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Improved drought and salt stress tolerance in transgenic tobacco overexpressing a novel A20/AN1 zinc-finger *"AlSAP"* gene isolated from the halophyte grass *Aeluropus littoralis*

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Abstract We describe here the isolation of a novel gene, designated *AlSAP,* from *A. littoralis* in a first step to exploit the potential of this halophyte grass as a genetic resource to improve salt and drought tolerance in plants and, particularly, in cereals. The *Aeluropus* genome contains a single *AlSAP* gene which has an intron at its 5'UTR. Sequence homology analysis showed that the AlSAP protein is characterized by the presence of two conserved zinc-finger domains A20 and AN1. *AlSAP* is induced not only by various abiotic stresses such as salt, osmotic, heat and cold but, also by abscisic acid (ABA) and salicylic acid (SA). Tobacco plants expressing the *AlSAP* gene under the control of the duplicated CaMV35S promoter exhibited an enhanced tolerance to abiotic stresses such as salinity (350 mM NaCl), drought (soil Relative Water Content (RWC) = 25%), heat (55°C for 2.5 h) and freezing (−20°C for 3 h). Moreover, under high salt and drought conditions, the transgenic plants were able to complete their life cycle and to produce viable seeds while the wild-type plants died at the vegetative stage. Measurements of the leaf RWC and of the root and leaf endogenous Na^+ and K^+ levels in *AlSAP* transgenic lines compared to wild-type tobacco, showed an evident lower water loss rate and a higher Na^+ accumulation in senescent-basal leaves, respectively. Finally, we found that the steady state levels of transcripts of eight stress-related genes were higher in *AlSAP* transgenic lines than in wild-type tobacco. Taken together, these results show that *AlSAP* is a potentially useful candidate gene for engineering drought and salt tolerance in cultivated plants.

Keywords *Aeluropus littoralis* · *AlSAP* · Salt stress · Drought stress · Heat stress · Freezing stress · Transgenic tobacco

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Introduction

As plants have a sessile nature, they are constantly exposed to various biotic and abiotic (e.g., drought, salinity, high and low temperature and light intensity) stresses, which have a great impact on their growth and productivity. To cope with these stresses, plants trigger a network of events. These events start with stress signal perception, followed by transduction cascades that eventually lead to the expression of target genes which participate in stress tolerance (Shinozaki and Yamguchi-Shinozaki 2000) by promoting morphological, biochemical and physiological changes (Pastori and Foyer 2002).

The "Stress Associated Protein" (SAP) gene family is composed of genes encoding proteins containing A20/AN1 zinc-finger domains. Their number ranges from 19 in *Populus trichocarp*, 18 in *Oryza sativa* (OsSAP) and

Sorghum bicolour, 14 in *Arabidopsis thaliana* (AtSAP), 11 in *Zea mays*, 10 in *Physcomitrella patens* and *Vitis vinifera*, to 3 in *Chlamydomonas reinhardtii* (Jin et al. 2007; Vij and Tyagi 2008). The A20/AN1 protein family has been divided into 2 types. Type I harbours the traditional pattern $Cx_2Cx_{9-12}Cx_{1-2}Cx_4Cx_2Hx_5HxC$. Type II has an expanded pattern $Cx_4Cx_{9-12}Cx_{1-2}Cx_4Cx_2Hx_5HxC$ (Jin et al. 2007). Moreover, most of type I members lack intron and have an A20 domain while most of type II members have a single intron but do not exhibit an A20 domain. It has been demonstrated that most rice type I SAP genes are induced by one or more abiotic stresses and perform functions in a stress-specific or tissue-specific manner (Jin et al. 2007; Vij and Tyagi 2006). The term 'zinc finger' represents the sequence motifs in which cysteines and/or histidines coordinate a zinc atom(s) to form local peptide structures required for their specific functions (Takatsuji 1998). The zinc finger domain enables different proteins to interact with or bind to DNA, RNA or other proteins. Some classes of zinc-finger motifs such as TFIIIA- and GATA-types (in most cases) are part of the DNA-binding domains of transcription factors. However, other classes such as LIM- and RING-finger types are mostly implicated in protein–protein interactions (Takatsuji 1998).

In animals, the family of proteins containing A20/AN1 zinc-finger domain is well-characterized and plays a central role in regulating the immune response by acting as a negative regulator of inflammation and apoptosis (Heyninck and Beyaert 2005; Hishiya et al. 2006; Huang et al. 2004). The A20 zinc-finger domain characterized by multiple Cys2/Cys2 finger motifs was first identified in the TNFα-inducible protein of the human endothelial cells (Dixit et al. 1990; Opipari et al. 1990). On the other hand, one putative zinc-finger domain AN1 was identified in the proteins encoded by the *Xenopus laevis* animal hemisphere 1 (AN1) maternal RNA (Linnen et al. 1993). This AN1 zinc-finger domain is usually associated with the A20 zinc-finger. Such type of proteins is present in all eukaryotes (Evans et al. 2004).

Recently, the A20/AN1 zinc-finger domain proteins have been shown to be involved in stress response in plants. However, their cyto-protective molecular mechanism remains elusive (De Valck et al. 1999; Evans et al. 2004; Lee et al. 2000). Yet, Vij and Tyagi (2008) suggested "these proteins most probably belong to the regulatory class of proteins in the stress signalling cascade as they lack any typical nuclear localization signal". In animals, such proteins have already been shown to function in the cytosol. These proteins may use their zinc-finger domains for protein–protein interactions as shown in the case of the human ZnF216 protein (Scott et al. 1998). The *indica* rice OsSAP1 was identified as the first plant protein containing both zinc-finger domains A20 (present at the N-terminus) and AN1 (present at the C-terminus). It was found that the *OsSAP1* transcript is induced in response to different types of stresses including cold, desiccation, salt, submergence, heavy metals, wounding and ABA (Mukhopadhyay et al. 2004). It was also shown to be an intron-less and present as a single copy gene in the rice genome. Furthermore, overexpression of this gene in transgenic tobacco plants increased their tolerance to cold, salt, and dehydration stresses (Mukhopadhyay et al. 2004; Vij and Tyagi 2006). More recently, it has been shown that expression of *OsSAP8*, a member of the SAP gene family in rice, is induced by various abiotic stress treatments like *OsSAP1*. Subcellular localization of OsSAP8-GFP fusion protein indicated that OsSAP8 is a cytoplasmic protein. Yeast two-hybrid analysis indicated that A20 and AN1 zinc-finger domains of OsSAP8 interact with each other. Taken together, these data prove that SAP gene family products are cytoplasmic proteins and might carry out their functions via protein–protein interactions mediated by A20 and AN1 zinc-finger domains. Overexpression of *OsSAP8* in transgenic rice and tobacco plants conferred tolerance to high salt (800 mM NaCl), drought (water deficit for 23 days during anthesis) and cold (4 ± 1°C for 4 days) stresses (Kanneganti and Gupta 2008). On the other hand, overexpression of the rice zinc finger A20/AN1 gene *ZFP177* (identical to *OsSAP9)* in tobacco plants led not only to an increased tolerance to both high and low temperature and H_2O_2 stresses but also to an over sensitivity to dehydration and salt stresses (Huang et al. 2008).

In an objective of improving drought and salt tolerance in cereals, especially wheat and barley, we investigate the potential of *A. littoralis* to isolate genes involved in key mechanisms of environmental stress response. *A. littoralis* is a perennial, monocotyledonous halophyte (can endure up to 600 mM NaCl) growing in dry salty areas or marshes (Li and Liu 1994; Gulzar et al. 2003). It is also a C4 photosynthesis plant, salt-secreting and rhizomatous. This plant is diploid ($2n = 2X = 14$) and has a relative small genome around 342 Mb (Zouari et al. 2007). Thus, *A. littoralis* has the potential to become a precious genetic resource, not only for understanding the molecular mechanisms of stress-responses in monocots but also for improving tolerance to abiotic stresses in economically important crops. To understand the genetic basis of salt tolerance mechanisms in *A. littoralis* at the genomic level, we have isolated, sequenced and annotated 492 transcripts with a size ranging from ESTs (Expressed Sequence Tag) to full length cDNAs (Zouari et al. 2007). Among these, we report here the isolation and characterization of a gene (*AlSAP*, **DQ885218**) encoding for an A20/AN1 zinc- finger protein induced by salt, drought, cold, heat, ABA and SA. By stress assays, we have found that the overexpression of the *AlSAP* gene in tobacco improved

tolerance to continuous salt and drought stresses under greenhouse conditions.

Materials and methods

Plant materials

Aeluropus littoralis and *Nicotiana tabacum var. Xanthi* were used in this study. Seeds of an *A. littoralis* ecotype were collected from salt marshes near "Sfax", a coastal town in the middle of Tunisia.

AlSAP gene isolation

The *AlSAP* cDNA was isolated from an *A. littoralis* root cDNA library (cloned in pDNR-LIB vector, CLONTECH) prepared by using plants stressed for 15 days with 300 mM NaCl as described previously (Zouari et al. 2007). The total cDNA sequence of 707 bp was obtained by end sequencing (ABI 3100 automatic DNA sequencer, APPLIED BIOSYSTEMS) of the cDNA clone using M13F and M13R (supplementary Table 1) primers. The sequence was deposited to the Genbank with the accession number **DQ885218** (UniProtKB: **A1YAQ3**). Bioinformatics analysis has revealed significant homology with different A20/AN1 zinc-finger proteins that belong to the Stress Associated Protein (SAP) gene family and that are isolated from different plants. Therefore, the gene was designated as *AlSAP* (*A. littoralis* Stress Associated Protein). The primers AlF5' and AlR3' (supplementary Table 1) were used to amplify the genomic clone of *AlSAP* gene. The generated fragment of 2,230 bp was cloned in the pGEM T-easy vector (PROMEGA) and three different positive clones were sequenced using SP6 and T7 primers.

Stress assay in *Aeluropus littoralis*

Surface sterilized seeds of *A. littoralis* (1% sodium hypochlorite solution for 15 min, followed by six washings with autoclaved Milli-Q water) were germinated in Eppendorf tubes containing 500 µl half strength MS (Murashige and Skoog 1962) solid medium under 16 h photoperiod at 25°C. The tubes containing the seedlings were perforated and their caps were cut when the plants' roots reached their bottom. Later, the seedlings were transferred to a nutrient solution as described by Zouari et al. (2007) and grown for 2 months before treating them with different stress factors: high salinity (300 mM NaCl), high osmotic pressure (10% PEG 8000), low temperature (4°C) and high temperature (37°C). The plants were also treated with 100 µM abscisic acid (ABA) and 10 mM salicylic acid (SA) for hormonal stress. Plants were sampled at 0, 1, 2, 3 and 6 days after each treatment, frozen in liquid nitrogen and stored at −80°C for RNA extraction.

Semi-quantitative RT-PCR

Total RNA was isolated from the *A. littoralis* plants subjected to various stress treatments using Trizol reagent (INVITROGEN) according to the manufacturer's protocol. The RNA was treated with DNase I (MBI, FERMENTAS) at 37°C for 15 min in order to remove the remaining genomic DNA. For semi-quantitative RT-PCR analysis, 5 µg of treated total RNA was reverse-transcribed using the oligo-dT (18 mer) primer and SuperScript reverse transcriptase (INVITROGEN) according to the manufacturer's instructions. The PCR amplification of *AlSAP* gene was performed using 2 µl of 1/10th dilution cDNA as a template and two specific primers Al5'UTR and Al3'UTR (supplementary Table 1). The reaction included an initial 3 min of denaturation at 94°C, then 30 cycles of 30 s at 94°C, 40 s at 55°C and 1 min at 72°C, and finally 10 min extension at 72°C. As an internal control, a fragment of actin gene (380 bp) was amplified using the following primers ACTF and ACTR (supplementary Table 1). To ensure reproducibility, experiments were repeated three times with similar results.

The transcript accumulation was monitored for eight stress-related genes in transgenic lines and wild-type tobacco plants. These genes encode catalase (CAT1, **U93244.1**) (Takahashi et al. 1997), manganese superoxide dismutase (MnSOD, **AB093097.1**), ascorbate peroxidase (APX, **U15933.1**) (Orvar and Ellis 1995), osmotin (**M2979**) (Singh et al. 1989) and four encoding group 2 LEA proteins NtERD10A (**AB049335.1**), NtERD10B (**AB049336.1**), NtERD10C (**AB049337.1**), NtERD10D (**AB049338.1**) (Kasuga et al. 2004). Total RNA was extracted from wild-type tobacco and transgenic lines and then reverse-transcribed using oligo-dT (18 mer) as described above to generate first strand cDNA. The amplification of stress-related genes was performed using 2 µl of 1/10th dilution cDNA as a template and two specific primers with 30 PCR cycles. This reaction included an initial 3 min denaturation at 94°C, then 30 cycles of 30 s at 94°C, 40 s at 60°C, 45 s at 72°C, and finally 10 min extension at 72°C. The following gene-specific primers were used: *CAT1* (cat1F, cat1R), *MnSOD* (MnsodF, MnsodR), *APX* (apxF, apxR), *osmotin* (osmF, osmR), *NtERD10A* (NtEAF, NtEAR), *NtERD10B* (NtEBF, NtEBR), NtERD10C (NtECF, NtECR) and NtERD10D (NtEDF, NtEDR) (supplementary Table 1). RT-PCR experiments were repeated three times to validate the results. To determine if equal amounts of total RNA were used in the RT-PCR reactions among samples, the RT-PCR for the house-keeping actin gene was performed under the same above-mentioned conditions.

Overexpression of *AlSAP* in *Saccharomyces cerevisiae* and stress-tolerance assays

For overexpression of *AlSAP* gene in *S. cerevisiae*, strain W303 (*MATa ade2 ura3 leu2 his3 trp1*), the cDNA fragment was released from the plasmid pDNR-LIB by digestion with *Eco*RI and *Xba*I. To obtain the plasmid pYES2-*AlSAP*, the resulting product was inserted into the *Eco*RI/*Xba*I sites of pYES2 vector (INVITROGEN) which is a 2-μm-based multi-copy yeast plasmid and contains the *URA3* gene and the *Gal1* promoter for selection and expression in yeast. Yeast cells were grown overnight in YPD medium (1% yeast extract; 2% peptone and 2% dextrose, DIFCO) at 30°C in a rotary shaker (180 rpm) to mid-exponential phase. They were then transformed with 1 μg of pYES2 empty vector or pYES2-*AlSAP* constructs using the EZ-transformation kit (Q-BIOGENE) according to the manufacturer protocol. The recombinants colonies were selected on Yeast Nitrogen Base plates lacking uracil (YNBUra$^-$). This medium is the same as for YNB except that uracil-lacking CSM was added instead of the CSM mix. For the stress-tolerance assays, positive colonies were grown overnight to mid-exponential phase in YNBUra$^-$ medium, adjusted to an $OD_{600} = 1$ and serially diluted (10^{-2}, 5×10^{-2} or 10^{-3}) with fresh medium. Finally, 5 μl aliquots of each dilution were cultivated onto YNBUra$^-$Gal 2% solid or liquid medium supplemented with NaCl (1.5 M), LiCl (0.1 M) KCl (1.5 M) or Mannitol (1.5 M) and incubated at 30°C. The growth rate was evaluated visually or by measuring the OD_{600} for solid or liquid medium respectively. The galactose at 2% was added to YNBUra$^-$ plates to induce the expression of *AlSAP* gene under the control of Gal1 promoter. For quantitative RT-PCR, yeast cells were cultivated in YNBUra$^-$ liquid medium containing 2% galactose at 30°C. After the cell growth reached the exponential growth phase (OD_{600} = about 1), cells were harvested by centrifugation, frozen in liquid nitrogen immediately and stored at −80°C until the isolation of total RNA samples. Total RNA was extracted using Trizol reagent (INVITROGEN) according to the manufacturer's protocol. Total RNA (10 μg) were treated with DNase I (MBI FERMENTAS) for 30 min at 37°C and further incubated at 65°C for 10 min. The treated total RNA was reverse-transcribed using the oligo-dT (18 mer) primer and SuperScript reverse transcriptase (INVITROGEN) according to the manufacturer's instructions. The *AlSAP* gene was amplified using 2 μl of 1/10th dilution of cDNA as a template and two specific primers Al5'UTR and Al3'UTR. As an internal control, a fragment of 18S rRNA (600 pb) was amplified using the following primers: 18SF and 18SR (supplementary Table 1).

Construction of binary vector and transformation of tobacco

The plasmid pDNR-LIB was digested with *Xba*I and *Sma*I to release the cDNA fragment of *AlSAP* gene which was purified of the gel and finally cloned in the binary vector pCAMBIA 2300 (CAMBIA, Canberra, Australia) under the control of the CaMV35S promoter and CaMV35S terminator. The obtained construct pCAMBIA2300-*AlSAP* was then mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) by freeze–thaw transformation method (Chen et al. 1994). *Agrobacterium*-mediated leaf disc transformation of tobacco was carried out per standard protocol (Horsch et al. 1988). Transformants (T0) were selected on MS agar medium containing 250 mg/1 kanamycin. The integration and expression of *AlSAP* was ascertained by Southern and northern blot analysis, respectively (see below).

Southern and northern blot analyses

Genomic DNA was isolated using the CTAB (*N-acetyl-N, N, N-trimethylammonium bromide*) method (Murray and Thompson 1980). For Southern blot analysis, 20 μg of genomic DNA extracted from PCR-positive transgenic tobacco lines or from *A. littoralis* were digested with *Hind*III and with *Eco*RI, *BamH*I, *Bgl*II, *Hind*III respectively. The digested DNA was separated by electrophoresis on a 1% agarose gel and then transferred onto Hybond-N$^+$ nylon membrane (AMERSHAM-PHARMACIA). The *AlSAP* cDNA fragment amplified by PCR with a pair specific primer (Al5'UTR, Al3'UTR) and labeled with [α-^{32}P]dCTP (AMERSHAM-PHARMACIA) was used as a probe. After hybridization at 65°C, the membrane was washed once with 2XSSC plus 0.1% SDS at 65°C for 20 min and then twice with 1XSSC plus 0.1% SDS at 65°C for 30 min. For RNA blot analysis, total RNA was extracted from 4-week-old seedlings of transgenic tobacco using Trizol reagent (INVITROGEN). About 20 μg of total RNA samples were resolved on a 1.5% formaldehyde gel and blotted onto a Hybond-N$^+$ membrane (AMERSHAM). Hybridization and washing conditions were identical to the above-mentioned DNA blot. Hybridization was detected by autoradiography. The RNA also served for RT-PCR analyses, as described in the semi-quantitative RT-PCR section.

Evaluation of transgenic tobacco plants for abiotic stress tolerance

In vitro assays: The *AlSAP* transgenic tobacco plants (T9, T16 and T22) of homozygous T2 generation were used in the subsequent abiotic stress assays. Seeds of WT and

transgenic plants were surface-sterilized and plated on MS0, MS0 m (MS0 plus 300 mM mannitol) and MS0 s (MS0 plus 200 mM NaCl) culture medium plates. The plates were placed in a growth chamber under a 16 h light/8 h dark cycle at 25°C. Germination rates were scored after 4 weeks. To evaluate the growth rate under osmotic and salt stress conditions, 10 day-old transgenic and wild-type seedlings were transferred to MS0, MS0 m, MS0 s and to MS0sm (MS0 plus 300 mM mannitol and 200 mM NaCl). Plates were held vertically. After 1 month of incubation, root length was monitored. For ionic stress tolerance analysis, 15 day-old seedlings of WT and transgenic lines were transferred on MS medium supplemented with 50 or 100 mM LiCl. After incubation in growth chamber for 10 days, the fresh and dry weights of the plants were determined. For freezing and heat stresses, 1 month-old seedlings of transgenic and WT plants grown in peat under greenhouse conditions were incubated at −20°C for 3 h or at 55°C for 2.5 h, respectively. The plant's fresh and dry weights were determined after 1 week of recovery in greenhouse.

Leaf disk floating assays: One cm diameter leaf disks (5 disks per treatment) were prepared from leaves of identical development stage of both wild-type and transgenic plants and floated on 0, 300 and 800 mM NaCl solution for 72 h. The total chlorophyll content in each sample was calculated after extraction in aqueous 80% acetone using the following formulae (Arnon 1949) which express [Chl *a*], [Chl *b*] and [Chls $a + b$] in $\mu g \cdot ml^{-1}$: [Chl *a*] = 12.70·*A*663–2.69·*A*645, [Chl *b*] = 22.90·*A*645–4.68·*A*663, [Chls $a + b$] = 20.21·*A*645 + 8.02·*A*663. The *A*663 and *A*645 represent absorbance values read at 663 and 645 nm wavelengths, respectively.

Greenhouse assays: To monitor the effects of drought and salt tratments on *AlSAP* overexpressing plants under greenhouse conditions, seeds of three homozygous transgenic lines (T9, T16 and T22) and WT plants were germinated and grown on MS medium supplemented with 250 mg/l kanamycin for 1 month. The seedlings were then transferred to pots filled with a 3:1 mixture of soil and peat and grown in a greenhouse for two more weeks before exposure to stress treatments. Transgenic and WT plants were irrigated with tap-water using a program that maintained the RWC of soil at 75% under normal conditions. For salt stress treatment, the same irrigation program was used for watering the plants with a solution containing 350 mM NaCl until the end of plant cycle. This NaCl concentration was increased gradually from 200 to 350 mM within the first 15 days of stress treatment. Leaves (young (top) and old (bottom)) and roots were collected from salt stressed treated plants and then dried at 80°C for 24 h. Finally, the dried material was incubated in 0.5% HNO_3 for a week. The Na^+ and K^+ contents were analyzed in the filtrate using atomic absorption spectrophotometry.

For drought stress, plants were watered with tap-water using a program that maintained the RWC of the soil at 25%. The RWC of the leaves was estimated every 6 days for the whole period (30 days) of withholding water according to the method of Turner (1981): $RWC(\%) = (FW-DW)/(TW-DW) \times 100$. Leaves of uniform position were taken from WT and transgenic lines and were immediately weighed (fresh weight: FW), hydrated to full turgidity for 12 h at 4°C by floating them on distilled water in a closed Petri dish. They were also weighed to obtain the turgid weight (TW). Finally, the leaves were oven-dried at 80°C for 24 h and were weighed to determine the dry weight (DW). For all treatments, plant height, root length, number of fertile pods and yield of seeds were determined.

Sequence analysis

We performed the BLAST search of the *AlSAP* gene at NCBI (http://www.ncbi.nlm.nih.gov/Blast). Various tools from Expasy (http://www.expasy.org/tools) were used to deduce the translated product and compute theoretical pI and molecular weight. The putative domains were identified using the InterProscan search (http://www.ebi.ac.uk/interproscan). The multiple sequence alignments and the degree of amino acid sequence identity were determined by the use of the biological sequence editor software BioEdit 7.0.0. Finally, to predict the phylo-genomic relationships between *AlSAP* gene and *O. sativa/A. thaliana* genes, we have used the Greenphyl Orthologs Search Tool (GOST, http://greenphyl.cines.fr/cgi-bin/gost.cgi).

Results

Analysis of the *AlSAP* sequence

A cDNA clone, *AlSAP*, was isolated from roots of salt treated *A. littoralis* plants (300 mM for 15 days). Sequence analysis using the web-based annotation tool linked to BioEdit software revealed that *AlSAP* cDNA was 707 bp long, including a complete open reading frame of 477 bp with 5'-UTR and 3'-UTR regions of 118 and 112 bp, respectively (supplementary Fig. 1a). *AlSAP* encodes a predicted polypeptide of 159 amino acids with a molecular weight of 17.69 kDa and a pI of 8.01. The amino acid sequence analysis by NetPhos 2.0 server showed the presence of some predicted phosphorylation sites with a high score (>0.9) at serine (S-5, S-63, S-64, S-97, S-101), threonine (T-70, T-102) and tyrosine (Y-136) (supplementary Fig. 1a). It has been shown that the protein phosphorylation at these residues affects a multitude of cellular signaling processes (Blom et al. 1999). In addition, one kinase-specific phosphorylation site PKC (protein

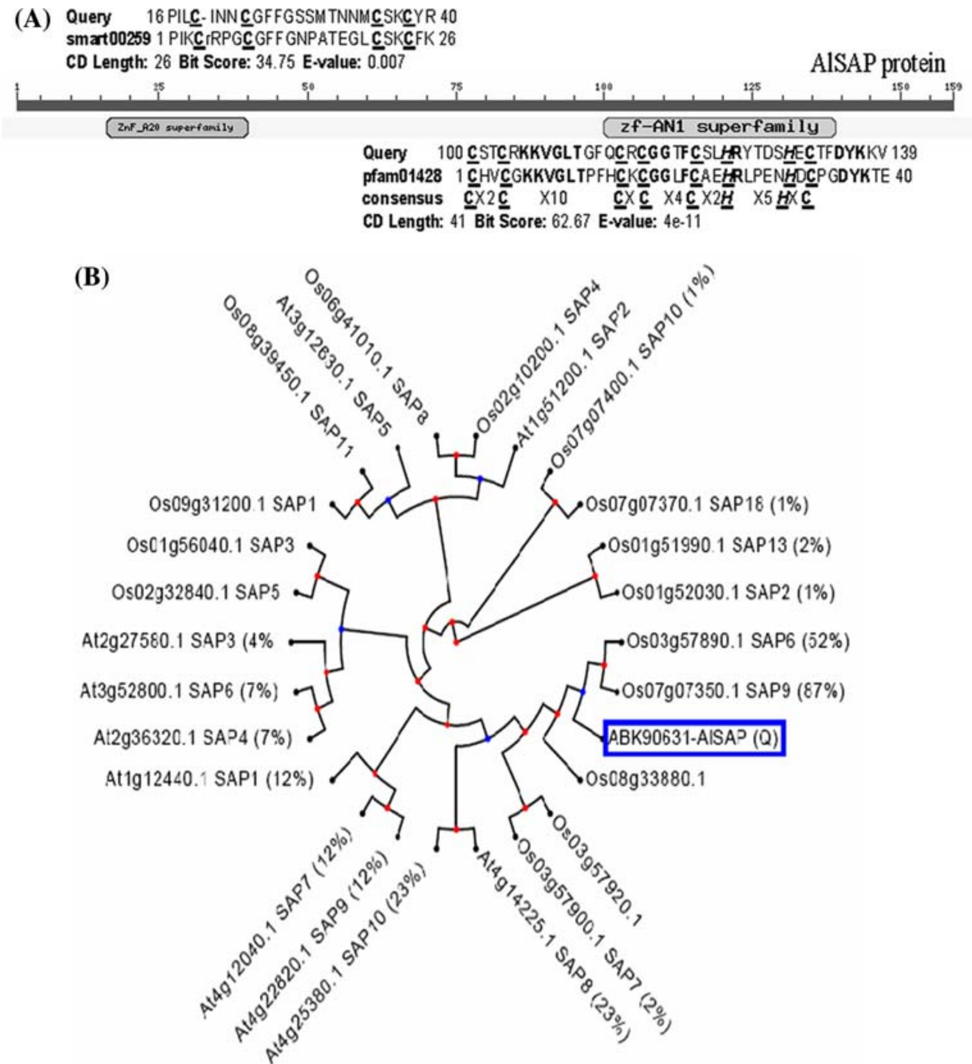

Fig. 1 Sequence analysis of the AlSAP protein highlighting the A20/AN1 conserved domains and the prediction of phylogenomic relationships between *AlSAP* gene and *O.sativa*/*A.thaliana* SAP genes. **a** The amino acids at N-terminal A20 and the C-terminal AN1 type zinc-finger domains are conserved in the AlSAP protein. Conserved cysteines and histidines are indicated in *bold* type. **b** Phylogenetic tree of rice and *Arabidopsis SAP* gene families encoded A20/AN1 zinc-finger proteins. The unrooted tree was generated using the Greenphyl Orthologs Search Tool (GOST, http://greenphyl.cines.fr/cgi-bin/gost.cgi) which predicts the phylogenomic relationships (percentage of orthology) between *AlSAP* gene and *O. sativa*/*A. thaliana* SAP genes

kinase C) was predicted at threonine (T-102, score of 0.9) (supplementary Fig. 1a) by NetPhosK 1.0 server (Blom et al. 2004).

The conserved domain search of AlSAP amino acid sequence in NCBI and the InterProScan in EMBL-EBI server predicted the presence of two zinc-finger domains as a characteristic of the *SAP* gene family in plants (Fig. 1a). The first one was located at the N-terminus region between amino acids 16 and 40 and was similar (E-value of 0.007) to the zf-A20 domain (smart00259) of the human A20 protein with a consensus of $Cx_{2-4}Cx_{11}Cx_2C$, where x represents any amino acid. The second domain was found at C-terminus of the protein, covering the region from amino acid 100–139, which was similar (E-value $= 4 \times 10^{-11}$) to the zf-AN1 domain (Pfam01428) having a consensus sequence of $Cx_{2-4}Cx_{9-12}Cx_{1-2}Cx_4Cx_2Hx_5HxC$.

Using blastp in NCBI, the homology searches run with the translated full-length amino acid sequences of the *AlSAP* cDNA clone revealed a significant similarity to the A20/AN1 zinc-finger proteins isolated from plants, human (ZNF216 and AWP1) and animals. The prediction of orthologs to the *AlSAP* gene in the Greenphyl database using the GOST tools (GOST, http://greenphyl.cines.fr/cgi-bin/gost.cgi) revealed the presence of two orthologs in *O. sativa*, *OsSAP9* (**Os07g0168800**) and *OsSAP6* (**Os03g0792900**) with a high percentage of 87 and 52% orthology, respectively (Fig. 1b). AlSAP showed the highest identity values at the amino acid levels with the two rice SAP proteins, OsSAP9 (**Q7Y1W9**, 79%) and OsSAP6 (**Q852K5**, 63%) (Vij and Tyagi 2006), whereas, for other SAPs from rice, *Arabidopsis* and maize the identity values were much lower, ranging from 25 to 40%. Thus, AlSAP showed 37 and 41% of identity to OsSAP1 (Mukhopadhyay et al. 2004) and OsSAP8 (Kanneganti and Gupta 2008), respectively. These authors have also shown that OsSAP1, OsSAP6, OsSAP8 and OsSAP9 are members of the SAP gene family characterized by the presence of zf-A20 and zf-AN1 zinc-finger domains in their putative

encoded proteins. Apart from the plant homologues, the *AlSAP* showed 35% identity to the human ZNF216 and AWP1 proteins, concentrated in the zf-A20 and zf-AN1 zinc finger domains.

Comparison of the *AlSAP* full-length cDNA with a genomic fragment (2,230 bp) generated by PCR amplification and sequencing, revealed the presence of two exons of 83 and 624 bp separated by one intron of 1,523 bp (supplementary Fig. 1b).This long intron is located in the 5'UTR region while the coding sequence is continuous. In addition, Southern blot analysis revealed that *AlSAP* is a single copy gene (Fig. 2). A RAP-DB database-search (http://rapdb.dna.affrc.go.jp) showed that *AlSAP* gene has the same genomic structure than its two rice orthologous genes, *OsSAP9* and *OsSAP6*, located on rice chromosome 7 and 3 respectively. The *OsSAP9* and *OsSAP6* genes also harbour an intron of of 1,537 and 1,430 bp length, respectively in their 5'UTR. It was also reported that *OsSAP8* possesses two introns (176 and 739 bp) located in the 5'UTR region (Kanneganti and Gupta 2008).

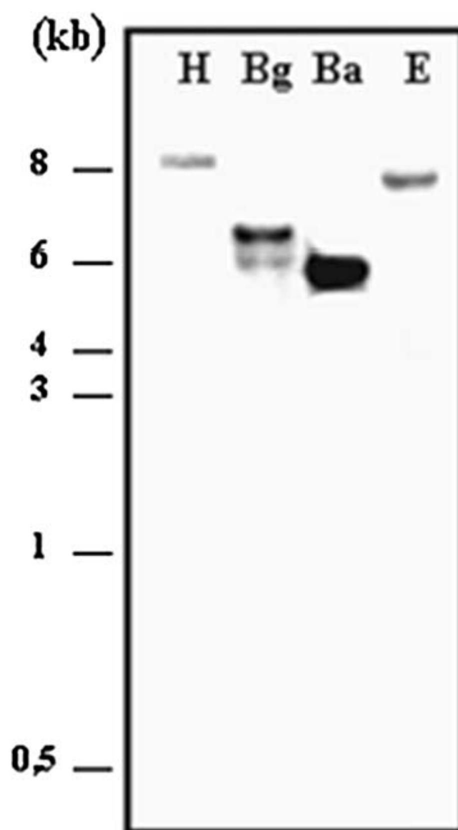

Fig. 2 Genomic organization of *AlSAP* gene. The *AlSAP* gene is present as a single copy in the genome of *A. littoralis*. Total genomic DNA from *A. littoralis* plant was digested with *Hind*III (H), *BamH*I (Ba), *EcoR*I (E) (do not cut in the sequence of *AlSAP* gene) and *Bgl*II (Bg) (cut once at position 2,115), and analyzed by Southern blot using the full-length cDNA of *AlSAP* as a probe. The positions of the molecular size standards are indicated

Stress-induced transcription of *AlSAP* gene

We have investigated the accumulation of *AlSAP* transcripts in response to multiple abiotic stresses (salt, osmotic, heat and cold) as well as to hormonal stresses (ABA and SA). The transcript levels increased gradually as a function of exposure time of *A. littoralis* plants to PEG, heat and cold stresses and remained at elevated levels for 6 days (Fig. 3b, d and e). In the case of salt stress, transcript accumulation reached a high level after a 2 day treatment and then gradually declined back to its basal level from days 3–6 (Fig. 3a). A comparable pattern of transcript accumulation was observed during the ABA treatment, except that the mRNA level remained high both on days 2 and 3 following the treatment (Fig. 3c). For SA treatment, the transcripts started to accumulate within 1 day of treatment, reached the maximum level on the 2nd and 3rd days and then declined on the 6th day (Fig. 3f).

Heterologous expression of *AlSAP* gene in yeast cells

The fact that the *AlSAP* gene is induced in response to multiple abiotic stresses, led us to further determine whether the expression of this gene can protect cells against environmental stresses. In that aim, we used yeast (*Saccharomyces cerevisiae*) as a fast heterologous model system. Wild-type yeast strain W303 was transformed with the plasmid pYES2 containing the *AlSAP* gene driven by the galactose inducible promoter GAL1 or by the empty pYES2 vector. The cells overexpressing *AlSAP* (as confirmed by RTPCR, Fig. 4a) exhibited a better growth than control cells under NaCl, LiCl, KCl and mannitol stresses (Fig. 4b). The number of transgenic *AlSAP* cells was two-fold and six-fold higher than control cells under LiCl and NaCl stresses, respectively (Fig. 4c). Finally, in hyperosmotic liquid medium containing KCl or mannitol at 1.5 M, the growth rate of *AlSAP* cells was two-fold higher than that of control cells (Fig. 4c). These findings suggest that overexpression of *AlSAP* in yeast increased cell tolerance to ionic and osmotic stresses. Based on these results, we further investigated whether *AlSAP* can operate the same role in transgenic tobacco plants.

Heterologous expression of *AlSAP* gene in transgenic tobacco plants

Generation and characterization of AlSAP tobacco plants

To investigate the physiological function of *AlSAP*, we generated 7, independent transgenic tobacco lines overexpressing *AlSAP* under the control of the duplicated cauliflower mosaic virus 35S promoter (Fig. 5a). The copy

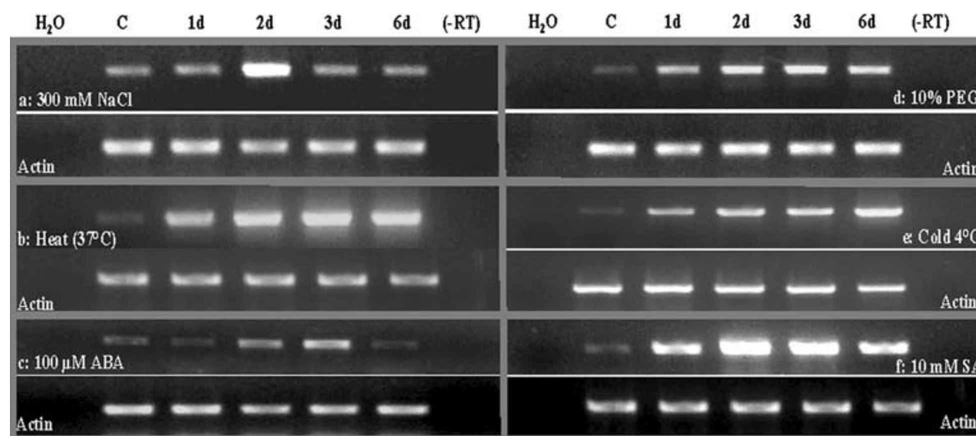

Fig. 3 Expression pattern of the *AlSAP* gene in response to different stresses applied to *A. littoralis* plants. RT-PCR analysis was performed with *AlSAP* specific primers using the RNA isolated from the *A. littoralis* plants subjected to normal conditions (C), **a** 300 mM NaCl, **b** heat at 37°C, **c** 100 µM ABA, **d** 10% PEG, **e** Cold at 4°C and **f** 10 mM SA. Actin amplification was used as an internal control (*lower panel*).1–6 day represents the duration of treatment in days. (-RT) represents the amplification of the RNA without RT-reaction. (H_2O) represents the amplification in the absence of templates

number of the integrated *AlSAP* gene was determined by Southern blot analysis (Fig. 5b). The constitutive expression of *AlSAP* in the selected transgenic lines was established by northern blot and semi-quantitative RT-PCR analyses (Fig. 5c, d). The absence of obvious phenotypic difference between AlSAP-expressing plants and WT plants under normal growth conditions indicates that ectopic expression of *AlSAP* does not affect the overall plant morphology.

Evaluation of AlSAP tobacco for stress tolerance under in vitro conditions

To evaluate the level of stress tolerance of the generated events, we selected 3 transgenic lines, T9, T22 and T16 that harboured 1, 2 or 3 copies of the *AlSAP* gene respectively (Fig. 5b). Homozygous T2 seedlings were first evaluated in vitro for their tolerance to salt (NaCl), ionic (LiCl) and osmotic (mannitol) stresses.

Seeds of Wild-type tobacco and of the three homozygous transgenic lines T9, T16 and T22 were germinated on different culture media -MS0 (control), MS0 s (salt) and MS0 m (osmotic). Germination rates of wild-type and transgenic seeds cultured on the control MS0 medium (Fig. 6a) were similar, indicating that *AlSAP* gene expression has no detrimental effect on seed germination. On the other hand, germination of wild-type seeds was significantly impaired under stress conditions when compared to transgenic seeds. Whereas only 29% of the wild-type seeds germinated on MS0 s medium supplemented with 200 mM NaCl after 1 month,.seeds of the T16, T22 and T9 lines germinated at a rate of 96, 89 and 80%, respectively (Fig. 6b). Along the same line, 55% of the wild-type seeds germinated on MS0 m medium supplemented with 300 mM mannitol while 74, 95 and 98% of the T9, T22 and T16 transgenic seeds, respectively, did so (Fig. 6b). The transgenic lines harboring more than one copy of *AlSAP* gene (T16 and T22) showed a higher germination rate under salt and osmotic stresses than the transgenic line carrying a single copy (T9). This could be related to the higher level of accumulation of *AlSAP* transcripts in lines 16 and 22 than in line 9 (Fig. 5d). In addition, the germination experiments revealed that the wild-type seedlings stopped their growth, producing small (osmotic stress) or yellow (salt stress) leaves and less root biomass than transgenic seedlings. Interestingly, shoots and roots of all the transgenic ssedlings continued to grow after germination under these conditions (Fig. 6a). To further investigate the effect of salt and mannitol on the growth rate, 10-day-old wild-type and transgenic seedlings were transferred to MS0 s medium (NaCl 200 mM), MS0 m (mannitol 300 mM) or a combination of the two chemicals. After another month, root and leaf growth of wild-type seedlings were strongly retarded, whereas, growth inhibition was less pronounced in transgenic lines (Fig. 7a). Root lengths in wild-type plants exhibited severe reduction of 78, 88 and 89% under osmotic, salinity and a combination of the two stresses respectively, compared to control conditions. On the other hand, in transgenic lines, root elongation inhibition reached 35% for the two types of stresses (osmotic or salinity) and 62% for the combination of the two stresses (Fig. 7b). More importantly, we noticed that the root length reduction observed under osmotic or salt stress in transgenic plants was compensated by an increase in root number when compared to control conditions (Fig. 7a). Transgenic seedlings were able to produce a fourth pair of leaves during the stress treatment whereas WT seedlings had produced only the second pair of leaves.

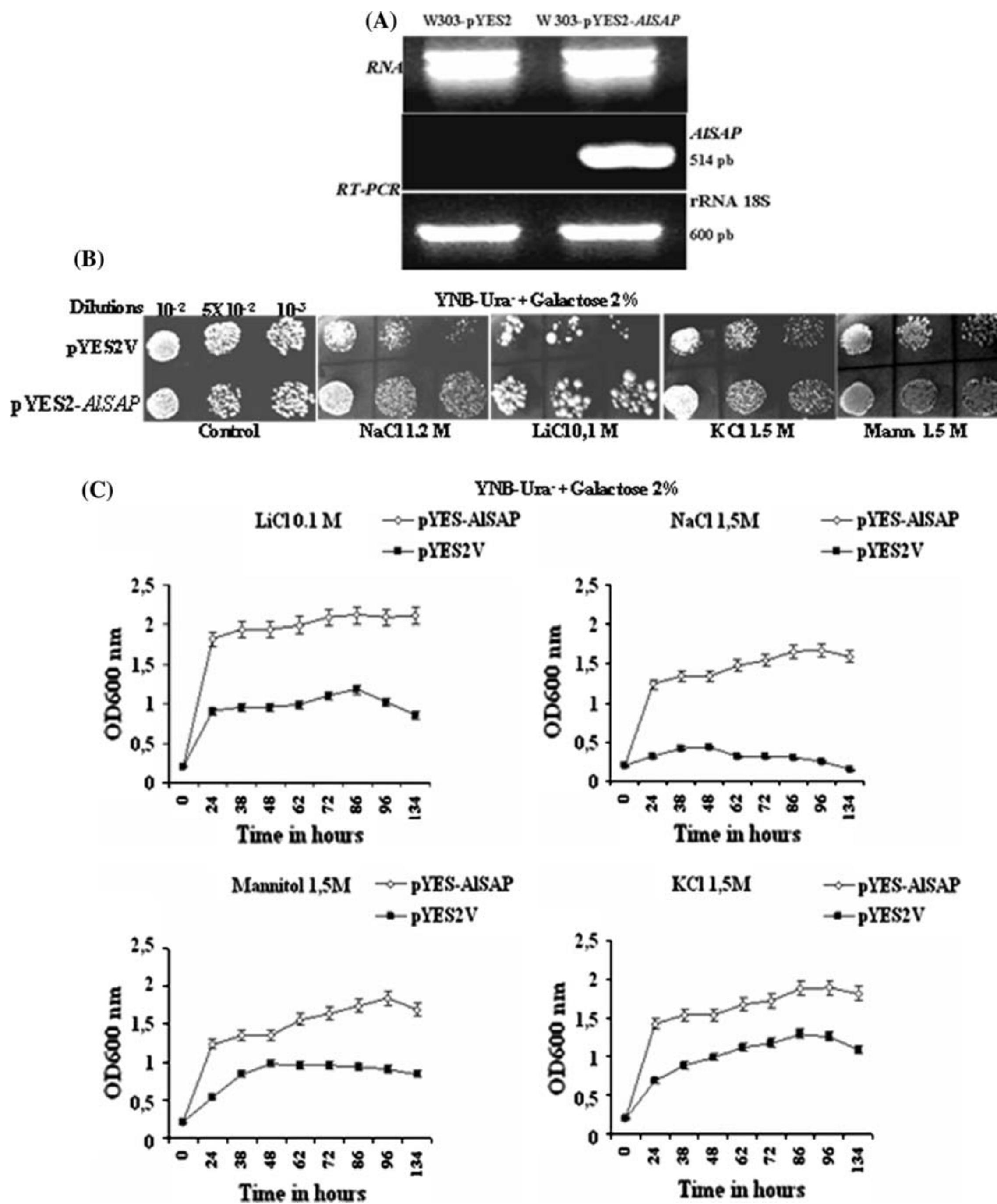

Fig. 4 Abiotic stress tolerance of yeast cells overexpressing the *AlSAP* gene. **a** The overexpression of *AlSAP* in yeast, grown in liquid YNB-Ura^{-}/Gal2% medium, was confirmed by RT-PCR analysis. The 18S rRNA used as an internal control. **b** A culture with an $OD_{600} = 1$ of transformed cells with empty plasmid pYES2 as control or pYES2 containing *AlSAP* was serially diluted to 10^{-2}, 5×10^{-2} or 10^{-3}. 5 µl of each dilution were plated onto solid YNB-Ura/Gal2% plates (control) or supplemented with different concentrations of NaCl (1.2 M), LiCl (0.1 M), KCl (1.5 M) or Mannitol (1.5 M). Colonies were photographed after 5–6 days of incubation at 30°C. **c** Yeast cells containing pYES2-*AlSAP* and pYES2 were incubated in liquid YNB-Ura/Gal2% medium at 30°C till OD_{600} reached 0.2, then the stresses were started by adding different concentrations of NaCl (1.5 M), KCl (1.5 M), LiCl (0.1 M) or Mannitol (1.5 M) to the cultures and finally the yeast cell growth was monitored by measuring the OD_{600} at different periods

Finally, the alteration in chlorophyll content under salt stress was evaluated using a detached leaf disk assay. This assay can be taken as a reliable index of the damage to the photosynthesis apparatus under stress. When leaf disks were floated on a salt solution containing 300 or 800 mM NaCl for 72 h, disks of WT plants bleached more intensely

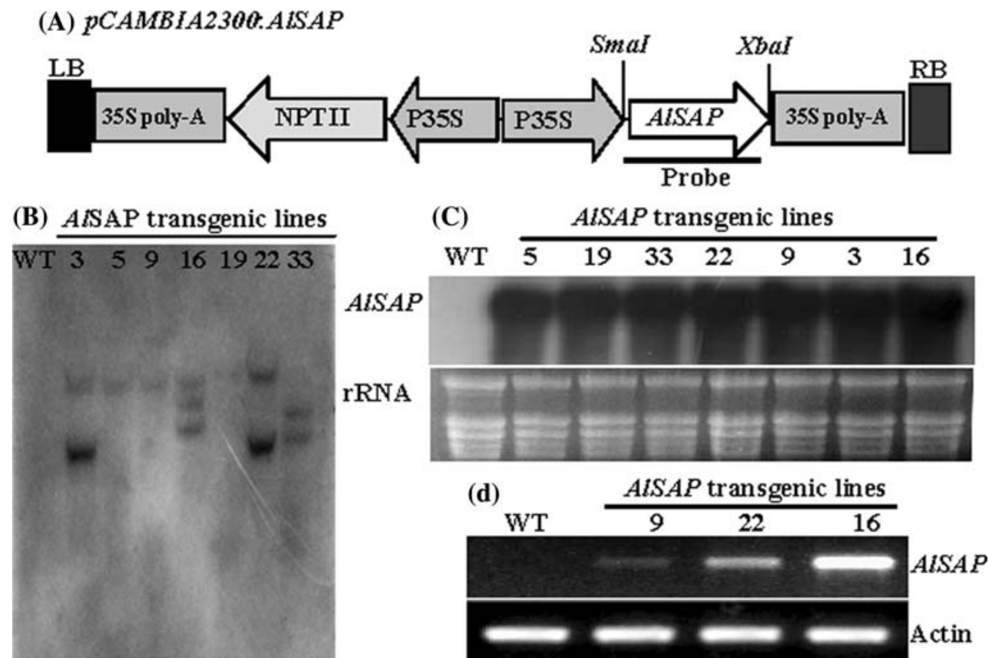

Fig. 5 Analysis of transgenic tobacco lines overexpressing the *AlSAP* gene. **a** Schematic map of the T-DNA inserted in the binary vector pCAMBIA2300: *AlSAP*, used for tobacco transformation. **b** Southern blot analysis of transgenic and wild type tobacco plants determining the T-DNA copy number. Genomic DNA was digested with *Hind*III, separated by electrophoresis on a 1% agarose gel and transferred onto a nylon membrane. The transferred DNA was hybridized with the [α- ^{32}P]dCTP labeled *AlSAP* full-length cDNA probe. **c** Northern blot analysis of *AlSAP* transcript levels in wild-type tobacco and in the transgenic lines. RNA was transferred onto a nylon membrane and hybridized with the [α-^{32}P]dCTP labeled *AlSAP* full-length cDNA probe. Equal loading in each lane was confirmed by ethidium bromide staining (*lower panel*). **d** Expression pattern of the *AlSAP* gene in three transgenic lines (9, 16 and 22) used for tolerance assays. RT-PCR analysis was performed with *AlSAP* specific primers using the RNA isolated from the three transgenic lines and WT plants. Actin amplification was used as an internal control (*lower panel*)

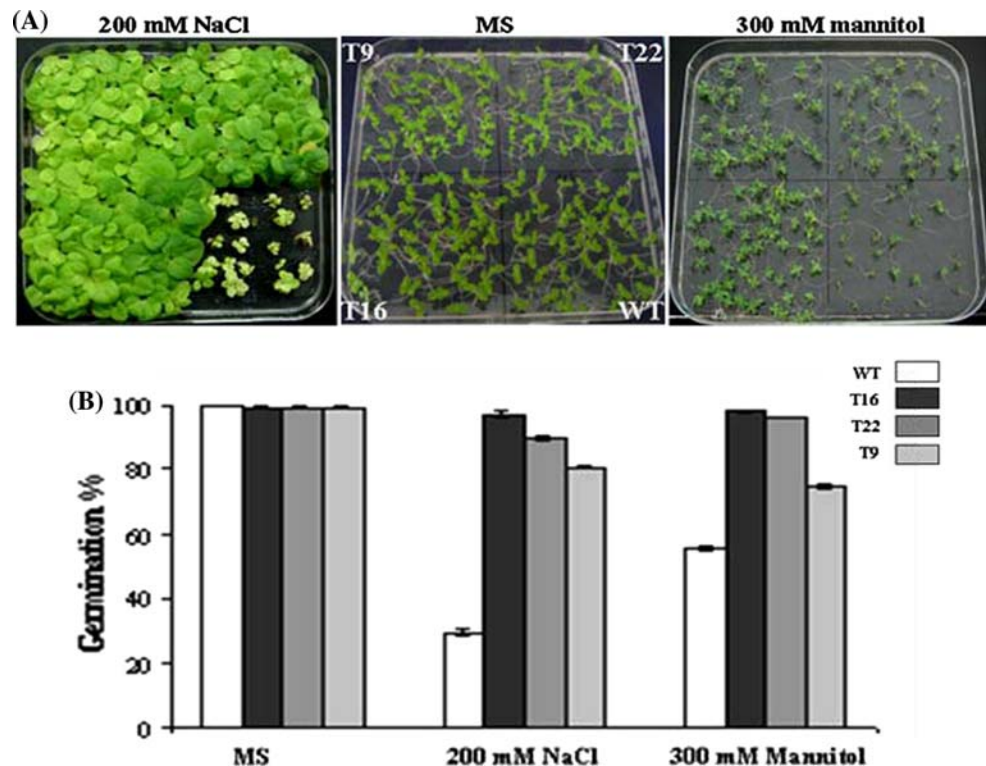

Fig. 6 Effect of salt (NaCl 200 mM) and osmotic (mannitol 300 mM) stresses on seed germination and plant phenotype in transgenic tobacco plants overexpressing the *AlSAP* gene. **a** Photographs were taken 4 weeks (control and mannitol) and 2 months (salt) after seed germination. **b** Percentage of seed germination in the case of WT and T2 homozygous transgenic plants (T9, T16 and T22) growing on MS0 medium (control), MS0 s (NaCl 200 mM) or MS0 m (mannitol 300 mM). Results are presented as means and standard errors from three independent experiments (150 seeds of each line were sown for each experiment)

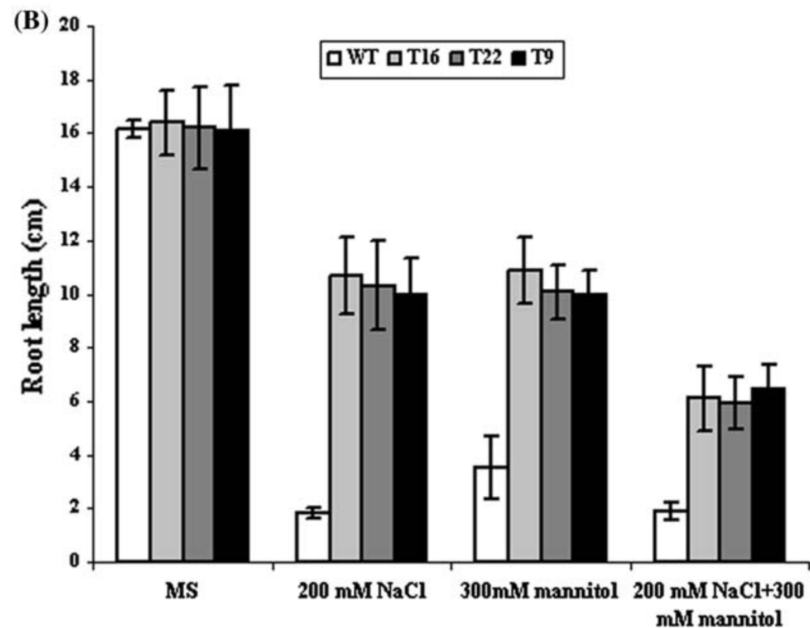

Fig. 7 Effect of salt (NaCl 200 mM), osmotic (mannitol 300 mM) and the combined stresses on shoot and root growth of WT plants and three transgenic *AlSAP* tobacco lines. **a** Photographs were taken 4 weeks after incubation in vitro. **b** Root length expressed in cm in the case of WT and transgenic plants (T9, T16 and T22) grown on MS medium (control) or on MS medium supplemented with NaCl (200 mM), mannitol (300 mM) or the two chemicals. Results are presented as means and standard errors from three independent experiments (10 plants of each line were grown for each experiment)

than those of transgenic lines (Fig. 8a). This was later found consistent with the measurement of the chlorophyll content which dropped dramatically in WT plants (4–6%) while the *AlSAP* transgenic lines retained almost 30–50% of their chlorophyll under similar conditions (Fig. 8b). Taken together, these results demonstrate that transgenic tobacco plants overexpressing *AlSAP* have a better ability to tolerate salinity stress than wild-type plants. To investigate if this tolerance to salinity was due to osmotic and/or ionic mechanisms, 15 day-old WT and transgenic seedlings were challenged with a strictly ionic agent such as 50 or 100 mM LiCl. Stress tolerance was evaluated by monitoring the plant fresh and dry weights, 10 days following stress application. As shown in Fig. 9a, a significant

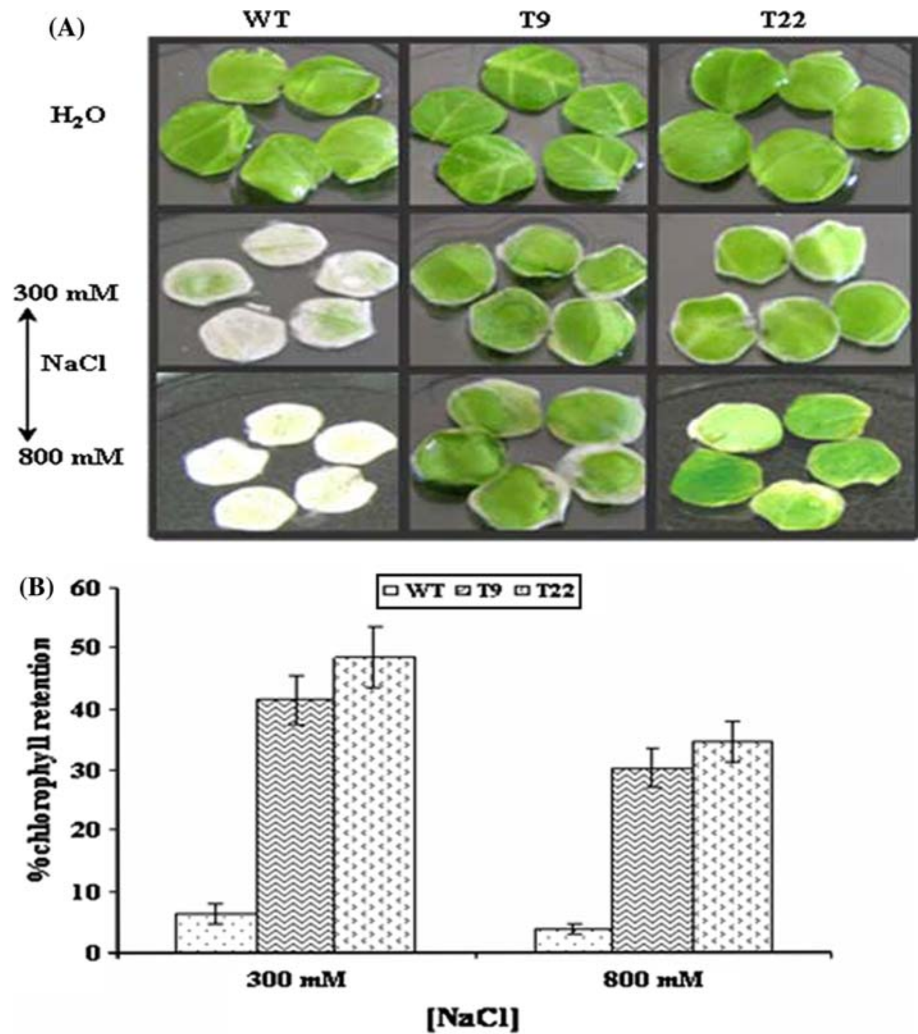

Fig. 8 Leaf disk floating assays as an evaluation of salinity tolerance in transgenic *AlSAP* tobacco lines (T2 generation). **a** Representative picture that shows phenotypic differences in leaf disks. **b** % of chlorophyll retention from leaf segments of WT and two transgenic lines plants (T9 and T22) after incubation in 300 and 800 mM NaCl solution. Leaf disks floating in water served as experimental control. After 72 h of incubation, the % of chlorophyll retention in the leaf discs was measured

difference in root and leaf growth rates was observed between the transgenic and WT seedlings. In fact, 10 days after stress application (50 mM LiCl), the transgenic seedlings reached the 4th leaf pair stage whereas the WT seedlings were at the 3rd leaf pair stage. In addition, the biomass production of WT plants, measured by fresh and dry weights, was four-fold to five-fold lower than that of transgenic lines (Fig. 9b, c). These results indicate that expression of the *AlSAP* gene conferred not only osmotic but also ionic stress tolerance.

Expression of stress-associated genes in AlSAP tobacco plants

Semi-quantitative RT-PCR was used to compare expression of some stress-related genes between wild-type and *AlSAP* overexpressing tobacco plants under normal conditions. Transcript accumulation of the eight stress-related genes tested (CAT1, MnSOD, APX, osmotin and NtERD10 A, B, C or D) were higher in *AlSAP* transgenic plants than in wild-type plants in the absence of stress (Fig. 10a, b). Based on these results, it could be concluded that *AlSAP* might act as an upstream activator of some stress-related genes, which could lead to the tolerance to salt and drought stresses observed in transgenic lines. The induction of *CAT1*, *MnSOD* and *APX* genes in *AlSAP* transgenic lines is probably related to an increase of the plant capacity to eliminate the reactive oxygen species (ROS) generated by salt and drought stresses. In addition, the upregulation of the osmotin gene (a dehydrin) and the four genes of group 2 LEA protein (NtERD10 A, B, C or D) in *AlSAP* transgenic lines is another element that could explain the tolerance observed to drought and salt stresses.

Evaluation of AlSAP tobacco for stress tolerance under greenhouse conditions

Because *AlSAP* expression in transgenic plants was found to allow their growth in vitro under salt and osmotic stresses, it was crucial to investigate its ultimate effect on

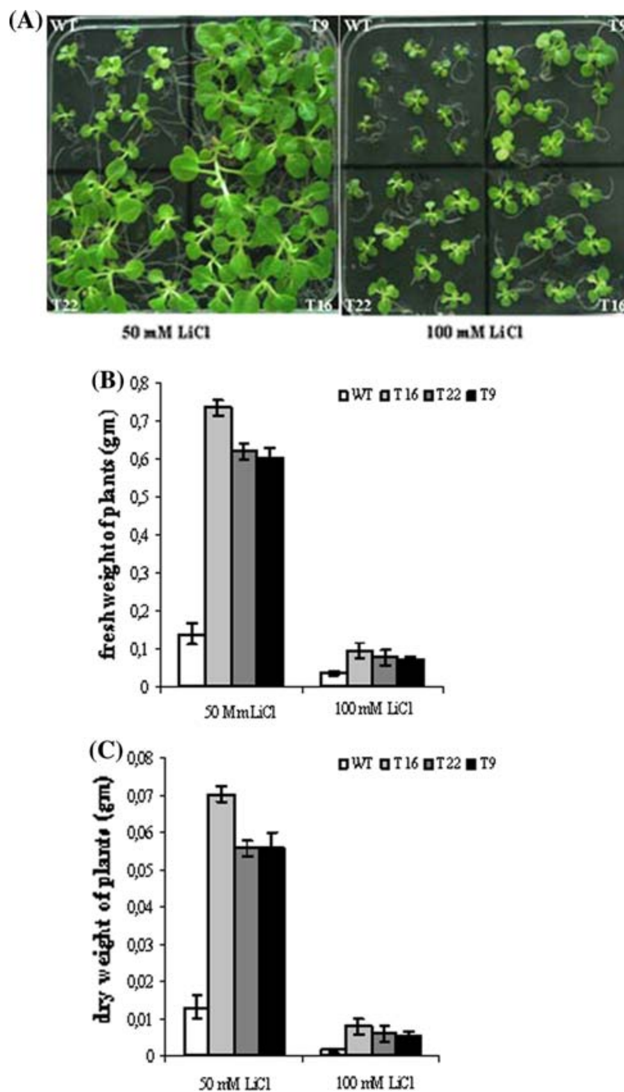

Fig. 9 Effect of strict ionic stress treatments on growth rate of WT and transgenic seedlings. Tobacco seedlings grown in MS medium for 15 days were transferred to culture plates containing 50 or 100 mM LiCl. **a** The photograph was taken 10 days after transplantation**. b** The fresh and **c** dry weight of WT and three transgenic plants (T9, T16 and T22) were determined

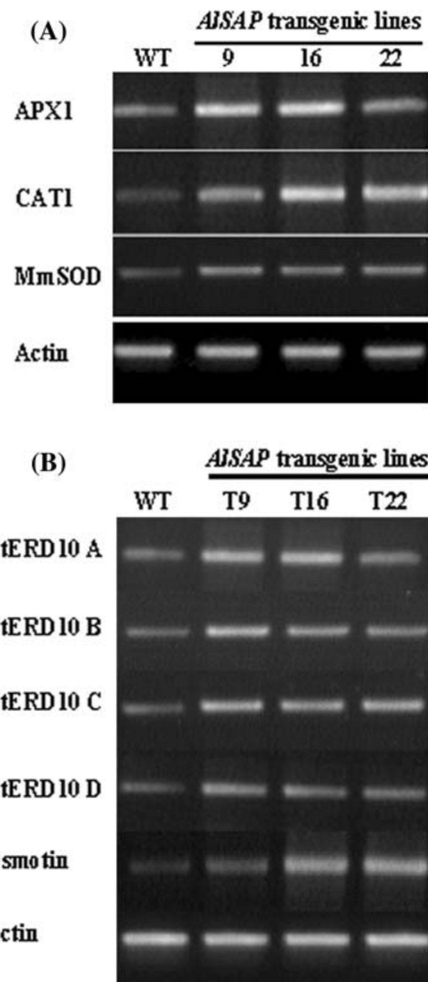

Fig. 10 Semi-quantitative RT-PCR assay of stress-related genes in WT and transgenic *AlSAP* tobacco lines grown under normal conditions. Total RNA was extracted from wild-type tobacco and transgenic lines (9, 16 and 22) and then reverse-transcribed using oligo-dT (18 mer) as described in materials and methods to generate first strand cDNA. The amplification of stress-related genes was performed using 2 µl of 1/10th dilution cDNA as a template and two specific primers. Actin amplification was used as an internal control. The stress-related genes used for the test are as follows: **a** genes encoding for ROS scavenging enzymes: catalase (CAT1, **U93244.1**), manganese superoxide dismutase (MnSOD, **AB093097.1**), ascorbate peroxidase (APX, **U15933.1**). **b** Genes encoding for protecting proteins: osmotin (**M2979**) and group 2 LEA proteins NtERD10A (**AB049335.1**), NtERD10B (**AB049336.1**), NtERD10C (**AB049337.1**) and NtERD10D (**AB049338.1**)

seed production and yield. For this purpose, we performed salt and drought tolerance experiments with WT and the three T2 generation transgenic lines (T9, T22, T16) grown in soil in the greenhouse. Plants were grown either under optimal water supply (control conditions: RWC of the soil was maintained at 75%), under drought stress (the RWC of the soil was maintained at 25%) or continuous salt stress using NaCl (350 mM) for irrigation until the end of the plant cycle. As an indicator of salinity and drought stress tolerance in T2 transgenic lines, several critical growth parameters (plant fresh weight, root and shoot length, leaf and fertile pod numbers, time to flowering and seed weight) were scored (Table 1). The T2 generation transgenic plants behaved similarly to the WT counterparts for all these parameters when grown under non-stress conditions, confirming that *AlSAP* overexpression does not induce any growth or yield penalty in transgenic plants (Table 1; Fig. 11-control). In contrast, we have noticed a slight increase in the root and shoot length and in leaf number in transgenic lines when compared to WT plants (Table 1). This constrasts with the almost 50% yield penalty observed in transgenic rice lines overexpressing *OsSAP8* under normal growth conditions (Kanneganti and Gupta 2008).

Table 1 Comparison of various growth parameters of WT and *AlSAP* T2 transgenic plants grown under either normal condition, a continuous presence of 350 mM NaCl or a continuous drought stress (soil RWC of 25%) until the end of the plant cycle

Parameter	Control				Salt stress (350 mM NaCl)				Drought stress			
	WT	T16	T22	T9	WT	T16	T22	T9	WT	T16	T22	T9
Plant weight (gm)	115 (0.31)	119 (0.31)	116.3 (0.48)	117.3 (0.18)	20 (0.31)	86 (0.31)	81.3 (0.48)	84 (0.31)	29 (0.31)	61.6 (0.18)	57.3 (0.66)	62.6 (0.48)
No. of leaves per plant	22 (0.18)	24 (0.31)	26 (0.18)	28 (0.48)	7 (0.31)	22 (0.31)	28 (0.18)	24 (0.31)	8 (0.48)	24 (0.63)	20 (0.66)	24 (0.84)
Shoot length (cm)	51 (0.48)	56 (0.48)	57 (0.18)	62 (0.66)	10 (0.63)	45 (0.18)	51 (0.36)	49 (0.18)	11 (0.18)	42 (0.18)	42 (0.31)	45 (0.18)
Root length (cm)	33.6 (0.18)	37.3 (0.36)	36 (0.31)	36.3 (0.18)	12.3 (0.18)	30.3 (0.48)	26.6 (0.18)	25.6 (0.18)	15.6 (0.36)	26.3 (0.48)	24.3 (0.18)	25 (0.31)
Flowering time (days)	60 (0.36)	59 (0.31)	60 (0.22)	59 (0.55)	48 (0.18)	60 (0.48)	59 (0.31)	64 (0.63)	48 (0.31)	80 (0.36)	82 (0.18)	79 (0.36)
No. of pods per plant	47 (0.55)	47 (0.48)	49 (0.31)	51 (0.31)	2 (0.48)	46 (0.18)	48 (0.48)	49 (0.48)	5 (0.31)	24 (0.74)	20 (0.62)	24 (0.81)
No. of fertile pods per plant	45 (0.36)	43 (0.55)	44 (0.18)	46 (0.36)	ND	42 (0.31)	41 (0.48)	41 (0.8)	ND	19 (0.8)	18 (0.55)	18 (0.66)
Seed weight per plant (gm)	3.8 (0.18)	3,9 (0.25)	3.7 (0.35)	3.4 (0.22)	ND	2.8 (0.12)	2.7 (0.23)	2.6 (0.34)	ND	2.4 (0.21)	2 (0.16)	1.8 (0.36)
Yield penalty compared to control conditions (%)	–	–	–	–	100	26.28	28.41	21.47	100	36.81	47.87	45.98

Each value represents the mean of three different samples. *SD* is indicated in brackets

ND WT plants did not survive under salt and drought stresses

Under salt and drought stresses, the transgenic plants clearly performed differently than the WT. They indeed continued to grow, reached maturity, flowered and set seeds while the WT plants showed chlorosis, a stunted phenotype, were not able to produce viable seeds and ultimately died (Fig. 11). It should be noted that the biggest difference between transgenic and WT plants was observed in seed production. Thus, the three lines overexpressing the *AlSAP* gene produced under salt and drought stresses, between 55 and 75% of their respective seed yield observed under control conditions (Table 1). This could be related to the fact that the transgenic plants exposed to the continuous salt or drought stresses showed a relatively normal growth (Fig. 11) reflected by average plant weights, shoot and root lengths, leaf and fertile pod numbers remaining not deeply altered compared to control conditions (Table 1). Conversely, growth of WT plants was severely affected by a continuous exposure to salinity or drought stress, and their flowers were not able to produce any seed (Table 1; Fig. 11). Importantly, the yield of transgenic plants was more affected by the drought stress than by the salt stress. While the flowering time in transgenic plants remained unchanged under salt stress, flowers appeared with a delay of nearly 20 days under drought stress compared to control conditions. On the other hand, WT flowered 12 days earlier under both stresses than under control conditions. Such behaviour is considered as a normal response of sensitive plants to escape stress conditions.

As the transcription of the *AlSAP* gene is induced by high- and low-temperature stress (Fig. 3b, e), we also evaluated T2 plants of T9, T16 and T22 transgenic lines along with WT plants, for freezing (−20°C for 3 h) and heat (55°C for 2.5 h) tolerance. After a recovery period of 1 week all WT seedlings were wilted, whereas all transgenic seedlings recovered following the two types of stresses (Fig. 12a, b). Analysis of the seedling fresh weight after 1 week recovery period revealed that the transgenic seedlings had a three- and eight-fold higher fresh weight than WT seedlings following heat and freezing stresses, respectively (Fig. 12c). Additionally, the dry weights of transgenic seedlings was two- and four- fold higher than those of WT in the case of heat and freezing stresses, respectively (Fig. 12d).

Characterization of the physiological status of AlSAP plants under stress treatment

To examine the basic mechanism conferring the salt-tolerant phenotype of *AlSAP*-overexpressing lines, we have measured the endogenous Na^+ and K^+ levels in leaves (young and old) and roots of plants grown in the absence or presence of NaCl for 60 days. Under normal conditions, no significant difference is observed in Na^+ or K^+ accumulation and

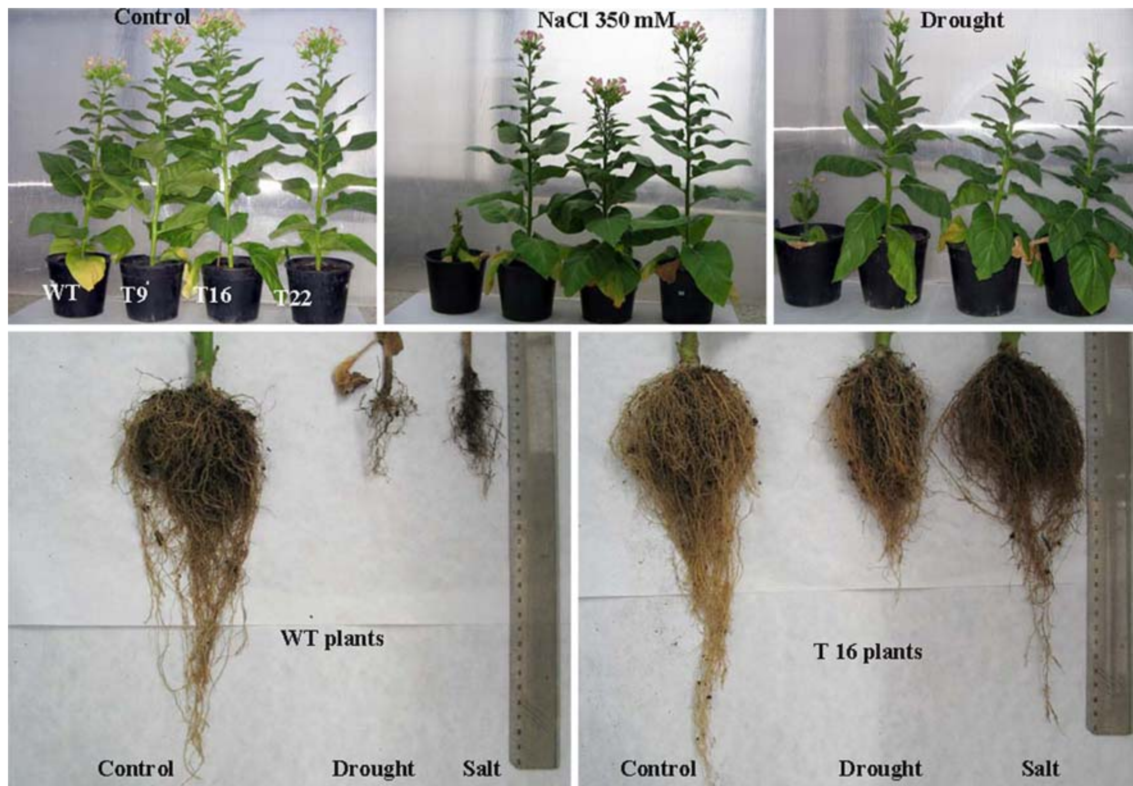

Fig. 11 Relative salt tolerance of WT and T2-*AlSAP*-overexpressing transgenic tobacco lines (events T9, T16 and T22) at the adult stage under greenhouse conditions. WT and transgenic plants were grown under either normal condition, a continuous presence of 350 mM NaCl or a continuous drought stress (soil RWC of 25%) until the end of the plant cycle. Note that WT plants could not sustain growth under these stress conditions while transgenic plants were able to produce viable seeds (*upper panel*). Photographs show the root growth of WT plants and T16 plants grown under control, salt and drought conditions at the end of their life cycle

partitioning for young/old leaves and roots of WT and transgenic plants (Fig. 13a, b). However, a dramatic change is observed under stress conditions, especially, in the partitioning of Na^+ and K^+. Thus, in transgenic plants, the Na^+ concentration in old leaves was 3.5 fold higher than in young leaves (Fig. 13a). Conversely, in WT plants, the Na^+ content in the young leaves was at least twice that of old leaves. In addition, Na^+ accumulation in WT roots was three-fold higher than in those of transgenic plants. Moreover, K^+ accumulation was ten-fold higher in transgenic roots compared to WT ones (Fig. 13b). All these findings suggest that transgenic plants were able to maintain a higher selectivity of K^+ over Na^+ in roots and sequestered almost 65% of the Na^+ uptake in senescent leaves, thereby keeping young leaves and roots protected from Na^+ ion toxicity. This could be one factor explaining the relatively normal growth of transgenic plants and their ability to produce viable seeds under salt stress. On the other hand, WT plants sequestered 70 and 20% of the Na^+ uptake in roots and young leaves, respectively.

To validate and dissect the physiological mechanisms of drought stress tolerance afforded by *AlSAP* overexpression in tobacco plant, the RWC of leaves (WT and T16 line) was estimated every 6 days after withholding water for 30 days. The leaves tested were of similar size and developmental stage. After stopping irrigation for 12 days and until the end of stress, the RWC of detached leaves overexpressing *AlSAP* was almost double than that of WT leaves (Fig. 13c). These results clearly showed that the water content of transgenic leaves was higher than that of WT plants. Water loss in plants occurs via stomata and cuticula, and under well-watered conditions, stomata remain open**.** Most of the water loss is due to stomatal transpiration. Under stress conditions, stomata are closed and then cuticular transpiration rate becomes the most important component for water loss and consequently for plant survival (Savé et al. 1993). The results obtained on RWC of detached leaves suggest that the constitutive expression of *AlSAP* gene in plant tissues may alter stomatal behaviour or increase water uptake. In order to explain the difference in the water loss rate between the WT and transgenic plants, key parameters (water use efficiency (WUE), mean transpiration rate (MTR), stomatal conductance and net carbon assimilation rate (NAR)) must be determined.

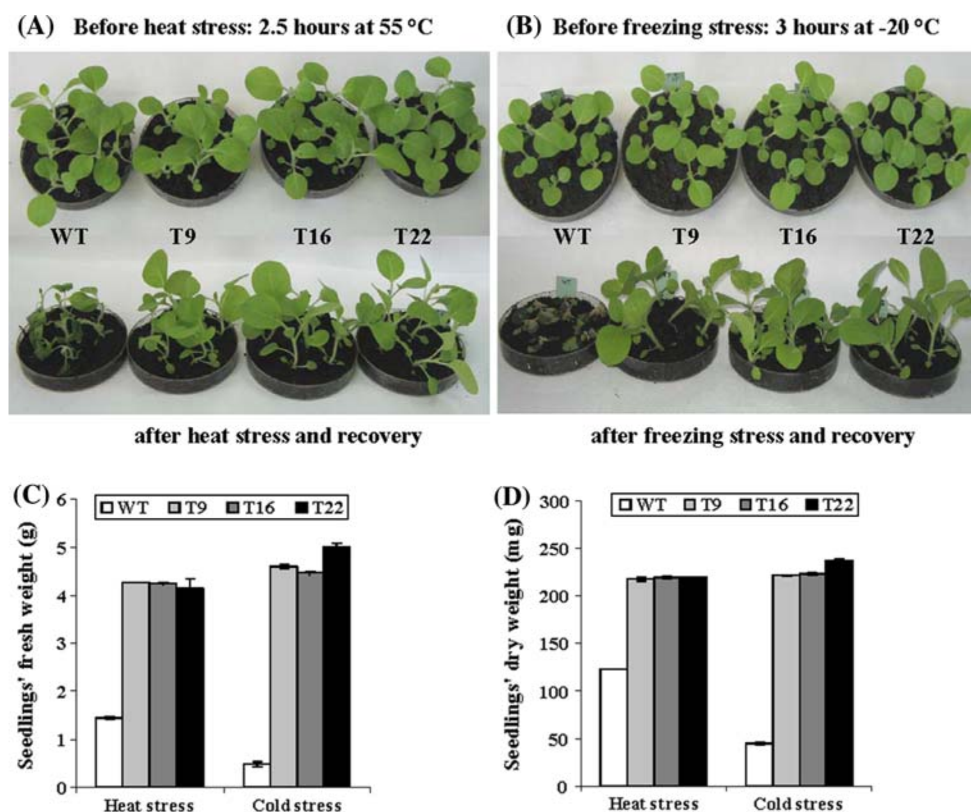

Fig. 12 Overexpressing *AlSAP* gene in transgenic tobacco confers tolerance to freezing (−20°C for 3 h) and heat (55°C for 2.5 h) stresses under greenhouse conditions. One-month-old seedling of WT plants and three *AlSAP* transgenic tobacco lines (T9, T16 and T22) were grown **a** at 55°C for 2.5 h or **b** at −20°C for 3 h and then transferred back to the normal condition for recovery. Photographs were taken before stress treatments and after 1 week of recovery. The *AlSAP* transgenic lines recovered better and growth of new leaves with elongated internodes was observed. **c** The fresh and **d** dry weights of WT and three *AlSAP* transgenic plants (T9, T16 and T22) were measured 1 week after recovery. Results are presented as means and standard errors from three independent experiments (10 plants of each line were grown for each experiment)

Discussion

AlSAP belongs to the SAP gene family, is a single gene and has no ortholog in dicots

We reported here the isolation of the *AlSAP* gene, a single copy gene of the halophyte grass *A. littoralis* and demonstrated that it belongs to SAP gene family. This family is composed of genes encoding proteins containing A20/AN1 zinc-finger domains. AlSAP contains both A20 and AN1 domains at the N-terminus and C-Terminus regions of the protein, respectively. In animals, the A20 protein has been characterized as an inhibitor of both apoptotic and necrotic cell death (De Valck et al. 1996). These authors also suggest that self-association of A20 is mediated by its zinc finger domain. The AN1 domain was found at the C-terminus of AN1, an ubiquitin-like protein in *Xenopus laevis* (Linnen et al. 1993). In humans, two A20/AN1 proteins, ZNF216 and AWP1 (associated with PRK1) have been widely studied. In animals, the A20/AN1 zinc-finger domain-containing family of proteins is well-characterized and plays a central role in regulating the immune response and inflammation as well as in inhibiting apoptosis (Heyninck and Beyaert 2005; Hishiya et al. 2006; Huang et al. 2004). In plants, the A20/AN1 zinc-finger domain proteins have been shown to be involved in stress responses (Huang et al. 2008; Jin et al. 2007; Kanneganti and Gupta 2008; Mukhopadhyay et al. 2004; Vij and Tyagi 2006). Using the GOST tools in Greenphyl database, we found that the most likely rice ortholog of *AlSAP* is the *OsSAP9* gene (87% of orthology). No ortholog was identified in dicots. Jin et al. (2007) recently reported that most zf-AN1 genes expanded in a monocot or eudicot-specific manner according to their phylogenetic tree. This suggests that SAP gene expansion could have occurred after the divergence of monocots and dicots and could explain the absence of orthologs to *AlSAP* in *A. thaliana*.

AlSAP is induced in response to a range of stresses

We have shown that *AlSAP* gene was induced by salt, drought, cold, heat, ABA and SA. It was reported that all members of the rice SAP family are also induced by at least

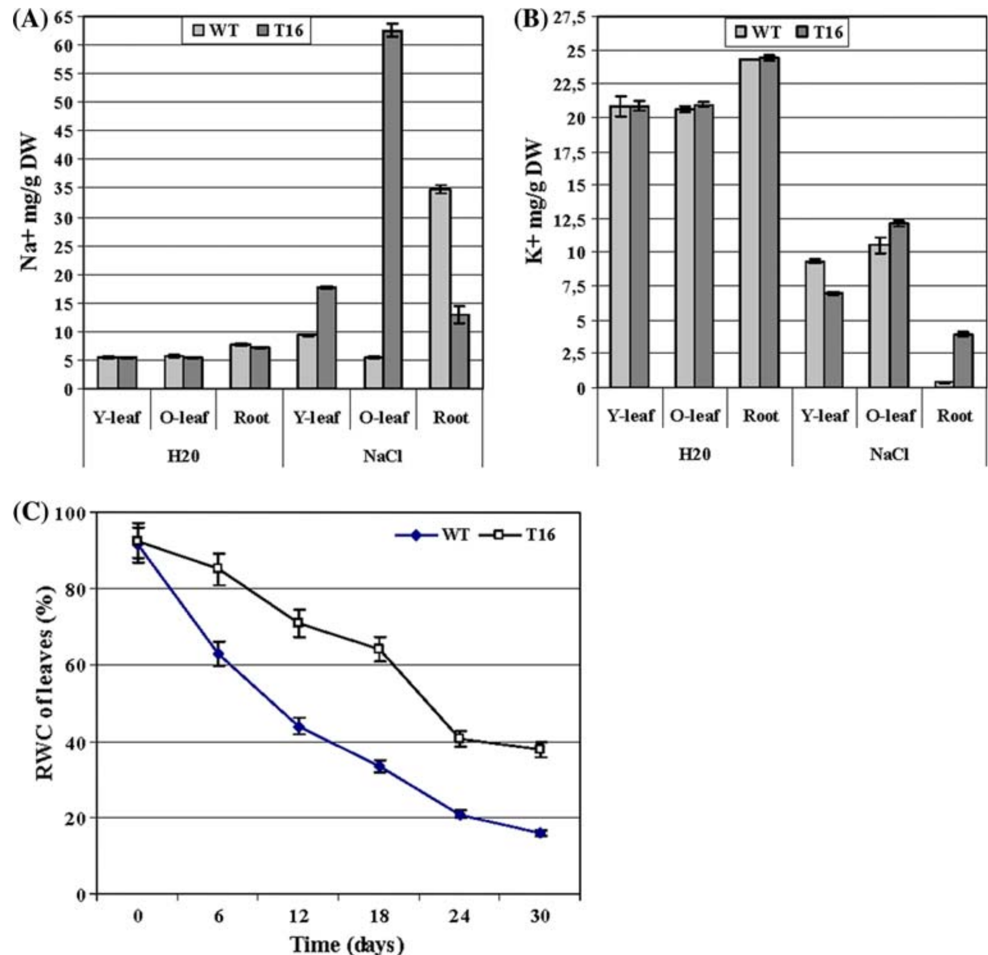

Fig. 13 Physiological characterization of WT and transgenic *AlSAP* tobacco plants grown under normal and stress conditions. **a** Time-course of relative water content (RWC) during plant dehydration. **b** Na^+ and **c** K^+ content (calculated as mg of ions per g of dry weight (DW) of the tissue) in young (Y) and old (O) leaves and roots of the WT and *AlSAP* transgenic plants (WT, T16) grown under the continued presence of 350 mM NaCl or in H_2O. In the histogram for each determination, roots, old leaf (*bottom*), young leaf (*top*), and roots were collected from three different plants of each type. Values are the mean ± SD ($n = 3$). Each value represents the average of independent measurements in three plants. *Error bars* represent SD

one type of abiotic stress, indicating that the *OsSAP* genes are a key component of stress response in rice (Vij and Tyagi 2006). The *OsSAP1* and *OsSAP8* genes were found to be induced by salt, drought, cold, desiccation, submergence, wounding, ABA and heavy metals (Kanneganti and Gupta 2008; Mukhopadhyay et al. 2004). An early accumulation of transcripts for both genes was observed within 30 min after stress treatment in 1 week-old seedlings of *indica* rice but it declined rapidly within 24 h later. This time course transcription profile differs from that observed for the *AlSAP* gene. The induction of *AlSAP* gene started later after application of the stress (1–2 days) but remained at a high level for a longer period (1–6 days). The *OsSAP9* (ZFP177) which is the nearest ortholog to the *AlSAP* gene (87% of orthology and 79% of amino acid identity) was demonstrated to be induced by heat and cold stresses but down-regulated by salt stress in *japonica* rice (Huang et al. 2008). These authors have also shown that following drought stress, *OsSAP9* is upregulated as early as 2 h after treatment and then down-regulated at 6 h. Our results, along with those reported by Huang et al. (2008), show differential regulation of *AlSAP* and its close rice ortholog *OsSAP9*, indicating that evolutionary changes in promoter structure occur between closely-related glycophytic (rice) and halophytic (*A. littoralis*) species and may have contributed to develop a salt tolerance mechanism in *A. littoralis*. Many evolutionary biologists consider that evolution often proceeds by changes in the spatial and temporal patterns of gene expression (Doebley and Lukens 1998). A detailed analysis of the transcripts accumulation under abiotic stresses of *AlSAP* in *A. littoralis* along with its ortholog *OsSAP9* in rice can throw more light on the importance of the temporal expression patterns of these genes in stress tolerance. Indeed, it has been hypothesized that differences in salt tolerance mechanisms between salt-sensitive *A. thaliana* and salt-tolerant *Thellungiella halophila* result from a differential regulation of the same basic set of genes involved in salt tolerance (Xiong and Zhu 2002; Zhu 2000, 2001).

Heterologous expression of the *AlSAP* gene in yeast cells and tobacco enhance their tolerance to abiotic stresses

The overexpression of *AlSAP* gene in yeast cells or in transgenic tobacco conferred tolerance to ionic and osmotic

stress under in vitro conditions. In addition, the expression of *AlSAP* gene conferred freezing- and heat-stress tolerance in transgenic tobacco. This contrasts with the salt resistance conferred by the transgenic expression of Rab17 in *Arabidopsis* which was due to a mechanism of osmotic rather than ionic tolerance (Figueras et al. 2004). Similarly, the transcript of *PDH45* (Pea DNA helicase 45) was shown to accumulate following Na^+ but not Li^+ treatments (Sanan-Mishra et al. 2005). The increased ability to grow under NaCl stress of the transgenic plants overexpressing this gene could likely be explained by an osmotic tolerance mechanism. The dual osmotic and ionic tolerance conferred by the *AlSAP* strongly supports the proposition of this candidate gene for improving salt and drought tolerance in cereals.

Under greenhouse conditions, *AlSAP* transgenic tobacco exposed to continuous salt or drought stress showed a higher level of tolerance. Indeed, these plants under salt or drought stress were able to maintain 75 and 55% of the yield observed under unstressed conditions, respectively. Under salinity stress, WT plants sequestered 70 and 20% of the Na^+ uptake in roots and young leaves, respectively while *AlSAP* plants sequestered Na^+ in senescent basal leaves. Similar differential Na^+ sequestration between old and young leaves was observed in tobacco and rice plants overexpressing glyoxalase (Singla-Pareek et al. 2003, 2008).

To our knowledge, experimental evidence for conferring abiotic stress tolerance has so far been reported in three genes belonging to the SAP gene family: *OsSAP1*, *OsSAP8* and *OsSAP9* (Mukhopadhyay et al. 2004; Huang et al. 2008; Kanneganti and Gupta 2008). In these reports, tolerance evaluation was performed either under in vitro conditions or using the recovery system in a greenhouse. By contrast, in our study, along with in vitro tolerance assays, we carried out experiments under greenhouse conditions applying a continuous stress during the whole plant cycle in order to mimic the conditions found in semi-arid and arid regions. By comparing our results and those reported by Mukhopadhyay et al. (2004) and Kanneganti and Gupta (2008), it is clear that overexpressing of *AlSAP*, *OsSAP1* or *OsSAP8* in tobacco conferred tolerance to drought and salt stresses. However, it is impossible to answer if transgenic tobacco plants expressing *AlSAP* are more or less tolerant than those transformed with *OsSAP1* or *OsSAP8*. In fact, the experimental procedures used for stress tolerance evaluation are different in the three cases. Furthermore, the three genes have a weak amino acid identity around 35% (no orthology relation). Finally, the *OsSAP1* and *OsSAP8* transcripts levels increased within 30 min after subjecting seedlings to various stresses while *AlSAP* transcript peaked within 2 days. This could suggest that the products of *OsSAP1* and *OsSAP8* are required in the early stage of stress response while *AlSAP* could be needed later. In contrast to these two above-mentioned genes (*OsSAP1*, *OsSAP8*), *OsSAP9* (Huang et al. 2008) showed 79% of amino acid identity and 87% of orthology with *AlSAP* (according to the phylo-genomic tree generated by GOST tools). It has been demonstrated that the overexpression of *OsSAP9* in transgenic tobacco conferred tolerance to both low and high temperature stresses but increased sensitivity to salt and drought stresses. The contrasting results about tolerance level to drought and salt stresses conferred by the two nearest orthologs genes *AlSAP* and *OsSAP9* could be due to either sequence/structural differences between these two proteins or to their origins (halophyte and glycophyte monocots). Our results show that AlSAP and its ortholog OsSAP9 act differently in transgenic tobacco despite their amino acid identity. In addition, the expression patterns of *AlSAP* in *A. littoralis* and of *OsSAP9* in rice are different in response to salt and drought stresses. For this purpose, further studies to clone the promoter of *AlSAP* gene and compare it with the one of its ortholog (*OsSAP9*) in rice are required.

We have shown that the steady state level of transcripts of some stress associated genes encoding proteins involved in anti-oxidative and protection activities are higher in unstressed *AlSAP* tobacco than in WT plants. It is well known that antioxidant enzyme activity is increased in plants in response to various environmental and chemical stresses (Allen 1995; Baek et al. 2006). 'Late Embryogenesis Abundant' or LEA proteins and osmotin are thought to protect macromolecules and membranes under stress conditions (Grover et al. 2001). In contrast to our findings, Huang et al. (2008) found that overexpression of *OsSAP9* in transgenic tobacco inhibited the expression of stress-related genes such as osmotin, NtERD10 A, C or D. Taken together, these results suggest that *AlSAP* from the halophyte grass (*A. littoralis*) and its nearest ortholog from rice *OsSAP9* act differently in tobacco plants.

A proposed role for AlSAP

The AlSAP protein has predicted serine, threonine, tyrosine and PKC phosphorylation sites, together with the presence of zf-A20 and zf-AN1 domains. It was suggested (as revealed by yeast two hybrid analyses) that A20 interacts not only with itself but also with the AN1 domain while the AN1 does not interact with itself (Kanneganti and Gupta 2008). Moreover, it was also suggested that OsSAP1 and OsSAP8 were cytoplasmic proteins and might carry out their functions via protein–protein interactions aided by their A20 and AN1 zinc finger domains (Kanneganti and Gupta 2008; Mukhopadhyay et al. 2004). It is therefore possible that AlSAP may interact with proteins needed for the phosphorylation cascade that targets proteins directly

involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes. This model is supported by several arguments based on mammalian A20/AN1 zinc finger data. Indeed, it was demonstrated that the human A20/AN1 zinc finger protein AWP1 interacts with a serine/threonine protein kinase PRK1 and thus may play a regulatory role in mammalian signal transduction pathways (Duan et al. 2000).

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