

MusaSAP1, a A20/AN1 zinc finger gene from banana functions as a positive regulator in different stress responses

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Abstract A20/AN1 zinc finger domain containing Stress Associated Proteins (SAP) are involved in diverse stress response pathways in plants. In the present study, a novel banana SAP gene, *MusaSAP1*, was identified from banana EST database and was subsequently characterized by overexpression in transgenic banana plants. Expression profiling in native banana plants showed that *MusaSAP1* was up-regulated by drought, salt, cold, heat and oxidative stress as well as by treatment with abscisic acid. Cellular localization assay carried out by making a *MusaSAP1::GFP* fusion protein indicated that *MusaSAP1* is incompletely translocated to nucleus. Copy number analysis performed using real time PCR and Southern blotting indicated that *MusaSAP1* occurs in the banana genome in a single copy per 11 chromosome set. Transgenic banana plants constitutively overexpressing *MusaSAP1* displayed better stress endurance characteristics as compared to controls in both in vitro and ex vivo assays. Lesser membrane damage as indicated by reduced malondialdehyde levels in transgenic leaves subjected to drought, salt or oxidative stress pointed towards significant role for *MusaSAP1* in stress amelioration pathways of banana. Strong up-regulation of a polyphenol oxidase (PPO) coding transcript in *MusaSAP1* overexpressing plants together with induction of *MusaSAP1* by wounding and methyl jasmonate treatment indicated possible involvement of

MusaSAP1 in biotic stress responses where PPOs perform major functions in multiple defense pathways.

Keywords Abiotic stress · *Agrobacterium*-mediated genetic transformation · Banana · *MusaSAP1* · Stress associated protein

Abbreviations

ABA	Abscisic acid
ACS	Acetosyringone
BAP	6-Benzylaminopurine
PCV	Packed cell volume
PPO	Polyphenol oxidase
RACE	Rapid amplification of cDNA Ends
ROS	Reactive oxygen species

Introduction

Environmental stresses such as drought, high salinity, extreme temperatures, high light intensities and exposure to heavy metals have a profound impact on the growth and productivity of major crop plants (Mahajan and Tuteja 2005; Munns 2005). To successfully survive these stresses, plants trigger a cascade of events starting with stress signal perception, followed by several parallel transduction pathways that eventually lead to synthesis of transcription factors and subsequently the up- or down-regulation of genes responsible for synthesis of effector proteins and metabolites which partake in stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2000). Detailed studies in model plants like *Arabidopsis* and rice have characterized several genes involved in stress perception, signal transduction, transcription and production of protective biomolecules (Yamaguchi-Shinozaki and Shinozaki 2006; Bhatnagar-Mathur et al.

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2008). Characterization of genes involved in signal transduction and in synthesis of transcription factors is important because they influence a wide range of stress-related genes. Genes coding for proteins containing zinc finger domains belong to this category as they contribute significantly towards protection against a variety of environmental stresses (Davletova et al. 2005; Ciftci-Yilmaz and Mittler 2008). In recent past, a zinc finger protein family containing an N-terminal A20 and a C-terminal AN1 zinc finger domain and collectively called as Stress Associated Proteins (SAP) has emerged as a vital gene family involved in processes related to stress response and its management (Mukhopadhyay et al. 2004). Although the exact role of these SAP genes in plants is still being elucidated, several such SAP genes from *Arabidopsis* and rice have been studied in great detail (Vij and Tyagi 2006).

Banana, which ranks as the world's second largest fruit crop, is listed among the world's 10 most important food commodities. In comparison with rice and maize, the research into banana stress response pathways, apart from a few recent studies, has historically not been commensurate with its inclusion among the most important food products of the world. Banana being a water intensive plant with shallow roots and permanent green canopy is especially sensitive to any kind of stress which limits the availability of water. Drought, salinity, cold as well as heat stress all limit the water uptake by an already shallow root system of banana and hence investigations into its stress response pathways and detailed studies on possible mitigation of the cellular consequences of such stress conditions are urgently warranted. We identified several A20/AN1 zinc finger domain containing putative SAP sequences in banana EST database available with NCBI. One such SAP gene (*MusaSAP1*) was found to be inducible in response to a variety of abiotic stress treatments in a relatively robust and hardy triploid cultivar of banana (cv. *Karibale Monthan*, ABB group, n = 11). Banana cultivars having greater number of B genomes are generally considered to be more tolerant to various types of environmental stresses (Bakry et al. 2009). Thus, *MusaSAP1*, which was found to be inducible in banana in response to different abiotic stress treatments, was postulated to be involved in stress perception, signaling and its mitigation in banana. To confirm this postulate, we overexpressed *MusaSAP1* in an elite edible cultivar of banana (cv. *Rasthali*, AAB Group) using a constitutive promoter, which in turn enabled investigations into the possible roles played by *MusaSAP1* in the stress response pathways in banana plant.

Materials and methods

Primers

The primer sequences used in this study are shown in Supplementary file 1.

Amplification and sequence analysis of *MusaSAP1* gene

Young leaves derived from banana cv. *Karibale Monthan* were used to extract total RNA using Concert Plant RNA Reagent (Invitrogen, USA). This RNA was cleaned up and further treated with DNase using RNeasy Plant Mini Kit (Qiagen, Germany). Five µg of this RNA was used for synthesizing first strand cDNA using Oligo (dT)₁₂₋₁₈ primer (Invitrogen, USA) and AccuScript Reverse Transcriptase (Stratagene, USA). Full-length coding sequence of *MusaSAP1* was amplified from this cDNA using Pfu Ultra AD DNA Polymerase (Stratagene, USA) under the following thermal cycling conditions 94 °C for 5 min and subsequently 30 cycles each comprising of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 15 min. Putative protein sequence for *MusaSAP1* cDNA was established using the online ExpASY translate tool (<http://au.expasy.org/tools/dna.html>). Predicted *MusaSAP1* protein sequence was aligned with its closest homologs by using ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The A20 and AN1 zinc finger domains of *MusaSAP1* protein were identified using ExpASY prosite server (<http://au.expasy.org/prosite/>). Phylogenetic relationships for *MusaSAP1* protein were established using MEGA 5 software. Genomic sequence of *MusaSAP1* was determined by amplifying its sequence from banana cv. *Karibale Monthan* genomic DNA isolated using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). Further, the 5' upstream sequence of *MusaSAP1* in banana cv. *Karibale Monthan* was deciphered by employing TAIL-PCR methodology (Liu et al. 1995) and using degenerate primers as described in Shekhawat et al. 2011a. Sequence for the 3' UTR of *MusaSAP1* in banana cv. *Karibale Monthan* was determined by using 5'/3' RACE kit (Roche Applied Science, Germany).

Copy number determination of *MusaSAP1* in banana genome

Copy number of *MusaSAP1* gene in banana genome was determined using real time PCR and by Southern blotting. Absolute quantification method as described by Bubner and Baldwin (2004) was applied to determine the number of copies of *MusaSAP1* in the banana genome. *MusaSAP1* 5' UTR partial genomic sequence was amplified from banana cv. *Karibale Monthan* genomic DNA and from a serial dilution series of a plasmid with *MusaSAP1* 5' UTR partial genomic sequence using SYBR Green Extract-N-Amp PCR ReadyMix (Sigma, USA). Absolute quantification was then achieved by relating the measured Ct of genomic DNA sample to the standard curve obtained from serial dilution series of the plasmid with *MusaSAP1* 5' UTR

sequence. Numbers of copies of *MusaSAPI* in the banana genome were then calculated based on the molecular weights and concentrations of the plasmid and the banana genomic DNA as explained in Bubner and Baldwin (2004).

For Southern blotting approx. twenty micrograms genomic DNA isolated from young banana cv. *Karibale Monthan* leaves was separately digested overnight with *Bam*HI, *Eco*RI, *Hind*III and *Xho*I restriction enzymes. Following enzyme deactivation, the fully restricted DNA was purified using High Pure PCR Product Purification Kit (Roche Applied Science, Germany) and electrophoresed for 12 h at a field strength of 1.25 V/cm in a 0.9 % (w/v) agarose-TAE gel. DNA was subsequently transferred to a positively charged nylon membrane by capillary method using 20X SSC buffer. Transferred restricted DNA was immobilized on the membrane by exposing it to 302 nm UV for 3 min followed by drying at 120 °C for 30 min. The membrane was then treated with DIG-labeled probes generated using *MusaSAPI* 5' UTR partial genomic sequence as a template. Chemiluminescent detection of hybridization signals was performed using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) according to manufacturer's instructions.

Determination of cellular localization of *MusaSAPI* protein

MusaSAPI cDNA without the native stop codon (amplified from banana cv. *Karibale Monthan* leaf derived cDNA) was inserted in-frame at the N-terminal end of GFP in the binary vector pCAMBIA-1302 (GenBank accession no. AF234298) using *Nco*I and *Spe*I restriction enzymes. The resulting binary vector (p*MusaSAPI*-1302) was electroporated into *Agrobacterium tumefaciens* strain EHA105 and then used to transform onion peels (cut into 1 cm × 1 cm pieces) at an OD_{600nm} of ~ 0.1. Three days post cocultivation with *Agrobacterium*, cellular localization of the *MusaSAPI*::GFP fusion protein in onion peel cells was determined using a fluorescence microscope (Eclipse 80i, Nikon, Japan). To visualize the nuclear targeting of the fusion protein, onion peels were incubated for 10 min in dark in a staining solution containing 10 mM tris, 1 mM EDTA (pH 8), 0.005 % (v/v) triton X-100 and 1 µg ml⁻¹ DAPI. *MusaSAPI*::GFP fusion protein was visualized by employing GFP filter set (excitation 460–500 nm, emission 515–550 nm) whereas DAPI was seen using DAPI filter set (excitation 340–380 nm, emission 435–485 nm). Several onion peel pieces were analysed and the best representative cells were photographed. Online prediction of subcellular localization was performed using cNLS mapper utility (<http://nls-mapper.iab.keio.ac.jp>).

Expression profiling of *MusaSAPI* in different banana tissues by Northern blotting

Total RNA was extracted from banana cv. *Karibale Monthan* leaf, root and pseudostem tissues as described above and separated in a 1.2 % (w/v) FA-MOPS agarose gel. The samples were allowed to migrate in 1X MOPS buffer at a field strength of 5 V/cm for 2 h. Subsequently the gel was washed twice with 20X SSC (to ensure efficient equilibration) and then the RNAs were transferred onto positively charged nylon membrane. RNA was crosslinked to the membrane by baking at 120 °C for 30 min. DIG-labeled DNA probes generated using 220 bp 5' cDNA sequence were allowed to probe the membrane. Prehybridization was performed at 60 °C for 2 h followed by hybridization with DIG-labeled DNA probes at 55 °C overnight. The nylon membrane was then washed twice each with 2X SSC and 0.1 % (w/v) SDS at room temperature and 0.1X SSC and 0.1 % (w/v) SDS at 60 °C. Chemiluminescent detection of hybridization signals was performed as described above in Southern blot analysis.

Expression profiling of *MusaSAPI* in banana leaves in different stress conditions using real time PCR

In vitro plantlets of banana cv. *Karibale Monthan* were acclimatized in greenhouse for 2 months. Uniform sized plants with 4–5 leaves were treated with 250 mM NaCl (applied in gradual increments from 75 to 150 mM and then to 250 mM at 12 h intervals), 100 µM ABA, 10 µM methyl viologen and 200 µM methyl jasmonate. Cold and heat stress were exerted by exposing the plants respectively to 10 ± 2 °C or to 45 ± 2 °C inside a growth chamber set at 16 h light/8 h dark regime. For drought stress, plants were washed thoroughly to remove soil and then left to dry on blotting sheets in the greenhouse. For wounding stress, leaves were left on moist blotting sheets after making four longitudinal and four transverse cuts in each leaf. Plants treated only with sterile water were taken as experimental controls. Leaf samples sourced from treated plants at specific time points were frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated from the frozen samples as described above. The first-strand cDNA was synthesized using 5 µg total RNA, Oligo (dT)₁₂₋₁₈ primer and ThermoScript Reverse Transcriptase (Invitrogen, USA). For every treatment, three independent samples from separate plants were pooled prior to total RNA extraction to guarantee reproducibility of results. All cDNAs were diluted 1:50 with sterile water before they were used in quantitative real time PCR reactions performed in three technical replicates using SYBR Green Extract-N-Amp PCR ReadyMix (Sigma, USA) and primers specific for *MusaSAPI*. *Musa EF1α* cDNA (GenBank accession no.

DQ057979) was amplified along with *MusaSAPI* to enable gene-expression normalization between different treatment samples and subsequent quantification of relative expression levels of *MusaSAPI* in different samples (Chen et al. 2011, Podevin et al. 2012). All real-time quantitative RT-PCR reactions were performed on Qiagen Rotor Gene platform (Qiagen, Germany). Cycling parameters for these reactions were 94 °C for 4 min initially followed by 30 cycles, each comprising of 94 °C for 25 s, 56 °C for 25 s and 72 °C for 25 s. Ct values obtained (using Rotor Gene software, Qiagen, Germany) were analyzed using REST-MCS utility (Pfaffl et al. 2002) to calculate relative expression levels of *MusaSAPI* gene (normalized against *Musa EF1 α* expression levels) in response to various stress treatments. All cDNA samples were established to be genomic DNA-free by performing 40 cycles of PCR amplification with *Musa actin* primers.

Construction of plant expression vector for overexpression of *MusaSAPI*

Expression cassette for achieving overexpression of *MusaSAPI* in transgenic banana plants was assembled in the MCS region of plant expression vector pCAMBIA-1301. Firstly, the nos 3' UTR amplified from pBI121 binary vector was cloned in the multiple cloning site of pCAMBIA-1301 using *SacI* and *EcoRI* restriction enzymes. Then, the *Zea mays* polyubiquitin promoter amplified from *Zea mays* genomic DNA and the *MusaSAPI* coding sequence amplified from the banana cv. *Karibale Monthan* leaf cDNA were cloned in this modified pCAMBIA-1301 binary vector in a three-way ligation reaction by utilizing restriction sites specific for *HindIII*, *PstI* and *KpnI* restriction enzymes. The new binary vector obtained was denoted as p*MusaSAPI*-1301. This expression vector, having the expression cassette [pZmUbi-*MusaSAPI*-nos], was sequenced to confirm the sequence of *MusaSAPI* coding region. p*MusaSAPI*-1301 binary vector was then transformed into *Agrobacterium tumefaciens* strain EHA 105 (Hood et al. 1993) by electroporation and later used for transformation of banana embryogenic cells.

Agrobacterium-mediated genetic transformation of banana embryogenic cells and generation of transgenic banana plants

Dense culture derived from a single *Agrobacterium tumefaciens* colony grown overnight at 27 °C in liquid YENB medium (0.75 % w/v yeast extract with 0.8 % w/v nutrient broth) added with 50 mg l⁻¹ kanamycin was resuspended in the same medium at an OD_{600 nm} of 0.1 in the morning and grown further until an OD_{600 nm} of 0.6–0.8 was achieved. This culture was pelleted at 6,500×g for 10 min following

which the *Agrobacteria* were suspended in M2 medium (Cote et al. 1996) supplemented with 100 μM ACS at a final OD_{600 nm} of ~0.2. This bacterial suspension was used for transformation of banana cv. *Rasthali* embryogenic cells (initiated from male flower buds) as described previously (Ganapathi et al. 2001). Five to seven days post-subculture cells (0.5 ml PCV) sieved through a 85-μm sieve were used for cocultivation with *Agrobacterium* for 30 min with intermittent shaking. Further the cells were aspirated onto glass filter discs using a Buchner apparatus and transferred on to solidified M2 medium supplemented with 100 μM ACS. The plates were incubated in dark for 3 days at 25 ± 1 °C. After 3 days the filters along with the cells were transferred to fresh solidified M2 medium supplemented with cefotaxime (400 mg l⁻¹). After further 3 days the cells were removed from the filters and cultured on banana embryo induction medium added with cefotaxime (400 mg l⁻¹) and hygromycin (5 mg l⁻¹). Embryo development was evident in 3 to 4 weeks and the developing embryos were cultured on the same medium for three subcultures of 3 weeks duration with hygromycin selection. The embryos developed were transferred to MS medium supplemented with BAP (0.5 mg l⁻¹) for germination. The germinated embryos were subcultured onto banana multiplication medium (Ganapathi et al. 2008) to generate the copies of the same transformed line. The shoots were isolated individually from the multiple shoots and were transferred to MS medium added with NAA (1 mg l⁻¹) for root development. The complete plantlets thus obtained were hardened in the greenhouse for 2 months and then used for all the analysis.

Molecular analysis of transgenic banana plants

Ten putative transgenic banana lines selected on hygromycin supplemented medium were subjected to GUS staining (performed as described in Ghosh et al. 2009). Also, genomic DNA isolated (as described above) from young leaves of different transgenic lines was used as template in PCR reactions with primers specific for hygromycin phosphotransferase gene present within the T-DNA borders of p*MusaSAPI*-1301 binary vector. Genomic DNA isolated from untransformed banana plant served as a control in these PCR reactions. Further, the exact quantum of overexpression of *MusaSAPI* in four selected transgenic lines (S2, S6, S7 and S8) was deduced by real time quantitative RT-PCR analysis. Total RNA extraction from young leaves and first strand cDNA synthesis were performed as described above. cDNA obtained from untransformed banana plants served as a control in these RT-PCR reactions. The above said four p*MusaSAPI*-1301 transformed lines were analyzed by southern blot analysis (carried out by using a DIG-labeled probe against hygromycin phosphotransferase gene) for determination of

T-DNA copy number in these lines. Lines S2, S6 and S7 were finally chosen for further biochemical analysis as transgenic line S8 had multiple insertions of the T-DNA which was not desirable.

Assessment of abiotic stress responses of *MusaSAP1* overexpressing lines

Assays for improved drought and salt tolerance of the *MusaSAP1* overexpressing lines were performed in a two way mode utilizing both the in vitro maintained multiple shoot cultures of the transgenic lines and the detached leaves of hardened transgenic plants growing in the green house. Uniform individual in vitro shoots approx. 2 cm in length and 7–8 mm in diameter (derived from multiple shoot cultures of the selected transgenic lines) were cultured for 10 days on rooting media added separately with mannitol (100 mM) and NaCl (100 mM). These shoots were then transferred to plain rooting media and cultured in vitro for further 20–25 days. Leaves derived from 2 to 3 months old transgenic green house maintained plants were detached along with their petiole and their cut ends were immersed in 1/10 MS medium added separately with mannitol (250 mM) and NaCl (250 mM) for 7 days in 16 h light/8 h dark regime. For analyzing the response of *MusaSAP1* overexpressing lines to oxidative stress, which is a common component of drought and salt stress, similar leaves derived from greenhouse maintained plants were immersed in 100 μ M methyl viologen for 24 h under continuous bright light followed by recovery in 1/2 MS medium for 24 h in 16 h light/8 h dark regime. All in vitro and ex vitro assays were conducted in triplicates and after their completion representative samples for each assay were photographed. Detached leaves treated with mannitol, NaCl and methyl viologen were analysed for membrane damage by determining their malondialdehyde (MDA) content (performed as described in Shekhawat et al. 2011b; Hodges et al. 1999). In vitro shoots and hardened greenhouse maintained plants of untransformed banana cv. *Rasthali* were used as controls in all the above assays.

Analysis of PPO gene transcription and protein activity

Total PPO protein activity was determined in transgenic and untransformed control plants using catechol as substrate. Briefly, 500 mg leaf tissue was crushed with liquid nitrogen and then resuspended in 4 ml sodium phosphate buffer (pH 6). This suspension was centrifuged at 12,000 \times g for 30 min at 4 °C. 20 μ l of this extract was then used to determine the PPO activity by monitoring its reaction with catechol at 475 nM. Leaf samples from three independent plants of each of the three selected transgenic lines along with three independent control plants were used to perform the assay.

Banana EST sequences homologous to known sequences of PPOs of other related plant species were identified using tBLASTn program of NCBI. Specific primers were designed for four such identified sequences (Genbank accession nos. ES436231, ES437160, FL664618, ES436966) and quantitative real time RT-PCR reactions were carried out to determine the transcript levels of these putative PPO coding genes in *MusaSAP1* overexpressing lines as compared to their level in untransformed plants. *Musa EF1 α* cDNA was also amplified along with the putative PPO coding genes to enable gene-expression normalization and subsequent quantification of relative expression levels.

Results

Isolation and sequence analysis of *MusaSAP1*

A novel SAP gene was identified from banana EST library (Genbank accession no. FF557701). The same was amplified from banana cv. *Karibale Monthan* leaf cDNA and then sequenced (Supplementary File 2). This being the first SAP gene to be identified in banana, it was named as *MusaSAP1*. Full length mRNA sequence of *MusaSAP1* consisted of a coding region of 516 nucleotides along with a 154 nucleotides long 5' UTR (predicted through in silico analysis of the upstream genomic fragment isolated by TAIL-PCR) and a 182 nucleotide long 3' UTR (identified by 3' RACE). *MusaSAP1* cDNA encoded a protein of 171 amino acids having a molecular weight of 18.27 kDa and a theoretical pI of 8.28. *MusaSAP1*, like other related SAP proteins has an A20 zinc finger domain towards the N-terminal side and an AN1 zinc finger domain towards the C-terminal side. Among the annotated and characterized SAP proteins, *MusaSAP1* shows the best homology with OsiSAP8 from *Oryza sativa* (Genbank accession no. A2YEZ6). This homology is more pronounced at the genomic level as both these genes have two introns each in their respective 5' UTR regions and both of them have continuous coding regions without any intervening introns. Alignment of *MusaSAP1* with four of its closest homologs revealed that the two zinc finger domains were highly conserved but the region between was highly variable indicating specific function of each of these proteins in respective species (Fig. 1).

Copy number determination of *MusaSAP1* in banana genome

Copy number determination performed by absolute gene quantification method predicted *MusaSAP1* to be a single copy gene in banana genome (detailed calculations in Supplementary File 3). Southern blot analysis of banana cv. *Karibale Monthan* genomic DNA restricted with *Bam*HI, *Eco*RI, *Hin*-DIII and *Xho*I enzymes (which do not cut inside the partial

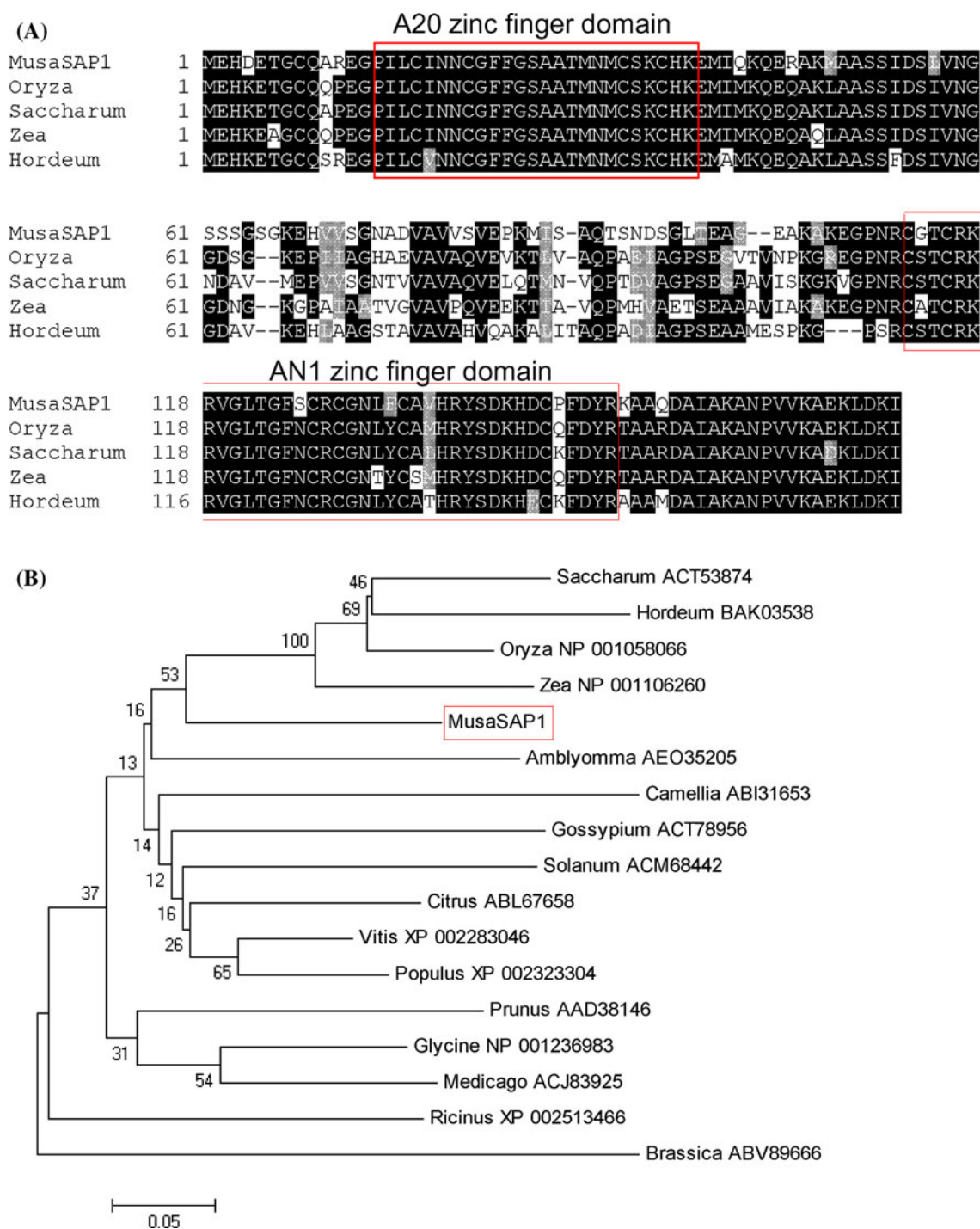


Fig. 1 Sequence analysis of *MusaSAP1* isolated from banana cv. *Karibale Monthan* and depiction of its phylogenetic relationships. **a** Alignment of *MusaSAP1* protein sequence with sequences from *Oryza sativa* (A2YEZ6), *Saccharum officinarum* (ACT53874), *Zea mays* (ABS83245), *Hordeum vulgare* (BAK03538). The two conserved zinc finger domains, A20 towards the N-terminus and AN1

towards the C-terminus are marked by red boxes, **b** Phylogenetic relationship of *MusaSAP1* with other closely related SAP sequences. The accession numbers of the respective protein sequences used for constructing the phylogenetic tree are given alongside. This bootstrapped tree with 1,000 replicates was made using ClustalW2 and MEGA 5 tools

MusaSAP1 5' UTR genomic sequence used to generate the probes) and performed using DIG-labeled probe against *MusaSAP1* 5' UTR also indicated that *MusaSAP1* is present in a single copy in the banana genome (Supplementary File 4).

Cellular localization of *MusaSAP1* protein

A single bipartite nuclear localization signal was predicted (RCGTCRKR VGLTGFSCRCGNLFCAVHRYSDKHDCQFDYRKAADATAKANPVVKAEKLDKI),

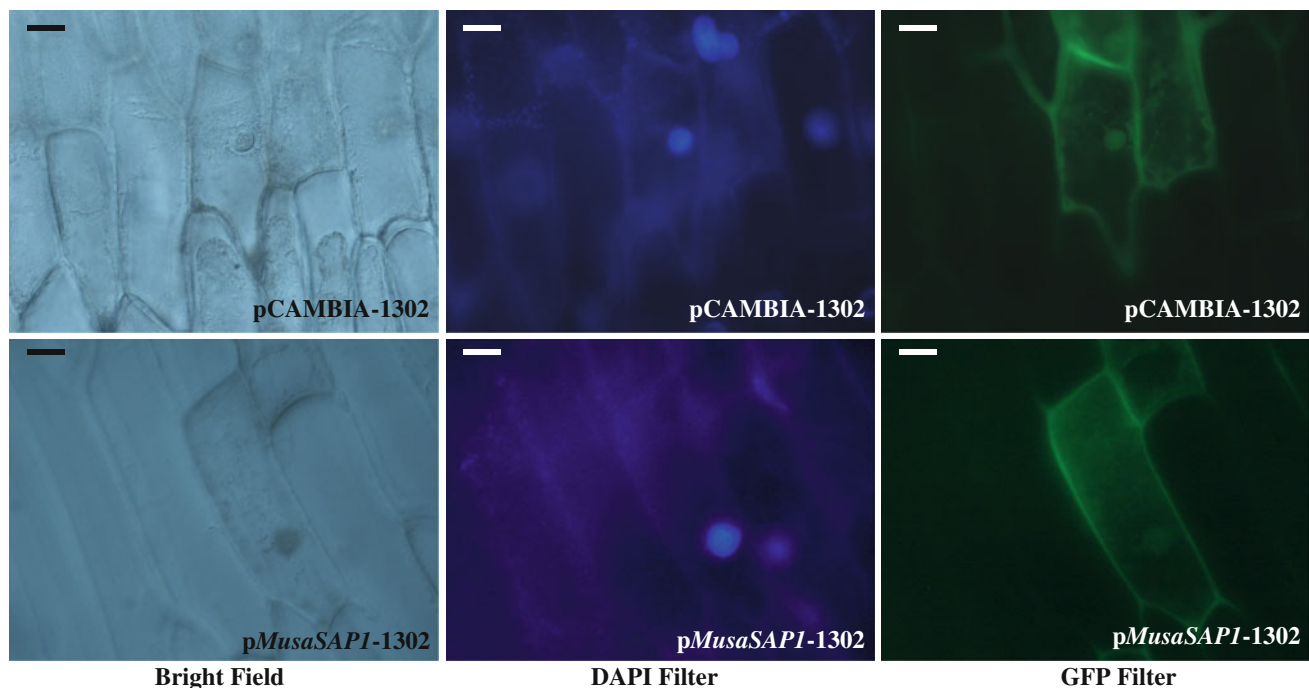


Fig. 2 Cellular localization of *MusaSAP1* in onion peel cells. Onion peel cells transformed with pCAMBIA-1302 and p*MusaSAP1*-1302 were visualized under a fluorescent microscope. Nuclei of the onion cells were stained using DAPI. Bars correspond approx. to 100 μm

amino acids 111–143) using cNLS mapper although its score indicated that *MusaSAP1* protein was incompletely localized to nucleus. To confirm this prediction, an expression cassette comprising of *MusaSAP1::GFP* fusion cDNA driven by CaMV 35S promoter was assembled (p*MusaSAP1*-1302) and transformed into onion peels. GFP fluorescence was visualized both in nucleus and cytosol in p*MusaSAP1*-1302 transformed onion cells thus validating the results of cNLS mapper prediction. GFP fluorescence in pCAMBIA-1302 transformed cells was also seen on similar lines, probably because the GFP due to its small size diffuses into the nucleus even in the absence of a defined nuclear localization signal (Fig. 2).

Expression profiles of *MusaSAP1* in different banana tissues and under different stress treatments

Northern blot of *MusaSAP1* performed using total RNA isolated from different banana tissues clearly indicated that the expression of *MusaSAP1* is the strongest in banana leaves as compared to roots and pseudostem (Supplementary File 5). Hence, banana leaves were chosen for expression profiling of *MusaSAP1* under different stress conditions. Previous studies on *SAP* genes of related monocot plants like rice have shown that these genes are induced in response to different abiotic stresses. Therefore, we also studied the expression pattern of *MusaSAP1* in response to various stress conditions for different periods of time. Real-time quantitative RT-PCR carried out using

cDNA derived from stressed and non-stressed leaves of banana cv. *Karibale Monthan* plantlets showed the inducibility of *MusaSAP1* expression in response to salt, drought, cold, heat and wounding stress as well as upon ABA, methyl viologen and methyl jasmonate treatment (Fig. 3). Drought, salt, ABA and methyl viologen induced the expression of *MusaSAP1* in small time of exposure after which the expression came down towards un-induced levels. In contrast, under cold, heat or wounding stress or after methyl jasmonate treatment *MusaSAP1* expression increased gradually towards higher levels. As similar *SAP* genes in related plant species are known to have transcription factor activity, induction of *MusaSAP1* in response to different stress conditions in banana indicates potential role of this *SAP* gene in modulating effector gene expression during diverse stress conditions in banana.

Generation and analysis of transgenic banana plants

Banana cv. *Rasthali* embryogenic cells (Fig. 4a) were transformed using *Agrobacterium* harboring an expression vector designed to overexpress *MusaSAP1* constitutively in the transgenic banana plants (Fig. 4b). Whitish embryos started developing on banana embryo induction medium supplemented with hygromycin (5 mg l^{-1}) within 3 to 4 weeks after transformation (Fig. 4c). These embryos later gave rise to secondary embryos upon subculturing on the fresh medium of the same composition. Embryos so developed were germinated on embryo germination

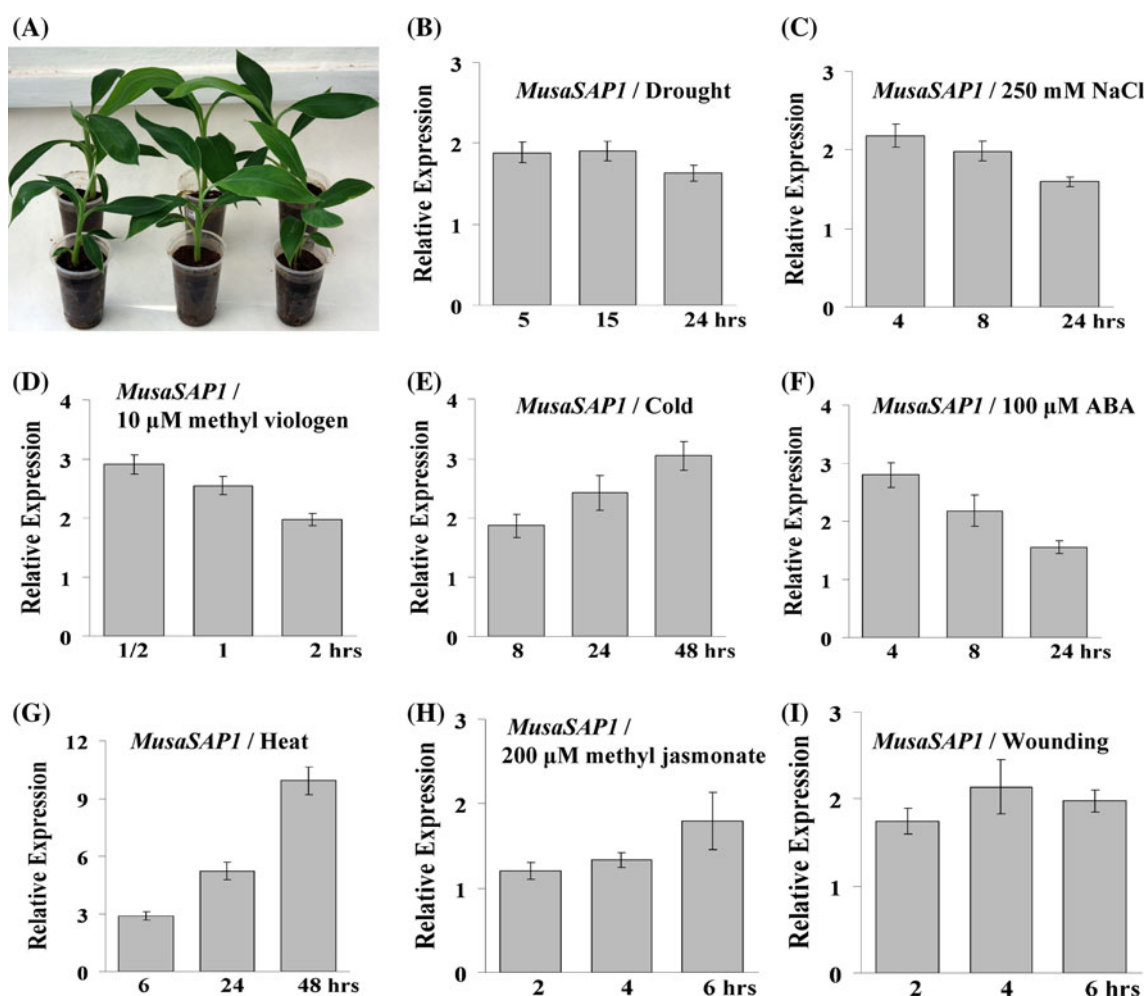


Fig. 3 Expression profiles of *MusaSAPI* in banana cv. *Karibale Monthan* leaves in response to different stress treatments as determined by quantitative real-time RT-PCR. **a** Two months old greenhouse maintained plants used for various stress treatments. Time-course pattern of *MusaSAPI* induction after exposure to drought (**b**), 250 mM NaCl (**c**), 10 μ M methyl viologen (**d**), cold

(**e**), 100 μ M ABA (**f**), heat (**g**), 200 μ M methyl jasmonate (**h**) and after wounding (**i**). All expression values have been normalized against *Musa EF1 α* gene expression levels. Further expression of *MusaSAPI* in control untreated plants is assumed to be 1 for calculating the level of induction of *MusaSAPI* in response to various stress conditions. Values are mean \pm SE

medium (Fig. 4d). The germinating embryos were later subcultured on to banana multiplication medium for multiple shoot induction (Fig. 4e). These shoots were later separated and rooted on MS medium added with NAA (Fig. 4f). Rooted transgenic plantlets were hardened in a contained greenhouse (Fig. 4g).

All the ten transgenic lines regenerated based on their vigorous growth in hygromycin containing medium stained positive for GUS expression (data not shown) indicating that the T-DNA(s) containing the GUS, 'pZmUbi-*MusaSAPI*-nos' and the hygromycin phosphotransferase expression cassettes were integrated in these lines in transcriptionally active euchromatin regions of the banana genome. Genomic DNA PCR analysis of these *MusaSAPI* overexpressing lines showed a single 788-bp amplified fragment derived from hygromycin phosphotransferase coding sequence whereas it

was absent in untransformed control plants (Fig. 5a). Four of these ten lines, selected based on their intense GUS staining were analysed by quantitative real-time RT-PCR to determine the exact quantum of overexpression of *MusaSAPI* in these lines (Fig. 5b). The overexpression as determined by using *Musa EF1 α* gene for expression normalization was found to be 1.799 times (relative to the expression of *MusaSAPI* in untreated control) in S2, 6.498 times in S6, 3.864 times in S7 and 2.902 times in S8 line. To further characterize the selected transgenic lines before submitting them for different biochemical assays, Southern blotting of restricted genomic DNA was carried out using a DIG-labeled probe generated using hygromycin phosphotransferase gene coding region. Restriction enzyme *KpnI* was used to digest the genomic DNAs as it is expected to cut the T-DNA of the overexpression vector only once and hence

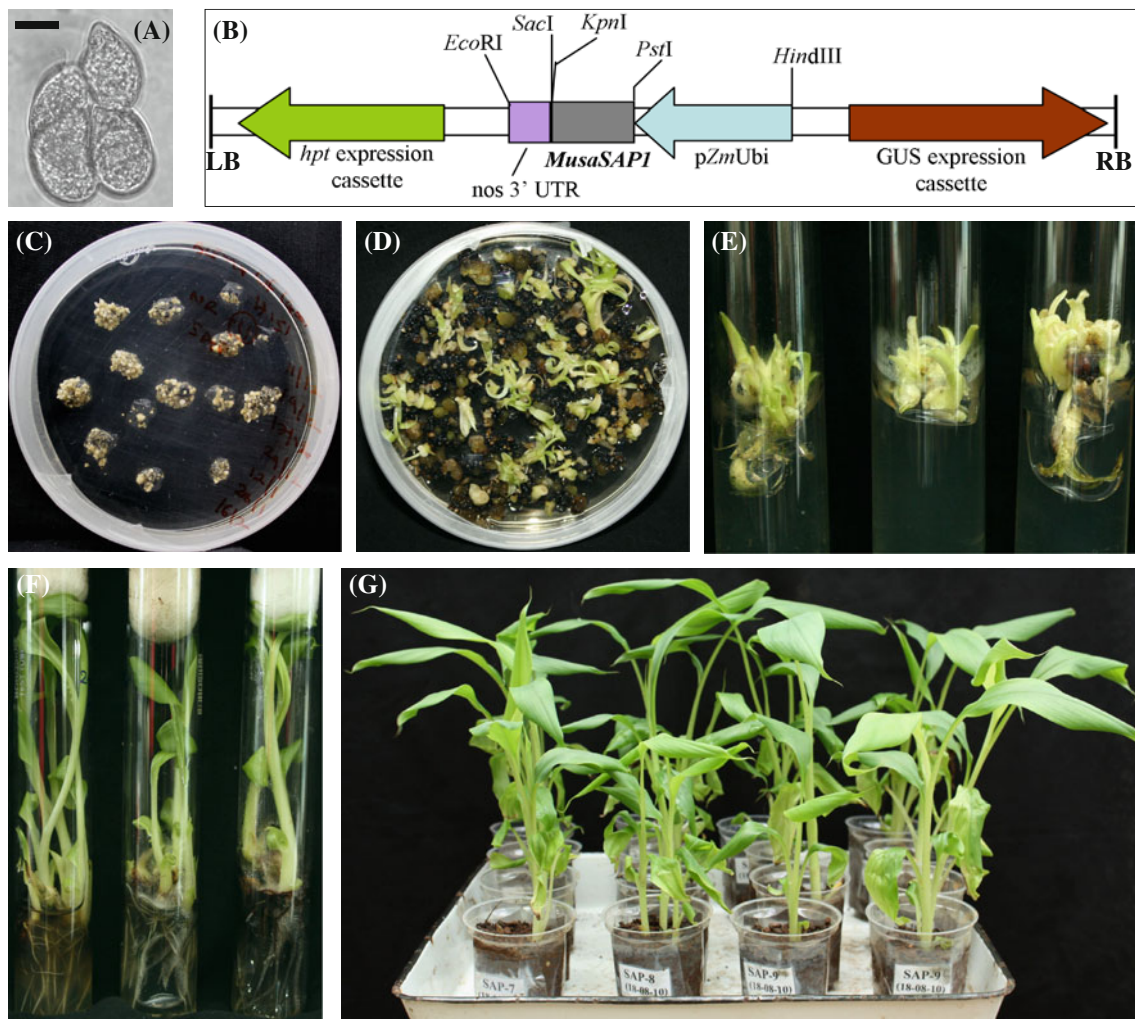


Fig. 4 Generation of transgenic banana cv. *Rasthali* plants overexpressing *MusaSAPI* by *Agrobacterium*-mediated genetic transformation. **a** Densely cytoplasmic embryogenic cells of banana cv. *Rasthali*. Bar correspond approx. to 16.67 μm . **b** Schematic representation of T-DNA region of binary vector p*MusaSAPI*-1301 designed to constitutively overexpress *MusaSAPI* in transgenic banana plants.

c Transformed embryos cultured on hygromycin supplemented embryo induction medium. **d** Germinating embryos growing on embryo germination medium. **e** Transgenic multiple shoots growing in multiple shoot induction medium. **f** Transgenic rooted plantlets on NAA supplemented rooting medium. **g** 2-Months old transgenic hardened plants in greenhouse

the number of bands finally obtained on the autoradiographs can be directly considered as the copy number of the T-DNAs transferred to banana genome in these transgenic lines. T-DNA copy numbers ranging from 1 to 3 were found in three of the four lines analysed (Fig. 5c). The different sizes and numbers of the bands detected in these three lines proved that these transgenic lines originated out of independent transformation events. Line S8 had multiple insertions of T-DNA and hence it was not analyzed any further.

Assessment of abiotic stress responses of *MusaSAPI* overexpressing lines

The transgenic banana plants overexpressing *MusaSAPI* were phenotypically normal in both in vitro and ex vitro

conditions. As most cultivated edible varieties of banana are triploid and hence do not produce seeds, small uniformly sized transgenic shoots derived from in vitro maintained multiple shoot cultures were used in place of seeds as our explants for various stress treatments. As these multiple shoot cultures were each obtained from single transgenic embryos they were expected to be clonal in nature. Shoots were imparted drought and salt stress by supplementing the rooting media with additives namely mannitol and NaCl (Fig. 6a–d). Roots being the first and the most sensitive plant organ which respond to abiotic stress conditions, the ability of in vitro shoots to produce roots in the presence of stress-inducing components like mannitol and NaCl gives the best indication of their superior stress tolerance. Shoots derived from S2, S6 and S7 transgenic lines were markedly fresh

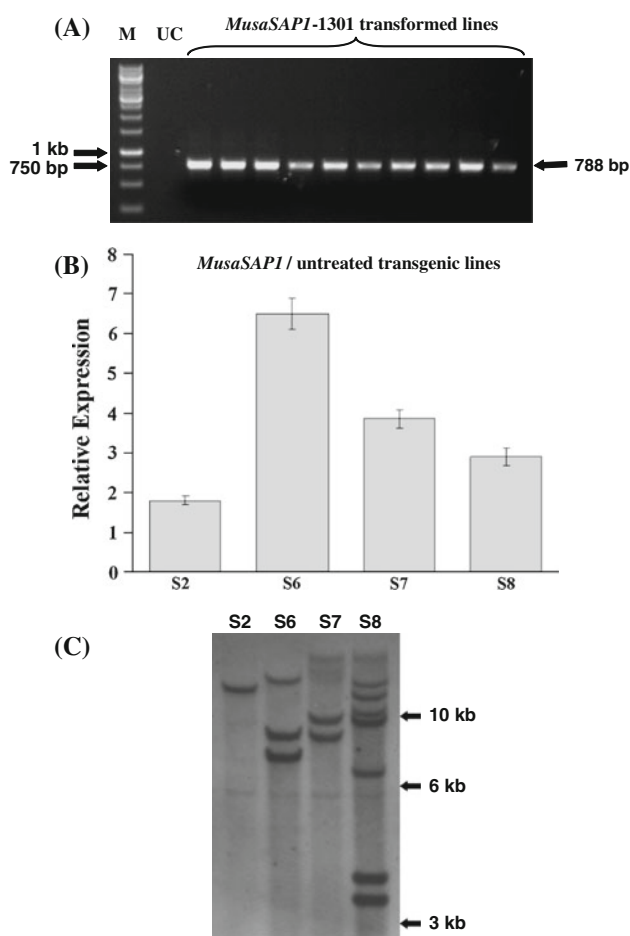


Fig. 5 Molecular analysis of *MusaSAPI* overexpressing transgenic banana plants. **a** Genomic DNA-PCR analysis of untransformed control and transgenic plants. A 788-bp amplification product derived from hygromycin phosphotransferase gene was amplified from genomic DNA derived from p*MusaSAPI*-1301 transformed lines whereas it was absent in untransformed banana plants (UC). **b** Quantum of *MusaSAPI* overexpression in transgenic banana lines (S2, S6, S7 and S8) ascertained using real-time quantitative RT-PCR. All expression values have been normalized against *Musa EF1 α* gene expression levels. Further expression of *MusaSAPI* in control untransformed plants is assumed to be 1 for calculating the level of overexpression of *MusaSAPI* in different transgenic lines. Values are mean \pm SE. **c** Southern blot analysis of *MusaSAPI* overexpressing banana plants. Genomic DNA isolated from young leaves was digested with *Kpn*I and then immobilized and subsequently filter hybridized with DIG-labeled probes generated using hygromycin phosphotransferase gene sequence as template. Approx. positions of the marker bands are indicated

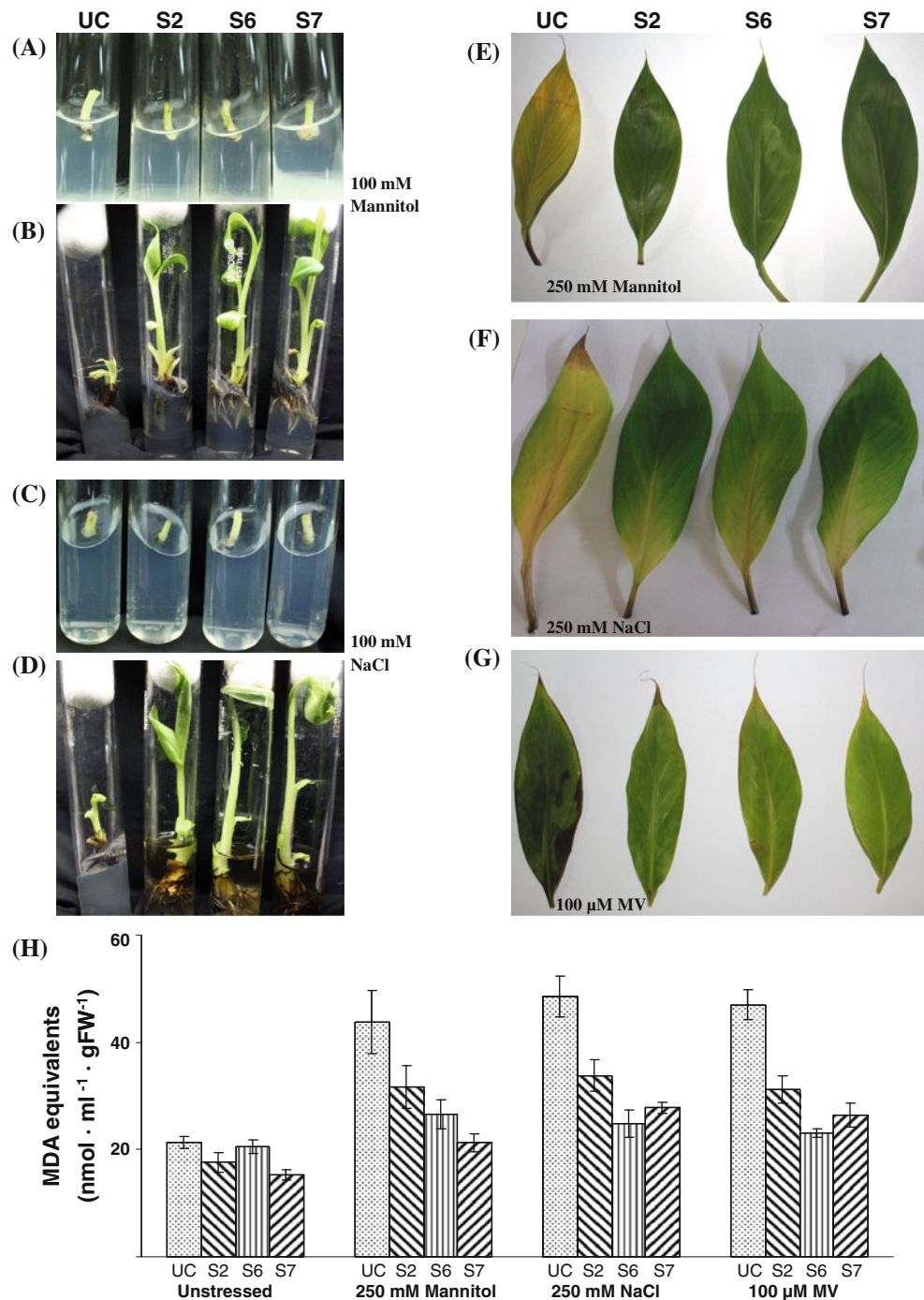
after 10 days in rooting medium supplemented with 100 mM mannitol or 100 mM NaCl as compared to untransformed controls. Further, the transgenic lines grew vibrantly in the recovery medium by generating prolific adventitious roots followed by elongation of leaves and leaf stalks. The untransformed control shoots grown under similar conditions displayed stunted root and shoot growth. To further validate the findings obtained by using in vitro grown shoots, we also used detached leaves derived from greenhouse maintained

hardened transgenic plants in stress assays (Fig. 6e–g). The detached leaves along with their petioles were treated with 250 mM mannitol or 250 mM NaCl for 7 days to check their stress hardness as compared to equivalent untransformed controls. Leaves derived from *MusaSAPI* overexpressing transgenic plants were significantly less damaged by the drought or salt stress applied as compared to control leaves which showed marked chlorosis. Treatment with mannitol and NaCl is a standard procedure widely used to analyze improved tolerance to drought and salt stress in plants and the ability of the single shoots to tolerate and grow robustly in their presence proves the increased tolerance of transgenic lines towards drought stress. Similarly, the fact that detached leaves remained green and looked healthy even after 7 days in mannitol or NaCl solution clearly indicated that overexpression of *MusaSAPI* provided these transgenic plants with improved ability to tolerate these stress conditions. Oxidative stress exerted mainly through ROS is common component of both drought and salt stress. Hence, to further assess the performance of *MusaSAPI* overexpressing plants in response to oxidative stress, detached leaves derived from greenhouse maintained transgenic and control plants were treated with methyl viologen in continuous light. Methyl viologen, in the presence of light accepts electrons from photosystem I and transfers them to oxygen causing the production of ROS. Transgenic leaves were almost unaffected after the methyl viologen treatment as compared to the untransformed control leaves which got darkened and looked visibly damaged. To quantify the damage caused to the cellular membranes by oxidative stress during detached leaf assays, leaves treated with mannitol, NaCl and methyl viologen were analysed by TBARS (Thiobarbituric Acid Reactive Substances) assay which quantifies the level of MDA produced by lipid peroxidation of cellular membranes (Fig. 6h). Untransformed control leaves suffered the maximum membrane damage as the MDA levels were the highest in these leaves. Constitutively increased expression of *MusaSAPI* in transgenic plants thus imparted these plants with improved capacity to scavenge the free radicals, thereby reducing the damage caused by these radicals on cellular membranes of transgenic banana plants. Further, the MDA levels after exposure to different abiotic stress treatments broadly correlated (in salt and oxidative stress treatments) with level of overexpression of *MusaSAPI* in the three transgenic banana lines (S2, S6 and S7).

Increased PPO gene transcription and protein activity

Induction of *MusaSAPI* transcripts in leaves of banana cv. *Karibale Monthan* in response to methyl jasmonate treatment and wounding stress indicated towards possible involvement of this SAP gene in biotic stress responses as well as in specific antiherbivore (anti-insect) defenses of

Fig. 6 Assessment of abiotic stress responses of *MusaSAP1* overexpressing lines (S2, S6 and S7) along with untransformed control plant (UC). **a** Single in vitro shoots cultured on rooting medium (MS added with 1 mg l^{-1} NAA) added with 100 mM mannitol. **b** Recovery of shoots in plain rooting medium (20–25 days) after 10 days in mannitol supplemented medium. **c** Single in vitro shoots cultured on rooting medium added with 100 mM NaCl. **d** Recovery of shoots in plain rooting medium (20–25 days) after 10 days in NaCl supplemented medium. **e–g** Detached leaves derived from greenhouse maintained transgenic and control plants respectively after exposure to simulated drought (250 mM mannitol), salt (250 mM NaCl) and oxidative (100 μM methyl viologen) stress. All in vitro and ex vitro assays were performed in triplicates. **h** MDA equivalents in leaves of *MusaSAP1* overexpressing lines and untransformed control plants subjected to drought, salt and oxidative stress



banana plant. Further, as PPOs are known to be important players in plants in the context of biotic defense pathways, we wanted to assess the effect of overexpression of *MusaSAP1* in transgenic banana plants on total PPO activity in these transgenic plants. Total PPO activity was found to be higher in *MusaSAP1* overexpressing transgenic banana plants as compared to controls (Fig. 7a). Further, the transcript level of one of the four putative PPO coding sequences (Genbank accession no. ES437160) was found to be highly upregulated in transgenic banana plants

(Fig. 7b). This partial PPO coding transcript contains the three characteristic PPO domains namely tyrosinase domain, PPO1_DWL domain and PPO1_KFDV domain. Also, the quantum of this gene upregulation was much higher as compared to the increase in total PPO protein activity probably due to low mRNA stability of the PPO transcripts in question. This also explains the fact that wide differences in mRNA abundance between the three transgenic lines was not correlated with the total PPO activities of the transgenic leaves which were not very different from

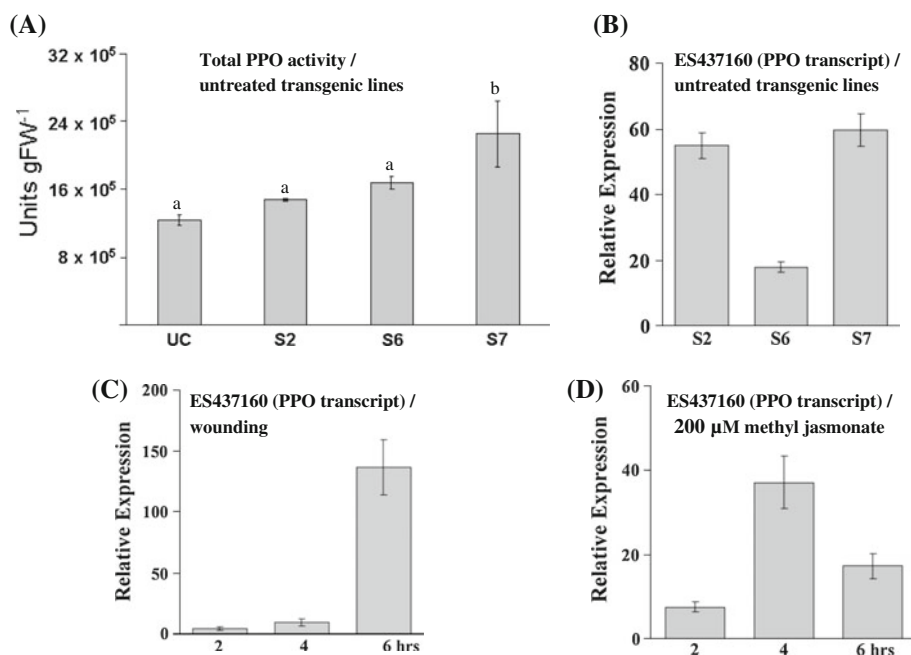


Fig. 7 Analysis of PPO gene transcription and protein activity. **a** Enhanced total PPO activity in transgenic leaves (derived from S2, S6 and S7) as compared to untransformed controls (UC). *Different letters* denote significant differences in total PPO activity at $P \leq 0.05$ between different lines. Values are mean \pm SE. **b** Up-regulation of a putative PPO encoding transcript (GenBank accession no. ES437160) in *MusaSAP1* overexpressing lines as determined by quantitative real-time RT-PCR. All expression values have been normalized against *Musa EF1 α* gene expression levels. Further expression of the transcript corresponding to EST ES437160 in control untransformed plants is assumed to be 1 for calculating the level of induction of this

transcript in *different transgenic lines*. Values are mean \pm SE. **c**, **d** Up-regulation of putative PPO encoding transcript (GenBank accession no. ES437160) in native banana plants respectively in response to wounding and 200 μ M methyl jasmonate as determined by quantitative real-time RT-PCR. All expression values have been normalized against *Musa EF1 α* gene expression levels. Further, expression of the transcript corresponding to EST ES437160 in control untreated plants is assumed to be 1 for calculating the level of induction of this transcript in response to wounding treatment or methyl jasmonate application. Values are mean \pm SE

each other. This particular transcript was also found to be upregulated in native banana plants in response to wounding and methyl jasmonate treatment (Fig. 7c, d) thereby pointing towards a significant role for *MusaSAP1* in the wound signaling in banana plants.

Discussion

Diverse members of SAP family have recently been shown to be involved in a variety of stress response and other developmental pathways in plants (Solanke et al. 2009). Several studies have demonstrated expression induction of SAP genes in response to different abiotic stress conditions suggesting their function in ameliorating the cellular effects of these stresses (Jin et al. 2007). Studies on SAP genes have mostly been concentrated on *Arabidopsis*, rice, maize or poplar with important food security plants like banana with huge economic importance being largely ignored. Banana ranks among the key food crops in the world and India stands first in its production with 31.89 million metric tonnes which make up 34.5 % of total production of top 20 banana growing countries. Because of the huge economic

dimension attached with control of banana ripening, most of scientific attention till now has been directed at understanding the mechanisms underlying ripening and associated processes (Bapat et al. 2010). Nevertheless, a few studies have been conducted for studying banana abiotic stress responses (Feng et al. 2009; Liu et al. 2010; Dai et al. 2011; Shekhawat et al. 2011a; Shekhawat et al. 2011b).

The present report describes cloning and characterization of a novel banana SAP gene, *MusaSAP1*, by generating transgenic banana plants overexpressing this SAP gene. *MusaSAP1* was selected from among a few A20/AN1 zinc finger domain containing sequences identified in banana EST database based on its inducibility in response to several stress treatments including drought, salinity, cold, heat, wounding and oxidative stress and application of ABA and methyl jasmonate. Southern blot analysis indicated that *MusaSAP1* is present in the banana genome as a single copy gene signifying a specific but important role for this gene. Homology and phylogenetic analysis indicated that *MusaSAP1* like other related SAP sequences has highly conserved zinc finger domains whereas regions outside these domains are mostly specific for specific plant species. The presence and the position of the two introns in the 5' UTR region and the

absence of any intron in the protein coding region of *MusaSAPI* further established its closeness with characterized members of SAP family like *OsiSAP8* (Kanneganti and Gupta 2008). Anticipating functional conservation also between these closely related SAP sequences, overexpression of *MusaSAPI* was undertaken in its native species, that is, banana. Further, the cellular localization of several members of SAP family has been described in past and both cytosolic and nuclear localization has been proposed for different SAP sequences. AtSAP5 was demonstrated to be localized exclusively in nucleus (Kang et al. 2011) whereas *OsiSAP8* was found to be exclusively localized in the cytosol (Kanneganti and Gupta 2008). Further, AtSAP10 was found to be distributed both in nucleus and cytosol (Dixit and Dhankher 2011). In our studies performed using onion peels and a GFP fusion platform, we found that *MusaSAPI* is localized both to the cytosol and nucleus. The single bipartite nuclear localization signal could not ensure complete translocation of *MusaSAPI* in the nucleus of onion cells. As indicated by previous studies, *MusaSAPI*, like other related SAP sequences may also be functioning by protein–protein interactions in the cytosol and nucleus in addition to its expected transcription factor activity in the nucleus. Further detailed studies are needed to clarify this aspect of *MusaSAPI* function.

Involvement of SAP genes in stress response pathways has been demonstrated in recent past by achieving improved tolerance to abiotic stress treatments by overexpression of select SAP genes in model plants like *Arabidopsis*, tobacco or rice. Three such genes from rice namely *OsiSAPI* (Mukhopadhyay et al. 2004), *OsiSAP8* (Kanneganti and Gupta 2008) and *ZFP177* (Huang et al. 2008) have been shown to give stress tolerance upon their overexpression in transgenic plants. Entire families of such genes have been demonstrated to be involved in various stress response pathways in *Arabidopsis*, rice and maize (Jin et al. 2007). AtSAP5 possessing E3 ligase activity has recently been demonstrated to act as a positive regulator of stress responses in *Arabidopsis* (Kang et al. 2011). AtSAP from halophyte grass *Aeluropus litoralis* when overexpressed in transgenic tobacco improved drought and salt stress tolerance (Ben Saad et al. 2010). Similarly, AtSAP10, showing differential regulation in response to heavy metals, high and low temperatures, cold and ABA has been shown to impart heavy metal and high temperature stress tolerance in *Arabidopsis* (Dixit and Dhankher 2011).

The ideal way to investigate the functions of a protein is its overexpression in the parent plant species which makes possible direct study of its interactions with various other macromolecules present in the native plant. When *MusaSAPI* was overexpressed in transgenic banana plants, we observed that the transgenic banana plants had improved stress survival characteristics. Banana transgenic tissues transformed with *MusaSAPI* overexpression vector grew normally in vitro under hygromycin selection such that no

gross abnormalities or stunting effects were observed. Small uniform in vitro transgenic shoots were able to tolerate simulated drought and salt stress in a much better fashion as compared to untransformed controls as indicated by their limited damage in mannitol and NaCl supplemented medium and later swift growth in recovery rooting medium. In vitro assays for stress tolerance using shoots have been performed previously wherein the rooting capacity has been taken as an indicator for stress tolerance (Shekhawat et al. 2011b; Hamrouni et al. 2008). When leaves derived from transgenic and untransformed controls were exposed to drought, salt or oxidative stress, the injury and visible damage caused to detached leaves derived from transgenic plants was much less than leaves derived from untransformed controls. Thus, both in vitro as well as ex vivo assays proved the positive participation of *MusaSAPI* in abiotic stress responses of banana. These results also proved our earlier assumption that inducibility of *MusaSAPI* in response to different stress treatments can be affirmatively correlated with its positive role in stress amelioration pathways in banana plant. Further, both drought and salt stress lead to generation of ROS in plants. Decreased stomatal conductance for avoiding excessive water loss from the plants reduces the internal CO₂ concentrations in the plant leading to rapid depletion of the oxidized NADP⁺ (final acceptor of electrons in photosystem I). Because of this there is an increase in the leakage of electrons to O₂ leading to formation of ROS and concomitant peroxidation of thylakoid membrane lipids (Abogadallah 2010). Considering the commonality of incidence of oxidative stress in any water limiting condition in plants, we tested *MusaSAPI* overexpressing plants for tolerance to oxidative stress by exposing them to methyl viologen in the presence of continuous light. The damage caused to the untransformed control was much more severe as compared to that seen in transgenic plants. Further, the MDA levels in leaves exposed to drought, salt or oxidative stress were significantly lower in transgenic plants as compared to equivalent controls thereby confirming the role of *MusaSAPI* in effective amelioration of stress injury in banana plants.

In addition to abiotic stress factors, inducibility of *MusaSAPI* in response to methyl jasmonate and wounding indicated possible role for this SAP gene in biotic stress responses including antiherbivore defenses. Also, the role of PPOs has been studied in detail in plants in context of biotic defense pathways (Constabel et al. 2000). PPOs released upon plant wounding catalyze the conversion of o-diphenolic compounds to o-quinones, which in turn covalently modify free amino and sulfhydryl groups in plant proteins. These phenolic adducts later thwart efficient assimilation of the alkylated amino acids in the insect gut, and hence reduce the nutritive value of protein. Based upon

this correlation between wounding and PPO function and also the induction of *MusaSAPI* transcripts in response to wounding and methyl jasmonate (known to be involved in wound signaling) treatment, we investigated the total PPO activity in healthy unwounded transgenic plants. Higher total PPO activity coupled with increased transcript levels of at least one PPO coding sequence in *MusaSAPI* transgenic plants signaled towards an important role for *MusaSAPI* in biotic defense pathways of banana plant. Among all the plant SAP sequences characterized so far, this is probably the first report providing evidence for involvement of SAP genes in biotic stress response pathways in plants. Overall, true to its naming as a stress associated protein, *MusaSAPI* modulates a wide variety of cellular processes in banana plants upon its induction during non optimal stress conditions.

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