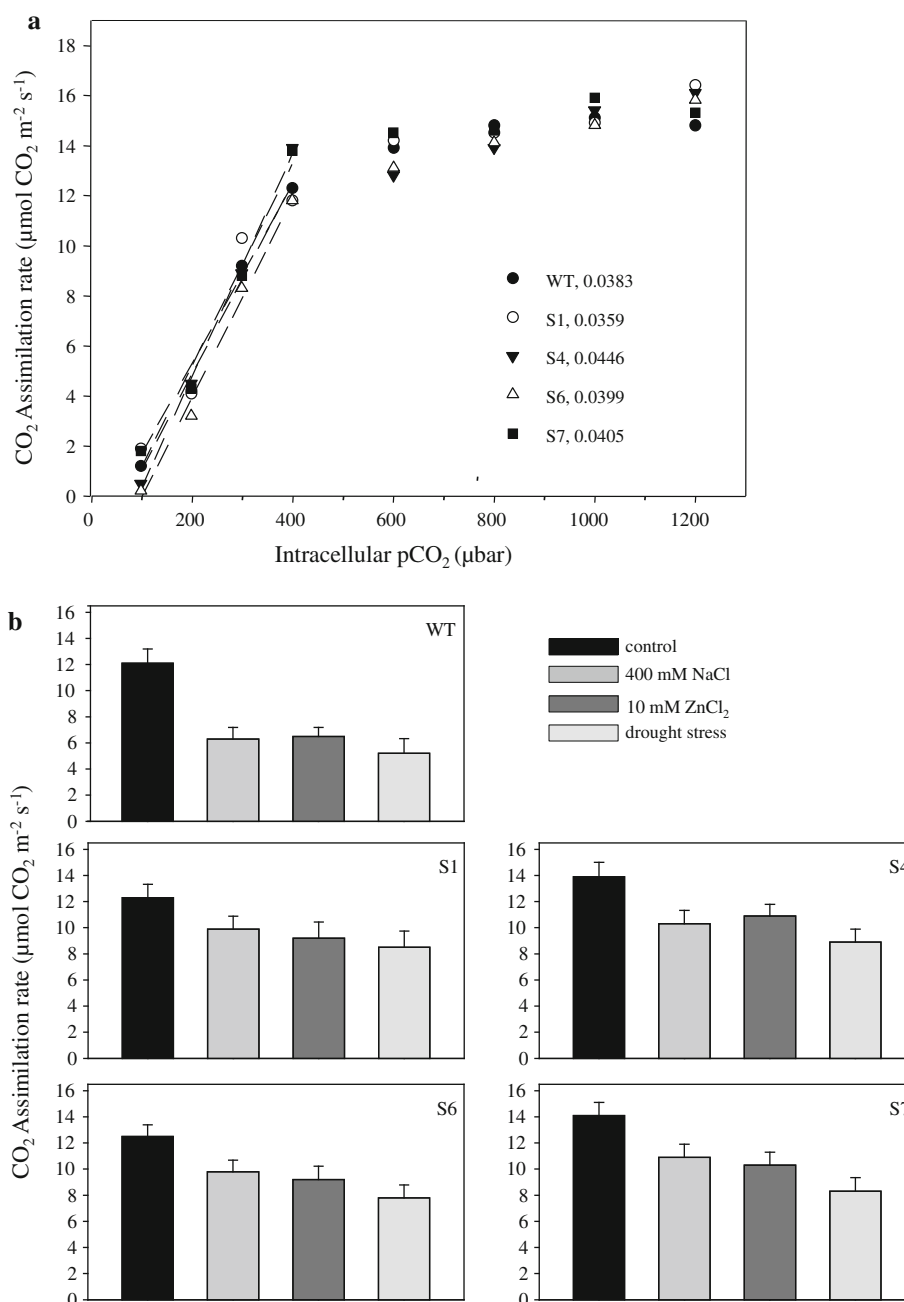
3 days of treatment, and presented as mean $\pm$ SD of three independent experiments (WT wild-type untransformed tobacco control; S7, T1 transgenic line.)

than WT controls in presence of 200 mM NaCl, 100 mM mannitol and 5 mM ZnCl₂, respectively. Additionally, leaf disc senescence assays showed bleaching and significant loss of chlorophyll in WT control leaf discs as compared to S7 transgenic leaves under high salt (200–600 mM NaCl) (Fig. 8a, b), mannitol (200–600 mM) (Fig. 8c, d) and heavy metal (5–20 mM ZnCl₂) (Fig. 8e, f) treatments, thus implicating a role of *AhSIPR10* overexpression in mitigating stress-induced damage to the photosynthetic apparatus, health and vigor of transgenic plants. Similar data were obtained for transgenic lines S1, S4 and S6 (data not shown).

Overexpression of *AhSIPR10* maintains optimal photosynthetic CO₂ assimilation rates in transgenic tobacco under abiotic stress conditions

The transgenic lines overexpressing *AhSIPR10* were also subject to gas-exchange measurements for determining photosynthetic CO₂ assimilation rates. The initial slopes of the CO₂ response curves (measured between 100 and 400 μbar) were comparable for the WT and transgenic tobacco lines, reflective of the comparable amount and activity of rubisco under optimal non-stress environments (Fig. 9a). However, at 350 μbar leaf CO₂ concentration,

Fig. 9 a CO₂ response curves for transgenic tobacco lines overexpressing *AhSIPR10* under 21% O₂. The initial slopes of photosynthesis rates (dotted lines) determined from linear regression of the CO₂ assimilation data across 100–400 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \mu\text{bar}^{-1}$ are noted, and represent comparable photosynthetic efficiencies of transgenic tobacco lines and WT untransformed control under non stress conditions. **b** Effect of abiotic stress environments on photosynthetic CO₂ assimilation rates (at 350 μbar leaf CO₂ concentration) in transgenic and WT tobacco plants. The stress treatments were as described in “Materials and methods”. The data were collected for eighth and ninth fully expanded leaves per plant, and presented as average $\pm$ SD for three plants per treatment. (WT wild-type untransformed tobacco control; S1–S7, T1 transgenic lines)



untransformed WT control plants displayed significantly compromised photosynthetic rates, 47.9, 46.2 and 58.7 decrease following 400 mM NaCl, 10 mM ZnCl₂ and drought stress treatments, respectively (Fig. 9b). Transgenic lines sustained adequate CO₂ assimilation rates, with only 19.5–25.8, 21.6–26.9 and 30.9–41.13% reduction in photosynthetic gas exchange under similar salt, heavy metal or drought-induced stress environments, respectively. Notably, *AhSIPR10*-mediated arbitration of drought stress effects on leaf photosynthesis rates were less pronounced in comparison to salt or heavy metal stress.

Discussion

In the present study, a novel salt-inducible PR10 cDNA (*AhSIPR10*) from a callus cell line of peanut was isolated and characterized. The deduced *AhSIPR10* protein revealed structural and functional conservation of all the hallmark features of known PR10 proteins, such as small size, acidic pI, putative phosphorylation sites as well as a putative nucleic acid binding motif. Several PR10 proteins, including *AhSIPR10* and *Ara h 8*, contain the plant polyketide cyclase/dehydrase-like signature domain

(polyketide_cyc, Pfam:PF03364) that contains a minimal Bet v 1 (birch pollen allergen)—like fold, an evolutionary ancient, versatile and ubiquitous domain implicated in binding of large hydrophobic ligands (Radauer et al. 2008). Polyketide cyclase domain containing proteins catalyze the cyclisation of polyketides (poly- β -keto adducts of short-chain carboxylic acids) in the biosynthesis of a diverse group of compounds including pigments, antibiotics and anti-tumor drugs, and are also involved in lipid transport.

Transcriptional induction of *AhSIPR10* transcripts across various abiotic stress treatments confirmed an involvement in plant defense mechanisms under abiotic stress conditions. Drought stress-induced accumulation of *PR10* transcripts was observed in maritime pine (Dubos and Plomion 2001), barley (Muramoto et al. 1999) and birch (Paakkonen et al. 1998). While *PR10* transcriptional activity was induced by cold and salt stress in hot pepper (Hwang et al. 2005), and during cold-hardening in western white pine (Liu et al. 2003), *SsPR10* expression was reduced by cold treatment in *Solanum surattense* (Liu et al. 2006). In a recent study on an arctic adapted plant species *Oxytropis*, *PR10* genes were among those overexpressed along with defensin and cold dehydrin genes (Archambault and Stromvik 2011). Notably, even though accumulation of *PR-10c* protein was discernible in response to heavy metal stress, *PR10c* proteins did not directly confer metal-tolerance in birch (Koistinen et al. 2002).

Induction of *AhSIPR10* was observed in a time-dependent manner following ABA, MeJA, SA and H_2O_2 treatment in peanut cell lines, that was further enhanced by salt as well as mannitol treatments. Ample evidence exists to show crucial role of several plant growth regulators and elicitor molecules such as ABA, SA, jasmonic acid (JA) and ethylene in adaptive responses to abiotic and biotic stresses (Singh et al. 2002). ABA is involved in many aspects of water-limiting stresses such as drought, salt and cold stress, whereas, JA function is mainly attributed to wounding and pathogen response. Previous studies also reported up-regulation of *PR10* genes by JA in rice (Rakwal et al. 1999; Jwa et al. 2001; Hashimoto et al. 2004) and saffron (Gomez-Gomez et al. 2011). Induction of *PR10* transcripts in lily (Wang et al. 1999) and *S. surattense* (Liu et al. 2006) was observed by both ABA and JA. However, no effect of ABA application was observed on expression of jasmonate-inducible (Jwa et al. 2001) and root specific (Hashimoto et al. 2004) *PR10* genes in rice. Root-specific rice *PR10* gene (*RSOsPR10*) transcripts accumulated rapidly across drought, NaCl, JA and probenazole treatments, but not by exposure to low temperature, ABA or SA (Hashimoto et al. 2004). Xie et al. (2010) studied the differential expression of two *PR10* genes *ZmPR10.1* and *ZmPR10* in *Zea mays* and observed that SA, $CuCl_2$, H_2O_2 , wounding, cold and dark treatments upregulated, whereas,

ABA transiently down regulated *ZmPR10.1* and *ZmPR10* expression. However, expression of both *ZmPR10s* was upregulated briefly, but reduced when exposed to treatments such as kinetin, gibberellic acid, MeJA and NaCl. SA inhibited MeJA and ABA induced expression of *AhSIPR10* in peanut callus cultures. Given that SA and JA biosynthetic pathways are antagonistically inhibited by JA and SA, respectively, evidence for an antagonistic relationship of SA and JA in regulation of *PR* gene expression also exists (Niki et al. 1998; Gupta et al. 2000; Salzman et al. 2005 and the references therein). However, comparative transcriptome analyses revealed one-way and mutually antagonistic, as well as synergistic effects on regulation of SA and MeJA responsive genes in sorghum (Salzman et al. 2005). *AhSIPR10* gene was transiently upregulated and antagonistically downregulated later on due to combined SA + MeJA, in comparison to either SA or MeJA treatments. Notably, a type 2C protein phosphatase homolog implicated in negatively regulating ABA responses was synergistically upregulated by combined MeJA and SA treatments (Salzman et al. 2005). Recently, a regulatory component of ABA receptor complex in *Arabidopsis*, RCAR1 sharing structural similarity with birch pollen *PR10* protein Bet v 1, was shown to mediate ABA-dependent inactivation of type 2C protein phosphatases (ABI1 and ABI2) that negatively regulate ABA responses (Ma et al. 2009). In essence, while an axiomatic role of *PR10* genes in plant defense mechanisms against biotic or abiotic stress environments is imperative, a concerted adaptive role of *PR10* proteins is manifested through diversely regulated *PR10* isoforms (Liu and Ekramoddoullah 2006) along with an intricate and discreet involvement of various interconnecting signaling pathways.

A role of *AhSIPR10* in alleviation of abiotic stress was functionally validated through genetic manipulation of tobacco. Several independent transgenic events were obtained following *Agrobacterium*-mediated transformation, and confirmed for transgene integration and expression.

Transgenic tobacco plants over-expressing *AhSIPR10* gene showed higher tolerance to salt, mannitol and heavy metal stress as indicated by seed germination, leaf disc senescence and chlorophyll estimation data. Previously, amelioration of the growth inhibitory effects of salinity and cold stress during germination and early seedling development has been reported in transgenic *Brassica napus* and *Arabidopsis thaliana* plants constitutively over-expressing a pea *PR10* (*PR10.1*) and an ABA-inducible *PR10* gene *ABA17*, respectively (Srivastava et al. 2004, 2006, 2007). As observed from stress-induced expression profiles of *AhSIPR10* in cell lines, it is safe to presume that constitutive expression of *AhSIPR10* protein alleviates electrophilic and oxidative stress in tobacco plants exposed to saline, heavy metal, or drought stress environments,

presumably mediated through ABA and/or JA-mediated signaling cascades. Oxidative stress-induced activation of *PR10* gene promoters in *A. thaliana* and *Asparagus officinalis* (Mur et al. 2004) lends support to the above argument. The transgenic tobacco lines also displayed higher CO₂ assimilation rates under salt, heavy metal and drought stress conditions. It is plausible that PR10 proteins, by virtue of their high binding affinity for cytokinins (Fernandes et al. 2008), may support adequate photosynthetic responses through provision of bioactive cytokinins under stress conditions. A role for cytokinins has earlier been implicated in promoting photosynthesis (Haisel et al. 2008) and protection of photosynthetic apparatus (Rivero et al. 2009) in transgenic tobacco, and polarization of sink-source relationship in favor of younger leaves (Cowan et al. 2005) during drought stress conditions. Furthermore, cytokinins have been shown to stimulate stomatal opening and supersede stress-induced ABA-mediated effects on stomatal closure and leaf abscission (Pospíšilová and Dodd 2005) and induction of *PR* genes (Pasquali et al. 2009).

In conclusion, we reiterate that cell lines offer a tangible system to investigate molecular responses to stress environments at the cellular level. To the best of our knowledge, this is the first report of isolation of a salt-inducible *PR10* gene from a callus cell line, and its functional accreditation in imparting tolerance to salt, drought, heavy metal and cold temperature stress. Further characterization of *AhSIPR10* gene and its regulation under ambient and stress environments will enhance our understanding of molecular cross-talk operative between various signaling pathways mediating plant defense responses. Further efforts for engineering abiotic/biotic stress tolerance into economically important crop plants by transformation of *AhSIPR10* gene, are currently underway in our laboratory.

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