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RESEARCH PAPER

The soybean sucrose binding protein gene family: genomic organization, gene copy number and tissue-specific expression of the *SBP2* promoter

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Abstract

The sucrose binding protein (SBP) from soybean has been implicated as an important component of the sucrose uptake system. Two *SBP* genomic clones, *gsS641.1* and *gsS641.2*, which correspond to allelic forms of the *GmSBP2/S64* gene, have been isolated and characterized. As a member of the seed storage protein superfamily, it has been shown that the *SBP* gene structure is similar to vicilin genes with intron/exon boundaries at conserved positions. Fluorescence *in situ* hybridization (FISH) suggested that the soybean *SBP* gene family is represented by at least two non-allelic genes corresponding to the previously isolated *GmSBP1* and *GmSBP2/S64* cDNAs. These two cDNAs share extensive sequence similarity but are located at different loci in the soybean genome. To investigate transcriptional activation of the *GmSBP2* gene, 2 kb 5′-flanking sequences of *gsS641.1* and *gsS641.2* were fused to the β-glucuronidase (*GUS*) reporter gene and to the green fluorescent protein (*GFP*) reporter gene and independently introduced into *Nicotiana tabacum* by *Agrobacterium tumefaciens*-mediated transformation. The *SBP2* promoter directed expression of both *GUS* and *GFP* reporter genes with high specificity to the phloem of leaves, stems and roots. Thus, the overall pattern of *SBP–GUS* or *SBP–GFP* expression is consistent with the involvement of SBP in sucrose translocation-dependent physiological processes.

Key words: FISH, gene family, *Glycine max*, phloem-specific expression, promoter activity, sucrose binding protein.

Introduction

A central characteristic of plants is the capacity to convert light energy through photosynthesis into carbohydrate. These molecules can be stored, directly consumed as an energy source or utilized as structural components of the cells. Because of that plants are considered as basic producers of the energy flux of the planet trophy chain. Although carbon autotrophy is a typical feature of plant cells, whole plants behave as physiological mosaics in which photosynthetically active tissues, such as mesophyll cells from mature leaves (source tissues), export carbohydrate to photosynthetically less active or inactive tissues, such as stems, flowers and roots (sink tissues) (Frömmer and Sonnewald, 1995; Stitt, 1996). The processes that regulate carbon allocation to the various sink organs directly impact plant development. Sucrose is the main carbohydrate that is transported cell-to-cell and used for long-distance transport in the vascular system of a large number of higher plants. Sucrose not only functions as a transport metabolite, but also contributes to the osmotic driving force for phloem translocation (mass flow) and serves as a signal to activate or repress specific genes in a variety of different tissues (Lalonde *et al*., 1999). The cell-to-cell sucrose transport occurs either directly via plasmodesmata (symplastic transport) or across plasma membranes mediated by protein carriers (apoplastic transport)

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(Lemoine, 2000; Williams *et al*., 2000). Both symplastic and apoplastic transports can contribute for phloem loading and unloading processes, although in some plants one route of sucrose loading may predominate over the other (Frömmer and Sonnewald, 1995). In fact, in several species, sucrose transporters have been identified as essential for phloem loading into sieve elements, the phloem-specific photoassimilate-transporting cells (Riesmeier *et al*., 1994; Bürkle *et al*., 1998).

Complementation assays using an invertase-deficient yeast mutant have been used to identify and isolate the sucrose transporter *SoSUT1* cDNA from spinach leaves (Riesmeier *et al*., 1992) and *StSUT1* from potato leaves (Riesmeier *et al*., 1993). The subsequent identification of sucrose transporters in other species has been accomplished by hybridization screening using heterologous probes or PCR-amplification from these initial sequences (Lemoine, 2000). The members of the *SUT* family encode highly hydrophobic disaccharide transporters with two sets of six membrane-spanning domain structures, separated by a large cytoplasmic loop (Williams *et al*., 2000). Many of these transporters have been shown to mediate sucrose transport through a proton-coupled transport mechanism. The SUT1 protein has been described as the proton-motive-force-driven sucrose symporter that mediates phloem loading and long-distance transport, the key transport step in assimilate partitioning for many plants (Riesmeier *et al*., 1994; Bürkle *et al*., 1998). SUT1 serves as a high-affinity transporter, whereas SUT4, a second member of this sucrose transporter family, corresponds to the low-affinity/high capacity saturable component of sucrose uptake found in leaves (Weise *et al*., 2000). A third structurally related-member of the family has been identified and designated SUT2 (Barker *et al*., 2000). Although the whole family of sucrose transporter genes of a given species has not been identified, the sucrose transporters make a large gene family, as at least seven distinct sequences that encode putative sucrose transporters are present in the *Arabidopsis* database (Williams *et al*., 2000).

A sucrose binding protein (SBP), structurally unrelated to the members of the SUT family, was first identified in soybean cotyledons and has been demonstrated to be involved in sucrose translocation-dependent physiological processes in plants (Ripp *et al*., 1988). *SBP* repression studies in tobacco have indeed shown some of the typical phenotypes caused by impairment of sucrose translocation (Riesmeier *et al*., 1994; Kühn *et al*., 1996), such as the accumulation of carbohydrates within source leaves, the inhibition of photosynthesis and stunted growth (Pedra *et al*., 2000). Furthermore, manipulation of SBP levels in transgenic cell lines correlated with the efficiency of radiolabelled sucrose uptake by the cells and altered sucrose-cleaving activities in a metabolic compensatory manner (Delú-Filho *et al*., 2000). Direct evidence implicating SBP in sucrose transport has been obtained with complementation studies using a secreted invertase-deficient mutant yeast strain, incapable of growth on medium containing sucrose as the only carbon source (Overvoorde *et al*., 1996, 1997; Pirovani *et al*., 2002). The SBP-mediated specific sucrose uptake in yeast displays linear, non-saturable kinetics up to 30 mM external sucrose, being relatively insensitive to the pH gradient across the membrane (Grimes and Overvoorde, 1996; Overvoorde *et al*., 1996). These biochemical features closely resemble the kinetic properties of the previously characterized linear component of sucrose uptake in higher plants (Maynard and Lucas, 1982; Lin *et al*., 1984). Nevertheless, these relevant data do not allow the apparent inconsistency between the absence of typical membrane transporter structural motifs on SBP and an SBP-mediated sucrose transport mechanism to be reconciled and, as a consequence, a scenario for SBP function remains elusive.

Homologous *SBP* genes have been isolated from pea (GeneBank™ accession number Y11207) and *Vicia faba* (GeneBank™ accession number VFA292221). In spinach, an SBP homologue was immunolocalized in the plasma membrane of sieve elements in fully expanded leaves, shoots and roots (Warmbrodt *et al*., 1989, 1991) and, in tobacco, it was detected in the microsomal fraction of young leaves from *Nicotiana tabacum* (Pedra *et al*., 2000). In soybean, two *SBP* genes have been identified (Grimes *et al*., 1992; Pirovani *et al*., 2002), but an extensive analysis has not been performed to provide a global view of the *SBP* gene content. In this investigation, the isolation of *SBP2* genomic clones is reported and *in situ* hybridization was performed on isolated nuclei to characterize the soybean *SBP* gene family. Data are also presented on the tissue-specific expression of the *SBP2* promoter.

Materials and methods

Screening of soybean genomic libraries and DNA sequence analysis

A size-selected genomic library propagated in λZAPII (9.75×10^9 pfu) was screened by plaque hybridization, as described by Sambrook *et al*. (1989), using the complete *SBP2/S64* cDNA (GeneBank™ accession number AF191299) as probe. The hybridization probe was radiolabelled with [α-^{32}P]dCTP by random primed labelling (Amersham Pharmacia Biotech). Two positive clones, *gsS641.1* and *gsS641.2*, were plaque purified, excised and sequenced. Sequencing was carried out with a combination of insert subcloning and primer walking. The identity of these clones was obtained by sequence comparison analysis using the BLAST program (Altschul *et al*., 1990). The computer program ClustalW was used for sequence alignment.

Genomic DNA gel blot analysis

DNA was extracted from young leaves, digested overnight with *Bam*HI or *Eco*RI, precipitated with ethanol 70% (v/v) and separated on a 1% (w/v) agarose gel. The gel was washed with 250 mM HCl followed by alkaline denaturation (Sambrook *et al*., 1989). After neutralization, the DNA was transferred to nylon membranes and UV fixed (Stratalinker, Stratagene). The *SBP2* cDNA was labelled

using the Primer-It Fluor Fluorescence Labelling Kit (Stratagene) according to the instructions of the supplier. Hybridization and washing of the blots were performed using standard procedures (Sambrook *et al*., 1989). The hybridization signals were revealed by the Iluminator™ Nonradioactive Detection System (Stratagene) according to the instructions of the supplier.

Probe preparation for in situ *hybridization*

Two DNA fragments were used as probe for *in situ* hybridization. The first one corresponds to the 4 kb *Eco*RI insert from pgsS641.2. The second one corresponds to a 1.5 kb DNA fragment extending from position 313 to 1695 of the *GmSBP1* cDNA (GeneBank™ accession number L06038). This DNA fragment corresponds to the insert of the soy25Z12 cDNA clone, previously isolated in the laboratory through the screening of a soybean seed cDNA library using the *SBP2/S64* cDNA (GeneBank™ accession number AF191299) as probe. Both DNA fragments, used as FISH probes, were released from the vector by *Eco*RI digestion, gel-purified and labelled using the Primer-It Fluor Fluorescence Labelling Kit (Stratagene) according to the recommendations of the supplier.

Cytological preparations and in situ *hybridization*

Soybean seeds were germinated in Petri dishes containing a film of distilled water, and incubated at 29 °C in the dark. Seedlings with 0.5–1 cm long roots were fixed in a fresh ice-cold methanol:acetic acid solution (3:1, v:v) and kept at –20 °C for 24 h. Then, the roots were excised at 0.1 cm from the root tip and macerated with a freshly prepared Flaxzyme (NOVO) enzymatic solution (1/10), and incubated at 35 °C for 90 min. The macerated cells were dissociated in a clean slide with a fresh fixative solution, air-dried and stained with a 2% Giemsa solution in phosphate buffer, pH 6.8, for 5 min (Carvalho and Saraiva, 1997).

The slides containing soybean interphase nuclei were treated with 100 μg ml^{-1} RNase-A in 2× SSC (150 mM NaCl, 15 mM Na_3 citrate, pH 7.0) at 37 °C for 1 h, washed with the same buffer, then with PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0), dehydrated sequentially in 70%, 80% and 100% ethanol (2 min each) at room temperature and finally air-dried. The probe (2 ng μl^{-1}) and salmon sperm DNA (200 ng μl^{-1}) were denatured in the hybridization solution [50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 50 mM sodium phosphate, pH 7.0, and 2× SSC] for 75 °C for 15 min and, then, further co-denatured with the nuclei for 4 min at 75 °C. The denatured nuclei were hybridized for 12 h at 37 °C in HYBAID OmiSlide termal cycler. After hybridization, the slides were washed twice in 2× SSC containing 50% (v/v) formamide for 2 min each at 45 °C; four times for 2 min in 2× SSC at room temperature and then rinsed in PBS for 2 min. The slides were air-dried and mounted in 15 μl of detection buffer (12.5 mg ml^{-1} triethylenediamine in glycerol, 1/2× PBS and 200 ng ml^{-1} propidium iodide).

Microscopy and analysis of the hybridization signal

The images of isolated nuclei were captured with a CCD video camera and digitalized by an image analysis system attached to the Olympus BX 60 reflected-light fluorescence microscope with objective 100× and WU excitation (cube U-MWU) fluorescence filter, BA 420 barrier filter and DM400 dichroic mirror. Image analyses were conducted using the public domain Image SXM 1.68 software (Rasband, 1997). The original colour TIFF images were converted to a grey scale up to a 255 grey value for the relative density plot analysis. Using the colour table tool of the software, the final plot images were digitally pseudo-coloured with the filter colour spectrum.

Construction of SBP2 *promoter–reporter gene constructs*

A *SBP2* promoter–*GUS* fusion gene was constructing by cloning a 2.0 kb *Eco*RI/*Nco*I fragment from pgsS641.1 into the *Eco*RI/*Nco*I sites of pCAMBIA 1381Z (Roberts *et al*., 1996) to give pUFV335, also referred to as –2000p*SPB2–GUS*. To construct an *SBP2* promoter–*GFP* fusion gene, a Klenow-repaired *Kpn*I/*Spe*I GFP cDNA fragment was transferred from pCAMBIA 1302 (Roberts *et al*., 1996) to the Klenow-repaired *Nco*I site of pUFV335. The resulting clone, pUFV419 (–2000p*SBP2–GFP*), contains a *GFP* cDNA under the control of 2.0 kb 5′-flanking sequence of *gsS641.1*.

Generation of transgenic plants

The pCAMBIA-derived recombinant plasmids or pCAMBIA 1381Z 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 the transformed plants were regenerated on medium containing hygromycin (50 mg l^{-1}) (Buzeli *et al*., 2002). For the –2000p*SBP2–GUS* constructs, plantlets were assayed for GUS activity and primary transformants were either maintained *in vitro* or transferred into soil and grown in standardized greenhouse conditions (T_0 plants) to generate seeds. Most of the rooted plants were tested for the incorporation of the hygromycin gene and *SBP2* promoter–reporter gene fusions by PCR analysis. The intensity of GUS staining among the selected lines was similar and the expression pattern was consistent between independent transgenic lines. Detailed sectional analyses for tissue-specific expression were carried out on eleven independent *SBP2–GUS* transgenic lines and five independent *SBP2–GFP* transgenic lines. One hygromycin-resistant plant for the pCAMBIA 1381Z incorporated binary vector was used as control.

Analysis of expression patterns

Protein extraction and fluorometric assay for GUS activity were performed essentially as described by Jefferson *et al.* (1987) with methylumbelliferone (MU) as a standard. Extracts were prepared from the tissue that had been frozen in liquid nitrogen and kept at –80 °C until processing. For the standard assay, plant tissues were ground in 0.5 ml of GUS assay buffer [100 mM $NaH_2PO_4.H_2O$ (pH 7.0), 10 mM EDTA, 0.1% (w/v) sarcosyl, 0.1% (v/v) Triton X-100] and 50 μl of this extract were mixed with 50 μl of GUS assay buffer containing 2 mM of 4-methylumbelliferyl-β-D glucuronide (MUG) as a substrate. The mixture was incubated at 37 °C for 15 min and GUS activity was measured using a DYNA Quant 200 Fluorometer (Amersham Pharmacia Biotech).

For the transgenic lines carrying *SBP2–GUS* fusion, histochemical analysis of β-glucuronidase activity was performed as previously described (McCabe *et al*., 1988). The tissues (roots, stems and leaves) were sampled and sectioned using a hand microtome. Tissue sections were embedded in the GUS assay buffer [100 mM $NaH_2PO_4.H_20$ (pH 7.0), 0.5 mM $K_4Fe(CN)_6.3H_2O$, 10 mM $Na_2EDTA.2H_2O$, 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, v:v). After extensive washing, the clarified tissues were stored in 50% (v/v) glycerol until photodocumentation. The micrographies were taken under an Olympus AX-70 microscope.

Transformed plants containing *SBP2–GFP* construct were harvested and mounted. An Olympus BX 60 fluorescence microscope with objectives 10×, 20×, and 40× and B excitation (WB cube U-MWU) fluorescence filters were used to view plants. Images were captured with a CCD video camera and analysed using the public domain Image SXM 1.68 software (Rasband, 1997). The original

colour TIFF images were processed as previously described. The images were digitally pseudocoloured using the colour filter number 5 (cyan, yellow and green).

Results

The genomic clones, gsS641.1 *and* gsS641.2*, are allelic forms of the* SBP2 *gene*

Two genomic clones have been isolated, *gsS641.1* and *gsS641.2*, carrying an *SBP* gene from a soybean genomic library constructed in λZAPII. The insert of *pgS641.1* is 6.4 kb long, possesses 1963 bp of 5′-flanking sequences and covers the complete sequence of a gene, which is interrupted by five introns (Fig. 1A). The introns have a length of 318, 598, 110, 575, and 529 bp. All the intron/exon junctions deduced by comparison with the *SBP* cDNA sequence possess the consensus GT/AG dinucleotide and keep conserved positions as compared with those of *VfSBPL* and other genes belonging to the vicilin superfamily (Braun *et al*., 1996; Heim *et al*., 2001). Although the coding region has not been completely sequenced, partial sequence analysis implicated the *gsS641.1* as the *SBP2/S64* gene (Pedra *et al*., 2000; Pirovani *et al*., 2002). The six exons give a continuous open reading of 1469 bp, which is almost identical to the *SBP2* cDNA sequence, differing by just one nucleotide at position 94 of the *SBP2* cDNA, in which a C was replaced by a G in the *gsS641.1* sequence. This nucleotide difference led to a glutamine to glutamate replacement in gsS641.1 deduced protein.

With respect to *gsS641.2*, its insert is 4.0 kb long, possesses 1963 bp of 5′-sequence and covers half of the coding region sequence (2037 bp), which is interrupted by three introns (Fig. 1B). The four exons give a continuos open reading frame encoding a partial SBP protein of 330 amino acid residues that shares 99% identity with the corresponding SBP2 protein sequence.

Comparison of *gsS641.1* and *gsS641.2* nucleotide sequences revealed that they retain identical sequences, except for the presence of an *Eco*RI site in the fourth exon of *gsS641.2*, that is absent in the *gsS641.1* sequence. The high identity of the sequences extends to include the 5′-upstream regulatory sequences and the 5′-untranslated sequence. In fact, the genomic clones share identical promoter sequences. Very likely *gsS641.1* and *gsS641.2* correspond to allelic forms of the *SBP2* gene.

The nucleotide sequence of a 1.0 kb 5′-flanking sequence from *gsS641.1* is presented in Fig. 1C. Sequence analysis of these promoter regions revealed a number of conserved motifs of most eukaryotic promoters, in addition to several potential regulatory elements of plant promoters. Typical TATA box and CCAAT box were identified on *gsS641.1* at position –73 and –337 upstream of the ATG translation start codon, respectively. The potential regulatory elements found in the soybean *S64* promoters fall into three categories: (1) tissue-specific controlling elements, such as the GLUB1 sequence AACAAAC (Wu *et al*., 2000) in sense (coordinates –68 to –74) and reverse orientation (–33 to –39), the legumin box CATGCAY (–116 to –127; Fujiwara and Beachy, 1994), and a reverse SEF1 motif ATATTTAWW (–519 to –527; Allen *et al*., 1989); (2) light-responsive elements, such as the GT1 core sequence GGTTAA in reverse orientation (coordinates –767 to –756; Zhou, 1999) and a reverse IBOX core sequence GATAA (–275 to –281; Terzaghi and Cashmore 1995); (3) stress-responsive elements, such as a MYB2AT sequence TAACTG in reverse orientation (–418 to –428; Urao *et al*., 1993), a WBOXATNPR1 sequence TTGAC (–215 to –221; Yu *et al*., 2001) and two repeated ASF1 sequences, TGACG, positions –541 and –575 (Katagiri *et al*., 1989). The conserved stress–responsive elements are found in a number of genes, involved in sugar metabolism or transport.

The SBP *gene family is represented by few copies in the soybean genome*

Two distinct *SBP* cDNAs which share 85% nucleotide sequence identity have been isolated from a seed cDNA library (Grimes *et al*., 1992; Pirovani *et al*., 2002), but precise information about the copy number of *SBP* genes in the soybean genome is not available. Sequence comparison against the soybean EST database revealed multiple hits for SBP-like genes that may reflect transcript abundance in the soybean cDNA libraries rather than gene copy number. In fact, the majority of these sequences are repeated and some sequence variation of these ESTs may also be attributed to allelic polymorphisms. In direct comparison with SBP2, the soybean ESTs may be grouped into two major clusters, one ranging from 94–100% sequence identity with SPB2 and the other in the range of 85–89% identity. These results may indicate the presence of two major classes of SBP genes in the soybean genome. Reconstruction genomic DNA blot analysis was performed in order to estimate the *SBP* gene copy number (Fig. 2). DNA digested with endonucleases that recognize one site in the genomic clone, resulted in banding patterns in which more than two bands were detected. The endonuclease *Eco*RI yielded one major band and three bands of lower intensity. Using the enzyme *Bam*HI, which does not cut within the two isolated cDNAs and the genomic clones, a major band of high intensity and a low intensity, higher band poorly resolved on a 1% agarose gel were detected. The number of *SBP* genes was further estimated to be 2–3 copies by comparing the signal intensity of the hybridizing bands (lanes E and B) with that of standard amounts of cDNA (lanes 1, 5 and 10).

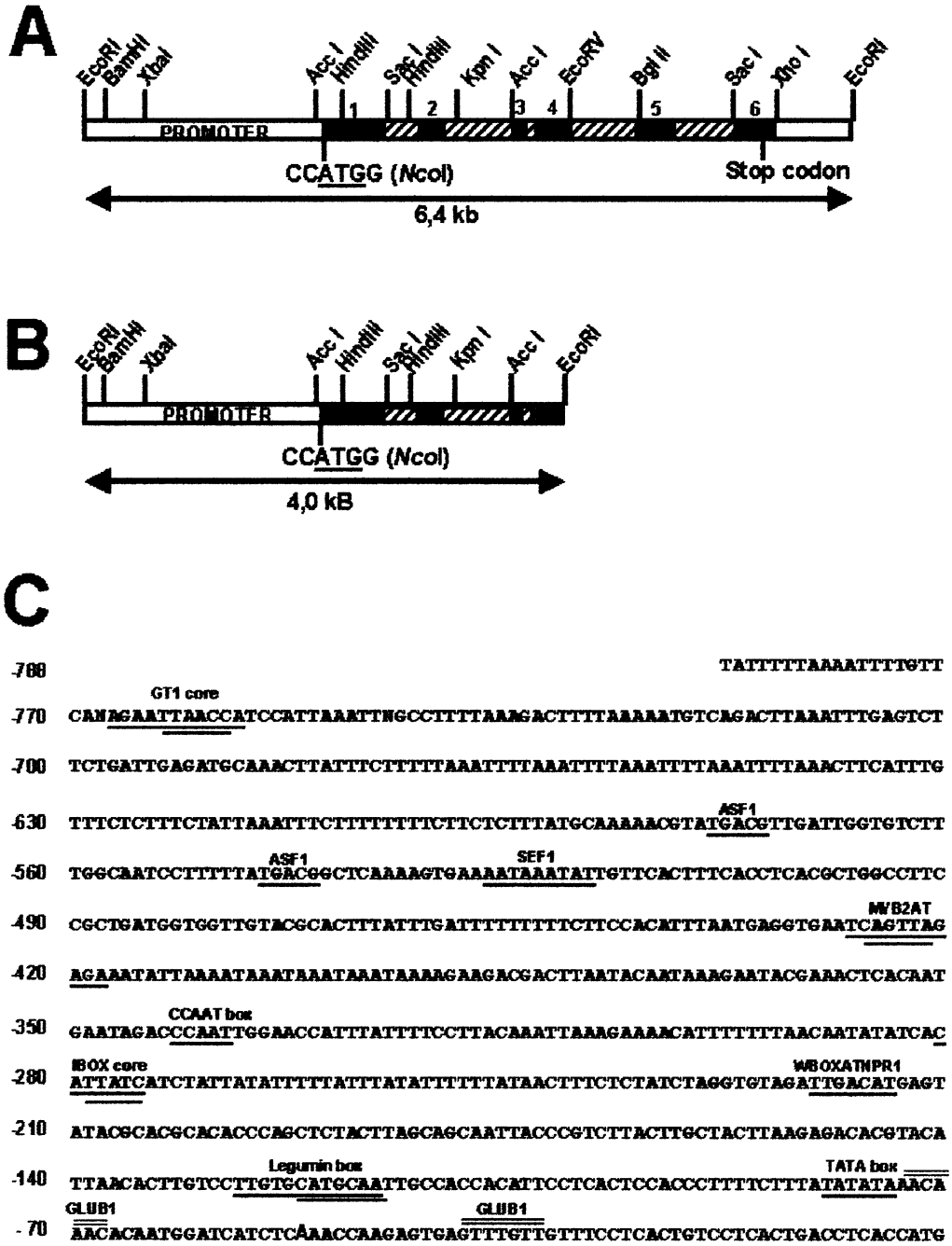

Fig. 1. Gene strucuture of soybean *SBP2*. (A) Schematic diagram of the intron–exon organization of *GmSBP2* in the soybean genome (*gsS641.1*). The position of the translation start codon (ATG) in the first exon and the translation stop codon within exon 6 are indicated. The exons (black boxes) are numbered from 1 to 6. Promoter region and 3′-flanking genomic sequence are indicated by white boxes and introns as hatched boxes. The positions of some restriction enzyme sites are indicated. (B) Schematic representation of the *gsS641.2* allele. An extension of 4.0 kb sequences representing the genomic clone *gsS641.2* is presented in which the promoter sequences, intron and exons are indicated as in (A). The positions of some restriction enzyme sites are indicated. (C) Putative *cis*-regulatory elements on *gsS641.1* promoter regions. The sequences presented extend until the ATG translational initiation codon of *gsS641.1*. Numbers indicate the position relative to the translation start codon. The putative transcriptional start site +1 is indicated, followed by the putative TATA box. Several putative *cis*-regulatory elements are underlined and indicated by their appropriate names.

The S64/SBP2 *and* SBP *genes are located in distinct regions on the soybean genome*

To estimate the *SBP* gene copy number in the soybean genome further, fluorescence *in situ* hybridization was performed on interphase nuclei using a fragment of the *SBP* cDNA as probe (Fig. 3A, B, C). At moderate stringency, four hybridizing spots on the genome were detected, suggesting the presence of two distinct loci or

two highly homologous genes. The intensity of the hybridizing signals was very similar and may reflect the high degree of sequence conservation of the *SBP* genes. In fact, the probe covers the most conserved region between *SBP* and *S64/SBP2* cDNAs. These results were consistently repeated in different nucleus preparations.

A *gsS641.2* genomic probe has also been used in FISH assays for specific labelling of the *S64/SBP2* locus (Fig. 3D, G). The genomic probe cross-hybridized to a different extent with the two pairs of genes. The intensity of the hybridizing signals was estimated by a semi-quantitative method based on the quantification of the pixel values (8 bits) of each labelling signal of the digitalized image. The original colour of the images was converted to a grey scale up to a 255 value for the analysis of relative density of the labelling signals (Fig. 3E, H). The density of each signal was three-dimensionally plotted using software for image analysis, as described in the Materials and methods (Fig. 3F, I). The values of each labelling signal were obtained as a function of the highest peak value. The calculated values grouped the signals in two pairs of similar intensity. Based on the high homology of the probe to *SBP2*, the stronger hybridizing signals may correspond to the *SBP2/S64* alleles, whereas the *SBP1* alleles may be represented by the lower intensity spots. Thus, the *gsS641.2* (*pgSBP2*) genomic fragment probe seems to be able to discriminate quantitatively between *GmSBP1* and *GmSBP2* genes. Collectively, these results may indicate that soybean *SBP* is encoded by a small gene family that is represented by at least two highly conserved copies in the soybean genome.

Tissue-specific expression of the SBP2 *promoter*

Transgenic plants expressing the *SBP2–GUS* fusion genes were examined for promoter activity and tissue-specific GUS activity. Because the expression patterns of *gsS641.1–GUS* (–2000p*SBP2–GUS*) and *gsS641.2–GUS* fusion genes were indistinguishable, only the results of the expression of the first construct (-2000p*SBP2–GUS)* are presented. RNA blot analyses have previously demonstrated that SBP mRNA accumulates predominantly in immature seeds (Grimes *et al*, 1992; Overvoorde *et al*., 1997). Consistent with this observation, the SPB promoter directed high levels of GUS activity in transgenic seeds and in developing fruits from independent transgenic lines (Fig. 4). By contrast, quantitative measurements of GUS activity in other organs demonstrated a low level of GUS expression (Fig. 4, roots and data not shown). Northern analysis and RT-PCR have detected low levels of SBP mRNA accumulation in other organs, such as leaves, seedlings, seed coat, pods, and roots (Grimes *et al*., 1992; Heim *et al*., 2001; and data not shown). In view of this observation, the SBP promoter activity was histochemically assayed in leaves, stems and roots. Histochemical examination of different independently transformed *SBP2–GUS* lines indicated that in all the organs analysed (leaf, stem and root) the expression of the *SBP2–GUS* fusion was restricted to the vascular tissue (Fig. 5). In the shoot apex, reporter gene activity was also mostly expressed in vascular tissues (Fig. 5B) and transverse sections through the leaf revealed an intense GUS staining associated exclusively with the vascular bundle (Fig. 5E). Detailed analysis of leaf sections revealed that the *SBP2–GUS* expression was restricted to the phloem (Fig. 5F) with clear staining of the sieve elements (Fig. 5G). Likewise, in cross-sections of petiole and stems, high levels of expression were mostly associated with the phloem, especially the inner phloem tissues (Fig. 5L, N). An intense GUS staining was also observed in the vascular cylinder in roots (Fig. 5R). Microscopic analysis of root sections revealed that this expression was restricted to the phloem of the vascular tissue (Fig. 5S). In control assays, GUS activity was not detected in shoots and roots from transgenic plants harbouring the promoterless *GUS* gene (Fig. 5A, D, K, M, Q).

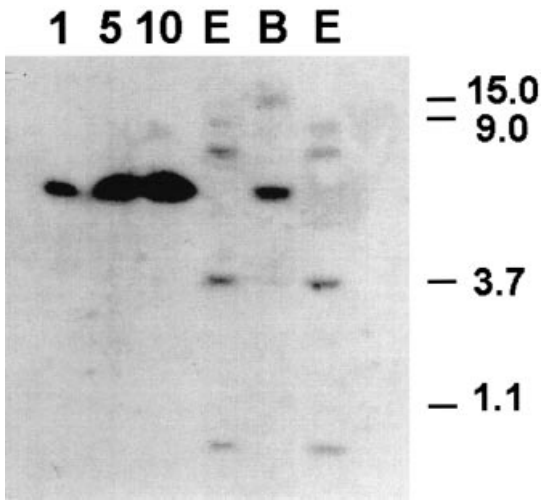

Fig. 2. DNA gel blot analysis. Soybean genomic DNA (10 μg) was digested with *Eco*RI (E) and *Bam*HI (B), separated by electrophoresis, transferred to nylon membrane and hybridized with a fluorescein-labelled *GmSBP2/S64* cDNA sequence. The numbers at the top correspond to the relative gene copy number. Migration positions of molecular weight standards are indicated on the right in kb.

The tissue-specific expression from the *SBP2* promoter was also analysed in transgenic plants containing the green fluorescent protein (*GFP*) cDNA fused to *SBP2* upstream sequences. Analyses of the vascular tissues from transverse sections of untransformed, control leaves (Fig. 5I), stem (Fig. 5O) and roots (data not shown) demonstrated that the xylem tissue from tobacco contains very high levels of yellow fluorescent background, whereas the phloem tissue does not autofluoresce. By contrast, microscopic analysis of the vascular tissue from the apical meristem (Fig. 5C), leaves (Fig. 5H, J), stem (Fig. 5P), and root (Fig. 5T) of *SBP2–GFP* transgenic lines clearly demonstrated a high level of *GFP* expression in the

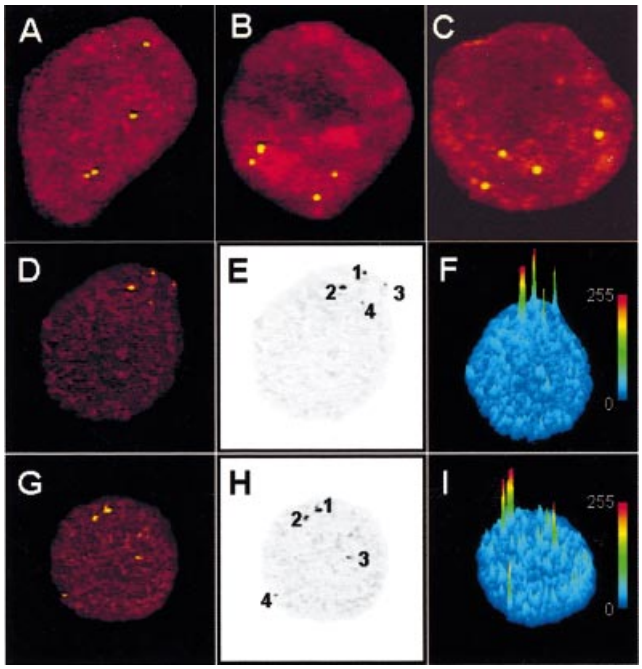

Fig. 3. FISH on soybean interphase nuclei with *GmSBP1* cDNA probe or *gsS641.2* genomic probe (*SBP2/S64*). Interphase nuclei were hybridized with a fluorescein-labelled *GmSBP1* cDNA fragment (A, B, C) or with a fluorescein-labelled *gsS641.2* genomic fragment (D, G) and counterstained with propidium iodide. The originally coloured TIFF images were converted into a grey scale up to 255 value (E, H) and the fluorescence intensity of the hybridizing signal was pseudocoloured for graphic representation (scale from 0 to 255 points) (F, I). Using a *GmSBP1* cDNA sequence as probe, four hybridization sites (two loci) with similar intensity were consistently observed in different preparations (A, B, C), whereas the *gsS641.2* genomic probe discriminated between the two loci.

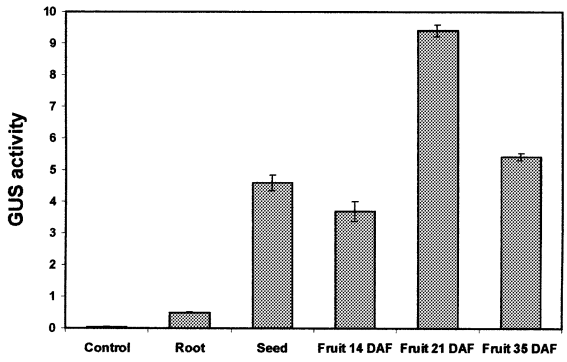

Fig. 4. GUS activity in mature seeds and developing fruits of transgenic plants expressing *SBP2–GUS* fusion genes. Specific GUS activity was determined by fluorometric assays with total extracts from seed, root and fruit at 14, 21 and 35 d after flowering (DAF) and is expressed as nmol of 4-methylumbelliferone μg^{-1} protein min^{-1}. The bars in the histogram represent average (±SE) of three independent measurements using extracts from independent transgenic lines.

phloem tissue. Thus, the pattern of *SBP2–GFP* expression was similar to the *SBP2–GUS* expression pattern, further confirming that the *SBP2* promoter exhibits a phloem-specific expression. In addition, these results provided evidence that the intense activity of the *GUS* reporter gene associated with vascular tissue was indeed a result of *SBP2* promoter activity and not due to the enzymatic product diffusion to the phloem or availability of the substrate.

Discussion

The structure of the soybean *SBP* gene has been described. The *SBP* gene is approximately 6.4 kb long and contains six exons with five introns. The exon–intron boundaries follow the 5′-donor and 3′-acceptor consensus sequence (GT … AG) and conserve identical positions as those of genes belonging to the proposed vicilin-like protein superfamily (Braun *et al*., 1996; Heim *et al*., 2001). This observation underscores the evolutionary relatedness between SBP and seed storage proteins, which has been previously considered based on conservation of primary and possibly tertiary structure (Overvoorde *et al*., 1997). The 5′-flanking region upstream of the initiation codon is 2000 bp and the 3′-flanking non-coding region is about 800 bp. The 5′-flanking region of the soybean *SBP2* gene contains typical eukaryotic promoter elements, such as CCAAT box (position –337 upstream of the ATG translational start codon) and TATA box (position –73). Furthermore, it harbours several potential *cis*-regulatory elements found in plant promoters, such as tissue-specific expression controlling elements, light-responsive elements and stress-induced elements. Experiments to determine whether these *cis*-acting promoter elements are functionally relevant are under progress in this laboratory.

In addition to *SBP* and *SBP2* cDNA from soybean, two other members of the *SBP* family have been described, a pea *SBP* (Castillo *et al*., 2000) and the faba bean *VfSBPL* cDNA (Heim *et al*., 2001). Sequence comparison in the EST database of other plant species revealed the presence of multiple EST sequences from *Medicago truncatula* that share with SBP an average of 70–75% sequence identity along 700 nucleotides (EST636009, GeneBank™ accession number CA858754, for example). Nevertheless, sequences of such a high degree of conservation were not found in the *Arabidopsis thaliana* genome. At the amino acid level, SBP was most related to cupin domain-containing proteins and vicilin-like seed storage proteins (about 35% identity and 50% similarity) from *Arabidopsis*. Likewise, SBP shares 40–45% partial sequence identity with globulin-like protein from *Oryza sativa* and vicilin-like proteins from *Zea mays* and *Picea glauca*. Although the function of these homologue proteins is unknown, as putative members of the vicilin-like protein superfamily, they may be storage proteins or may perform an SBP-like function. The *Arabidopsis* protein (GeneBank™ accession number AY058085) that is most related to SBP contains a predicted transmembrane domain and has been classified as a putative membrane protein, suggesting that it may be

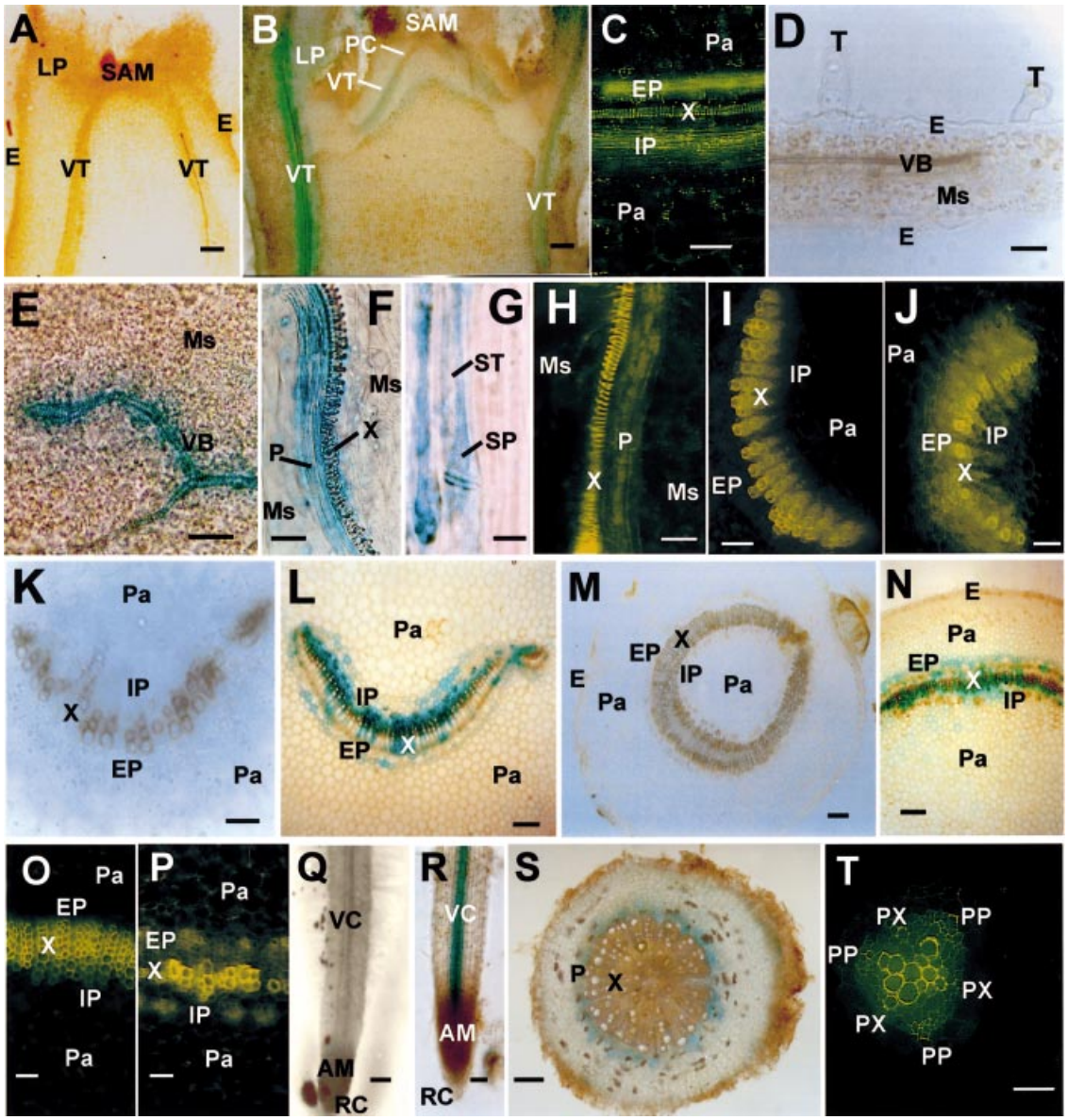

Fig. 5. Tissue-specific regulation of *SBP2–GUS* and *SBP2–GFP* fusion gene expression in transgenic plants. Photographs of transgenic organs plants harbouring –2000p*SBP2–GUS* fusion gene stained for GUS activity or *SBP2-GFP* fusion gene visualized for GFP fluorescence. The expression of –2000p*SBP2–GUS* fusion gene was determined in longitudinal section through the shoot apex (B); transverse sections of leaf (E, F, G); transverse section of petiole (L); transverse section through the stem (N); root tip (R), and transverse sections of roots (S). The expression of the –2000p*SBP2–GFP* fusion gene was visualized in a transverse section of the shoot apical meristem (C); transverse sections of leaves (H, J); transverse section through the stem (P), and a transverse section of roots (T). (A), (D), (K), (M), and (Q) correspond to pCAMBIA-transformed, control plants stained for GUS activity. (A) Longitudinal section through the shoot apex, (D) transverse section of leaves, (K) transverse section of petiole, (M) transverse section of stems, and (Q) root tip. The transverse section of leaf (I) and transverse section through the stem (O) are originated from pCAMBIA-transformed control plants but visualized for fluorescent background. Abbreviations: LP, procambium; SAM, shoot apical meristem; E, epidermis; VT, vascular tissue; Pa, parenchyma; X, xylem; EP, external phloem; IP, internal phloem; T, trichome; VB, vascular bundle; Ms, mesophyll; P, phloem; ST, sieve tube; SP, sieve plate; AM, apical meristem; VC, vascular cylinder; RC, root cap; PX, primary xylem; PP, primary phloem. Bars in (A), (B), (E), (L), (M), (N), and (S)=200 μm; bars in (D), (K), (Q), and (R)=100 μm; bars in (F), (I), (J), (O), (P), and (T)=50 μm; bars in (C) and (H)=20 μm; bar in (G)=10 μm.

functionally distinct from the vicilin-like seed storage proteins.

By contrast with *Vicia faba* SBP that is represented by a single copy in the faba bean genome, as judged by DNA gel blot analysis, the soybean SBP is encoded by a small gene family. These data, based on nuclei *in situ* hybridization using cDNA and genomic probes, confirmed that the soybean *SBP* family is represented by at least two non-allelic genes. These distinct hybridizing loci may correspond to the previously isolated *GmSBP1* (Grimes *et al*., 1992) and *GmSBP2/S64* cDNAs (Pirovani *et al*., 2002), as judged by the intensity of the hybridizing signal

using either a *GmSBP1* cDNA probe or a *gsS641.2* (*pgSBP2*) genomic fragment probe harbouring promoter, exon and intron sequences. The exon sequences on the gsS641.2 probe give a continuous SBP sequence that is highly related to *GmSBP1* (90% sequence identity) and *GmSBP2* (99% sequence identity) cDNAs. While the *GmSBP1* cDNA probe cross-hybridized with both loci with the same efficiency (Fig. 3), the genomic probe was more specific to its cognate *GmSBP2* alleles. The distinct intensities of the cross-hybridizing spots may reflect significant divergence between the *SBP1* and *SBP2* genomic sequences, which most likely lies in their 5′-flanking and intron sequences, contrasting with the high homology found in their coding region (85% sequence identity by comparing the full-length *GmSBP1* and *GmSBP2* isolated cDNAs). In fact, among genes of the same family, the conservation of sequence is expected to be lower when 5′ upstream and intron sequences are used as the basis for comparison. Thus, the capacity of the genomic probe to discriminate quantitatively between *GmSBP1* and *GmSBP2* genes makes it well suited for simultaneously FISH mapping these genes on the soybean chromosome.

Despite the structural relationship between *VfSBPL* and *GmSBP/SBP2* (65% and 63% identity between the former and the soybean DNA sequences, respectively), functional studies in yeast and transgenic plants have indicated that these proteins may not be functionally analogues. The soybean SBP has been shown to facilitate sucrose uptake when ectopically expressed in a yeast mutant strain (Grimes and Overvoorde, 1996; Pirovani *et al*., 2002) and to alter plant growth and carbohydrate partitioning in leaves of tobacco plants expressing the *SBP2* cDNA in sense or antisense orientation (Pedra *et al*., 2000). By contrast, expression of *VfSBPL* in yeast cells failed to show sucrose transport properties and its overexpression in potato plants did not cause any detectable alteration in the carbohydrate status of the transgenic tubers (Heim *et al*., 2001). Consistent with the lack of functional similarity, *GmSBP* from soybean and *VfSBPL* from faba bean display distinct expression patterns. While the expression of the *VfSBPL* has been demonstrated to be confined to seeds, *GmSBP1* transcripts have also been detected in young sink leaves (Grimes *et al*., 1992). Furthermore, the *VfSBPL* promoter failed to drive expression of a reporter gene in any tissues other than cotyledons (Heim *et al*., 2001). By contrast, it was shown that the *GmSBP2* promoter directed expression of *GUS* and *GFP* reporter genes to the vascular tissues of roots, stems and leaves, corroborating with the involvement of SBP in the long-distance sucrose translocation pathway. These results further support the notion that *GmSBP* from soybean and its homologue *VfSBPL* from faba bean may perform distinct functions.

Analysis of the *SBP* promoter activity in tobacco provides indirect evidence that *SBP* is expressed in sieve element–companion cell complex of phloem and supports the hypothesis that the protein operates in the sucrose translocation pathway. In fact, the transport of sucrose between source and sink tissues occurs in the vascular tissue and is mediated by the sieve-elements and the closely associated companion cells of the phloem tissue (Truernit, 2001). Nevertheless, these results, based on expression of *SBP*-reporter gene transcriptional fusions in transgenic plants, did not allow the possibility to be ruled out that the accumulation of SBP2 protein in soybean tissues might be different from the reporter gene activity pattern due to the lack of transcriptional regulatory elements and post-transcriptional control. However, the spatial regulation of the *SBP-GUS* and *SBP-GFP* reporter genes accurately reproduced the accumulation of SBP that has been observed in the phloem cells of soybean leaves (Grimes *et al*., 1992). Likewise, in spinach, an SBP homologue was immunolocalized in the plasma membrane of sieve elements in fully expanded leaves, shoots and roots (Warmbrodt *et al*., 1989, 1991). These observations support the argument that the *SBP2* derived sequence may control tissue-specific regulation of *SBP2* expression.

In conclusion, the data presented here confirm that the *SBP* gene family from soybean is represented by at least two non-allelic genes corresponding to the previously isolated *GmSPB1* and *GmSBP2/S64* cDNAs. As a member of the vicilin-like seed storage protein superfamily, the *GmSPB2* gene structure is similar to that of other members of the family and retains the conserved positions of the introns. This investigation on the spatial regulation of *SBP2* expression during plant development demonstrated that the *SBP2* promoter drives expression of linked reporter genes to the phloem tissue of tobacco transgenic lines, which is in marked contrast with the *VfSBP* promoter expression. The results of the *SBP2* promoter analysis are consistent with the involvement of SBP in the long-distance sucrose transport pathway and further support the notion that *GmSBP* and its homologue from *Vicia faba* performs distinct functions.

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