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Article *in* Plant Cell Tissue and Organ Culture · April 2014

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ORIGINAL PAPER

Overexpression of *Artemisia annua* sterol C-4 methyl oxidase gene, *AaSMO1*, enhances total sterols and improves tolerance to dehydration stress in tobacco

Alka Singh · Sunita Jindal · Bendangchuchang Longchar · Feroz Khan · Vikrant Gupta

Received: 25 July 2014 / Accepted: 10 December 2014

Abstract Biosynthesis of sterols is a multistep process in higher plants where the precursor cycloartenol gets converted into functional phytosterols after removal of two methyl groups at C-4 by an enzyme complex involving a sterol C-4 methyl oxidase (SMO). We identified and cloned a cDNA from *Artemisia annua* designated as *AaSMO1* showing similarity to SMO. The cDNA predicted to encode a polytopic protein with characteristic histidine-rich motifs and an ER retrieval signal. *GFP-AaSMO1* fusion protein was localized in endoplasmic reticulum of transformed protoplast and onion epidermal cells. *AaSMO1* expression was drastically induced upon osmotic/dehydration stress and its promoter showed the presence of abscisic acid responsive element. Transgenic tobacco plants ectopically overexpressing *AaSMO1* were raised, and various biochemical and physiological analyses of transgenics revealed increased total sterol, better germination and growth in subsequent generations. They also exhibited reduced sensitivity towards osmotic/dehydration stress which may be attributed to enhanced *SMO1* activity. Our studies demonstrated that apart from acting as phytohormones, plant sterols also participate in providing capability to plants for improved growth and adaptation during stress conditions. *AaSMO1* can be used as an excellent candidate for generating dehydration/drought tolerant plants.

Keywords Plant sterols · Sub-cellular localization · *AaSMO1* · Dehydration stress · RT-qPCR

Abbreviations

4-MU	4-Methylumbelliferone
ABA	Abscisic acid
ABRE	Abscisic acid responsive element
DRE/CRT	Dehydration-responsive element/C-RepeaT
ER	Endoplasmic reticulum
GA	Gibberellic acid
GFP	Green fluorescent protein
GUS	β-Glucuronidase
MeJa	Methyl jasmonate
MS	Murashige and Skoog medium
MUG	4-Methylumbelliferyl glucuronide
RT-qPCR	Reverse transcription quantitative real-time PCR
SMO	Sterol-4α-methyl-oxidase/sterol C-4 methyl oxidase
TCA	*Trans*-cinnamic acid
TLC	Thin layer chromatography

Key message

A stress-inducible sterol biosynthesis gene from *Artemisia annua* improves osmotic (desiccation) tolerance in transgenic tobacco plants.

Electronic supplementary material The online version of this article (doi:10.1007/s11240-014-0692-0) contains supplementary material, which is available to authorized users.

A. Singh · S. Jindal · B. Longchar · V. Gupta (✉)
Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India
e-mail: vikrant.gupta@gmail.com

F. Khan
Department of Metabolic and Structural Biology, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India

Published online: 17 December 2014

Introduction

Steroids are major class of compounds amongst which fat soluble sterols are essential components in fungal, animal and plant membranes. Unlike animal and fungal cells which synthesize one type of major sterol i.e. cholesterol and ergosterol, respectively, plant cells produce a wide array of sterols with sitosterol, stigmasterol and 24-methylcholesterol as predominating ones. Cycloartenol generated from mevalonate pathway produces sterols through a series of enzyme-mediated steps. Plant sterols being essential component of membranes are involved in several functions such as controlling membrane fluidity and permeability, adaptation of membranes to temperature, morphogenesis, cell differentiation, cell polarity and cellular patterning. Apart from the structural roles of sterols, plant campesterol act as precursor for biosynthesis of brassinosteroids (BRs) hormones (Bishop and Yokota 2001) which stimulate cell division and differentiation in plants. Plant sterols can be broadly classified into 4-desmethyl sterols (sitosterol, stigmasterol and campesterol), 4-methyl sterols and 4,4-dimethyl sterols. During their synthesis in plants, the first C-4-methyl group is removed from a 4,4-dimethyl-9β,19-cyclopropylsterol precursor followed by elimination of second C-4-methyl group several steps later rendering the sterol molecule functional as a membrane component (Rahier 2011). For the removal of 4α-methyl group from 4,4-dimethyl- or 4α-methyl-sterol precursors in animals and higher plants, an enzymatic complex is involved which contains sterol-4α-methyl-oxidase (SMO), 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase, a NADPH-dependent sterone reductase and a membrane-bound cytochrome b5 as an electron carrier (Pascal et al. 1994; Rahier et al. 1997; Gachotte et al. 1998; Rondet et al. 1999). Sterol C-4 methyl oxidases belong to a family of membrane-bound non-heme iron oxygenases which catalyze hydroxylation, desaturation, epoxydation or acetylation of hydrophobic substrates in higher plants. These enzymes possess three characteristic histidine-rich motifs ($HX_{(3–4)}H$, $HX_{(2–3)}HH$ and $HX_{(2–3)}HH$) and an endoplasmic reticulum (ER) retention signal (Jackson et al. 1990; Shanklin et al. 1994). Sterol C-4 methyl oxidases of ergosterol biosynthesis were reported and characterized from *Saccharomyces cerevisiae* (Bard et al. 1996) and fungus *Penicillium chrysogenum* which differed extremely in their copy number in the genome (Wang et al. 2008). Two distinct families of methyl oxidases termed *SMO1* and *SMO2* imparting distinct biochemical phenotypes were reported from *Arabidopsis thaliana* and *Nicotiana benthamiana* (Darnet et al. 2001; Darnet and Rahier 2004). Several developmental mutants defective in sterol biosynthesis in plants have been emerged from several screens (Lindsey et al. 2003). Arabidopsis *hydra* and *fackel* mutants showed defective cell division, cell expansion, embryogenesis and abnormal sterol composition which indicated a critical role of sterols in embryonic patterning and meristem programming (Jang et al. 2000; Schrick et al. 2000; Souter et al. 2002). Any mutant with defective *SMO* function in plants has not been reported so far probably due to the reason that blocking of *SMO* catalyzed step can compromise plant viability.

Environmental conditions of abiotic stresses such as drought, salinity, cold, high temperature, nutrient imbalances etc. lead to dehydration or osmotic stress through reduced availability of water to plant cells which further hamper vital cellular functions. To deal with water-deficit stress conditions, plants synthesize and accumulate various osmoprotectants. A close relative of Arabidopsis, i.e. *Thellungiella salsuginea*, is more tolerant to high salinity and dehydration because of much higher levels of most metabolites upon abiotic stress treatments (Lugan et al. 2010). Genetic mutations and their screens have been helpful in identifying genes essential for osmotic tolerance and its associated signal transduction (Borsani et al. 2002). Genomic and molecular tools have facilitated gene discovery and enabled genetic engineering for improved water-deficit stress tolerance in plants by introducing functional genes (Zhai et al. 2013; Yang et al. 2014) or regulatory genes (Cai et al. 2014; Jiang et al. 2014; Yuan et al. 2014). An Arabidopsis mutant (*dry2/sqe1-5*) defective in *squalene epoxidase 1* gene was identified because of its extreme hypersensitivity to drought stress. The *dry2/sqe1-5* mutant showed reduced levels of sitosterol and stigmasterols due to the reduced availability of sterol biosynthesis intermediates (Posé et al. 2009). In the present study, we identified a sterol C-4 methyl oxidase gene (*AaSMO1*) in *Artemisia annua*, a plant that produces anti-malarial drug artemisinin. Artemisinin is synthesized via isoprenoid pathway where farnesyl diphosphate (FPP) serves as a central common precursor for the first committed steps towards sterols and sesquiterpene (artemisinin) biosynthesis. FPP is competed between squalene synthase for sterols biosynthesis and amorpha-4,11-diene synthase for diverting the carbon flux to artemisinin biosynthesis. This junction represents a crucial branch-point for keeping a balance between sterol and sesquiterpene biosynthesis in *A. annua*. Since there are indications for sterol biosynthesis as a central role in abiotic stress tolerance and regulation of reactive oxygen species, therefore we analyzed *A. annua SMO1* gene in relation to stress tolerance.

Materials and methods

Plant material

A. annua (var: CIM-Arogya) seeds were obtained from the National Gene Bank for Medicinal and Aromatic Plants

maintained at CSIR-CIMAP, Lucknow, India. The seeds were surface sterilized and germinated on sand:peat:vermiculite (2:2:1) mixture in a culture room maintained at 23 ± 1 °C with 60–75 % relative humidity and 16/8 h of day/night cycle. Tobacco (*Nicotiana tabacum* cv. Xanthi) was used for stable overexpression of *AaSMO1*. Seeds were germinated on MS medium, and maintained aseptically at 23 ± 1 °C in culture room at a photoperiod of 16/8 h.

Elicitor and abiotic stress treatments

Two-month-old *A. annua* seedlings were used for challenging with different abiotic stresses or elicitors. For elicitor treatment, 0.3 mM methyl jasmonate (MeJa), 150 mg/l chitosan, 1 mM salicylic acid (SA), 1 mM *trans*-cinnamic acid (TCA), 100 µM gibberellic acid (GA) and 10 µM abscisic acid (ABA) were sprayed on the leaves of seedlings. Appropriate control (mock) treatment was also given to separate seedlings. For salinity or osmotic/dehydration stress, the seedlings were immersed in 400 mM NaCl or mannitol solution, respectively. Leaf tissue was harvested at different time intervals of 0, 0.5, 1.5, 3, 6, 9, 24 or 72 h from the treated and control seedlings, frozen in liquid nitrogen and stored at −80 °C till RNA extraction.

Cloning of full-length *AaSMO1* gene and its promoter

A cDNA library (λ-ZAP Express) of trichome-enriched leaf tissue of *A. annua* (Nair et al. 2013) was used for generating ESTs. Randomly picked clones were sequenced with ABI3130XL or ABI3730 XL DNA analyzer using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). The EST sequences were assembled with CAP3 program (http://pbil.univ-lyon1.fr/cap3.php) and subjected to similarity searches in NCBI protein (nr) database by using BLASTX algorithm with default parameters. An EST showing similarity to sterol C-4 methyl oxidases in the database was identified. This cDNA was subjected to RACE by using gene-specific primers (Supplemental Table 1) and 5′ RACE System for Rapid Amplification of cDNA Ends, ver 2.0 (Invitrogen, USA) following the manufacturer's instruction. The resulting 5′ RACE amplicon was first ligated in pGEM-T Easy vector (Promega, USA) as intermediate vector and subsequently inserted in the original pBK-CMV (phagemid excised from λ-ZAP Express vector) clone upstream and in-frame to the partial EST.

In order to isolate promoter of *AaSMO1*, a genomic library from *A. annua* DNA was constructed. Genomic DNA from *A. annua* leaf tissue was extracted and library was prepared by using Genome Walker Universal Kit (Clontech, USA) as per the manufacturer's instruction. Promoter of *AaSMO1* was PCR-amplified by using adapter-specific forward primers (provided with the kit) and gene-specific nested reverse primers (SMOPrGSP1 and SMOPrGSP2; Supplemental Table 1) for primary and secondary PCR amplifications. Largest amplified fragment was cloned in pGEM-T Easy vector and sequenced completely. The sequence was analyzed by using 'PLACE' (http://www.dna.affrc.go.jp/htdocs/PLACE) to identify various *cis*-acting elements.

GFP-AaSMO1 plasmid construction and sub-cellular localization

Coding region of *AaSMO1* was PCR-amplified by using two oligonucleotides GFPSMO-F and GFPSMO-R (Supplemental Table 1) containing *Apa*I and *Xma*I restriction sites, respectively, for directional cloning. Initially the amplified product was cloned in pGEM-T Easy vector and confirmed by sequencing. Subsequently, recombinant pGEMT-Easy was double-digested with *Apa*I/*Xma*I restriction enzymes and the released fragment was sub-cloned into psGFPcs_1/pUC18 vector (Jiang et al. 2001) placing *AaSMO1* in-frame between green fluorescent protein (*GFP*) and a *nos* terminator. The resulting *GFP-AaSMO1* fusion plasmid was then introduced into *Catharanthus roseus* protoplast cells by using the method described by Yoo et al. (2007). The *GFP-AaSMO1* fusion construct and a control plasmid vector ER-rk CD3-959 (Nelson et al. 2007) which has an ER-marker were transformed into onion epidermal cells. The epidermal sheath peeled from onion bulbs were subjected to particle bombardment using PDS-1000 Helios Gene Gun (BioRad) as described by Citovsky et al. (2006).

Reverse transcription real-time PCR (RT-qPCR) analysis

To analyze the expression of *AaSMO1* against various stresses, RT-qPCR was done for RNA extracted from 2-month-old *A. annua* seedlings treated with various abiotic stresses and elicitors. Total RNA was extracted from the treated seedlings and 2–3 µg converted to cDNA by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). RT-qPCR was carried out as described previously (Rajakani et al. 2014) by using *AaSMO1*-specific primers. *Actin* was used as an endogenous control for normalization of cDNA and gene expression data analysis. The primer sequences used for RT-qPCR are listed in Supplemental Table 1. Three independent experiments with individual cDNA preparations, each one in triplicates, were carried out and relative gene expression was calculated by ddCT method.

Binary constructs and tobacco transformation

The full-length *AaSMO1* cDNA was PCR-amplified using original cDNA (pBK-CMV phagemid) as template and gene-

specific SMO-F and SMO-R primers (Supplemental Table 1) for directional cloning in pBI121 binary vector. The PCR product was digested with *Xba*I and *Sac*I restriction enzymes, and inserted into binary vector pBI121 replacing the *GUS* gene driven by constitutive CaMV35S promoter. The *AaSMO1* promoter was PCR-amplified by using previously cloned genomic fragment as template and oligonucleotides SMOproF and SMOproR (Supplemental Table 1). The amplified promoter was ligated in the correct orientation in context to a promoter-less *GUS* reporter gene in pBI101 binary vector.

The binary constructs were independently moved into *Agrobacterium tumefaciens* strain AGL1 through triparental mating. The *Agrobacterium*-mediated leaf disk transformation-regeneration method was used to transform 2 to 4-week-old *N. tabacum* according to standard method (Horsch et al. 1985). Primary transformants and subsequent generations (transgenics) were selected on kanamycin (200 mg/l) containing MS medium, and maintained at 28 ± 1 °C and 16/8 h light/dark photoperiod. Transgenic tobacco lines were screened by *nptII*- and *AaSMO1*-specific PCR amplifications.

Biochemical analysis of transgenic tobacco plants

Biochemical analysis was done for transgenic tobacco lines expressing *AaSMO1* transcript. The ectopic expression of transgene in kanamycin-resistant tobacco lines was confirmed by RT-qPCR by using *AaSMO1*-specific oligonucleotides. *Ubiquitin* of tobacco was used as an endogenous control for expression analysis. Total sterol content and profile in transgenic lines was analyzed by the method described by Darnet and Rahier (2004) with some modifications. Total sterol obtained from 100 to 800 mg leaf tissue was weighed and re-dissolved in 0.5–1.0 ml n-hexane, and equal amount of each was separated by TLC on silica gel 60F254 plates (Merck, Germany) using ethyl acetate/hexane (3:7, v/v) as a mobile phase. Free fatty acid (FFA), docosahexaenoic acid (DHA), ergosterol (Erg), stigmasterol (St), sitosterol (Si) and cycloartenol (Cy) from their stocks (1 mg/ml each) were also run as standards on the same TLC plate. Plate was air-dried, sprayed with anisaldehyde solution and baked at 120 °C for 10-15 min to develop the spots. The osmolyte proline content in 4 to 6-week-old seedlings treated with 200 mM mannitol was estimated by using the method described by Bates et al. (1973). Total protein content in leaf tissue of transgenic and wild type plants were estimated by standard Bradford method using a standard curve made from known amounts of bovine serum albumin.

Leaf disk senescence and seed germination assay

Wild type and transgenic tobacco plants were subjected to leaf disk senescence assay by using the method described by Tuteja (2010). For chlorophyll estimation, leaf disks (1 cm^2) were placed on MS medium containing NaCl or mannitol, and incubated for 7 days. Then the disks were individually submerged in 3 ml of 80 % acetone (in ethanol) and kept overnight for leaching of chlorophyll. Estimation of chlorophyll was done spectrophotometrically by recording absorbance (A) at 645 and 663 nm (Lichtenthaler 1987).

To conduct seed germination assays upon abiotic stress and elicitor treatment, wild type and T_2 seeds of three selected transgenic tobacco lines were surface sterilized and sown on 1/5 MS medium supplemented with 100 mM NaCl, 100 and 400 mM mannitol, 50 μM ABA or 100 μM GA. Petri-dishes in biological triplicates with 100 seeds per dish were used for every given treatment and germination was done in a growth chamber (25 ± 2 °C and 16/8 h of light/dark cycle). Seed germination was considered after emergence of radicle and green cotyledon, and percentages were evaluated after 15 days of sowing.

GUS histochemical staining and fluorometric assay

Tissue-specificity of *AaSMO1* promoter was qualitatively checked by *GUS* histochemical staining. Quantitative measurement of *AaSMO1* promoter-driven GUS activity in tobacco tissues and protoplast cells were carried out by method described by Jefferson et al. (1987). Reaction was done with 1 mM 4-methylumbelliferyl glucuronide (MUG) as substrate and 4-methylumbelliferone (4-MU) was used for preparing standard curve. Fluorescence was recorded at 365/455 nm excitation/emission and *GUS* activity was calculated in nmoles mg^{-1} h^{-1} for each sample.

Microscopy and imaging

For sub-cellular localization, protoplasts harboring *GFP-AaSMO1* fusion plasmid were visualized under a Zeiss LSM 510 Meta Laser Scanning Confocal Microscope (Zeiss, Germany) with excitation/emission wavelength of 488/510 nm for GFP at CSIR-National Botanical Research Institute, Lucknow, India. Transient expression in onion peel by particle bombardment and its visualization under TCS SP2 (AOBS) Laser Scanning Confocal Microscope (Leica Microsystems) was carried out at National Institute of Plant Genome Research, New Delhi, India. Excitation/emission wavelength of 587/610 nm (mCherry) was used for ER-rk CD3-959 marker vector. For taking stomatal images, impression of the stress-treated wild type and transgenic tobacco leaves were printed by placing on 3 % molten agarose. The leaves were then removed and their impressions on solidified agarose were photographed under a light microscope.

Results and discussion

Cloning of full-length *AaSMO1* from *A. annua*

Sterols are crucial components of eukaryotic membranes that control membrane fluidity, permeability and play an important role in cell signaling, polarity and sorting. A cDNA similar to sterol C-4 methyl oxidase was identified in a library made from trichome-enriched tissue of *A. annua*. The full-length *AaSMO1* cDNA sequence was found to be 1,374 bp long which was submitted to NCBI GenBank (Accession: GQ847864). The cDNA has 41.4 % GC content when poly(A) tail is not considered. It has a coding region of 918 bp with 5′ and 3′ UTRs of 65 and 391 bp, respectively. The deduced protein of *AaSMO1* has 305 amino acids with a predicted molecular weight and isoelectric point of 35.44 kDa and 7.75, respectively. Its instability index II is computed to be 42.46 which classify this protein to be unstable in nature (http://web.expasy.org/cgi-bin/protparam). BLASTP of AaSMO1 showed highest homology with a putative C-4 sterol methyl oxidase (88 % identity, GenBank accession BAC57961) of *Aster tripolium* in NCBI. Other close homologs include *A. thaliana* SMO1-2 (64 % identity, NP_567670), sterol-4α-methyl oxidase 1-1 of *Populus trichocarpa* (62 % identity, XP_006368716) and C-4 sterol methyl oxidase 1 of *N. benthamiana* (65 % identity, AAQ83691). AaSMO1 also showed similarity to SMO2-2 of Arabidopsis (45 % identity, NP_001077975) and ERG25 of *S. cerevisiae* (33 % identity, EDN61653). In a previous report, five cDNAs including three isoforms of *SMO1* and two isoforms of *SMO2* acting at two distinct steps of phytosterol biosynthesis branch were cloned and reported from Arabidopsis (Darnet et al. 2001; Darnet and Rahier 2004). Sequence alignment of AaSMO1 using ClustalW with representative plant and yeast SMO sequences available in database showed the presence of three distinct histidine-rich clusters (HRLFH, HKQHH and HHDLHHH) characteristic of this enzyme and a C-terminal ER retrieval signal KKXX (Supplemental Fig. S1a). All the three histidine-rich motifs exhibit specific topological spacing within the histidine-motif typical of yeast Δ^5-desaturase (*ERG3*) and SMO (*ERG25*) families. The histidine-rich motifs are considered to provide ligand for a presumed catalytic di-iron centre like other enzymes which possess these motifs and catalyze desaturations or hydroxylations (Shanklin et al. 1994; Lee et al. 1998). Site-directed mutational analysis of recombinant Δ^7-sterol-C5(6)-desaturase enzyme of *A. thaliana* depicted eight evolutionarily conserved histidine residues present in the three histidine-rich motifs to be essential for catalytic activity (Taton et al. 2000). Deduced protein sequence of *AaSMO1* showed the presence of eight histidine residues in the three conserved histidine-rich domains. Phylogenetic analysis of SMO and other related proteins showed distinct clades for SMO1 and SMO2 families. AaSMO1 clustered with SMO1 group comprising of sterol C-4 methyl oxidases reported from *A. thaliana*, *N. benthamiana* and *Vitis vinifera* etc. indicating its closeness with the AtSMO1 family, and within the group it sub-grouped with an uncharacterized putative C-4 sterol methyl oxidase from *A. tripolium* (Supplemental Fig. S1b). Existence of two distinct SMO families was apparent in the phylogenetic tree which indicates distinct substrate specificity for sterol C-4 methyl oxidase enzymes. BLAST analysis of *AaSMO1* in 'Transcriptome Shotgun Assembly (TSA)' of *A. annua* in NCBI database indicated the presence of two distinct SMOs with multiple isoforms. This supports the fact that in photosynthetic eukaryotes, cDNAs encoding two distinct C-4-methylsterol oxidases controlling the levels of 4,4-dimethylsterol and 4α-methylsterol precursors function independently. Hydropathy analysis of AaSMO1 shows that the regions between 15–25, 40–65, 85–135, 175–210 and 252–260 amino acids are hydrophobic in nature (Supplemental Fig. S2). A total of four transmembrane helices were predicted at 44–63, 86–105, 126–144 and 183–207 (excluding N-terminal) residue regions of AaSMO1 when subjected to HMMTOP (http://www.enzim.hu/hmmtop; Tusnády and Simon 2001). Hydropathy plot indicates that AaSMO1 is a polytopic protein like other known sterol methyl oxidases such as ERG25 (Li and Kaplan 1996). The grand average of hydropathicity (GRAVY) was found to be −0.014 for this protein. All these predictions were found to be congruent with characterized SMOs from yeast and other eukaryotes. In order to assess the functionality of the enzyme, in silico molecular docking of AaSMO1 with substrates of SMO1 and SMO2 (i.e. 24-methylene cycloartanol and 24-ethylidene lophenol, respectively) was done by homology modelling approach. Both substrates docked with significant scores with AaSMO1 which were also comparable to the docking scores obtained with AtSMO1-1 isoform (Supplemental Fig. S3).

Sub-cellular localization of AaSMO1

To study the sub-cellular localization, *GFP-AaSMO1* fusion plasmid construct was introduced into protoplast cells prepared from *C. roseus* leaf tissue. Confocal imaging of transformed protoplast cells showed diffused green fluorescent signal predominantly in ER due to the localization of GFP-SMO fusion protein. While protoplast cells transformed with control GFP plasmid showed green fluorescence throughout in cytosol of the cell (Fig. 1a–f). To avoid ambiguous signals emitted from autofluorescence of chloroplasts in protoplast cells and to further confirm GFP-SMO fusion protein's organellar localization, a

control vector ER-rk CD3-959 having ER-marker was used and transfected into onion epidermal tissue. Both ER-rk marker (control) and GFP-SMO fusion proteins showed consistent and similar patterns of fluorescence signals in the transfected onion peel cells. AaSMO1 protein showed striking co-localization with ER-marker in the extensive network of endoplasmic reticulum (Fig. 1g–p). These results further support the presence of functional ER retention signal and transmembrane nature of the protein. Since much of the sterol metabolism is localized to endoplasmic reticulum, association of AaSMO1 with ER is expected.

Expression of *AaSMO1* and its response to abiotic stresses/elicitor treatment

To examine the tissue-specificity of *AaSMO1*, RT-qPCR-based expression analysis was conducted on cDNAs prepared from leaf, stem and root of 1.5-, 3- and 6-month-old and inflorescence of 6-month-old *A. annua* plants. RT-qPCR revealed that *AaSMO1* transcript expressed in all the tested tissues with highest expression noticed in leaf of young-aged (1.5- and 3-month-old) plants which decreased as the plant grows older (Fig. 2a). Least transcript levels were seen in root and flower, while overall it exhibited relatively higher expression in leaf. The requirement of sterols is high when the plant is actively growing which is reflected by a high expression of *AaSMO1* in leaf tissue of young *A. annua* seedlings and subsequent decline upon maturity.

To investigate regulation of *AaSMO1*, we monitored its expression during abiotic stresses and elicitor treatment by RT-qPCR. Expression analysis revealed that *AaSMO1* consistently and significantly got down-regulated (up to ~5 fold) under salinity conditions. Osmotic/dehydration stress markedly induced the transcript levels during 6–9 h of treatment and decreased then after. Maximum induction of up to ~30-folds at 6 h time point was noticed against dehydration stress (Fig. 2b). Dehydration or drought condition leads to transcriptional changes and triggers ABA biosynthesis which in turn induce stress-related functional proteins (water channels, transporters, LEA, chaperons and osmolyte biosynthesis etc.) and regulatory proteins (DREB, MYC, MYB, bZIP and NAC transcription factors). Since many drought-inducible genes are reported to be responsive to exogenous elicitors, we tested the effect of various elicitors on the expression of *AaSMO1*. Transcript levels of *AaSMO1* were consistently increased upon ABA treatment and induced to >2.0-fold relative to the control seedlings. The dehydration challenge due to mannitol treatment triggers endogenous biosynthesis of phytohormone ABA which in turn causes stomatal closure and induction of stress-related genes. In this case, a high expression (~30-folds) of *AaSMO1* clearly indicates that it could be one of the downstream targets. While on the other hand, exogenous application of ABA could comparatively weakly induce *AaSMO1* gene probably due to lesser uptake or availability of ABA to affect transcriptional cascade. As ABA-dependent and ABA-independent pathways operate

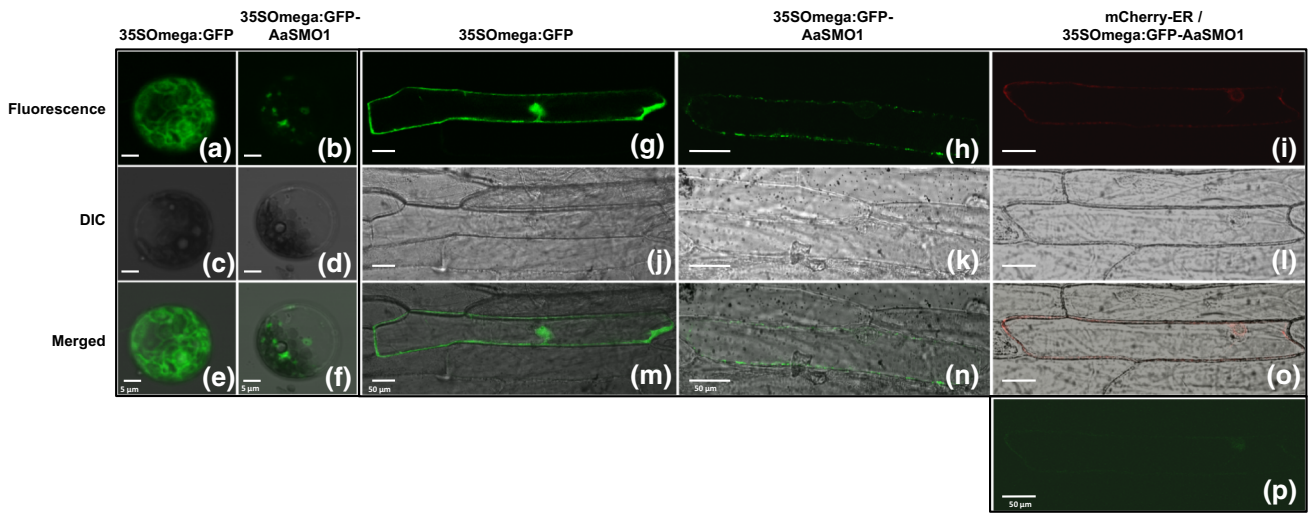

Fig. 1 Sub-cellular localization of AaSMO1. Protoplasts of *C. roseus* were transformed with *GFP-AaSMO1* fusion construct (*35SOmega:GFP-AaSMO1*) or GFP vector (*35SOmega:GFP*). Onion epidermal tissue was transfected with *GFP-AaSMO1* fusion and ER-rk CD3-959 (ER marker) plasmids. **a**, **c** and **e** Protoplast cell transformed with mock GFP vector. **b**, **d** and **f** Protoplast cell transformed with *GFP-AaSMO1* fusion construct. **g**, **j** and **m** Onion epidermal cell transformed with GFP vector. **h**, **k** and **n** Onion epidermal cell transformed with *GFP-AaSMO1* fusion construct. **i**, **l**, **o** and **p** Epidermal cell co-transfected with *AaSMO1* and ER-marker plasmid. Fluorescence images (**a**, **b**, **g–i** and **p**), Differential interference contrast (DIC) images (**c**, **d**, **j–l**), and merged images of fluorescence and DIC (**e**, **f**, **m–o**). *Scale bar*; 5 µm (**a–f**), 50 µm (**g–p**)

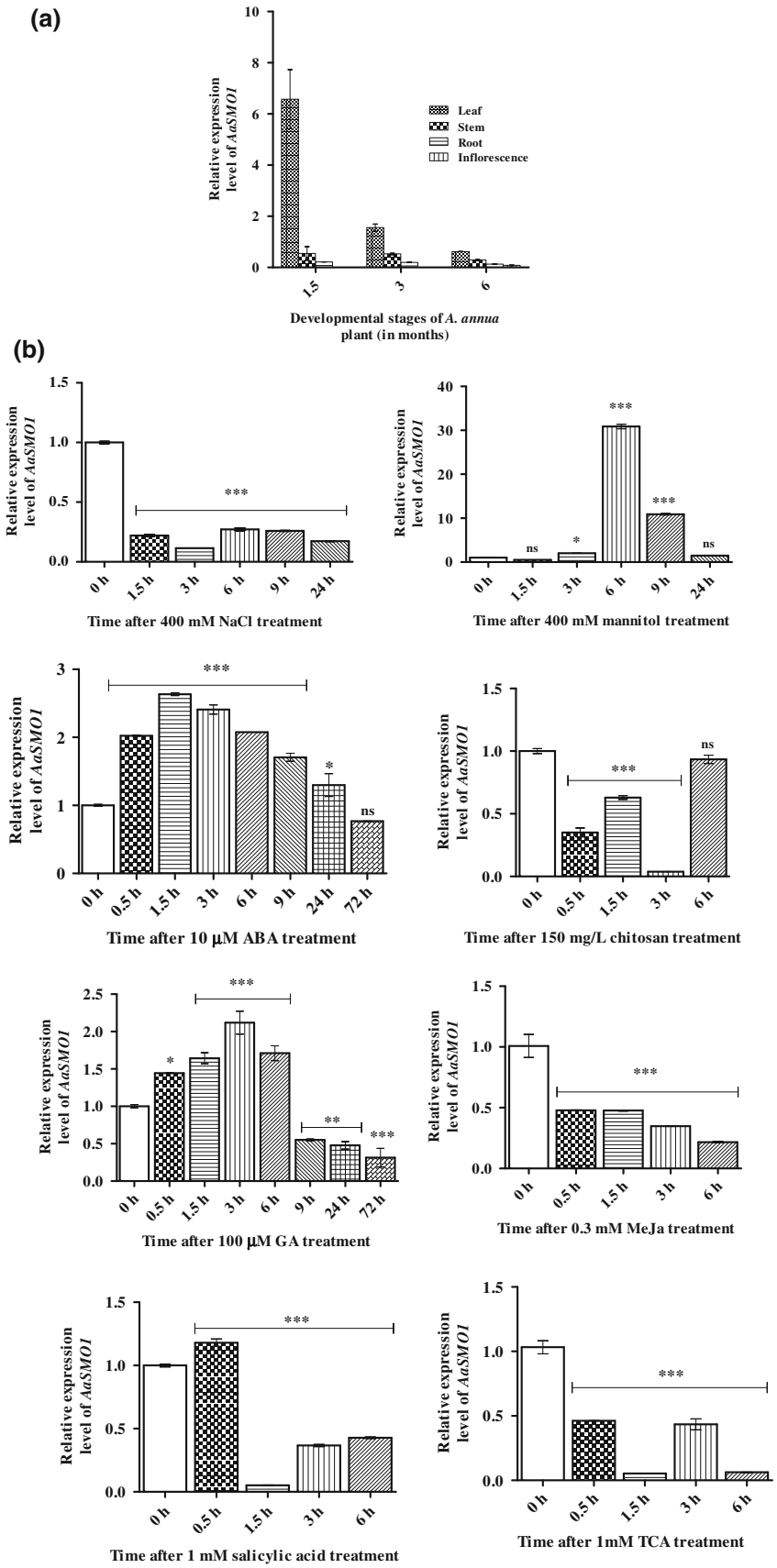

Fig. 2 Expression analysis of *AaSMO1* in *A. annua* by RT-qPCR. **a** Tissue-specific expression of *AaSMO1* in 1.5-, 3-, and 6-month-old wild type plants. **b** Relative expression of *AaSMO1* in response to salinity (NaCl), dehydration (mannitol) and various elicitor (ABA, chitosan, GA, MeJa, SA and TCA) treatments. The *actin* gene was used as an endogenous control for normalization. The statistical significance was analyzed by using one-way ANOVA in each case for variance in comparison to calibrator (*asterisk* indicates the values are significantly different from calibrator, $***P < 0.001$)

in plants, several stress-related genes are induced by exogenous application of ABA while others are not affected (Shinozaki and Yamaguchi-Shinozaki 2007). It is also established that a crosstalk between stress signaling cascades occurs which results in ultimate plant stress tolerance or response (Seki et al. 2003). Chitosan and TCA showed inconsistent levels of *AaSMO1* transcript induction which might be due to the overlapping physical and mechanical stresses (Fig. 2b). Jasmonates are regarded as endogenous elicitors that play crucial roles during stress, plant growth and development (Wasternack 2007). MeJa treatment significantly and consistently reduced *AaSMO1* transcript abundance at all the time points studied (Fig. 2b). GA induced the gene significantly during 3–6 h, and a subsequent reduction in transcript levels was seen. SA treatment significantly lowered the *AaSMO1* transcript levels at early hours (1.5 h) which gradually increased over time (Fig. 2b). Based on RT-qPCR data, we conclude that the expression of *AaSMO1* gene is induced by osmotic/dehydration, ABA and GA treatments while down-regulated by salinity, MeJa and SA treatments. An extensive expression analysis by using 22 K microarray identified 17 genes including a C-4 sterol methyl oxidase (CSMO) which expressed exclusively in two drought-tolerant barley genotypes under drought stress (Guo et al. 2009). *AaSMO1* might be a gene working downstream in abiotic stress pathways and its delayed response can be attributed to a tight regulation via other regulatory candidates (early responsive genes) involved in the concerned pathway. Our findings suggest that the transcriptional regulation of this gene is influenced by environmental conditions to some extent which also indicate a complex cross-talk network operates to regulate sterol biosynthesis in *A. annua* for combating stress.

Overexpression of *AaSMO1* in tobacco and analysis of transgenic plants

To study the physiological role of *AaSMO1*, it was overexpressed in *N. tabacum*. Out of several independently regenerated shoots, a total of seven lines which were kanamycin resistant and RT-qPCR-positive for *AaSMO1* foreign transcripts were maintained to maturity. In transgenic T_3 lines (*AaSMO1*-OE1, *AaSMO1*-OE2 and *AaSMO1*-OE3), *AaSMO1* transcripts were accumulated at significantly high levels while wild type and mock vector-transformed plants lacked expression (Fig. 3a). These lines were further used for biochemical and stress tolerance studies. All the transgenic lines did not show any significant morphological changes when compared with wild type except the in vitro-maintained transgenics were comparatively slightly darker in leaf color (data not shown). When compared with wild type, overexpression lines possessed lower amount of total soluble protein while they produced higher content of total sterols (Fig. 3b, c). The differences between mock vector-transformed and wild type plants were not significant for the parameters that were studied. The sterol fractions from transgenic and control plants were separated and analyzed by TLC. The TLC profile showed altered amounts of 4,4-dimethyl sterols and 4α-methyl sterols in transgenic lines in comparison to wild type plants (Fig. 3d). Altered pattern of sterol profile was found to be persistent in T_1, T_2 and T_3 generations. Overexpression of a rate limiting enzyme is expected to accumulate the immediate product as well as the products of subsequent steps to a certain degree (Verpoorte and Memelink 2002). Our results showed an increase in both substrate and product of *SMO1* enzyme i.e. 4,4-dimethylsterol and 4α-methylsterols, respectively, as well as elevated accumulation of desmethylsterols (sitosterol and stigmasterol; the final plant phytosterols) in transgenic lines. Higher accumulation of 4α-methylsterols in overexpression lines could be attributed to the similarity of *AaSMO1* with *AtSMO1* isoforms of Arabidopsis. Silencing of *NtSMO1* (71 % similar to *AtSMO1*) in *N. benthamiana* resulted in the accumulation of 4,4-dimethylsterol and unaltered level of 4α-methylsterols, whereas, *SMO2*-silenced plants largely accumulated 4α-methyl-Δ^7-sterols (i.e. 24-ethylidene lophenol and 24-ethyl lophenol) with no change in the levels of 4,4-dimethyl sterols (Darnet and Rahier 2004). Our data indicate that *AaSMO1* operates at conversion step of 4,4-dimethylsterol to 4α-methylsterols in *A. annua*. Increased accumulation of desmethylsterols in transgenic tobacco lines may be due to enhanced substrate influx for other SMO isoforms for catalyzing second step of methyl group removal. A relatively higher accumulation of cycloartenol/4,4-dimethyl sterol (24-methylene cycloartanol; a substrate of *SMO1*) in the transgenic tobacco lines is quite interesting as this step occurs upstream in the pathway. Higher accumulation of substrate as well as product prompted us to analyze the effect of this ectopic overexpression on the expression of other sterol pathway genes such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (*NtHMGR*), sterol 14α-demethylase (*NtCYP51*) and sterol C-24 methyltransferases (*NtSMT1* and *NtSMT2*) in transgenic lines by RT-qPCR. The formation of cycloartenol from suqalene epoxide is the first committed step in sterol biosynthesis pathway. The *HMGR* and *SMT1* play pivotal role in regulating the carbon flux into end sterol products (Chappell et al. 1995; Schaller et al. 1995; Holmberg et al. 2002, 2003). *NtCYP51* and *NtHMGR* showed remarkably higher transcript levels (>5 fold relative to wild type) in the transgenics while non-significant effect on relative expression of *NtSMT1* and *NtSMT2* was noted (Fig. 3e). High accumulation of sterols due to overexpression of *AaSMO1* probably induce *NtHMGR* (upstream to *SMO1*) and *NtCYP51* (downstream to *SMO1*) genes to higher levels through feedback for producing and diverting more precursors or intermediates for producing end product sterols in the pathway. A sterol-overproducing

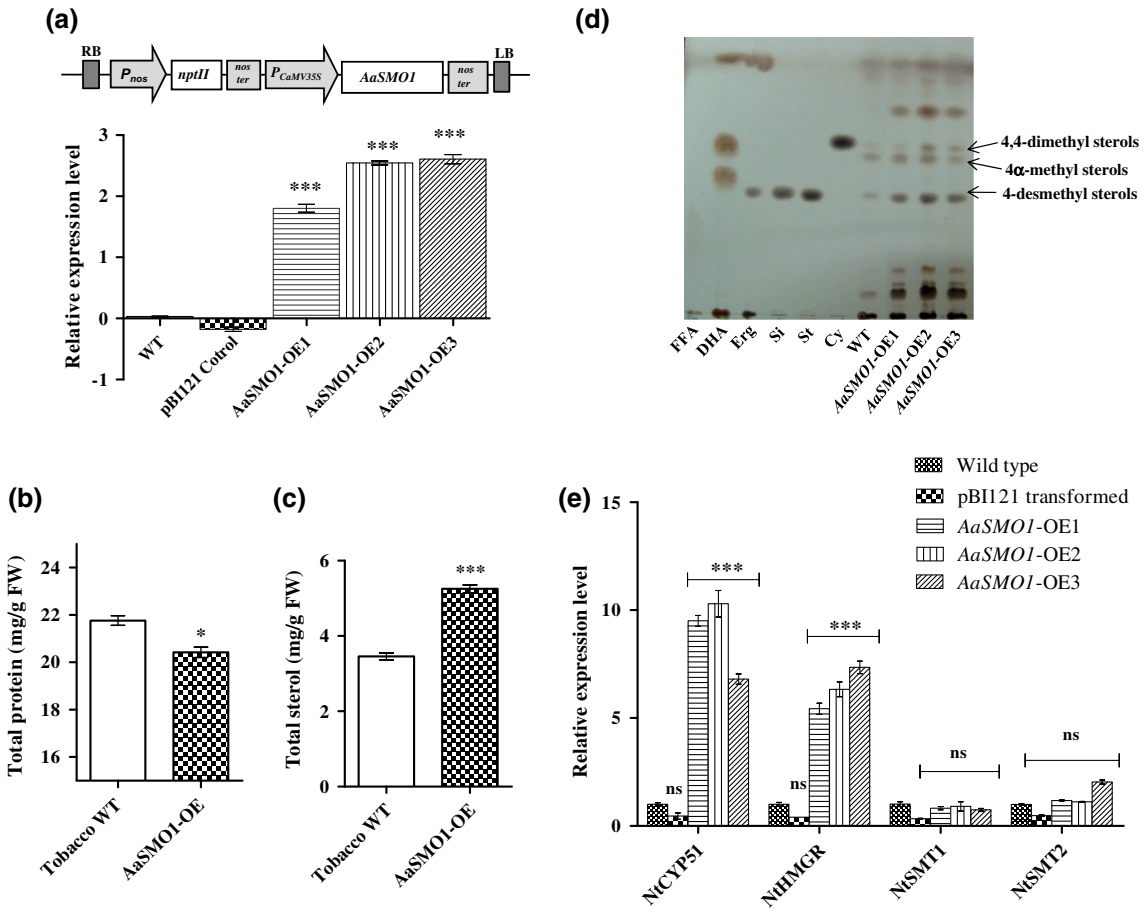

Fig. 3 Overexpression of *AaSMO1* in *N. tabacum* and analyses of transgenic plants. **a** Relative expression of *AaSMO1* in three representative T_3 transgenic plants (*AaSMO1*-OE1, *AaSMO1*-OE2 and *AaSMO1*-OE3) by RT-qPCR. The schematic diagram of the cassette used for transformation is shown above the graph. **b–d** Biochemical analyses of transgenic plants. Comparisons of total protein (**b**) and sterol contents (**c**) in transgenic (*representative line*) and wild type plants are depicted. A comparative TLC profiles are shown in (**d**). The positions of 4,4-dimethyl sterols, 4α-methyl sterols and 4-desmethyl sterols are indicated by *arrows*. **e** Effect of *AaSMO1* overexpression on the relative expression of sterol biosynthesis genes (*NtHMGR*, *NtCYP51*, *NtSMT1* and *NtSMT2*) in tobacco. RT-qPCR was carried out using tobacco *ubiquitin* as an endogenous reference gene for normalization. Statistical analyses were done by one-way ANOVA where *asterisk* indicates the values significantly different from calibrator (***$P < 0.001$) and 'ns' indicates non significance

tobacco mutant (*LAB1-4*) and a high-sterol mutant cell line of *Solanum xanthocarpum* displayed increased HMGR activity in relation to enhanced sterol biosynthesis (Gondet et al. 1992; Josekutty 1998). *SMT1* catalyzes formation of 24-methylene cycloartanol from cycloartenol and this has been proposed to be a critical step in sterol biosynthesis. Overexpression of *SMT1* in tobacco seeds resulted in elevated endogenous HMGR activity and higher sterol production (Holmberg et al. 2002, 2003). This effect of *SMT1* overexpression led to hypothesize that a reduced level of cycloartenol due to its conversion to next intermediate in the pathway provides a feedback to up-regulate HMGR activity to maintain constant carbon in-flux into the pathway (Holmberg et al. 2002). It can be postulated that overexpression of *AaSMO1* in tobacco leading to accumulation of 4α-methyl sterols and desmethylsterol provide a feedback to upstream enzymes (like *HMGR*) to increase their activity for maintaining the carbon flux in sterol pathway which is evident by enhanced levels of 4,4-dimethyl sterols observed in our transgenic lines. Concomitant with the overexpression of *AaSMO1*, we observed elevated transcript levels of *NtHMGR* which is in accord with Holmberg et al. (2002) and higher expression of *NtCYP51* in transgenic tobacco lines which further support this feedback hypothesis.

Transient overexpression and down-regulation of *AaSMO1* in 2-month-old *A. annua* by *Agro*-infiltration method did not show any detectable changes in the sterol content which could be attributed to early sampling of tissue because of transient expression (data not shown). Sterol C-4 methyl oxidases act downstream to *SMT1* in the sterol biosynthesis branch and has no direct effect on artemisinin biosynthesis. It cannot be ruled out that *AaSMO1*

might undergo tight regulation and act as rate limiting step for sterol biosynthesis and consequently diverting the flux (FPP) to sesquiterpene (artemisinin) biosynthesis.

Transgenic tobacco overexpressing *AaSMO1* show enhanced tolerance and better germination upon osmotic/dehydration stress

Tolerance to scarcity of water is enhanced by various mechanisms viz. through controlling stomatal closure, synthesizing the osmoprotectants, generating protectants against reactive-oxygen species, and stabilizing membranes and proteins in plants. We observed that the total sterol profile of transgenic tobacco ectopically expressing *AaSMO1* resembled to that of wild type exposed to dehydration/drought (data not shown). It gave a clue towards analyzing a possible role of sterol biosynthesis gene *AaSMO1* in conferring stress tolerance. In order to study dehydration and salt stress tolerance, leaf disk assays were carried out for wild type and transgenic lines. Dehydration stress-induced loss of chlorophyll was lower in transgenics as compared to wild type plants which were clearly reflected by the occurrence and degree of bleached regions at the margins (Fig. 4a). NaCl (salinity) did not show visible differences in the loss of chlorophyll in transgenic and wild type plants even at higher concentrations (Fig. 4a). Figure 4a shows images of a representative line *AaSMO1*-OE3 although all three tested lines depicted similar results. The damage caused due to stress was further evaluated by measurement of chlorophyll content in leaf disks of transgenic and wild type plants subjected to dehydration (mannitol) or salinity (NaCl) stress. Salinity stress showed non-significant marginal differences in the chlorophyll retention between transgenic and wild type plants (Fig. 4b). On the other hand, transgenic lines retained higher chlorophyll and survived to a greater extent than wild type plants when exposed to dehydration stress (Fig. 4b). Similar behavior of transgenic plants expressing transgenes have also been observed and reported earlier (Vivek et al. 2013; Sharma et al. 2014). Higher chlorophyll retention in transgenic plants indicated lesser damage in the chloroplast and thylakoid structure that may occur due to dehydration. It is evident that tobacco harboring *AaSMO1* show enhanced resistance to dehydration and our results further support to a positive relationship between higher accumulation of sterols and tolerance to dehydration. In a previous report, drought tolerance in transgenic tobacco was enhanced upon constitutive overexpression of *Zea mays* phospholipase C1 gene (*ZmPLC1*) which was mediated by maintaining the membrane stability and lipid levels (Zhai et al. 2013). Similarly, overexpression of a GST gene (*ThGSTZ1*) from *Tamarix hispida* in Arabidopsis improved the reactive oxygen species (ROS) scavenging ability which resulted in drought and salinity stress tolerance (Yang et al. 2014). Overexpression of certain transcription factor encoding genes has also been reported to be associated with improved abiotic stress tolerance in plants. A WRKY factor (*ZmWRKY58*) from maize conferred tolerance to salt and drought stress in transgenic rice (Cai et al. 2014). Transgenic tobacco harboring a *SbMYB8* (R2R3-MYB) gene from *Scutellaria baicalensis* had higher caffeoylquinic acid content and showed enhanced drought resistance (Yuan et al. 2014).

Several morphological features such as plant height, fresh weight of leaves, flowering time, pod size and shape, and quantity of seeds per pod were similar for *AaSMO1* transgenic and wild type plants. To further explore the effect of *AaSMO1* transgene, germination ability of transgenic seeds upon different stresses was evaluated. The percentage germination of transgenic seeds was found to be significantly higher than wild type on 100 mM NaCl and 100 mM mannitol i.e. salinity and dehydration, respectively, after 10–12 days of imbibition (Fig. 5a, b). At high salinity (400 mM NaCl) conditions, both transgenic as well as wild type seeds failed to germinate (data not shown). Similarly, transgenic seeds performed better over wild types on 100 µM GA (Fig. 5b). Under high osmotic stress conditions (400 mM mannitol), a low percentage of transgenic seeds were able to germinate after 10–12 days while wild type seeds either failed to germinate or got aborted after the emergence of radicle (Fig. 5a, b). Seed germination response similar to high osmotic stress was also observed at 50 µM ABA treatment (Fig. 5a, b). Under normal conditions (control), both transgenic and wild type seeds behaved in a similar fashion. When health and phenotype of seedlings germinated under stressed conditions were compared after 45 days, the wild type plants showed growth retardation whereas all the transgenic seedlings were found to be healthier. The root length was found to be higher in case of transgenic seedlings indicating towards a better absorption capabilities when grown under stressed conditions (Fig. 5a). The mock vector-transformed tobacco plants behaved similar to wild type and not shown here. The performance of transgenic plants during osmotic/dehydration stress was also tested at the seedling stage. After germination at normal conditions, 10 to 15-day-old transgenic and wild type seedlings were transferred to dehydration inducing conditions (200 mM mannitol solution). Seedlings of transgenic lines were found to be more resistant against dehydration as compared to wild type.

Physiological and biochemical assessment of transgenic plants under dehydration inducing conditions

Plants quickly respond to osmotic/dehydration or drought stress and adjust the photosynthetic machinery primarily by stomatal closure which ultimately leads to a decline in the

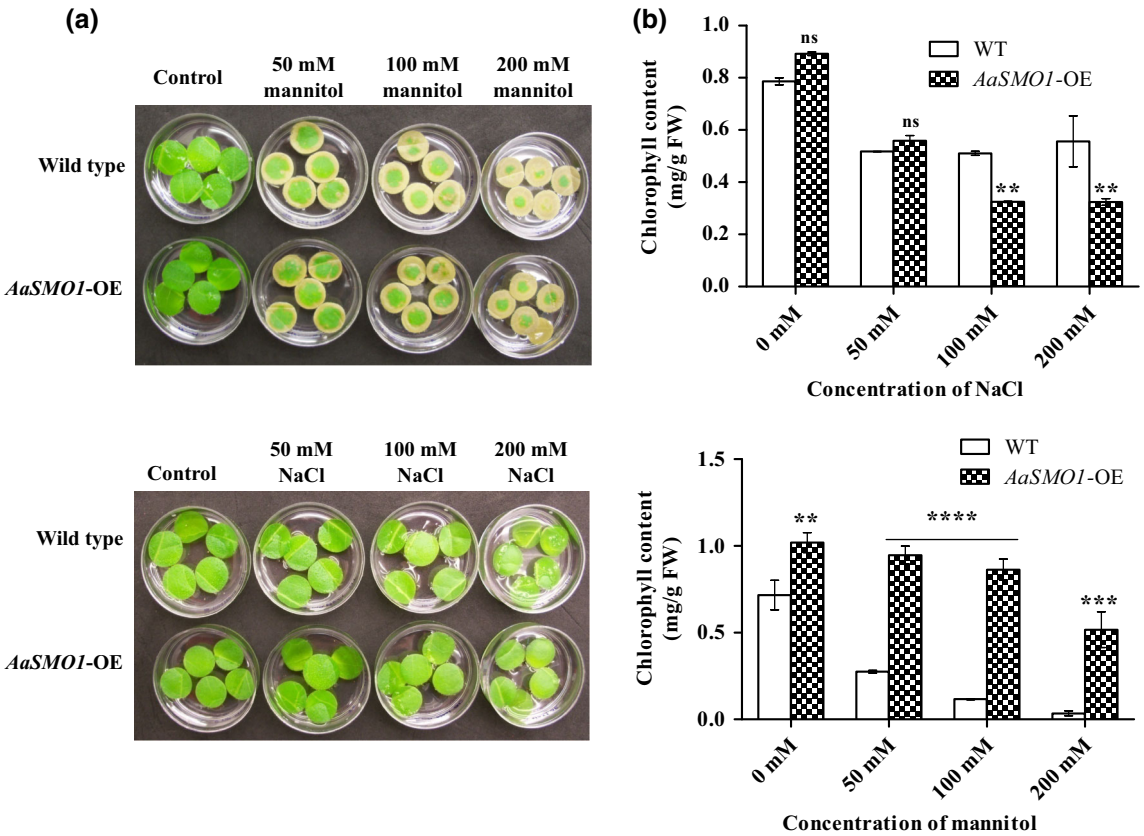

Fig. 4 Leaf disk senescence assay for dehydration and salinity stress tolerance in transgenic tobacco T_3 lines. **a** Representative pictures to show phenotypic differences between leaf disks of wild type and a representative *AaSMO1* overexpression line upon dehydration (mannitol) and salinity (NaCl) stress. **b** Bar diagram to show the chlorophyll content in leaf disks (1 cm^2) of wild type and a representative transgenic plant. Data presented as mean ± SE (n = 3) and *error bars* indicate deviation from the mean. Statistical analysis was done by one-way ANOVA where *asterisk* indicates the values are significantly different (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$) and 'ns' indicates non significance

rate of photosynthesis. Stomatal pores are surrounded by two guard cells, and serve as a gateway for exchange of CO_2 in the atmosphere and transpirational water loss in plants (Silva et al. 2010; Kim et al. 2010). The stomatal architecture of *AaSMO1* harboring transgenic and wild type tobacco plants were analyzed upon dehydration conditions. Leaves of transgenic plants showed reduced stomatal aperture than the wild type when treated with mannitol solution (200 mM) for 3 h (Fig. 5c). The reduced stomatal opening could be due to more rigidity of guard cells which ultimately is a consequence of higher accumulation of sterols in cells. We propose that the less stomatal opening and rigid cellular membranes in *AaSMO1* transgenic tobacco probably reduce the transpiration rate and contribute to dehydration stress tolerance.

Generally under stress conditions, the osmotic potential in plant cells increases due to higher accumulation of osmoprotectants such as sugars and amino acids. Most common effect seen is the accumulation of free proline in different tissues which functions as osmoprotectant of protoplasm. Higher production of proline or higher capacity of osmotic adjustment broadens the tolerance to osmotic stress in plants (Kishor et al. 1995; Mao et al. 2009). The transgenic tobacco T_3 lines (4 to 6-week-old) were subjected to dehydration (200 mM mannitol) stress and accumulation of free proline was measured. Stressed transgenic plants could produce and accumulate higher amounts (~3 fold) of free proline in leaf tissue than the untreated control. While only ~1.4-fold enhancement in the free proline content was observed in the stressed wild type plants with respect to unstressed control. Figure 5d shows the proline content during stressed and normal situations in representative transgenic and wild type tobacco. Overexpression of *OsCIPK12* in rice caused higher accumulation of proline and soluble sugars, and conferred tolerance to various abiotic stresses (Xiang et al. 2007). There is a clear indication that *AaSMO1* overexpressing transgenic tobacco is able to accumulate more proline to cope up with adverse environmental conditions which is in congruence with the better performance of transgenics over wild type. Moreover, dehydration-inducibility of *AaSMO1* indicates towards a cumulative effect of different pathways

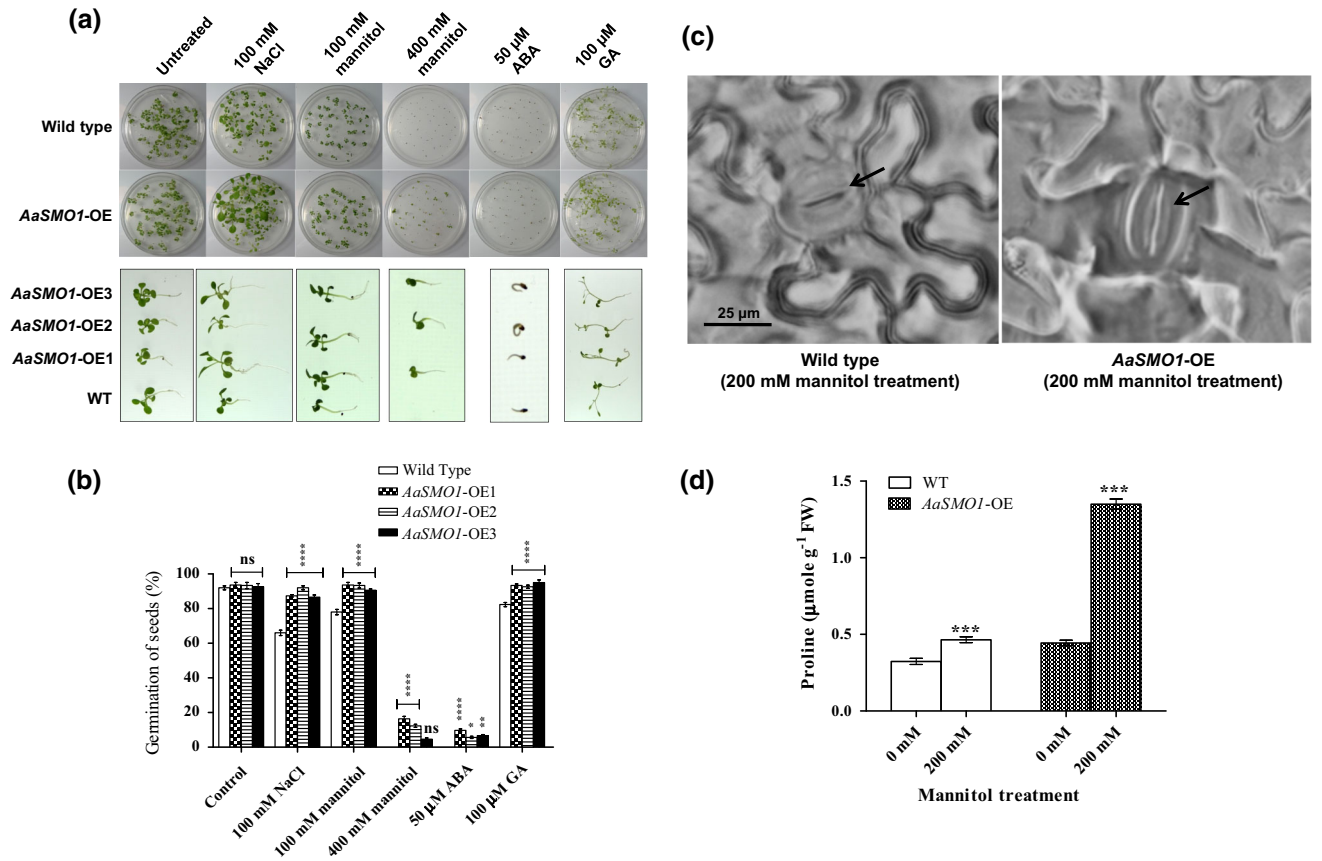

Fig. 5 Performance of *AaSMO1* tobacco transgenics during abiotic stress. **a** Pictures of plates showing germination of wild type (*upper row*) and transgenic seeds (*middle row*) under normal (control) and stressed conditions/elicitor treatment. Individual representative seedlings of wild type (WT) and three independent transgenic lines (*AaSMO1*-OE1, *AaSMO1*-OE2 and *AaSMO1*-OE3) were taken after 45 days of seed imbibition under normal and stressed conditions and are shown in the lower row. **b** Diagram showing percentage (%) germination of wild type and transgenic seeds after 15 days of imbibition. **c** Picture showing the stomatal opening (marked by *arrow*) in wild type and a representative transgenic plant after dehydration stress induced by mannitol treatment (200 mM) for 3 h. *Scale bar*, 25 µm. **d** Bar diagram showing the accumulation of free proline in wild type and a representative transgenic plant under normal and dehydration conditions. Statistical analyses were done by one-way ANOVA where *asterisk* indicates the values are significantly different ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$) and 'ns' indicates non significance

in combating abiotic stress. A possible crosstalk with ABA-independent pathways also cannot be ruled out since lower induction of *AaSMO1* was seen upon exogenous application of ABA.

Evaluation of *AaSMO1* promoter activity

To get an insight into the regulatory elements present in *AaSMO1* promoter, a 1,135 bp genomic fragment containing partial 5′ end of cDNA was PCR-amplified and cloned. To identify various *cis*-elements, 1,115 bp region upstream to start codon (ATG) was analyzed by using "PLACE" database. Putative TATA and CAAT boxes were identified at −25 and −213 bp positions, respectively, upstream to the first base of cDNA (TSS; transcription start site). Several *cis*-elements including CGC box, MYC core, MYB core, DOF responsive elements, W box, GT1 consensus, GATA box and rootmotiftapox1 were identified in the promoter fragment (Fig. 6a). Promoters of many drought/dehydration responsive genes contain *cis*-acting elements such as DRE/CRT, ABRE, and binding sites for MYC and MYB transcription factors. ABA mediates the response to osmotic or drought stress developed due to water-deficit which often relies on the presence of ABRE (Uno et al. 2000). Interestingly, an ABRE (Py-ACGTggc) could be recognized at −58 position in *AaSMO1* promoter which supports the high induction of this gene during osmotic/dehydration challenge.

To study the promoter activity, transgenic tobacco plants were raised harboring *AaSMO1* promoter-*GUS* construct (P_{SMO}*:GUS*). Positive transformants were identified by *nptII*- and *AaSMO1* promoter-specific PCR. Leaf and root of three transformed tobacco plants harboring P_{SMO}*:GUS* construct were evaluated for *GUS* activity by histochemical staining. GUS staining was observed in leaf as well as root, and quantitative GUS assays showed

overall weak promoter activity with a relatively higher activity in leaf tissue (Fig. 6b). Low promoter strength in tobacco leaves may be due to either weak recognition of this promoter in tobacco or the absence of quantitative element required for strong expression in leaf. Presence of diverse *cis*-acting elements including ABRE in the promoter region indicates that dehydration-induced elevation of *AaSMO1* expression may involve more than one signaling components. Therefore, the effect of exogenous application of ABA (10 µM) and GA (100 µM) on *GUS* activity was also studied in *C. roseus* protoplast cells separately transformed with pBI101 vector (promoter-less; negative control), pBI121 vector (*CaMV35S:GUS*; positive control) and P_{SMO}*:GUS* (*AaSMO1* promoter-*GUS*) construct. It was observed that *AaSMO1* promoter-driven GUS activity was significantly enhanced (induced) upon treating the P_{SMO}*:GUS*-transformed protoplast cells with ABA and GA which could be due to the presence of ABRE in the promoter (Fig. 6c). Presence of known dehydration responsive *cis*-elements in promoter and up-regulation of transcript upon exposure to water deficiency strongly support the role of *AaSMO1* in conferring dehydration tolerance.

In conclusion, we have cloned a sterol C4 methyl oxidase from *A. annua* which on ectopic overexpression in tobacco increases total sterol content and tolerance to osmotic/dehydration stress. *AaSMO1* is a dehydration-responsive gene and a target of ABA-mediated stress signal transduction. We speculate that *AaSMO1* gene and its promoter offer an opportunity towards understanding sterol partitioning and homeostasis in adverse environmental conditions more precisely, and it could prove as an excellent candidate for improving water-deficit or drought tolerance in plants.

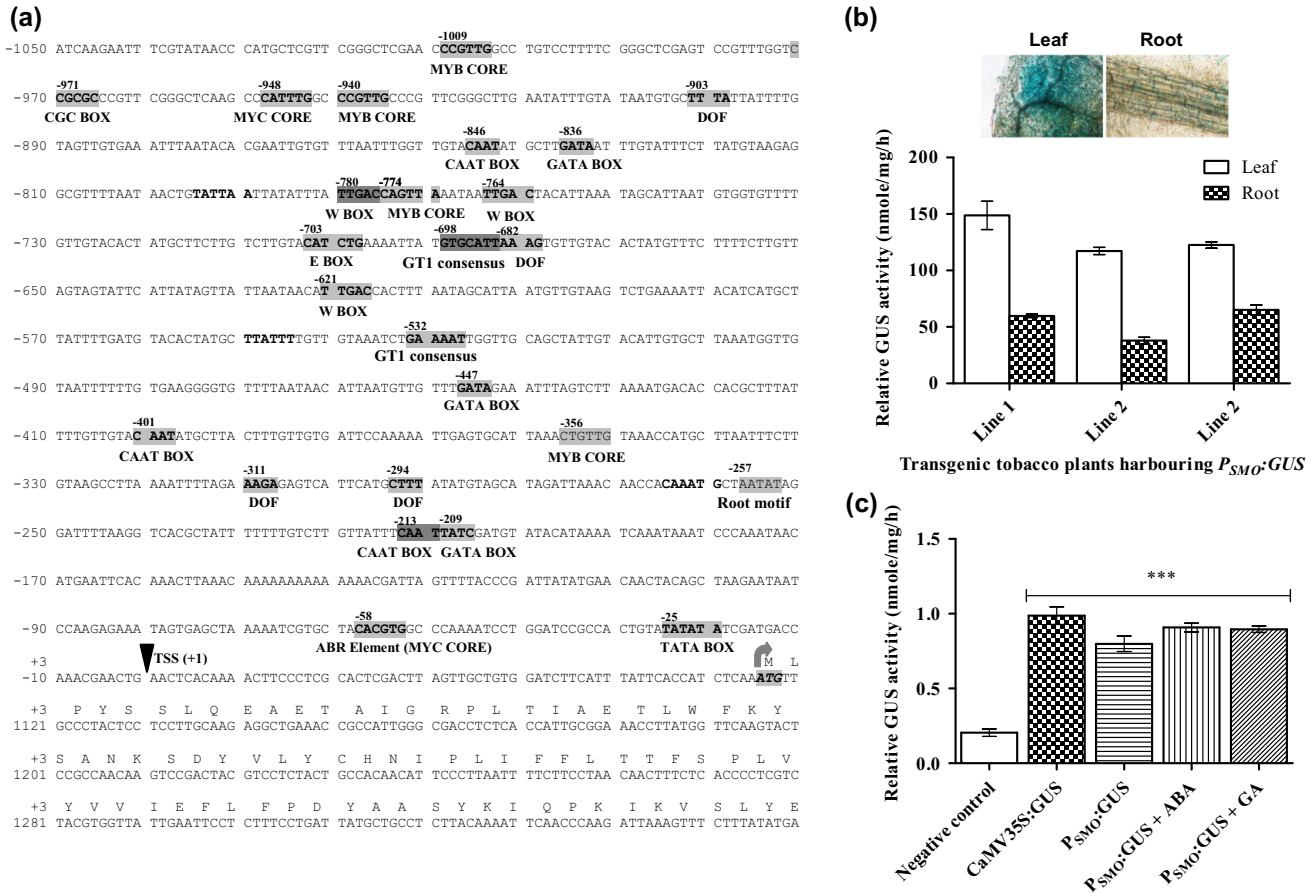

Fig. 6 Analysis of *AaSMO1* promoter. **a** Sequence of *AaSMO1* genomic fragment (1,360 bp) containing 1,115 bp region upstream to ATG. Various *cis*-acting elements identified in the promoter region are shaded in *grey color*. A putative TSS and ATG are marked in the sequence by a *black arrowhead* and a *curved arrow*, respectively. **b** Bar diagram depicting relative GUS activity in leaf and root of tobacco transformed with P_{SMO}*:GUS* construct. Any background GUS activity observed in the leaf and root of wild type plants were subtracted prior to plotting. GUS histochemical staining in leaf and root of a representative transgenic plant is shown in inset. **c** Analysis of the effect of exogenous supply of ABA and GA on *AaSMO1* promoter-driven *GUS* activity in transiently transformed *C. roseus* protoplast cells by fluorometric assay. Promoter-less-*GUS* (pBI101 vector) and *CaMV35S-GUS* (pBI121 vector) were taken as negative and positive controls, respectively. Statistical analysis was done by one-way ANOVA where *asterisk* indicates significantly different values (***$P < 0.001$)

Acknowledgments The initial work was supported by CSIR, Govt. of India, under NWP08 project, and further funding was provided by SERB, DST, Govt. of India under GAP265 project. Research fellowships from CSIR and UGC, Govt. of India, are acknowledged. The authors wish to express their sincere thanks to the Director, CSIR-CIMAP for providing necessary facilities. Help from Drs. R. K. Shukla, A. K. Shasany, A. S. Negi and Ms. Pooja Sharma is duly acknowledged. Dr. Samir Sawant from CSIR-NBRI, Lucknow and Dr. Jitendra Thakur from NIPGR, New Delhi, are acknowledged for extending their help in confocal microscopy.

Conflict of interest The authors declare no competing interests.

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