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Distinct repressing modules on the distal region of the *SBP2* promoter contribute to its vascular tissue-specific expression in different vegetative organs

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Abstract The *Glycine max* sucrose binding protein (*GmSBP2*) promoter directs vascular tissue-specific expression of reporter genes in transgenic tobacco. Here we showed that an SBP2-GFP fusion protein under the control of the *GmSBP2* promoter accumulates in the vascular tissues of vegetative organs, which is consistent with the proposed involvement of SBP in sucrose transport-dependent physiological processes. Through gain-of-function experiments we confirmed that the tissue-specific determinants of the *SBP2* promoter reside in the distal *cis*-regulatory domain A, CRD-A (position –2000 to –700) that is organized into a modular configuration to suppress promoter activity in tissues other than vascular tissues. The four analyzed CRD-A sub-modules, designates Frag II (–1785/–1508), Frag III (–1507/–1237), Frag IV (–1236/–971) and Frag V (–970/–700), act independently to alter the constitutive pattern of *–92pSBP2*-mediated *GUS* expression in different organs. Frag V fused to *–92pSBP2-GUS* restored the tissue-specific pattern of the full-length promoter in the shoot apex, but not in other organs. Likewise, Frag IV confined *GUS* expression to the vascular bundle of leaves, whereas Frag II mediated vascular specific expression in roots. Strong stem expression-repressing elements were located at positions –1485 to –1212, as Frag III limited *GUS* expression to the inner phloem. We have also mapped a procambium silencer to the consensus sequence CAGTTnCaAccACATTcCT which is located in both distal and proximal upstream modules. Fusion of either repressing element-containing module to the constitutive *–92pSBP2* promoter suppresses *GUS* expression in the elongation zone of roots. Together our results demonstrate the unusual aspect of distal sequences negatively controlling tissue-specificity of a plant promoter.

Keywords Transcriptional regulation · Tissue-specific regulation · Repressing elements · Promoter distal regions · Sucrose binding protein · *Cis*-acting elements · Vascular tissue-specific expression

Abbreviations

GmSBP2	*Glycine max* sucrose-binding protein 2
VfSBPL	*Vicia faba* sucrose-binding protein-like protein
PCR	polymerase chain reaction
CRD	*cis*-regulatory domain
GUS	*β*-glucuronidase

Introduction

The sucrose binding protein (SBP) was initially identified in soybean cotyledons by its capacity to bind the sucrose analogue 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamide-sucrose (Ripp et al. 1988). Members of the SBP family have also been identified in pea (Castillo et al. 2000), faba bean (Heim et al. 2001), spinach (Warmbrodt et al. 1989, 1991) and *Medicago truncatula* (Contim et al. 2003). At the amino acid level, SBP is most related to cupin domain-containing proteins and vicilin-like seed storage proteins

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(about 35% identity and 50% similarity) from other plant species. In addition to primary sequence conservation, homology-based molecular modeling has shown that SBPs are capable of folding into a cupin tertiary structure and they have therefore been classified as members of the cupin superfamily (Dunwell et al. 2004).

The cupin superfamily corresponds to a functionally diverse family of proteins that comprises seed storage proteins and enzymes as well as proteins that bind different sugars (Dunwell et al. 2000). The *Glycine max* SBP (GmSBP) binds sucrose (Rocha et al. 2007) and exhibits a low sucrose transport activity in the yeast heterologous system (Grimes and Overvoorde 1996; Overvoorde et al. 1996, 1997; Elmer et al. 2003). Furthermore, GmSBP interacts with GTP in a sucrose translocation independent manner and has been shown to be involved in sucrose-dependent physiological processes in transgenic tobacco plants (Delú-Filho et al. 2000; Pedra et al. 2000; Pirovani et al. 2002). In fact, *SBP* repression studies in tobacco resulted in some phenotypes typically consistent with inhibition of sucrose transport-translocation between source and sink tissues (Riesmeier et al. 1994; Kühn et al. 1996), such as sugar accumulation in source leaves, photosynthesis inhibition, stunted growth, delayed development, inhibition of sucrose exudation rate from detached source leaves and significant reductions in sucrose and hexose content in sink organs (Delú-Filho et al. 2000; Pedra et al. 2000; Waclawovsky et al. 2006b). Nevertheless, the observed variations in photosynthetic metabolism, sucrose exudation and growth in the tobacco transgenic plants are transient and are restricted to the vegetative phase of development (Waclawovsky et al. 2006b). This temporal restriction of the metabolic effects resulting from *SBP* repression indicates that SBP is probably functionally associated with temporal changes of sink strength and development rather than being directly involved in sucrose transport from source leaves.

In soybean, SBP is encoded by a small gene family, which is represented by at least two non-allelic genes, *GmSBP1* (GeneBank accession number—L06038) and *GmS64* cDNAs (AF191299), also designated *GmSBP2* (Pirovani et al. 2002). These have extensive sequence similarity but are found at different loci in the soybean genome (Contim et al. 2003). A third isolated *GmSBP* cDNA is 99% identical to the previously isolated *GmS64/GmSBP2* gene and may represent an allelic form (Pirovani et al. 2002; Elmer et al. 2003). Among other legumes, *SBP* genes have been characterized in pea (Castillo et al. 2000) and *Vicia faba* (*VfSBPL*, Heim et al. 2001). However, *GmSBP* from soybean and *VfSBPL* from fava bean display distinct expression patterns. While the expression of *VfSBPL* has been demonstrated to be confined to seeds, *GmSBP1* transcripts have also been detected in young sink leaves (Grimes et al. 1992; Heim et al. 2001). Furthermore, the *VfSBPL* promoter-mediated expression of a reporter gene has been shown to be restricted to cotyledons (Heim et al. 2001). In contrast, the *GmS64/GmSBP2* promoter drives expression of linked reporter genes to the vascular tissue of roots, stems and leaves from tobacco transgenic lines (Contim et al. 2003). We have recently shown that the *GmSBP2* promoter is functionally organized into a proximal region, with the combinatorial modular configuration of plant promoters, and a distal domain, which restricts gene expression to the vascular tissues in vegetative organs (Waclawovsky et al. 2006a). Here we further characterize the distal region on the *GmSBP2* promoter and demonstrate that it harbors discrete repressing *cis*-regulatory domains that act in a context-independent manner to control the tissue-specific expression of the *GmSBP2* promoter. We also analyze the expression of a SBP-GFP translational fusion under the control of the *GmSBP2* promoter to confirm the vascular accumulation of SBP protein in vegetative organs.

Materials and methods

Construction of *SBP2* promoter–reporter gene constructs

The *–2000pSBP2-GUS*, *–136pSBP2-GUS* and *–92pSBP2-GUS* constructs, which contain a *GUS* cDNA under the control of 5′-flanking sequences of *pgsS641.1* (*GmSBP2* genomic clone) up to positions –2000, –136 and –92 relative to the translational initiation codon, respectively, have been described previously (Contim et al. 2003; Waclawovsky et al. 2006a). The CRD-A region of *pgsS641.1*, spanning nucleotide position –2000 to –700 (Waclawovsky et al. 2006a), was amplified with the appropriate primers (Table 1), and inserted into *–136pSBP2-GUS*, *–92pSBP2-GUS* and pCAMBIA1381Z (CAMBIA, Black Mountain, Australia) to generate CRD-A/*–136pSBP2-GUS* (also designated pUFV652), CRD-A/*–92pSBP2-GUS* (pUFV651) and CRD-A-*GUS* (pUFV744), respectively (Fig. 1A). Deletions of the *SBP2* CRD-A sequences were obtained by PCR-based mutagenesis using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) and *pgS641.1* as the DNA template. The deleted fragments encompassing sequences –1785 to –1508 (Frag II), –1507 to –1237 (Frag III), –1236 to –971 (Frag IV) and –970 to –700 (Frag V) from the *SBP2* promoter were amplified using the appropriate primers (Table 1), digested with *Eco*RI and *Hin*dIII and inserted in the same sites of *–92pSBP2-GUS* (Waclawovsky et al. 2006a), resulting in the clones pUFV797 (Frag II/*–92pSBP2-GUS*), pUFV798 (Frag III/*–92pSBP2-GUS*),

Table 1 Oligonucleotides used to isolate distal regions on the *GmSBP2* promoter

Oligonucleotide	Sequences (5′–3′)	Coordinates	Restriction site created
pS64–2000/–1766HdR	CCCAAGCTTTAGGACCAACTGCCAAAAATG	–1766 to –1788	*Hin*dIII
pS642000F[a]	GTCGTGCTCCACCATGTTGGG	–	–
pS64–1765/–1485HdR	CCCAAGCTTCCTAGGCATGATAACAGTTAAAC	–1745 to –1765	*Hin*dIII
pS64–1765/–1485EcF	CCGGAATTCTTAGTGCTAGAGAAGACTTG	–1507 to –1487	*Eco*RI
pS64–1484/–1212HdR	CCCAAGCTTGACATGTGAGAAGACTGATC	–1463 to –1486	*Hin*dIII
pS64–1484/–1212EcF	CCGGAATTCATGCCTAGGAGTTGAATAAC	–1236 to –1215	*Eco*RI
pS64–1211/–945HdR	CCCAAGCTTTAGGTCCGAAATATCATAAGTGG	–1188 to –1212	*Hin*dIII
pS64–1211/–945EcF	CCGGAATTCTTTTCATCCAGTTCCAACAAAC	–971 to –948	*Eco*RI
pS64–944/–705HdR	CCCAAGCTTGCATCTCAATCAGAAGACTC	–927 to –946	*Hin*dIII
pS64–944/–705EcF	CCGGAATTCGACCTAAATGCAAAATGGTTTG	–743 to –720	*Eco*RI

[a] pS642000F anneals to pCAMBIA1381Z sequences

pUFV799 (Frag IV/*–92pSBP2-GUS*) and pUFV800 (Frag V/*–92pSBP2-GUS*) (Fig. 1B).

To obtain a translational fusion of SBP2 and GFP under control of the *SBP2* promoter, the 2-kb *SBP2* promoter fragment was amplified with the appropriate primers (Table 1) and inserted into *Eco*RI/*Sma*I sites of pCAMBIA1381Z to generate pUFV674. A *GFP* (*green fluorescent protein*) cDNA was amplified from pK7FWG2 (Karimi et al. 2002) with the primers eGFPPstR (5′-GCATGCCTGCAGGTCACTGGATTTTGG-3′, coordinates 8 and 34 of pK7FWG2, *Pst*I site underlined) and eGFPSalF (5′-GTGGTGGTCGACATGGTGAGCAAGGGC-3′, positions 978 to 951 of pK7FWG2, *Sal*I site underlined), digested with *Sal*I and *Pst*I, inserted into pUC118 to give pUFV714. The *GFP* cDNA was then relieved from pUFV714 with *Sal*I and *Pst*I and inserted into the same sites of pUFV674, yielding pUFV760 (also designated *–2000pSBP-GFP*). Likewise, the *SBP2* cDNA was

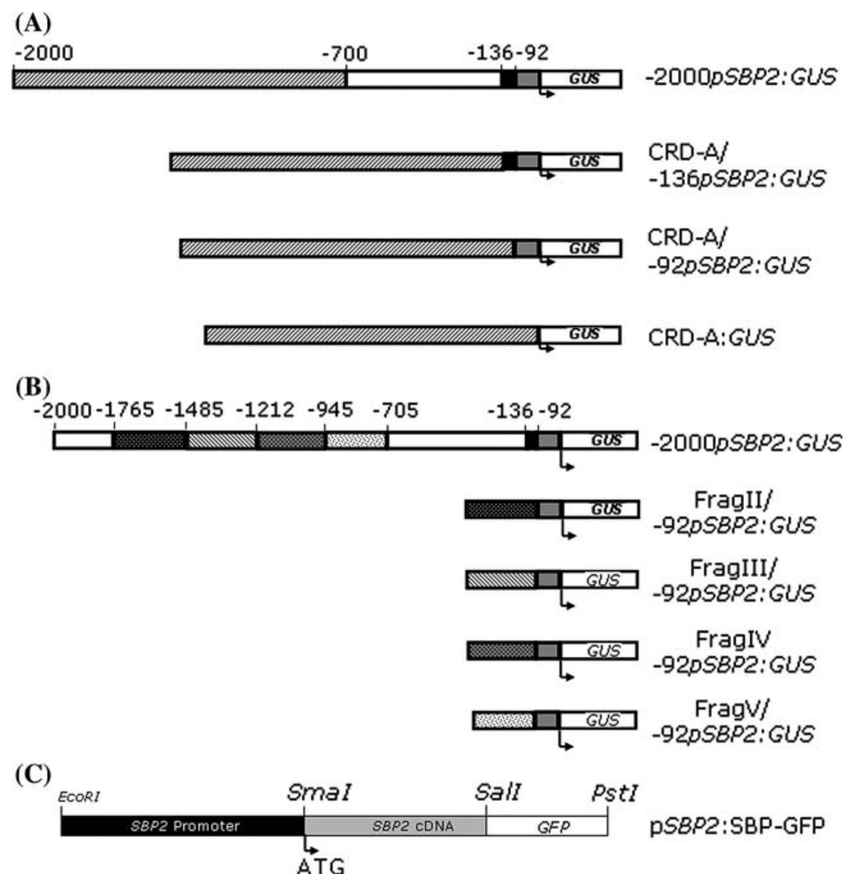

Fig. 1 DNA constructs used to transform *Nicotiana tabacum.* Numbers indicate the position relative to the translation start codon. (**A**) Schematic representation of 5′ flanking sequences of GmSBP2 (gsS641.1) fused to GUS. The CRD-A (–2000a to –700) fragment were generated by PCR-based mutagenesis and cloned into the appropriate sites of *–136pSBP2-GUS* construct, *–92pSBP2-GUS* construct and pCAMBIA1381Z. (**B**) Schematic illustration of CRD-A fragments fused to GUS. Sequential extensions of CRD-A (fragments II, III, IV, V) were isolated by PCR and individually fused to the 5′end of *–92pSBP2-GUS*. (**C**) SBP2-GFP fusion, under the control of *GmSBP2* promoter. The positions of restriction enzyme sites used for cloning are indicated

obtained by PCR from pUFV30, (Pirovani et al. 2002), with the primers S64BamF (5′-AGAGGATCCCCGGGTACCGAGCTC-3′, coordinates 93 and 116, creating a *Bam*HI site at position 96) and S64SalNSR (5′-CCTCCACACGTCGACCGCAACAGCGCG-3′, coordinates 1499 and 1473, creating a *Sal*I site at position 1484), inserted into *Sal*I and *Bam*HI sites of pUC118, generating pUFV713 and, then, transferred to the same sites of pUFV760. The resulting clone pUFV763 (also designated *–2000pSBP2:SPB2-GFP*) contains the *SBP2* cDNA fused in-frame to *GFP* cDNA, under the control of the *S-64/SBP2* promoter.

Generation and selection of transgenic plants

The pCAMBIA-derived recombinant plasmids or pCAMBIA1381Z binary vector alone were used to transform *Nicotiana tabacum* L. cv. Havana plants by *Agrobacterium tumefaciens*-mediated leaf disc transformation (Alvim et al. 2001) and transformed plants were selected and regenerated on medium containing hygromycin (Buzeli et al. 2002). Most of the rooted plants were tested for the incorporation of the hygromycin (*hptII*) and *SBP2* promoter-*GUS* fusion genes by PCR analysis and GUS activity, as previously described (Waclawovsky et al. 2006a). The transgenic lines were further selected by the transgene single-copy number criterion as judged by Southern blots. Detailed sectional analyses for tissue-specific expression were carried out on five independent *pSBP2-GUS* transgenic lines of each construct. One hygromycin-resistant plant for the pCAMBIA1381Z incorporated binary vector was used as a negative control.

Determination of GUS activity and histochemical in situ localization of *GUS* in tobacco organs

Protein extraction and fluorometric assay for GUS activity were performed as described by Buzeli et al. (2002) with methylumbelliferone (MU) as a standard. The histochemical analysis of *β*-glucuronidase activity was performed as previously described (McCabe et al. 1988). The tissues (roots, stems and leaves) were sectioned using a hand microtome. Tissue sections were embedded in the GUS assay buffer [100 mM $NaH_2PO_4 \cdot H_2O$ (pH 7.0), 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 10 mM $Na_2EDTA \cdot 2H_2O$, 0.1% (v/v) Triton X-100] containing 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide (X-Gluc) (McCabe et al. 1988) and incubated at 37°C in the dark for 4 h. Pigments were extracted from stained tissues with methanol:acetone (3:1). The micrographs were taken under an Olympus AX-70 microscope.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from soybean and tobacco leaves and stems as previously described (Polanco et al. 2002). The integrity of the nuclear extracts was monitored by SDS-PAGE. Fragment II, encompassing sequences positions –1765 to –1485 on *pgsS641.1* was amplified as described above, digested with *Hin*dIII and radiolabeled with [α-^{32}P]dCTP and one unit of *E. coli* DNA polymerase (Klenow fragment). An aliquot of nuclear extract was incubated with the radiolabeled probe in the presence of 2 μg of sonicated salmon sperm DNA and the binding buffer (12 mM Hepes, pH 7.9, 4 mM Tris-Cl, pH 7.9, 60 mM KCl; 1 mM EDTA, 1 mM DTT, 12% (v/v) glycerol) in a final volume of 15 μl for 30 min at room temperature. The reaction was resolved by electrophoresis in 5% acrylamide gel at 35 mA, for 4 h with the running buffer (6.7 mM Tris-Cl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) under circulation. The gel was dried and revealed by autoradiography at room temperature for 6 h.

Real-time RT-PCR analysis

Total RNA was extracted from frozen tissues with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and was further purified through silica columns. The quality and integrity of the RNA was monitored by spectrophotometry and agarose gel electrophoresis, respectively. We extracted RNA from leaves, roots and stems at different developmental stages. For the quantitative RT-PCR, 3 μg of total RNA were treated with DNase (Promega, Madison, WI) and fractionated through RNA purification columns (Qiagen, Valencia, CA). Reverse transcription was carried out using M-MLV reverse transcriptase (Invitrogen) and oligo-dT (18, IDT, Coralville, IA) primers (according to the protocol of the manufacturer). We used a set of primers, which was designed to anneal specifically to *SBP2* cDNA and another set of primers designed to anneal to all three isolated soybean *SBP* cDNAs (Table 2).

Real-time RT-PCR reactions were performed on an ABI7500 instrument (Applied Biosystems, Foster City, CA), using SYBR® Green PCR Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 94°C for 15 s and 60°C for 1 min. To confirm quality and primer specificity, we verified the size of amplification products after electrophoresis through a 1.5% agarose gel, and analyzed the T_m (melting temperature) of amplification products in a dissociation curve, performed by the ABI7500 instrument. Gene expression was quantified using the $2^{-\Delta C_T}$ method. The RNA helicase was used as a control

Table 2 SPP2-specific primers and SBP family-general primers

Oligonucleotide	Sequences (5′–3′)	Coordinates
SBP1322Rvs	TTG TCC TTC CCT GCA AAC GTA A	1322–1301 (family SBP)
SBP1200Fwd	GTT TGT TGT CCC TCC TGG TCA TC	1200–1222 (family SBP)
SBP2Rvs1514	CGT TAT CAG CCA CCT CCA CAC T	1514–1493 (SBP2-specific primer)
SBP2Fwd1398	ATG GTG AAC GGA GTC TTC GAA AG	1398–1420 (SBP2-specific primer)

gene to normalize all values in the real-time RT-PCR assays.

Tissue localization of *SBP2-GFP*

Confocal imaging was performed using a Zeiss inverted LSM510 META laser scanning microscope with an argon laser and a 40× oil immersion objective. For imaging GFP, the excitation line 488 nm and the 505–530 nm band pass filter were used. The pinhole was usually set to give a 1–1.5 μm optical slice. Post-acquisition image processing was done using the LSM 5 Browser software (Zeiss). We examined samples from ten independently transformed *–2000pSBP2:SBP-GFP* tobacco lines.

Results

The *GmSBP2* promoter directs accumulation of a SBP2-GFP fusion to the vascular tissues of *N. tabacum*

We have previously demonstrated that the *GmSBP2* promoter directs vascular tissue-specific expression of reporter genes in transgenic tobacco (Contim et al. 2003). These results were based on the expression of *pSBP2*-reporter gene transcriptional fusions in transgenic plants and may not reflect the accumulation of SBP2 protein in soybean tissues due to the lack of transcriptional regulatory elements and post-transcriptional control. To address these possibilities we have expressed a SBP-GFP fusion under the control of the full-length *GmSBP2* promoter in transgenic tobacco and analyzed the localization of the recombinant protein through confocal laser scanning microscopy in ten independently transformed lines. In the stem, intense GFP fluorescence was observed in the vascular tissue, both in the xylem tracheary elements and in the phloem fibers (Fig. 2A). In the shoot apex, the fusion protein accumulated in the procambium region (Pc), phloem (P) and xylem (X) (Fig. 2B). The phloem was clearly identified by the presence of the sieve plates between adjacent cells (see inset in Fig. 2B), whereas the xylem was distinguished by the annular and helical secondary wall thickenings. Collectively, these results indicate that the SBP2 protein is in fact located in the vascular system of vegetative organs which is consistent with the pattern of *SBP2* promoter activity in transgenic lines as well as with the SBP2 involvement in sucrose translocation-dependent physiological processes.

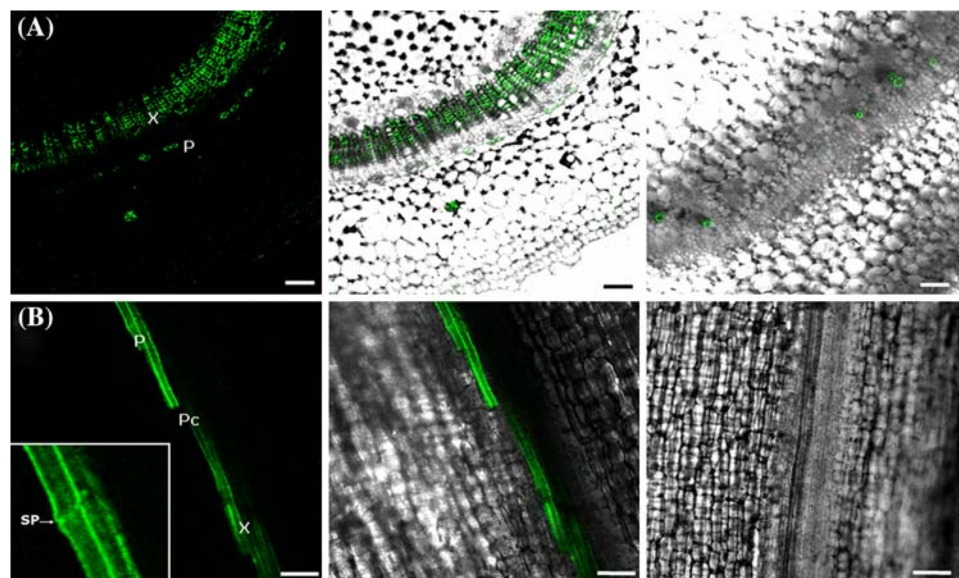

Fig. 2 Confocal images of *SBP2-GFP* stably expressed in shoot (**A**) and shoot apex (**B**) of tobacco plants. First and second columns show plants expressing *SBP2-GFP* and the last column shows wild type plants. Left images show the signal from the green channel. Right images display mixed green and transmission channels. X: xylem; P: phloem; Pc: procambium; SP: sieve plate. Size bars = 100 μm

The *SBP2* promoter distal region (position –2000 to –700) is essential for vascular tissue-specific expression of reporter genes

The vascular tissue-specific activity of the *SBP2* promoter in vegetative organs is confined to a distal region (–2000 to –700), designated CRD-A (*cis*-regulatory domain-A), which contains repressing sequences that prevent gene expression in tissues other than vascular tissues (Waclawovsky et al. 2006a). Having established that the tissue-specificity of the promoter-*GUS* transcriptional fusions reflected the accumulation of the protein, we confirmed the negative tissue-specific nature of CRD-A through gain-of-function experiments. The 1.3 kb CRD-A sequences were fused to the *–136pSBP2-GUS* construct, which has been shown to induce constitutive *GUS* expression in leaves (Fig. 1, CRD-A/*–136pSBP2-GUS*) and to the *SBP2* minimal promoter*–92pSBP2-GUS* (CRD-A/*–92pSBP2-GUS*), which has been shown to direct high levels of constitutive *GUS* expression in all vegetative organs (Waclawovsky et al. 2006a). These constructs were introduced into a tobacco plants and the GUS activity was determined in at least three independent transformants for each construct. CRD-A causes a drastic reduction in both *–136pSBP2*- and *–92pSBP2*-mediated *GUS* expression (Fig. 3). This quantitative measurements of GUS activity confirmed that the distal region on the *GmSBP2* promoter, CRD-A (–2000 to –700), possesses *cis*-regulatory modules capable of repressing gene expression.

For tissue-specific GUS activity, we analyzed more than five independent primary transgenic plants expressing the *SBP2* promoter-*GUS* fusion genes. We also included independent transgenic lines of the previously described *–2000pSBP2-GUS*, *–136pSBP2-GUS* and *–92pSBP2-GUS* constructs in the histochemical assays, which demonstrated the same expression pattern as described before (Contim et al. 2003; Waclawovsky et al. 2006a; Fig. 4). The fusion of the CRD-A sequences to the 5′ end of *–136pSBP2-GUS* caused a reduction in GUS activity in all organs analyzed. This reduction was clearly evident in the leaf surface, in which the GUS activity was restricted to the vascular tissue (Fig. 4, a4). In the stem, the *GUS* expression was also more pronounced in the vascular bundle, although the parenchymatic adjacent cells displayed a weak GUS staining (Fig. 4, compare b4 and b3). Likewise, in the shoot apex and roots, CRD-A reduced *–136pSBP2*-mediated *GUS* expression in all tissues except in vascular tissues (Fig. 4, c4 and d4, respectively).

The fusion of CRD-A to the 5′ end of *–92pSBP2-GUS* also reduced drastically GUS activity in all tissues analyzed (Fig. 4, a6, b6, c6, d6) and restored the tissue-specific expression pattern of the full promoter (Fig. 4, a2, b2, c2, d2), except in the shoot apex, in which the GUS staining was reduced but was not confined to the vascular tissue (Fig. 4, c6). In the leaf surface, a robust and restricted GUS staining was observed in the vascular bundle (Fig. 4, a6), while in the stem, *GUS* expression was restricted to the inner phloem (Fig. 4, b6). In roots, CRD-A abolished *–92pSBP2*-mediated *GUS* expression in the meristem (Fig. 4, compare d5 and d6) and confined the GUS staining to the vascular region (Fig. 4, d6). Taken together, these results confirmed the negative regulatory nature of CRD-A which promoted drastic reduction in the *SBP2* promoter activity in the majority of the tissues analyzed.

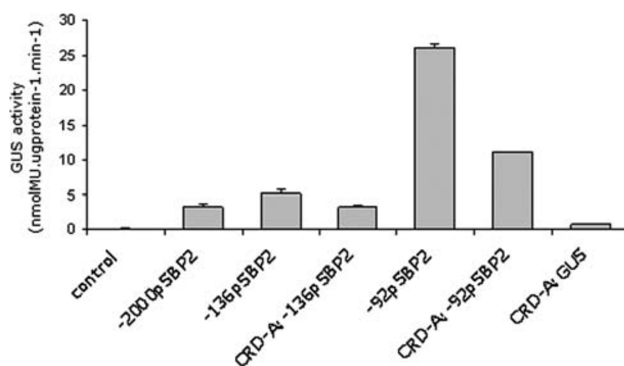

Fig. 3 Functional analysis of the *SBP2* promoter in mature leaves of transgenic tobacco plants expressing *SBP2-GUS* fusion genes. Specific GUS activity was determined by fluorometric assays with total extracts from leaves and expressed as nmol of 4-methylumbelliferone μg protein^{-1} min^{-1}. The bars represent average (±S.E.) of three independent measurements using extracts from independent transgenic lines. Control represents the promoter-less binary vector (pCAMBIA1381Z) transformed plants

The distal region CRD-A of the *GmSBP2* promoter functions independently on the proximal TATA-containing region

The CRD-A fragment was also inserted in the pCAMBIA1381Z binary vector in the absence of a minimal promoter (Fig. 1). This DNA construct was initially designed to serve as a negative control for *GUS* expression, but rather it directed *GUS* expression in a tissue-specific manner similar to the full-length promoter-mediated expression pattern. These analyses were performed in six independently transformed lines. Apart from the shoot apex, in which CRD-A failed to direct minimal promoter independent *GUS* expression (Fig. 4, c7), CRD-A functioned independently on the proximal TATA-containing region in all other tissues analyzed. Both in leaves (Fig. 4, a7) and roots (Fig. 4, d7), *GUS* expression was restricted to the vascular tissue, whereas in stem, GUS staining was detected only in the inner phloem (Fig. 4, b7). These results indicate that CRD-A harbors *cis*-regulatory

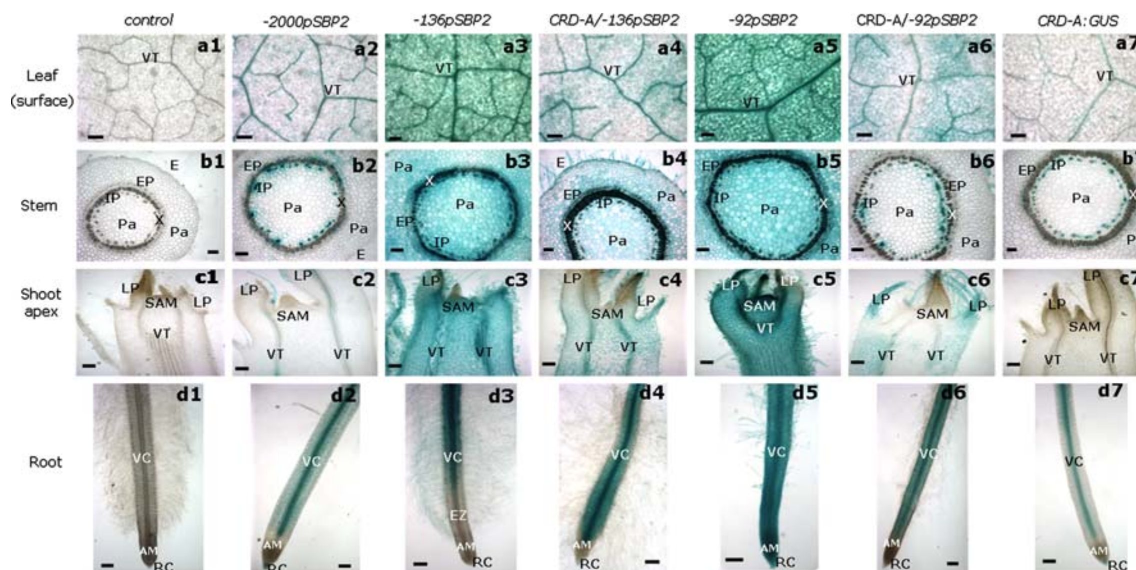

Fig. 4 Tissue-specific regulation of *SBP2-GUS* fusion gene expression in transgenic plants. Photographs of transgenic organs plants (as indicated) harboring the indicated *SBP2-GUS* fusion gene stained for GUS activity. Control corresponds to promoter-less pCAMBIA-transformed plants. Abbreviations: VT, Vascular Tissue; EP, Outer Phloem; IP, Inner Phloem; PA, Parenchyma; X, Xylem; LP, Leaf Primordia; SAM, Shoot Apical Meristem; E, Epidermis; VC, Vascular Cylinder; EZ, Elongation Zone; AM, Apical Meristem; RC, Root Cap. Bars in a1–a7 = 100 μm and in b1–b7, c1–c7, d1–d7 = 200 μm

elements capable of promoting basal transcription and is sufficient to drive *GUS* expression to the vascular tissue in a context-independent manner (Fig. 4). Furthermore, they indicate that sequences downstream of –92 are important for expression in the shoot apex, as their absence abolished detectable expression in the region (Fig. 4, c7).

The tissue-specific expression pattern is controlled by distinct *cis*-regulatory modules on the distal region of the *SBP2* promoter

Potential tissue-specific controlling elements were found on the distal region of the *GmSBP2* promoter (Fig. 5). These include the GLUB1 sequence AACAAAC, which has been associated with the negative tissue-specific control of the rice glutelin gene (Yoshihara et al. 1996), both in the sense (positions –1197 to –1203) and reverse orientation (positions –1053 to –1059); three NtBBF1 binding sites ACTTTA (positions –1474 to –1479, –1586a –1591, –1713 to –1718) which have been implicated in tissue-specific expression of the *rolB* promoter in tobacco (Baumann et al. 1999); the GATA box (positions –855, –987 and –1142) that has been shown to be involved in tissue-specific expression and in light regulation of plant promoters (Gidoni et al. 1989; Lam and Chua 1989). Furthermore, several potential DOF binding sites (AAG core sequence) were located on CRD-A (Fig. 5A, red).

To characterize functional regulatory *cis*-acting elements on the *SBP2* promoter, we performed gain-of-function experiments for distinct modules of the distal region. These modules, designated Frag II (–1785/–1508), Frag III (–1507/–1237), Frag IV (–1236/–971) and Frag V (–971/–700), were directly fused to the *–92pSBP2-GUS* construct (Fig. 1B). All modules were capable of reducing the *SBP2* promoter activity as their individual insertion at the 5′ end of –92*pSBP2* altered its constitutive expression pattern (Fig. 6). However, the intensity of reduction varied to different extents for the four modules and according to the organ analyzed. While Frag V was the least effective in reducing *–92pSBP2*-mediated *GUS* expression, Frag IV restored the vascular tissue-specific expression of the full-promoter in leaves (Fig. 6, a6). Likewise, in stem, Frag III sequences caused the most drastic reduction in *–92pSBP2*-mediated *GUS* expression, which was restricted to the inner phloem mimicking the full-promoter-mediated *GUS* expression (Fig. 6, b5). Although the other modules, Frag II, IV and V, retained a prominent GUS staining in phloem, they reduced but did not abolish *–92pSBP2* activity in the parenchymatic cells of stems (Fig. 6, b4, b6, b7). In the shoot apex, Frag II, III and IV abolished *–92pSBP2*-mediated *GUS* expression (Fig. 6, c4, c5, c6), whereas Frag V promoted a general reduction in expression with a predominant GUS staining in the vascular bundle (Fig. 6, c7). Finally, in roots, while all fragments were able to repress *–92pSBP2*-mediated *GUS* expression in the meristem (Fig. 6, d4, d5, d6, d7), Frag II and IV also abolished expression in the elongation zone (Fig. 6, d4 and d6). Furthermore, Frag II recovered the tissue-specific expression pattern of the full-length promoter, as Frag II/

Fig. 5 (**A**) Putative *cis*-regulatory elements on CRD-A region of *SBP2* promoter. Numbers indicate the position relative to the translational start codon and the arrows delimit the sequential extension of the CRD-A fragments as Frag II (bold), Frag III (underlined) Frag IV (italic) and Frag V (regular letter). Direct and inverted CCAAT boxes are shown in green and potential TATA boxes in orange. Direct and inverted GLUB sequences are boxed. Putative NtBBF1 binding sites are shown in blue; potential DOF binding sites, in red and GATA boxes, in purple. (**B**) Alignment of CRD-G with Frag II and Frag IV of CRD-A. A highly conserved sequence is shown in red

–*92pSBP2*-mediated *GUS* expression was confined to the root vascular tissue (Fig. 6, d4).

Negative *cis*-regulatory elements that prevent expression in the root elongation zone have been previously identified in the proximal promoter region, positions –136 to –92, designated *cis*-regulatory domain G, CRD-G (Waclawovsky et al. 2006a). To search for potential pro-cambium expression-repressing determinants, the CDR-G sequence was compared with Frag II and Frag IV sequences (Fig. 5B). Sequences matching the 44 bp-CRD-B were detected in the Frag II and Frag IV (about 50% sequence identity). This conserved DNA segment harbors

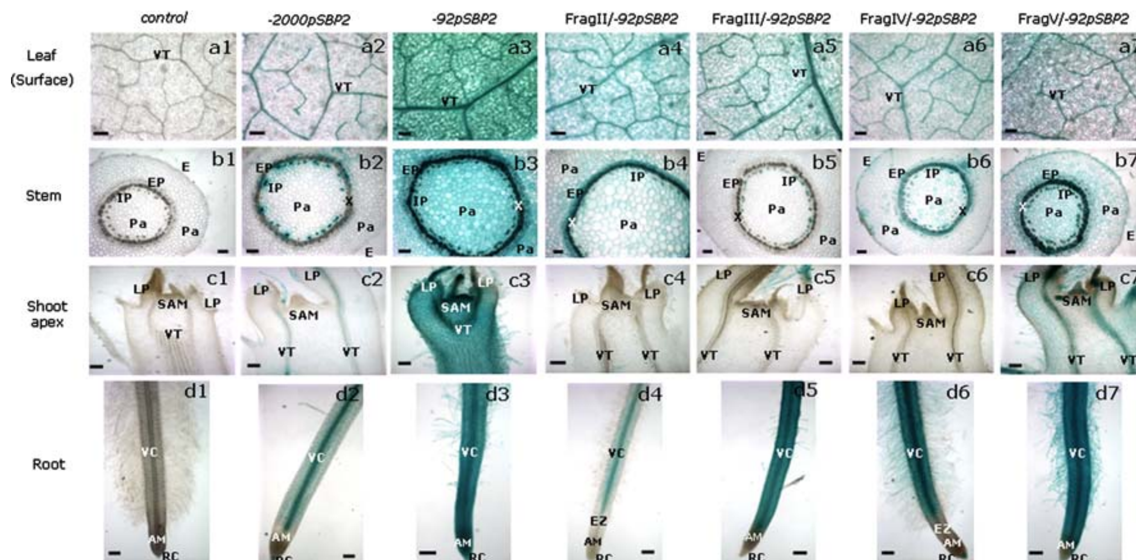

Fig. 6 Histochemical analysis of tissue-specific regulation of *SBP2-GUS* fusion gene expression in transgenic plants. Photographs of transgenic organs plants (as indicated) harboring the indicated *SBP2-GUS* fusion gene stained for GUS activity. Control corresponds to promoter-less pCAMBIA-transformed plants. Abbreviations: VT, Vascular Tissue; EP, Outer Phloem; IP, Inner Phloem; PA, Parenchyma; X, Xylem; LP, Leaf Primordia; SAM, Shoot Apical Meristem; E, Epidermis; VC, Vascular Cylinder; EZ, Elongation Zone; AM, Apical Meristem; RC, Root Cap. Bars in a1–a7 = 100 µm and in b1–b7, c1–c7, d1–d7 = 200 µm

at the 3′ end a nearly identical nucleotide sequence that may function as a root-specific repressing element.

Soybean nuclear activities interact with distal *cis*-regulatory sequences of *GmSBP2*

Because the promoter analyses were performed in the tobacco heterologous system, it was of interest to evaluate whether the *GmSBP2* promoter-driven expression pattern could reflect endogenous expression in soybean. To address this possibility, the accumulation of the *SBP2* transcripts was assayed by qRT-PCR (see below) in the same soybean organs as those in which the *SBP2* promoter activity was detected in tobacco. In addition, soybean nuclear activities were assayed for interactions with the distal region of the *SBP2* promoter by EMSA. Frag II was radiolabeled and incubated in the presence of leaf and stem nuclear extracts from soybean and tobacco and used for gel shift assays. As expected from the *GmSBP2* promoter analysis, tobacco nuclear extracts resulted in complex formation with Frag II (Fig. 7, lanes 4 and 5). Likewise, Frag II formed complexes with proteins of nuclear extracts prepared from soybean leaves (lane 2) and stems (lane 3), although with a different migration pattern from that found for tobacco extracts. The interactions were specific as the introduction of a ten-fold excess of unlabeled probe competed for binding (data not shown). Although these results clearly demonstrated that DNA binding activities from soybean nuclear extracts specifically recognized the *GmSBP2* promoter region, the significance and identity of the resulting complexes remain to be determined.

GmSBP2 transcripts accumulate in soybean vegetative organs

The accumulation of SBP2 in vegetative organs was analyzed by qRT-PCR with soybean *SBPs*-general primers (Fig. 8, SBP) and *GmSBP2*-specific primers (Fig. 8, SBP2). Consistent with the expression pattern driven by the *GmSBP2* promoter in tobacco, *GmSBP2* transcripts were detected in all soybean vegetative organs analyzed. In general, *GmSBP2* expression in mature plants was higher than in young plants. This result contrasts with the observation that SBP functions predominantly in the initial stages of plant development when the sink/source ratio is high (Waclawovsky et al. 2006b). Nevertheless, *GmSBP2* expression is higher in sink organs, such as young leaves and roots, than in source mature leaves (Fig. 8), which is consistent with the involvement of SBP in determining sink strength.

Discussion

In addition to its high activity in seeds, the *GmSBP2* promoter has been shown to direct phloem-specific expression of a linked reporter gene in vegetative organs of transgenic tobacco (Contim et al. 2003; Waclawovsky et al. 2006a). While the vascular tissue-specific *SBP2* promoter activity is consistent with the proposed involvement of GmSBP in sucrose transport-dependent physiological processes (Pedra et al. 2000; Waclawovsky et al. 2006b), the accumulation of the protein in vascular tissues of soybean vegetative organs is still a matter of debate. Although GmSBP had previously been immunolocalized in association with the plasma membrane of the sieve element–companion cell complexes of mature phloem (Grimes et al. 1992), these observations could not be reproduced using antibodies prepared against an *E. coli*-expressed truncated GmSBP1 (Elmer et al. 2003). Based on the specificity of the truncated SBP-antibody, the GmSBP family was found to accumulate exclusively in cotyledons and to be resolved in four p*I*-distinct cross-reacting polypeptides by immunoblotting assays of two-dimensional (2D) gels. In contrast, we demonstrated here that a GmSBP-GFP fusion protein

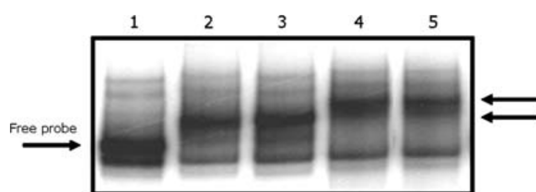

Fig. 7 Electrophoretic mobility shift assay. The radiolabeled –1785/–1508 fragment of the *SBP2* promoter was incubated with nuclear extracts from soybean leaves (lane 2) and stem (lane 3) as well as from tobacco leaves (lane 4) and stems (lane 5) and the complexes formed were resolved by non-denaturing polyacrylamide gel electrophoresis. The arrow on the left indicates the position of free probe, whereas the arrows on the right the positions of the retarded complexes

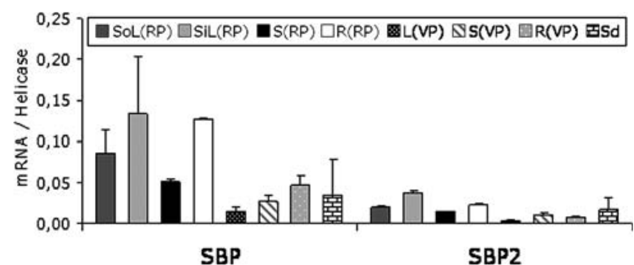

Fig. 8 *SBP* transcripts accumulate in soybean vegetative organs. *SBP2* expression was verified in soybean plants during the reproductive phase (RP) and vegetative phase (VP). Total RNA was extracted from source leaves (SoL), sink leaves (SiL), stem (S), roots (R), leaves (L) and seedlings (Sd) and quantified by qRT-PCR using SBP2-specific primers (SPB2) and SBP family-general primers (SBP)

accumulates in the vascular tissue of tobacco vegetative organs when the expression of the recombinant gene is driven by the full-length *GmSBP2* promoter. We have also detected by qRT-PCR the accumulation of *GmSBP* transcripts in soybean vegetative organs (Fig. 8). Consistent with the proposed role for SBP in sink strength (Waclawovsky et al. 2006b), *GmSBP2* transcripts were found to be predominantly expressed in sink organs, such as young leaves and roots. Taken together, these results confirm that the *GmSBP2* gene is expressed in soybean vegetative organs and the protein accumulates in the vascular tissue.

We have also determined by gain-of-function experiments that the distal region (CDR-A fragment) of the *SBP2* promoter harbors repressing elements that control the tissue-specific activity of the promoter. Fusion of CRD-A to *–136pSBP2-GUS* or *–92pSBP2-GUS* constructs diminished *GUS* expression and altered their constitutive pattern of expression. While the CRD-A/*–92pSBP2-GUS* restored the spatial pattern of the full-length promoter-mediated expression, fusion of CRD-A to *–136pSBP2-GUS* failed to confine the expression of the reporter gene to the inner phloem of stems and to the vascular tissue of roots (Fig. 4). This indicates that the region between –136 and –92 may harbor a positive element capable of attenuating but not abolishing the negative and tissue-specific effect of CDR-A. In fact, the region delimited by positions –136 to –92 contains a putative CCAAT box which has been associated with transcriptional activation and high levels of transcriptional activity of plant promoters (Kusnetsov et al. 1999; Buzeli et al. 2002).

Further characterization of the CRD-A fragment illustrates the silencer nature of distal elements on *SBP2* promoter and suggests that the tissue-specific control of *SBP2* gene expression requires a complex integration of multiple *cis*-acting regulatory elements, which are responsible for different levels of expression according to the organ analyzed. A direct comparison of the CRD-A/*–92pSBP2*-mediated *GUS* expression and the repressing effects of discrete modules from CRD-A allowed us to delimit the individual contribution of each *cis*-regulatory module to the general pattern of tissue-specific expression of *SBP2* promoter in vegetative organs. Frag V (–970/–700) contains the *cis*-elements responsible for the expression pattern of *GmSBP2* promoter in shoot apex, as when directly fused to the 5′ end of *–92pSBP2-GUS* it restored the expression pattern of the full-length promoter in shoot apex but not in the other organs analyzed. Likewise, in leaves, the *GmSBP2* promoter-mediated vascular tissue-specific expression is contribution of Frag IV (–1236/–971), whereas, in roots, it is directed by *cis*-regulatory elements presents in Frag II (–1782/–1508). In stems, the inner phloem-specific expression of the full-length promoter is mediated by Frag III delimited by positions –1507 and –1237. These results further substantiate the notion that tissue-specificity may be mediated by distal regions of plant promoters. Although in the majority of plant promoters the determinants for tissue-specificity are often located on proximal upstream sequences (Stougaard et al. 1987; Zhao et al. 1994; Hamilton et al. 1998; Ruiz-Rívero and Prat 1998), distal tissue-specific elements have previously been mapped in the distal 5′ region of the soybean *Msg* promoter and *Flaveria trinervia C4pppcA1* promoter (Stromvik et al. 1999; Gowik et al. 2004). Nevertheless, the negative nature of the *SBP2* distal repressing modules differs from other plant promoters as the determinants for tissue-specificity in distal upstream regions often act in a positive regulatory manner.

The gain-of-function experiments for individual modules of CRD-A also mapped several negative *cis*-regulatory domains on *SBP2* promoter distal regions. The sequences –1785 to –970 may contain shoot apex-specific expression-repressing elements, as all the three modules (Frag II, III and IV) were capable of abolishing GUS expression in this region. Likewise, strong silencers for root meristem expression are contained in the region –1785 to –700 because all modules repressed *–92pSBP2*-mediated root meristem expression. Frag II (–1785/–1508) tightly restricted the promoter activity to the vascular tissue of roots and may therefore harbor strong root expression silencers. Strong stem expression-repressing elements were located at positions –1507 to –1237, as Frag III confined *–92pSBP2*-mediated *GUS* expression to the inner phloem. Finally, the presence of any of the two modules (–1785/–1508 and –1236/–971) corresponding to Frag II and IV prevented *GUS* expression in the root elongation zone. Negative *cis*-regulatory elements that prevent expression in the root elongation zone have previously been identified in the proximal promoter region, positions –136 to –92, designated CRD-G (Waclawovsky et al. 2006a). A direct comparison among Frag II, Frag IV and CRD-G sequences identified the conserved consensus sequence CAGTTnCaAccACATTcCT present in all three *cis*-regulatory domains. This conserved sequence may represent a procabium expression-repressing element and hence a rational target for further functional promoter analysis in roots.

Among the four modules, Frag V (–970/–700) exhibited the most attenuated effects causing a slight decrease in *GUS* expression in the organs analyzed, except for the root meristem in which Frag V/*–92pSBP2*-mediated *GUS* expression was undetectable. The Frag V module harbors three TATA box-like sequences (positions –790, –783 and –761), in addition to an AT-rich region. We found that the CRD-A lacking the proximal 92-bp-minimal promoter mediates the similar tissue-specific expression pattern as the full-length promoter- and the CRD-A/*–92pSBP2*-mediated expression, although with weaker levels. The

presence of putative TATA box-like sequences, correctly positioned in Frag V when directly fused to an ORF, may explain the capacity of the CRD-A to sustain tissue-specific transcription in the absence of a minimal promoter. Furthermore, Frag V contains AT-rich regions which have been identified in numerous promoters as enhancers of transcription (Bustos et al. 1989; Rieping and Schoffl 1992). Fortuitous AT-rich regions on the distal region of promoters that can take over the function of TATA boxes have already been reported from other soybean genes (Stomvik et al. 1999). The capacity of the CRD-A to sustain basal transcription in the absence of an external minimal promoter, clearly demonstrated that the vascular tissue-specific determinants of the *GmSBP2* promoter are indeed contained in this region and function in a context-independent manner.

In summary, a detailed examination of the expression patterns of the *SBP2:GUS* constructs revealed that the vascular tissue-specific expression of the full promoter is conferred by the distal region, CRD-A, which is organized into repressing modules that act independently of one another to confer the vascular tissue-specific expression in distinct organs. The promoter activity reflected the accumulation of a SBP-GFP fusion protein into the vascular system of vegetative organs, which is consistent with the proposed involvement of GmSBP in sucrose transport-dependent physiological process. Our results also led to the identification of relevant *cis*-regulatory elements on *SBP2* promoter that suppress expression in tissues other than vascular tissues of vegetative organs.

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