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Molecular and functional characterization of the *HbSRPP* promoter in response to hormones and abiotic stresses

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Abstract Small rubber particle protein (SRPP) is a major component of *Hevea brasiliensis* latex, and obviously participates in the biosynthesis of natural rubber. However, little information is available about regulation of SRPP gene expression. In this study, the promoter region of *HbSRPP* was isolated and characterized. Its sequence included regulatory elements predicted to be responsive to hormones and other environmental cues. Promoter deletion analysis revealed that 219 nucleotides (nt) upstream of the transcription start site were sufficient for expression. The region from −1,055 to −219 nt positively regulated expression induced by methyl jasmonate (MeJA), abscisic acid (ABA), and wounding; the region from −734 to −528 nt positively regulated expression induced by gibberellic acid (GA); the region from −734 to −219 nt positively regulated expression induced by heat; the region from −1,055 to −4 negatively regulated expression induced by cold; the region from −219 to −4 nt was associated with negative regulation of expression induced by ABA and wounding; the region from −528 to −4 nt negatively regulated expression induced by GA. These results suggest the activity of the *HbSRPP* promoter is regulated by MeJA, ABA, GA, cold, heat, and wounding.

Keywords *Hevea brasiliensis* · Small rubber particle protein · Promoter · Regulatory elements · Hormone · Stress

Abbreviations

ABA	Abscisic acid
GA	Gibberellic acid
GUS	β-Glucuronidase
MeJA	Methyl jasmonate
NR	Natural rubber
nt	Nucleotides
SRPP	Small rubber particle protein
TSS	Transcription start site

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Introduction

The rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important tropical tree belonging to the Euphorbiaceae family, from which is obtained most of the world's natural rubber (NR) with variety of uses in industry. NR is a high-molecular-mass polymer of isoprene units with the *cis* configuration. Rubber molecules are produced and aggregated or packaged as rubber particles in latex vessels (laticifers) of the rubber tree. Latex is essentially the cytoplasmic content of the laticifer cell. Latex contains mainly rubber and non-rubber particles, organelles, proteins, and cytoplasmic C-serum (d'Auzac et al. 1989; Berthelot et al. 2012). The rubber particle is a specialized organelle in the laticifer cell and NR biosynthesis occurs on the surface of rubber particles (Cornish 2001). NR synthesis is regulated by the activity of rubber particle-associated proteins present on the surface of the membrane monolayer which surrounds the rubber particles (Siler et al. 1997; Cornish et al. 1999; Wood and Cornish 2000; Berthelot et al. 2012; Xiang et al. 2012). Small rubber particle protein (SRPP) from *Hevea* latex is an acidic protein of molecular mass 22.4 kDa (Yeang et al. 1996; Oh et al. 1999; Chow et al. 2007), which was believed not to be post-translationally modified (Yeang et al. 1996). SRPP is abundant on large rubber particles (LRP, generally above 0.4 mm in diameter) and small rubber particles (SRP, smaller than 0.4 mm in diameter) (Bahri and Hamzah 1996; Yeang et al. 1996; Singh et al. 2003; Ohya et al. 2000; Xiang et al. 2012). It obviously participates in the biosynthesis of natural rubber but its exact function is still unclear (Oh et al. 1999; Kim et al. 2004; Wititsuwannakul et al. 2008; Berthelot et al. 2012). Recently, SRPP was described as a glycoprotein which interacted with a *Hevea* latex lectin present on lutoids and was involved in latex coagulation (Wititsuwannakul et al. 2008). The gene encoding for SRPP proteins from *H. brasiliensis* (*HbSRPP*) has been cloned (Oh et al. 1999) and different isoforms have been identified (Chow et al. 2007). *HbSRPP* has been found to be highly expressed in *Hevea* latex and laticifers (Han et al. 2000; Ko et al. 2003; Chow et al. 2007) and is induced by ethylene (Chow et al. 2012). However, no information is available on regulation of the expression of *HbSRPP*.

In this paper we describe isolation and functional analysis of *HbSRPP* promoter regions. Deletion analysis with a GUS-reporter gene was performed by use of an *Agrobacterium*-mediated transient assay to identify regions of the *HbSRPP* promoter regulating transcription in tobacco leaves in response to abiotic stresses and hormones.

Materials and methods

Plant materials, growth conditions, and bacterial strains

Latex and leaf tissue were obtained from mature rubber plants (*H. brasiliensis* clone RRIM 600) growing at the experimental farm of the Chinese Academy of Tropical Agriculture. Tobacco (*Nicotiana benthamiana*) plants for agro-infiltration were raised in a growth chamber at 25 °C under a 16/8 h day/night cycle for six weeks. *Escherichia coli* strain DH5α was used for the cloning and propagation of all recombinant plasmid vectors. *Agrobacterium tumefaciens* strain GV3101 was used for tobacco leaf infiltration.

Cloning of the *HbSRPP* genomic sequence

Genomic DNA was isolated from rubber tree leaves by the method described by Dellaporta et al. (1983). On the basis of the *HbSRPP* cDNA sequence (GenBank accession no. AF051317), a pair of primers (srppF: 5′-CAGTGTTTTCCGAAAGGCAAATC-3′ and srppR: 5′-TTTTTAGTCCATCCATTCTTAATAA-3′) was designed, and used to amplify the full-length genomic sequence from genomic DNA templates. This fragment was amplified by use of LA Taq DNA polymerase (TaKaRa) in accordance with the manufacturer's recommended reaction conditions. The PCR product was cloned into pMD19-T simple vector (TaKaRa) and several clones were sequenced for each reaction.

5′-RACE for identification of the transcription start site of *HbSRPP*

Total RNA was extracted from the latex of *H. brasiliensis* by the method of Tang et al. (2007). The *HbSRPP* transcription start site was determined by a 5′-rapid amplification of cDNA ends (RACE) strategy,

on the basis of a SMART RACE cDNA Amplification Kit (Clontech, USA) procedure. Two specific primers 5RACE1 (5′-GAGTAACCACGGTCTTCACC ACATT-3′) and 5RACE2 (5′-AATCTACAGCATAA ACTCCAGCCGC-3′) were designed from the *HbSRPP* cDNA sequence. A primary PCR was performed with adaptor primer F1 (5′-AAGCAGTGG TATCAACGCAGAGT-3′) and specific primer 5RACE1. Fifty-times-diluted primary PCR products were then amplified by use of adaptor primer F2 (5′-ACGCAGAGTGGCCATTATGGCCGGG-3′) and nested primer 5RACE2. Second-round PCR products were introduced into the pMD19-T simple vector (TaKaRa) for sequencing. The promoter transcription start site was obtained by aligning the resulting sequences with that of the promoter.

Analysis of the *cis*-regulatory element content of the *HbSRPP* promoter

The promoter of *HbSRPP* was isolated from *H. brasiliensis* (Guo et al. 2010). The promoter sequence of *HbSRPP* was analyzed by use of PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp).

Construction of *HbSRPP* promoter–GUS fusions

For construction of *HbSRPP* promoter–GUS fusion genes, a series of *HbSRPP* promoter fragments (from −1,055 to +104 bp) of different lengths were obtained by PCR amplification, using the forward primers: P1: 5′-GCGGGATCCTCATAGTTGTTT-ATTTCATC-3′; P2: 5′-CGCGGATCCGCCCGTA ATGCAGTCAG CAT-3′; P5: 5′-CGCGAATTCTTA TCTTTATTGCC-3′; P4: 5′-GCG GGATCCAAGA TTTGAATTTTTAAGCG-3′; P5: 5′-GCGGG ATCC AACCATAATCAGTTGATA GC-3′ and the reverse primer: 5′-GCGAAGCTTGCAGCATCCTTCATT GA CTG-3′. A *Bam*HI restriction site was introduced into the forward primers and an *Hin*dIII restriction site was introduced into the reverse primer. The amplified sequences were then digested by *Bam*HI and *Hin*dIII and cloned into the *Bam*HI/*Hin*dIII digested binary vector pCAMBIA1381Z (Cambia, Australia). The five structures were denoted SP1 (−1,055 to +104), SP2 (−734 to +104), SP3 (−528 to +104), SP4 (−219 to +104), and SP5 (−4 to +104) (Fig. 1). All of the structures were sequenced and verified by use of PCR and digest reactions. In addition, pCAMBIA1301 was used as positive control and pCAMBIA1381Z was used as a negative control.

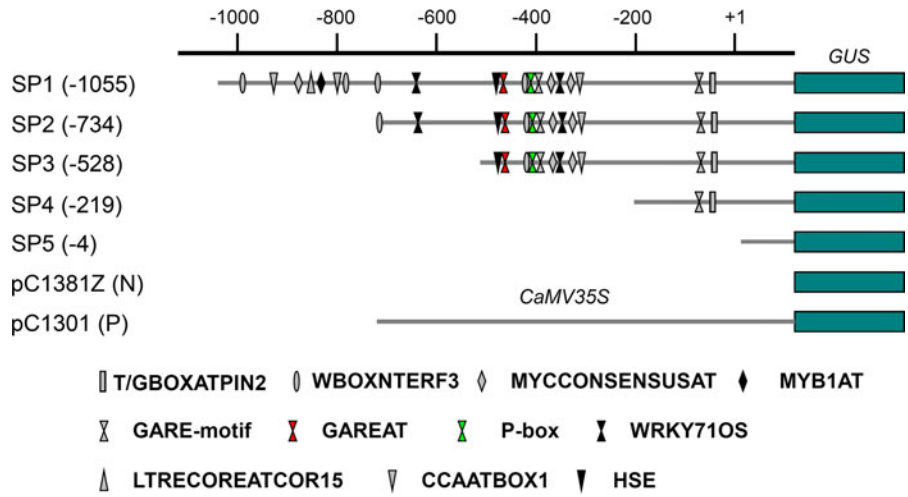

Fig. 1 Schematic diagram of the *HbSRPP* promoter deletion–GUS structures used for assay of reporter gene transient expression in tobacco leaves. The transcription start site is indicated as *+1*. Promoter fragments of different sizes were inserted into the pCAMBIA1381Z vector containing the GUS reporter gene. The *numbers* on each structure indicate the distance from the *HbSRPP* transcription start site. Plasmids pCAMBIA1381Z and pCAMBIA1301 were used as negative and positive controls, respectively

Agrobacterium-mediated transient expression assay

Agrobacterium-mediated transient expression assays were performed as described elsewhere (Xu et al. 2010; Yang et al. 2000). A single colony of *A. tumefaciens* strain GV3101 harboring a promoter structure was inoculated into 3 mL LB liquid medium supplemented with rifampicin (60 μg/mL) and kanamycin (50 μg/mL) at 28 °C for 24 h. This culture (1 mL) was transferred into 25 mL LB liquid medium containing the same antibiotics and incubated at 28 °C for 5 h, with shaking. *Agrobacterium* cells were harvested after centrifugation at 4,000×*g* for 15 min, resuspended in infiltration solution (10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 100 μM acetosyringone), incubated at 28 °C for 3 h, and adjusted to an OD_{600} of 0.8 for infiltration into tobacco leaves. After agroinfiltration at the abaxial surfaces of tobacco leaves by use of a needleless syringe (Yang et al. 2000), the tobacco plants were maintained in a moist chamber under a 16/8 h day/night cycle at 25 °C for 48 h. All stress treatments were applied 48 h after agroinfiltration.

Abiotic stress and hormone treatments

To characterize the induction of promoter activity by stress-responsive hormones, tobacco plants were treated with methyl jasmonate (MeJA), abscisic acid (ABA), and gibberellic acid (GA). For MeJA, ABA, and GA treatment the tobacco plants were sprayed with 100 μM MeJA, 100 μM ABA, or 50 μM GA in distilled water. Tobacco plants treated with MeJA were then incubated in a vinyl bag. The control plants were sprayed with water.

The tobacco plants subjected to agroinfiltration were exposed to a variety of environmental stresses, including low temperature, high temperature, and wounding, to determine the extent to which promoter activation occurred under these conditions. To induce cold and heat stress, the plants were transferred to an incubator at 4 or 42 °C, respectively, for 12 h. To induce mechanical wounding, the tobacco leaves were pricked with needles. All treated plants, except cold and heat-treated, were maintained in a moist chamber under a 16/8 h day/night cycle at 25 °C. Leaves were used for GUS analysis after 15 h. All experiments were repeated at least three times.

Histochemical and fluorimetric assays for GUS activity

Histochemical staining was performed in accordance with Jefferson (1987), using X-Gluc as a substrate. Tobacco leaves were incubated in GUS staining solution (80 mM sodium phosphate buffer (pH 7.0), 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 8 mM EDTA, 0.5 mg/ml 5-bromo-4-chloro-indolyl-β-D-glucuronide (X-Gluc), and 0.05 % Triton X-100) for 24 h at 37 °C, followed by removal of chlorophyll from the leaves by successive washes with 70 % ethanol at 37 °C. Stained tissues were examined by light microscopy.

Transient expression of GUS activity in the treated tobacco leaves was measured as described elsewhere (Jefferson 1988). Tobacco leaf tissue was homogenized in 600 μL extraction buffer (50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 0.1 % Triton X-100, 0.1 % (*w*/*v*) sodium dodecyl sulfate, 10 mM β-mercaptoethanol), and centrifuged at 12,000×*g* for 15 min at 4 °C. Supernatant (20 μL) was mixed with 180 μL pre-warmed (37 °C) GUS assay solution (1 mM methyl-4-umbelliferyl-D-glucuronide in extraction buffer) and incubated at 37 °C for 40 min. This mixture (50 μL) was then transferred into 950 μL stop buffer (0.2 M Na_2CO_3). GUS activity was measured with a Glomax Multi + detection system (Promega) at 365 (excitation) and 455 (emission) nm. Stop buffer and 50 nM to 1 μM 4-methylumbelliferone (4-MU) were used for calibration and standardization. The total concentration of protein extracts from the tested samples was normalized by dilution with extraction buffer, as described elsewhere (Bradford 1976). GUS activity was expressed as nM of 4-MU generated per min per mg soluble protein. GUS measurements were repeated at least three times, with similar results.

Results

HbSRPP genomic sequence analysis and determination of the TSS of the *HbSRPP* promoter

A 1,735 bp fragment was isolated from the genomic DNA of a rubber tree by use of the PCR strategy. Comparison of the cDNA with genomic

Table 1 Putative *cis* elements in HbSRPP as predicted by PLACE and PlantCARE

cis Element	Position (strand)	Sequence	Function
CCAATBOX1	−935 (+), −807 (+), −329 (+)	CCAAT	Act cooperatively with HSE to increase the activity of the promoter
TC-rich repeats	−894 (+), −618 (+), −557 (−)	GTTTTCTTAC	*cis*-Acting element involved in defense and stress responsiveness
T/GBOXATPIN2	−65 (−)	AACGTG	*cis*-Acting element involved in MeJA induction
OBP-1 site	−443 (−)	TACACTTTTGG	*cis*-Acting regulatory element
WRKY71OS	−648 (−), −350 (+)	TGAC	Core of W-box, transcriptional repressor of GA signal
GAREAT	−473 (+)	TAACAAR	Gibberellin-responsive element
GARE-motif	−408 (+), −75 (+)	AAACAGA	Gibberellin-responsive element
P-box	−418 (−)	CCTTTTG	Gibberellin-responsive element
ACE	−623 (−)	AAAACGTTTA	Light-responsive element
Box 4	−324 (+)	ATTAAT	Light-responsive element
Box I	−190 (−)	TTTCAAA	Light-responsive element
CATT-motif	−953 (+)	GCATTC	Light-responsive element
GATA-motif	−527 (−)	AAGATAAGATT	Light-responsive element
G-box	−887 (−)	CACATGG	Light-responsive element
GT1CONSENSUS	−819 (−), −590 (−), −412 (+), −333 (−), −229 (+), −186 (+), −132 (+), −44 (−)	GRWAAW	Light-responsive element
IBOXCORE	−659 (−)	GATAA	Light-responsive element
TBOXATGAPB	−430 (−)	ACTTTG	Light-responsive element
WBOXATNPR1	−770 (+)	TTGAC	Regulates NPR1, SA-induced
ARE	−753 (−), −84 (−), −6 (−)	TGGTTT	Response to anaerobic conditions
ARR1AT	−457 (−), −299 (−), −218 (+), −123 (−), −23 (+)	NGATT	Response for cytokinin
CPBCSPOR	−313 (−)	TATTAG	Response for cytokinin
HSE	−487 (+)	AAAAAATTTC	Response for heat stress
LTRECOREATCOR15	−860 (−)	CCGAC	Low-temperature responsive element
MBS	−961 (−)	CAACTG	Response to drought
MYB1AT	−832 (+)	WAACCA	Response to drought, ABA signal
MYCCONSENSUSAT	−885 (−), −378 (+), −340 (+)	CANNTG	Response to drought, ABA signal
WBOXNTERF3	−995 (+), −787 (−), −723 (−), −427 (−)	TGACY	Wound-responsive element

Symbols used in the sequences are: N: A, C, G, or T; R: A or G; Y: T or C; W: A or T

sequences revealed the *HbSRPP* gene contains three exons and two introns. The three exons (15, 204, and 396 bp in length) were separated by the two introns (492 and 349 bp in length) (Suppl. Fig. 1). The transcription start site (TSS) of the *HbSRPP* promoter was determined by 5′-RACE and use of total RNA extracted from the latex. Six positive clones were sequenced and aligned with the *HbSRPP* gene and promoter (Suppl. Fig. 2). The TSS is an adenine (A) at −104 bp upstream of the translation initiation codon. Analysis of the nucleotide sequence immediately to the 5′ side of the coding region revealed the presence of a putative CAAT box at position −79 and a putative TATA box at position −34 in good sequence context (Table 1).

cis-Regulatory element content of the *HbSRPP* promoter

The PLACE (Higo et al. 1999) and PlantCARE (Lescot et al. 2002) databases were used to identify matches in the *HbSRPP* promoter to the *cis*-regulatory elements of other plants species. A typical TATA-box and a CAAT-box were located −34 to −28 and −79 to −76 bp upstream from the transcription start site, respectively. Several types of regulatory element were found in the *HbSRPP* promoter (Table 1). These include: *cis*-acting elements involved in response to light, including ACE, Box 4, Box I, CATT-motif, GATA-motif, G-box, GT1CONSENSUS, IBOXCORE, and TBOXATGAPB; *cis*-acting elements involved in phytohormone responsiveness, for example WRKY71OS, GAREAT, GARE-motif, and P-box for responding to gibberellin; MYB1AT and MYCCONSENSUSAT for responding to ABA; and T/GBOXATPIN2 and WBOXATNPR1 for jasmonate and SA, respectively; WBOXNTERF3 elements involved in wound response, MBS response to drought, TC-rich repeats elements involved in defense and stress responsiveness; HSE and LTRECOREATCOR15, which function under conditions of high and low-temperature stress. CCAATBOX1 acts cooperatively with HSE to increase the activity of the promoter. Additional predicted *cis*-regulatory elements, including ARE response to anaerobic conditions, ARR1AT and CPBCSPOR response to cytokinin, and the OBP-1 site, were also identified in the promoter region.

Basal expression analysis of the *HbSRPP* promoter

Histochemical GUS assays showed the negative control was not stained and the 35S::GUS positive control was strongly stained. Strong activation of the GUS reporter gene by the −1,055 (SP1), −734 (SP2), −528 (SP3), and −219 (SP4) deletions was detected and weak activation of the GUS reporter gene by the −4 (SP5) deletion was observed in leaves (Fig. 2a). For more precise measurement of GUS expression, we performed quantitative GUS assays. Compared with CaMV35S-mediated GUS expression (pC1301), in plants transformed with SP5 the extent of basal expression was small (a factor of 0.16) whereas in plants transformed with SP1, SP2, SP3, and SP4 the extent of basal expression was greater (6.14, 3.57, 5.07, and 6.23-fold) (Fig. 2b).

Effects of phytohormone treatment on the *HbSRPP* promoter

To determine the role of phytohormone-responsive *cis*-acting elements found in the promoter of *HbSRPP* (Table 1), we tested the deletion structures of *HbSRPP* with regard to induction of GUS expression in tobacco leaves by treating the plants with MeJA, ABA, or GA. GUS fluorimetric assay was performed on agro-infiltrated tobacco leaves after treatment (Fig. 3). After treatment with MeJA, GUS activity of the SP1, SP2, and SP3 promoter structures was significantly increased (1.85, 1.92, and 2.04-fold, respectively; $P < 0.01$) compared with the mock-treated control whereas GUS activity of the SP4 promoter structure was not significantly increased. These results suggest that the promoter region from −1,055 to −219 contains the *cis* element that responds positively to MeJA. After ABA treatment, GUS activity was significantly increased for the SP1, SP2 (2.99-fold and 5.10-fold, respectively; $P < 0.01$), and SP3 (1.33-fold; $P < 0.05$) promoter structures compared with the mock-treated control whereas GUS activity of SP4 was significantly reduced ($P < 0.05$). These results suggest the promoter region from −1,055 to −219 contains the *cis* element that responds positively to ABA. After GA treatment, GUS activity increased significantly for SP2 ($P < 0.01$), was significantly reduced for SP3 ($P < 0.05$) and Sp4 ($P < 0.01$), and there was no significant effect for SP1. These results suggest that the promoter region from −734 to −528 contains the *cis* element that responds positively to GA.

Responsiveness of the *HbSRPP* promoter to stress treatment

To determine the activity of the *HbSRPP* promoter in response to environmental stress, five gene fusions with the promoter deleted were used for analysis of the effects of cold, heat, and wounding stress on expression of the GUS reporter gene. Results from fluorimetric assay of GUS activity are shown in Fig. 4. After cold treatment, GUS activity induced by SP1 to SP4 was lower than for the mock whereas that induced by SP5 was no different from the mock. GUS activity was significantly reduced for SP2 ($P < 0.05$), SP3 ($P < 0.01$), and SP4 ($P < 0.01$), indicating that the promoter region from −1,055 to −4 contains the *cis*

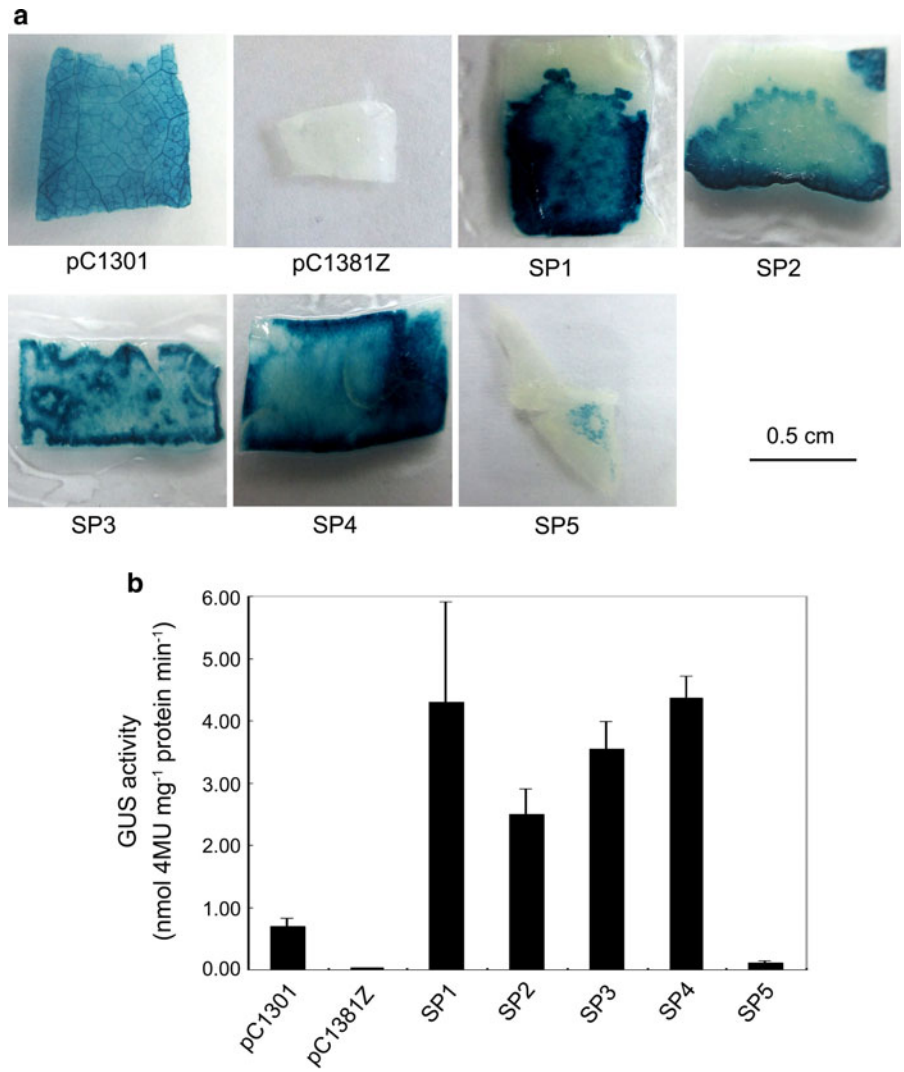

Fig. 2 Histochemical **a** and fluorimetric **b** analysis of GUS activity in transiently transformed tobacco leaves. GUS activity was analyzed fluorimetrically and is expressed as nmol 4-methylumbelliferone (*MU*) mg^{-1} protein min^{-1}. Data are mean values from three independent assays of tobacco leaf extracts, and *error bars* show the standard deviations of the replicates

element that responds to cold-stress treatment. After heat treatment, GUS activity induced by SP1, SP4, and SP5 was no different from that induced by the mock whereas that induced by SP2 and SP3 was 1.55 and 1.32-fold higher, respectively, than that induced by the mock, suggesting the promoter region from −734 to −219 contains the *cis* element that responds to heat-stress treatment. After wounding treatment, GUS activity induced by SP1, SP2, and SP3 increased 1.25, 1.10, and 1.68-fold, respectively, compared with the mock. GUS activity induced by SP3 was significantly increased whereas GUS activity induced by SP4 was significantly reduced ($P < 0.05$). These results suggest that the promoter region from −1,055 to −219 contains a major *cis* element that responds to wounding treatment.

Discussion

Little information was known about regulation of the expression of *HbSRPP*. To investigate the mechanism of regulation of *HbSRPP* expression, we cloned and characterized the 1,159 bp promoter of *HbSRPP*, which encodes small rubber particle protein, a key enzyme in natural rubber biosynthesis. Its sequence included regulatory elements predicted to respond to hormones and other environmental cues. The TSS was mapped to an adenine 104 bp upstream of the start cordon and 34 bp downstream of the TATA-box. Deletion analysis of the promoter of *HbSRPP*, by use of tobacco plants, revealed that the sequence within −219 nucleotides from the TSS was sufficient for expression of function; this region also contains some

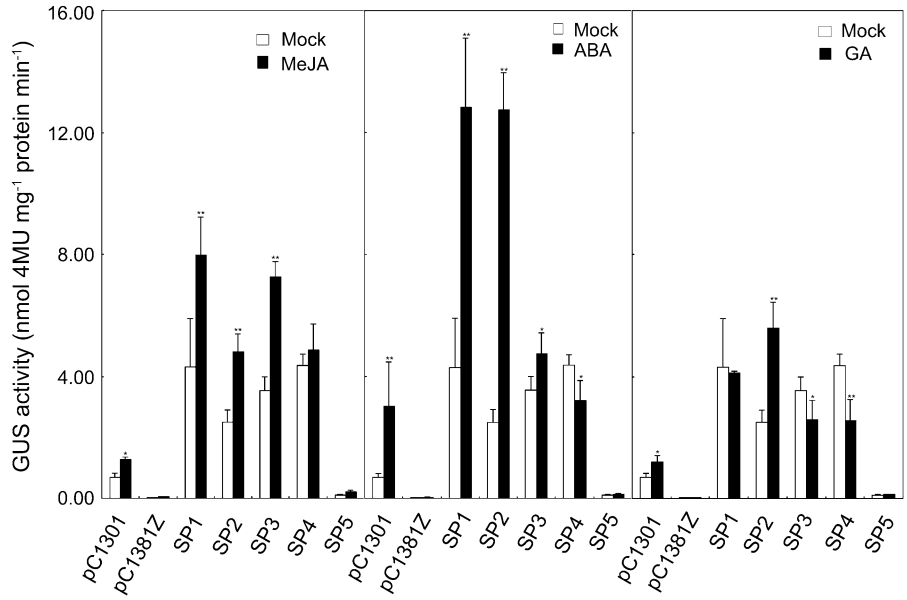

Fig. 3 GUS activity driven by *HbSRPP* promoter deletion–GUS chimeric structures after treatment with MeJA, ABA, and GA. Data are mean values from three independent assays of tobacco leaf extracts and *error bars* show the standard deviations of the replicates. Significant differences from the mock were assessed by use of a one-sided paired *t* test (*single* and *double asterisks* correspond to $P < 0.05$ and $P < 0.01$, respectively)

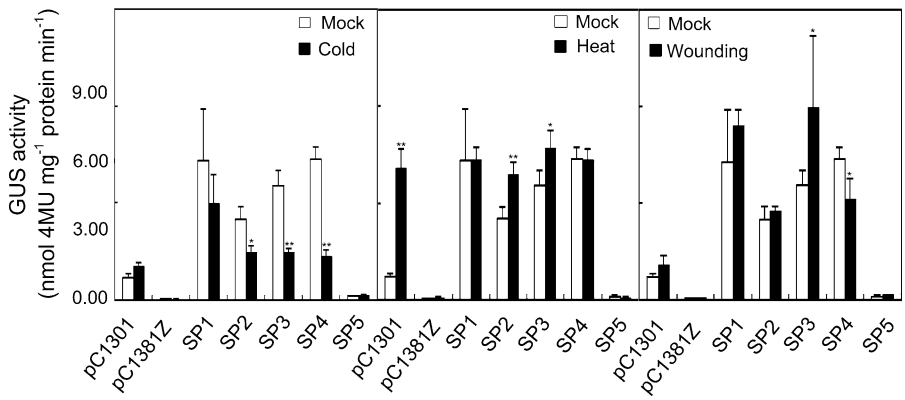

Fig. 4 GUS activity driven by *HbSRPP* promoter deletion–GUS chimeric structures after cold, heat, and wounding treatment. Data are mean values from three independent assays of tobacco leaf extracts and *error bars* show the standard deviations of the replicates. Significant differences from the mock were assessed by use of a one-sided paired *t* test (*single* or *double asterisks* correspond to $P < 0.05$ and $P < 0.01$, respectively)

responsive *cis* elements, for example the GARE-motif, GT1CONSENSUS, ARR1AT, and ARE. The region −1,055 to −219 nt positively regulated expression induced by MeJA, ABA, and wounding; the region from −734 to −528 nt positively regulated expression induced by GA; the region from −734 to −219 nt positively regulated expression induced by heat; the region from −1,055 to −4 negatively regulated expression induced by cold; the region from −219 to −4 nt was associated with negative regulation of expression induced by ABA and wounding; and the region from −528 to −4 nt negatively regulated expression induced by GA. These results suggest that the activity of *HbSRPP* promoter is regulated by MeJA, ABA, GA, cold, heat, and wounding.

Because the major role of plant secondary metabolites is protection against stress, treatment with a variety of elicitors, signal compounds, and abiotic stimuli can help to improve the yield of such metabolites (Zhao et al. 2000; Zhao et al. 2001a, 2001b). Adaptive stress responses in plants often involve cooperative and antagonistic interactions between signaling pathways activated by ABA and other plant hormones, for example MeJA, salicylic

acid, and ethylene (Mauch-Mani and Mauch 2005; Furihata et al. 2006; Hirayama and Shinozaki 2010). Natural rubber is a major secondary metabolite of the rubber tree. Exposure to exogenous MeJA and wounding can stimulate natural rubber production in the rubber tree (Hao and Wu 2000; Yu et al. 2007). Although we found a variety of *cis*-acting elements related to ABA, MeJA, GA, cold, heat, and wounding in the 5′-flanking sequence of *HbSRPP*, their effects on expression have not been previously described. As we expected, treatment with MeJA, ABA, GA, heat, cold, and wounding regulated transcription of *HbSRPP*. JA is believed to be a general inducer of biosynthesis of natural rubber metabolites; some genes related to rubber biosynthesis are activated by JA and wounding (Peng et al. 2009; Zeng et al. 2009; Tian et al. 2010; Zhao et al. 2011). Our results confirmed these earlier reports.

Taken together, the *HbSRPP* promoter sequence contains MeJA, GA and ABA responsive elements, and also cold, heat, and wounding responsive elements. Further deletion analysis will be required to elucidate the *cis*-acting elements of the *HbSRPP* promoter region. Finally, the fragment upstream from the transcription start site of *HbSRPP* may be of great utility to drive transgenic expression in the development of transgenic plants. This study provides useful information for further study of SRPP and its regulatory effect on the biosynthesis of NR, so researchers can improve the NR content of rubber trees.

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