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Expression patterns and subcellular localization of a 52 kDa sucrose-binding protein homologue of *Vicia faba* (VfSBPL) suggest different functions during development

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Abstract

A cDNA coding for a 54 kDa signal sequence containing protein has been isolated from a faba bean cotyledonary library and characterized. The deduced protein is designated *Vicia faba* SBP-like protein (VfSBPL) since it shares 58% homology to a 62 kDa soybean (*Glycine max*) protein (GmSBP) which has been described as a sucrose-binding and sucrose-transporting protein (SBP). VfSBPL as well as GmSBP are outgroup members of the large vicilin storage protein family. We were unable to measure any sucrose transport activity in mutant yeast cells expressing *VfSBPL*. During seed maturation in late (stage VII) cotyledons mRNA was localized by *in situ* hybridization in the storage parenchyma cells. At the subcellular level, immunolocalization studies proved VfSBPL accumulation in storage protein vacuoles. However, mRNA localization in stage VI cotyledons during the pre-storage/storage transition phase was untypical for a storage protein in that, in addition to storage parenchyma cell labelling, strong labelling was found over seed coat vascular strands and the embryo epidermal transfer cell layer reminiscent of sucrose transporter localization. The *VfSBPL* gene is composed of 6 exons and 5 introns with introns located at the same sites as in a *Vicia faba* 50 kDa vicilin storage protein gene. The time pattern of expression as revealed by northern blotting and the GUS accumulation pattern caused by a *VfSBPL*-promoter/*GUS* construct in transgenic tobacco seeds was similar to a seed protein gene with increasing expression during seed maturation. Our data suggest different functions of *VfSBPL* during seed development.

Abbreviations: DAF, days after flowering; GmSBP, *Glycine max* sucrose-binding protein; GUS, β-glucuronidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; VfSBPL, sucrose-binding protein-like protein of *Vicia faba*

Introduction

Plant seeds are typical sink organs dependent on source tissue assimilates. The most important photoassimilate transported in higher plants is sucrose. Therefore, understanding sucrose transport and its regulation is of great importance to understand seed development and sink strength determination. In legume seeds physiological studies defined a saturable and a non-saturable component responsible for sucrose transport into and within developing cotyledons (see Overvoorde *et al*., 1996; Patrick, 1997; Weber *et al*., 1998). Whereas the saturable component has been molecularly identified as a sucrose transporter with 12 membrane-spanning domains (Weber *et al*., 1997b) energized by an H^+-ATPase (Harrington *et al*., 1997), the non-saturable component is still somewhat elusive.

In 1988, Ripp *et al*. reported on the identification of a membrane protein associated with sucrose in cells of soybean cotyledons. The 62 kDa protein was termed sucrose-binding protein (here abbreviated GmSBP) since it exhibited several characteristics of a sucrose transporter such as affinity labelling with a sucrose analogue and preferential localization with antibodies in the plasma membrane. Interestingly, the same antibody labelled specifically sieve tube plasma membranes of spinach leaves (Warmbrodt *et al*., 1989), roots and shoots (Warmbrodt *et al*., 1991). In *Vicia faba* cotyledons only the plasma membranes of cell walls in growth regions of the transfer cells were found to be labelled with the antibody used (Harrington *et al*., 1997). These transfer cells are the embryonal location of a typical H^+-sucrose co-transporter (Weber *et al*.,1997b; Tegeder *et al*., 1999).

A soybean cotyledon cDNA of GmSBP contained a 29 amino acid residue leader peptide not present in the mature protein but no hydrophobic membrane-spanning domains (Grimes *et al*., 1992). Therefore, the protein should not be an integral membrane protein but several experimental treatments suggested tight binding to the external surface of the plasma membrane (Overvoorde and Grimes, 1994). To experimentally prove the sucrose transport capability of GmSBP, Overvoorde *et al*. (1996) expressed the protein in an engineered yeast strain dependent for growth on active sucrose uptake. The specific uptake mediated by GmSBP was very low but linear, non-saturable and H^+-independent and thus suggested to be similar to the linear component of the plant sucrose uptake system mentioned above.

Surprisingly, Braun *et al*. (1996) discovered extensive structural and sequence similarities of GmSBP with vicilin-like 7S storage proteins, an observation independently confirmed and extended by Grimes and Overvoorde (1996) and Overvoorde *et al*. (1997). Overvoorde and co-workers also demonstrated that a vicilin-type storage globulin of maize, GLB1, was unable to mediate sucrose uptake in the yeast cells described above.

In our ongoing studies on the role of especially soluble sugars in grain legume seed development (see Weber *et al*., 1997a, 1998; Wobus and Weber, 1999, for reviews), we set out to clone the *Vicia faba* homologue of GmSBP. We were able to isolate a cDNA which conceptually translates into a 54 kDa preprotein with 68% homology to GmSBP at the nucleotide level and 58% at the amino acid level but only around 38% at both levels with the most closely related storage protein, the 50 kDa vicilin. The protein did not mediate sucrose transport in yeast. In addition, mRNA was found by *in situ* hybridization in the storage parenchyma cells of the cotyledons during the seed storage phase as known for storage proteins. At the subcellular level the protein was localized mainly in storage protein vacuoles like storage proteins. However, mRNA localization in the prestorage/storage transition phase was untypical for a storage protein with respect to the labelling of the embryo epidermal transfer cell layer and of seed coat vascular strands. We discuss the available data and speculate on the possibly multiple functions. The postulated functional diversity is stressed by the most recent discovery of a pea nuclear protein of 16 kDa induced by dehydration which turned out to be the C-terminal part of a 54.4 kDa pea protein 85% homologous to the VfSBPL described here (Castillo *et al*., 2000).

Materials and methods

Plant material

Plants of the field bean *Vicia faba* L. var. *minor* cv. Fribo (Genebank, IPK Gatersleben, Germany) were grown in a growth chamber in 16 h light and 8 h dark at 20 °C. Pods were harvested in the middle of the light phase and chilled on ice immediately. Seeds were removed and either frozen in liquid nitrogen or used for experiments within 2 h. To study protein degradation during germination dry seeds were imbibed in wet soil and proteins isolated (see below) at different time points between 1 to 15 days after imbibition.

PCR-mediated isolation of SBP relatives of Vicia faba

The following primers were deduced from the GmSBP gene (accession number L06038): 5′-GAAGACCCTGAGCTCGTAACTTGCAAACAC- 3′ and 5′- AGTACTCATAGATCTCTGGGTGATGTTG GT-3′. Hot-start PCR was performed with 1.5 mM $MgCl_2$, 0.4 mM of each primer and an aliquot of a cDNA library specific for stage V to VI cotyledons obtained from T. Wohlfahrt, IPK Gatersleben (Zap Express; Stratagene, La Jolla, CA). The temperature regime was 98 °C for 5 min, 30 cycles at 50 °C for 0.5 min each, 72 °C for 0.5 min, 94 °C for 0.5 min and then 72 °C for 10 min. The amplified DNA band of about 980 bp was subcloned after end-filling and kinasing in pUC18. Homology of the PCR

fragment with the *GmSBP* gene was confirmed by sequencing. The fragment was used to screen the cDNA library under the following conditions: hybridization at 60 °C for 16 h, washing 3 times with 2× SSPE and 2 times with 1× SSPE for about 30 min each. From a large number of positive clones 27 were analysed and only one contained the complete open reading frame. Sequencing was carried out with the ALF DNA Sequencer (Pharmacia LKB Biotechnology, Uppsala, Sweden). For general sequence handling we used the software packages A.L.F. Manager V2.21, PC/GENE V6.85 (Intelli/Genetics, Mountain View, CA) and LaserGene (DNASTAR). The *VfSBPL* cDNA has the accession number AJ292221.

Isolation of nucleic acids and hybridization techniques

Genomic DNA was isolated from leaves and cotyledons of *Vicia faba* according to Pich and Schubert (1993), digested with restriction enzymes, separated on a 0.8% agarose gel and blotted onto a Hybond N^+ nylon membrane (Amersham, Braunschweig, Germany). RNA was isolated and hybridized as described in Heim *et al*. (1993). The complete cDNA fragment was used as probe after labelling with ^{32}P-dCTP (Feinberg and Vogelstein, 1983). Southern and northern hybridizations were performed according to Church and Gilbert (1984). *In situ* hybridization was performed under high-stringency conditions with full-length cDNA probes as described in Borisjuk *et al*. (1995).

Heterologous expression in yeast

The *VfSBPL*-cDNA was cloned in sense and antisense orientation into the *Saccharomyces cerevisiae/Escherichia coli* shuttle vector NEV-E (Sauer and Stolz, 1994). The resulting constructs NEV-S-VfSBPL (sense orientation) and NEV-A-*VfSBPL* (antisense orientation) were used to transform the *S. cerevisiae* strains DBY2617 (Kaiser and Botstein, 1986; yielding strains MGY22 (sense) and MGY23 (antisense)) and MGY14 (yielding strains MGY20 (sense) and MGY21 (antisense)). MGY14 is derived from strain SEY2102 (Emr *et al*., 1983), which has been transformed with a plasmid that drives the expression of the *Plantago major* sucrose transporter gene *PmSUC2* (Gahrtz *et al*., 1994).

RT-PCR analysis of expression patterns

RT-PCR was performed with the RT-PCR kit of Perkin Elmer according to the manual. A 250 ng portion of RNA from different tissues was used as templates with primers 479 and 502 to amplify a *VfSBPL*-specific fragment of 400 bp. After reverse transcription (70 °C for 15 min) the PCR reactions were carried out under the following conditions: 95 °C for 2 min; 35 cycles each 95 °C for 1 min, 59 °C for 1 min and 59 °C for 7 min. The PCR products were separated on a 1% agarose gel, blotted onto a Hybond N^+ nylon membrane and hybridized against the full-length cDNA fragment of *VfSBPL* at 65 °C.

PCR approaches to determine the exon/intron structure of the VfSBPL *gene*

For amplification of the genomic region containing intron I the primer pair 5′-CAAGGATCCCATGGCGAT TAAAAC-3′ (388) and 5′-TGGCTCGAGATTAACCG GTGC-3′ (768) was used. The region with introns II and III was amplified with the primer pair 5′-AATGTTCCACCAAAAGGCCA-3′ (479) and 5′-GCATAATTGGGTTGGTGGCAG-3′ (502). The PCR products produced with primers 5′-GGTCCATTCAAT ATTCGC-3′ (390) and 5′-CCAGGCTTCAACTTTGC ATTG-3′ (414) or 5′- GGGATCTATGTCTACTCCTC ACTATAACACAAATGC-3′ (461) and 5′-GGTGGAT CCATAACAGGCAGC-3′ (389) contain the introns IV and V, respectively. The PCR reactions were performed under the following conditions: 96 °C for 5 min, 30 cycles each 50 °C for 0.5 min, 67 °C for 2 min, 94 °C for 0.5 min and than 50 °C for 1 min and 67 °C for 10 min with *Tth* DNA polymerase (Clontech). After end-filling and kinasing all PCR fragments were subcloned in pUC18 and sequenced.

Protein analysis

After chilling in nitrogen 250 mg cotyledon tissue was homogenized in a mortar with 1 ml extraction buffer (PBS + 0.5 M NaCl, 5 mM EDTA, 1 mM PMSF pH 7.4) and 50 mg Polyclar AT. The extraction solutions were incubated for 30 min with shaking at room temperature. After centrifugation for 15 min the upper phase was collected and the protein content determined. For PAGE and western blot analysis usually 50 μg globulin proteins were used for PAGE followed by blotting.

Isolation of the promoter region of the VfSBPL *gene*

The following gene-specific primers were used for the isolation of the 5′ region of the *VfSBPL* gene according to the description of the Universal Genome Walker kit of Clontech: PSBP1, position 159 (5′-AATCCTCACACTTCTCCATGCATATCCGTTTGTC C-3′); PSBP2, position 118 (5′-GCCCTGCAGATCGC ATTTGTCTTTGCA-3′) and PSBP3, position 85 (5′-CTGGGTCCTTTTCTTTTCTGGC-3′). The genomic DNA of *Vicia faba* was restricted with the enzymes *Sca*I (a) or *Stu*I (b) and ligated with the adaptors of the kit. A two-step PCR reaction was performed with the following parameters: 7 cycles each at 94 °C for 2 s and at 72 °C for 3 min and 32 cycles each at 94 °C for 2 s, 67 °C for 3 min and then 67 °C for 4 min The PCR products were diluted 1:50 and 1 μl of the dilution was again amplified in a second PCR (5 cycles each 94 °C, 2 s and 72 °C, 3 min and 20 cycles each 94 °C/2 s, 67 °C/3 min and then 67 °C/4 min). A 1.7 kb band from (a) and a 1.9 kb band from (b) were detected in an agarose gel. After verification by Southern blotting the bands were cloned in pUC18 and sequenced. Both fragments, containing the 5′-flanking region of the *VfSBPL* gene were fused with the GUS-coding *uidA* gene in plasmid pBI101. This plasmid was transferred into the *Agrobacterium* strain EH105 used for transformation of tobacco. The two promoter fragments caused identical GUS staining patterns.

Histochemical and quantitative GUS analysis

The histochemical detection of GUS-activity was performed as described by Jefferson (1987). Seeds of different developmental stages were fixed with gluten on a teflon plate and cut by hand with a razor blade after drying. For quantitative assays the GUS Light kit (Promega) was used. The results are given as relative light units (rlu) per minute per μg protein.

Antibody production

The VfSBPL-cDNA was digested with *Hin*dIII yielding two fragments of 582 bp and 690 bp. The 582 bp fragment codes for a peptide of 194 amino acids (residues 6–199 of the VfSBPL protein sequence) and was used in the experiments reported. For formation of β-galactosidase fusion protein the fragment was cloned into the *Hin*dIII site of plasmid pTRB2 (Bürglin and De Robertis, 1987) and transformed into the *E. coli* strain BMH71-18 (Messing *et al.*, 1977). Production of fusion protein in transformed cells was induced with isopropyl-β-D-thiogalactoside. Cells were grown, harvested and disintegrated, and fusion protein was isolated as described (Sauer and Stadler, 1993). Eventually 2 mg of the fusion protein were used for the immunization of rabbits (BioGenes, Berlin, Germany).

Immunogold localization of VfSBPL

Vicia faba cotyledons were fixed in 3% w/v paraformaldehyde and 0.5% glutaraldehyde in 100 mM potassium phosphate buffer pH 7.2 at 4 °C overnight. Tissues were dehydrated with an ethanol series and low-temperature embedded in LR white resin, sectioned and immunogold-labelled with an affinity-purified anti-SBP polyclonal antibody produced as described above and 15 nm gold-conjugated secondary antibody (goat anti-rabbit IgG; Amersham). The grids were evaluated in a CEM 902A transmission electron microscope (Carl Zeiss, Oberkochen, Germany). Micrographs were taken on Kodak SO163 films (Kodak, Ostfildern, Germany).

Results

A 52 kDa soybean sucrose-binding protein homologue of Vicia faba, VfSBPL*: cDNA isolation and characterization*

To isolate a *Vicia faba* cDNA homologue of the soybean sucrose-binding protein (GmSBP) isolated by Grimes *et al*. (1992), primers were designed based on the *GmSBP* sequence and polymerase chain reactions (PCR) performed using as template double-stranded cDNA specific for mid-maturation stage cotyledons (for stageing, see Borisjuk *et al*., 1995). The amplified band was subcloned and several clones sequenced; all were identical except a few nucleotide exchanges, which could be technical errors. A comparison at the nucleotide level with the corresponding fragment of *GmSBP* revealed a homology of 70% . The PCR fragment was used to screen a cotyledon-specific cDNA library. We found a large number of positive clones, indicating a high expression level of the gene. Of a total of 24 clones only one harboured the complete coding sequence together with 6 bp of 5′-untranslated sequence, a TGA stop codon and 115 bp of the 3′-untranslated region followed by a poly(A) tail. Conceptual translation of the single open reading frame (Figure 1A) resulted in a 482 residue amino acid sequence with a predicted molecular mass of 54.6 kDa.

This value differs considerably from the 65.5 kDa predicted for the 524 amino acid GmSBP of Grimes *et al*. (1992). The overall homology of *VfSBPL* to *GmSBP* is 58% at the amino acid level and 68% at the nucleotide level. Like in GmSBP the N-terminus of the VfSBPL sequence is characterized by a hydrophobic leader peptide of 27 amino acid residues characteristic of known signal sequence structures. The sequenced 11 N-terminal residues of a protein from the seeds of *Vicia narbonensis* were found to be identical with the respective residues of the predicted mature VfSBPL (Wüstenhagen and Müntz, personal communication) indicating that the potential signal sequence is cleaved off at the predicted site during protein processing.

Sequence comparison at the amino acid level with several vicilin-like proteins from gymnosperm and angiosperm species clearly shows that the separation of vicilins and vicilin-like SBP proteins happened early during dicot evolution (Figure 1B).

VfSBPL *gene structure, 5′-flanking sequences and representation within the genome*

Since a clear homology of GmSBP to vicilin-like storage protein genes was found (see above) we decided to determine the intron-exon structure of the *VfSBPL* gene including 5′-flanking sequences by a PCR approach. Primers were designed as described in Material and methods, used in PCR reactions, and the amplified sequences were cloned and sequenced. This way we covered the whole gene region plus 1.9 kb of 5′-flanking sequences (hereafter called 'promoter'). Interestingly, several sequenced fragments from the same region of the gene showed sequence differences like base exchanges and small deletions/insertions preferentially in intron regions. This variation does not allow combining the sequences to a single authentic gene sequence but the general structure is evident (Figure 1C). The intron-exon structure exactly corresponds to that of the single completely sequenced *V. faba* vicilin gene (Weschke *et al*., 1988), i.e. all introns are found at the same places in the sequence (see Figure 1A) but all exons and introns are different in length in both genes. A few cluster of amino acid residues homologous between vicilin and VfSBPL are scattered through several exons (see Figures 1A and C).

Southern blotting data (Figure 2) are consistent with only one gene suggesting that the observed sequence variations may be due to allelic and/or individual sequence polymorphisms. This is not unexpected since DNA was isolated from pooled plant material and *Vicia faba* is genetically rather heterogeneous due to fertility problems in the homozygous state.

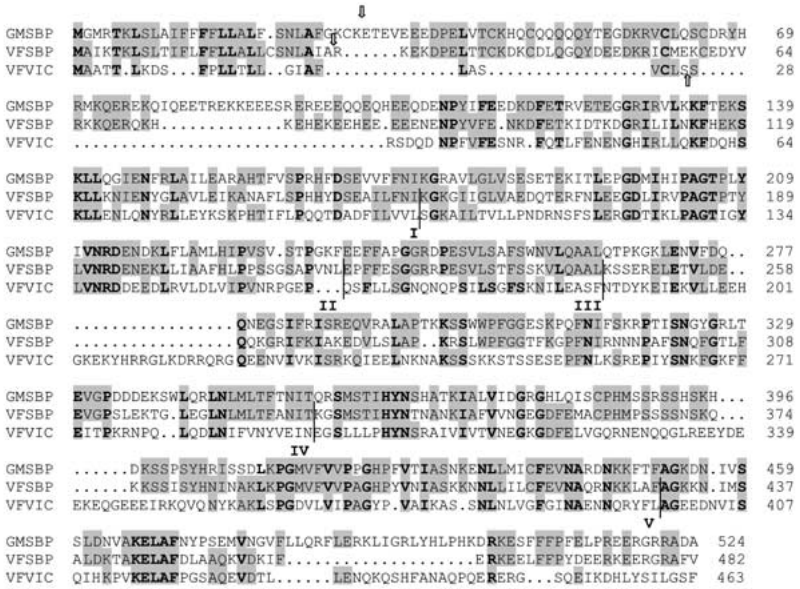
Computer analysis revealed 1 putative N-glycosylation site at amino acid residue 330 (see Figure 1A) in a region without homology to the 50 kDa vicilin sequence. The 5′-upstream sequence (Heim and Weber, patent application WO OO/26388, 1999) reveals a TATA box (–44 to –48 upstream of the ATG translational start codon) but no clearly defined transcription start site. At position –78 a RY motif (CACGTATG) is localized known to be involved in transcriptional regulation of genes expressed in seeds (cf. Bäumlein *et al*., 1991; Reidt *et al*., 2000), and at position –130 a short ACT element is localized, which is central to sugar response elements (SURE) in several plant and mammalian genes (see Nigel and Jenkins, 1997). Both elements could be functional since the gene is preferentially expressed in seeds and responds to sucrose (H. Weber, unpublished results).

Expression patterns of the VfSBPL *gene studied by northern and western blotting,* RT- PCR, in situ *hybridization and promoter-GUS fusions*

The full-length cDNA was used as a probe in RNA gel blot experiments. Figure 3A shows a northern blot of *VfSBPL* mRNA. Transcripts were only detected in the cotyledons where transcript levels increased from 22 days after flowering (DAF; stage V of Borisjuk *et al*., 1995) to reach highest levels during mid and late cotyledon stage. This pattern is typical of storage protein mRNA as legumin B4 mRNA but different from the expression profile of GmSBP in soybean seeds with a peak at the beginning of the storage phase and a continuous decrease during seed maturation (Grimes *et al*., 1992). In our northern experiments no signals were detected in RNA from the following tissues: seed coat, pod, sink leaf, source leaf, stem, flower and roots (Figure 3A). Only by using RT-PCR faint signals in seed coats and sink leaves could be detected (Figure 3B).

In situ hybridization detected the mRNA in stage VII cotyledons at relatively high levels mainly in the storage parenchyma cells but also in the axis (Figure 4I). Surprisingly, this pattern is strikingly different at earlier stages of seed development. In stages III and IV exclusively the thin-walled parenchyma of the seed coat and the endosperm are strongly labelled but not the embryo (Figure 4A). At stage V labelling begins to appear in the epidermis of the embryo, whereas

A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VFVIC MAATTLKDS...FPLLTLL..GIAF.....LAS.....VCLSS..... 28
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VFVIC .....RSDQD NPFVFESNR.FQTLFENENGHIRLLQKFDQHS 64
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VFSBP LVNRDENEKLLIAAFHLPPSSGSAPVNLEPFFESGGRRPESVLSTFSSKVLQAALKSSERELETVLDE.. 258
VFVIC LVNRDDEEDLRVLDLVIPVNRPGEP...QSFLLSGNQNQPSILSGFSKNILEASFNTDYKEIEKVLLEEH 201
GMSBP .....QNEGSIFRISREQVRALAPTKKSSWWPFGGESKPQFNIFSKRPTISNGYGRLT 329
VFSBP .....QQKGRIFKIAKEDVLSLAP..KRSLWPFGGTFKGPFNIRNNNPAFSNQFGTLF 308
VFVIC GKEKYHRRGLKDRRQRGQEENVIVKISRKQIEELNKNAKSSSKKSTSSESEPFNLKSREPIYSNKFGKFF 271
GMSBP EVGPDDDEKSWLQRLNLMLTFTNITQRSMSTIHYNSHATKIALVIDGRGHLQISCPHMSSRSSHSKH... 396
VFSBP EVGPSLEKTG.LEGLNLMLTEANITKGSMSTIHYNTNANKIAFVVNGEGDFEMACPHMPSSSSNSKQ... 374
VFVIC EITPKRNPQ..LQDLNIFVNYVEINEGSLLLPHYNSRAIVIVTVNEGKGDFELVGQRNENQQGLREEYDE 339
GMSBP ......DKSSPSYHRISSDLKPGMVFVVPPGHPFVTIASNKENLLMICFEVNARDNKKFTFAGKDN.IVS 459
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VFSBP ALDKTAKELAFDLAAQKVDKIF.....ERKEELFFPYDEERKEERGRAFV 482
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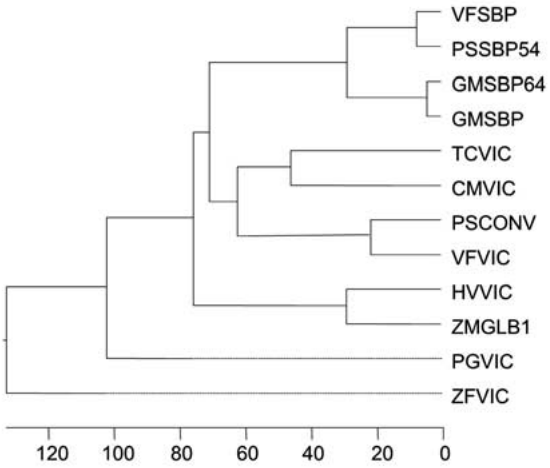
B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GMSBP
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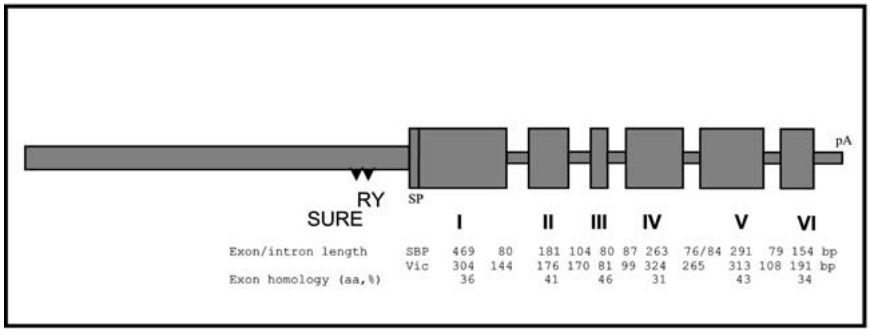
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Exon/intron length SBP 469 80 181 104 80 87 263 76/84 291 79 154 bp
Vic 304 144 176 170 81 99 324 265 313 108 191 bp
Exon homology (aa,%) 36 41 46 31 43 34

Figure 1. Sequence alignment (A), comparison with similar proteins (B) and gene structure (C) of VfSBPL. A. Multiple sequence alignment of VfSBPL (VFSBP) with the soybean sucrose-binding protein sequence (GmSBP) and a *Vicia faba* 50 kDa vicilin sequence (VFVIC). Open arrows in the N-terminal region indicate signal peptide cleavage sites and vertical lines plus a Roman number the location of introns within the genomic DNA sequence. Identical amino acid residues are underlain in grey and bold-face letters mark residues identical in all 3 proteins. B. Neighbour joining analysis of the amino acid sequences of vicilin-like proteins of selected species belonging to gymnosperm and angiosperm plant families including monocot and dicot species. PSSBP64, *Pisum sativum* SBP-type protein, accession number 2765097; GMSBP64, *Glycine max* SBP-type protein, AF191299; GMSBP, *Glycine max* sucrose-binding protein, 548900; TCVIV, *Theobroma cacao* vicilin-type protein, 384341; CMVIC, *Cucurbita maxima* vicilin-type protein, 3808062; PSCONV, *Pisum sativum* convicilin, 227928; VFVIC, *Vicia faba* 50 kDa vicilin, 226228; HVVIC, *Hordeum vulgare* vicilin-type protein, 421978; ZMGLB1, *Zea mays* 7S globulin, 542184; PGVIC, *Picea glauca* vicilin-type protein, 1350502; ZFVIC, *Zamia furfuracea* vicilin-type protein, 2148163. Note that monocot vicilin-type genes (HVVIV, ZMGLB1) separated before dicot genes diverged into 7S genes of the SBP-type and the typical vicilin storage protein genes. C. Scheme of *VfSBPL* gene structure. Exons are numbered by Roman letters and the location of the signal peptide cleavage site SP (see A), the polyadenylation site (pA) as well as of two *cis*-regulatory elements, RY and SURE (see text), indicated. The length of exons and introns in base pairs are given for the gene shown (SBP) and, for comparison, for the known *Vicia faba* vicilin gene (Vic; Weschke *et al*., 1988). Also given is the homology at the amino acid level (aa, %) between the two compared polypeptides.

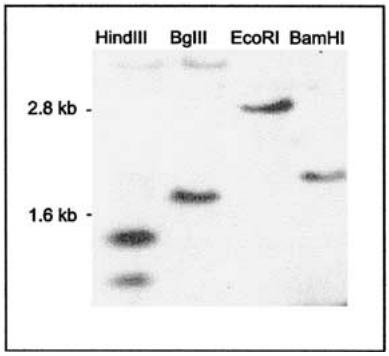

Figure 2. Southern analysis of *Vicia faba* genomic DNA. 25 μg DNA per lane digested with *Hin*dIII, *Bgl*II, *Eco*RI or *Bam*HI were separated in an agarose gel, blotted onto Hybond N^+ nylon membrane and hybridized with the ^{32}P-labelled full-length *VfSBPL* cDNA fragment. Fragment sizes in kb based on marker DNA fragment sizes are given.

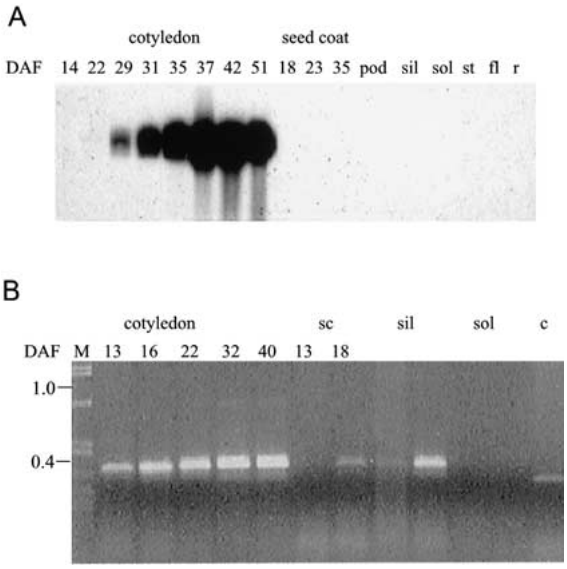

Figure 3. VfSBPL gene expression analysis. A. Developmental northern blot of *VfSBPL* expression in cotyledons, seed coat and other tissues. B. Low expression levels in other than cotyledon tissue can be detected only by RT-PCR analysis in the 18 DAF seed coat (sc) and in sink leaves (sil) but not in source leaves (sol). st, stem; fl, flower; r, root; M, molecular size (kb) marker lane; c, cDNA transcript control lane.

the pattern in the seed coat remains the same with the only difference that more cell rows of the parenchyma become labelled (Figure 4 B, C). At stage VI gene expression spreads to the storage parenchyma of cotyledons starting from the adaxial region (Figure 4F) and covering the whole embryo at stage VII (Figure 4I). Neither vascular tissues of the embryo nor meristematic tissues of the axis were labelled. However, we often observed labelling of the vascular bundle in the seed coat of stage V and VI seeds (Figure 4J, K). For comparison, the mRNA accumulation patterns of the faba bean 50 kDa vicilin (Figure 4E) and sucrose transporter VfSUT1 (Figure 4G) are shown.

5′-upstream sequences of *VfSBPL* were isolated as described in Materials and methods and fused at the ATG start codon via *Nco*I cleavage to the GUS gene in vector pBI101. The sequences upstream of the ATG site were 1539 and 1750 bp long and differed in 23 base substitutions and 2 deletions/insertions but produced identical results. After transformation of tobacco plants seeds from primary transformants were analysed. Histochemical staining (Figure 5) revealed a picture similar to a seed storage protein as exemplified by the faba bean legumin B4-promoter/GUS fusion also analysed in tobacco seeds (Bäumlein *et al*., 1991). The cotyledons were most intensively stained whereas the blue colour in the axis was less intensive; the endosperm was only pale blue and characterized by patchy colouring as noted before for the legumin B4 promoter (Bäumlein *et al*., 1991). No GUS activity was detected in sink and source leaves, in flowers,

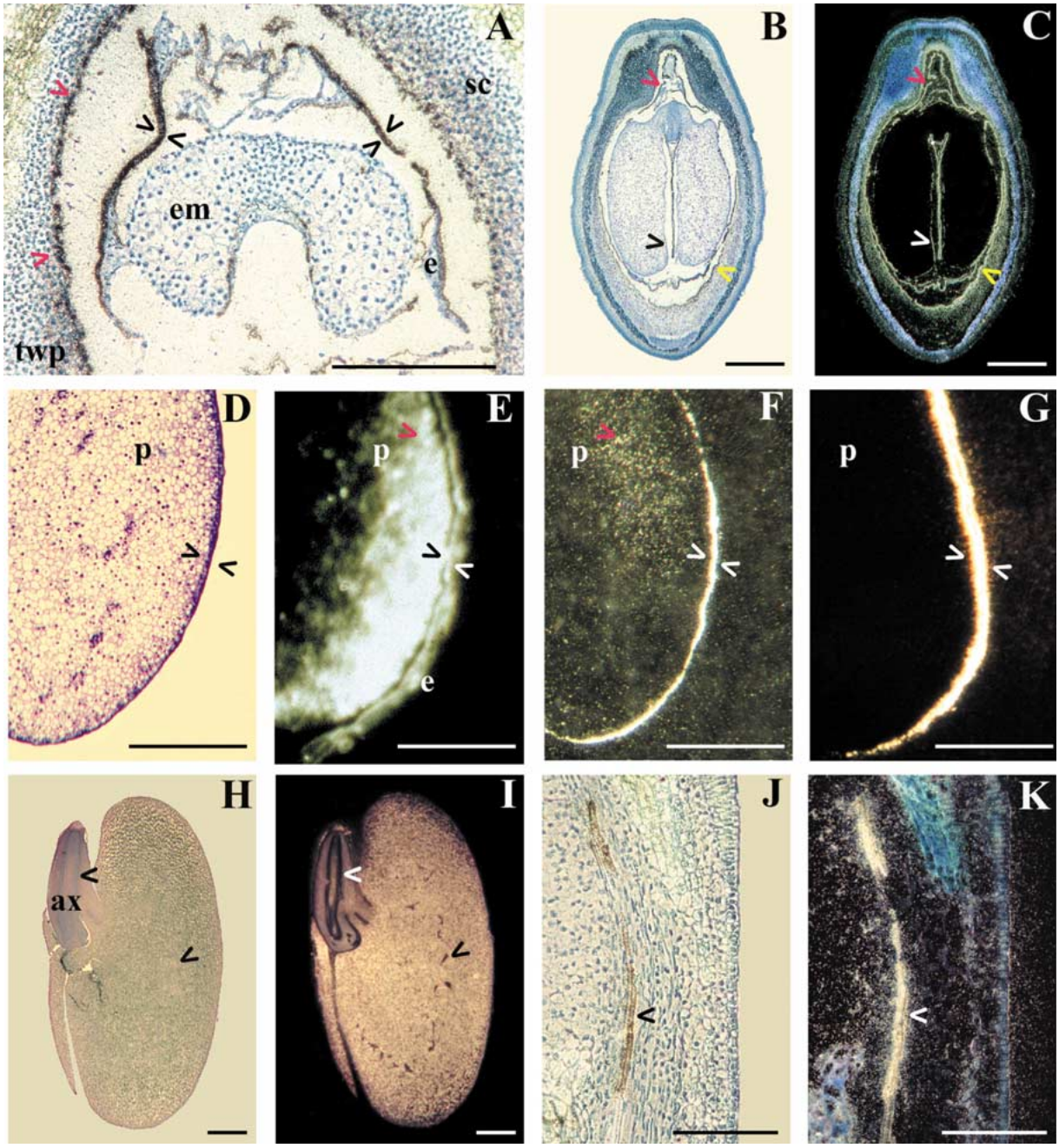

Figure 4. In situ localization of *VfSBPL* mRNA in developing seeds of *Vicia faba*. A. Developmental stage III: section including seed coat (sc), endosperm (e) and young embryo (em). The bright-field image of the section shows the strongly labelled thin-walled parenchyma (red arrowheads) and endosperm (black arrowheads). Bar: 500 μm. B, C. Bright-field and dark-field images of a seed section at stage V demonstrating strong labelling of the thin-walled parenchyma (yellow arrowhead), endosperm (red arrowhead) and epidermis of the embryo proper (black (B) or white (C) arrowhead). Bars: 1 mm. D, E, F, G. Sections through the outer adaxial region of a cotyledon at stage VI (D, toluidine-blue stain) showing localization of vicilin mRNA in the storage parenchyma (red arrowhead, E), *VfSBPL* mRNA not only in the storage parenchyma (red arrow) but also in the epidermis of the embryo (white arrowheads, F) and sucrose transporter mRNA (*VfSUT1*) in the transfer cells of the embryo (G). Bars: 1 mm. H, I. Longitudinal section through a seed at stage VII showing a fully developed embryo with axis (ax) in bright and dark field, respectively. Note that only vascular strands of axis and cotyledons (arrowheads) are not labelled. Bars: 1 mm. J, K. Bright- and dark-field images showing labelling of the vascular bundle in a stage VI cotyledon (arrowheads). Bars: 200 μm.

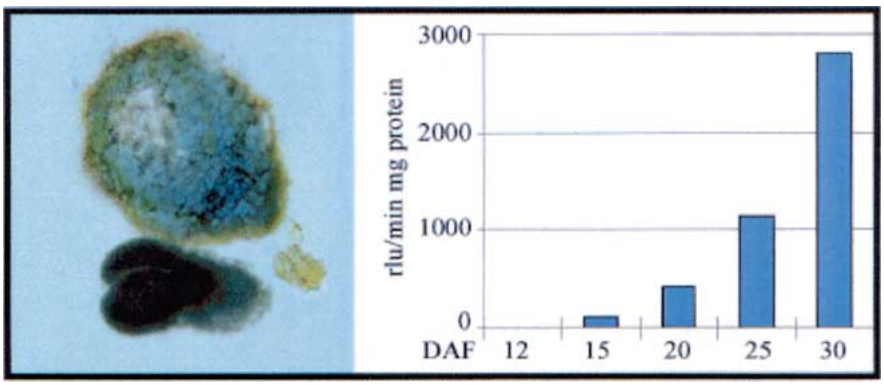

Figure 5. GUS activity analysis in seeds of transgenic tobacco plants expressing the *uidA* gene driven by the *VfSBPL* promoter. Left: GUS staining of a cotyledon-stage embryo taken out of the seed coat shown above the embryo. Right: GUS activity levels in developing tobacco seeds. The activity pattern is similar to that caused by a storage protein gene promoter-*uidA* gene construct (not shown).

anthers and pollen as expected from the northern data obtained in *Vicia faba*. Quantitative estimates of GUS activity during development revealed detectable activity first at 12 DAF followed by a steady increase up to 30 DAF (Figure 5).

Western blotting detected increasing amounts of SBP protein during development reflecting roughly the mRNA pattern (Figure 6A, right panel). In Coomassie-stained gels a band of about 50 kDa is most strongly stained (Figure 6A, left panel). It runs at the position of both 50 kDa vicilin and VfSBPL. In dry seeds the protein is still present at high amounts (Figure 6A, B). Control experiments using an enriched vicilin preparation in blotting experiments proved that the SBPL antibody did not react with vicilin migrating to a large part at the 50 kDa position (Figure 6C).

VfSBPL *is degraded during germination similarly to 50 kDa vicilin*

Since in the experiments described *VfSBPL* is accumulated and stored (see below) during seed maturation like a storage protein and not as expected for a membrane-associated sucrose-binding protein we determined the proteolytic degradation pattern during seed germination. As seen in Figure 6B, protein degradation as measured by SDS gel electrophoresis starts at about day 4 after imbibition and is thus similar to vicilin storage proteins (see Lichtenfeld *et al*., 1979 for *Vicia faba* and Schlereth *et al*., 2000 for *Vicia sativa*).

VfSBPL similar to storage proteins accumulates in protein storage vacuoles

Globulins, the most prominent storage proteins of legume seeds, are typically synthesized at the rough endoplasmic reticulum and transported through the Golgi apparatus into protein storage vacuoles (Robinson and Hinz, 1999) where they form protein lumps. During late maturation protein bodies are formed by gradual fragmentation of these vacuoles and membrane evaginations around the protein lumps (Bewley and Black, 1994). We have applied a specificity-tested fusion-protein VfSBPL antibody in electron microscopic immunolocalization studies. At the beginning of the storage phase (stages V and VI) label was mainly detected in vacuoles but at low levels also at the plasma membrane and the endoplasmic reticulum (not shown). At stage VII, i.e. in the middle of the storage phase, protein lumps are formed in storage vacuoles and only those were found to be labelled (Figure 7). Exactly the same labelling pattern has often been described for typical storage proteins like legumin and vicilin (see, for instance, Fischer *et al*., 2000).

Expression of VfSBPL *in yeast cells fails to show sucrose transport properties*

VfSBPL cDNA was cloned into the vector NEV-E and expressed in two different strains of baker's yeast in sense and antisense orientation (see Materials and methods). Expression in strain DBY2617 was performed to identify a possible increase in the otherwise negligible transport activity for sucrose. In contrast to the results of Overvoorde *et al*. (1996), who described GmSBP from soybean as a non-saturable, H^+-independent sucrose transporter, we were unable to measure any difference in the sucrose transport capacity of MGY22 (*VfSBPL* in sense orientation) and MGY23 (*VfSBPL* in antisense orientation) cells (Figure 8). Expression in strain MGY14, which ex-

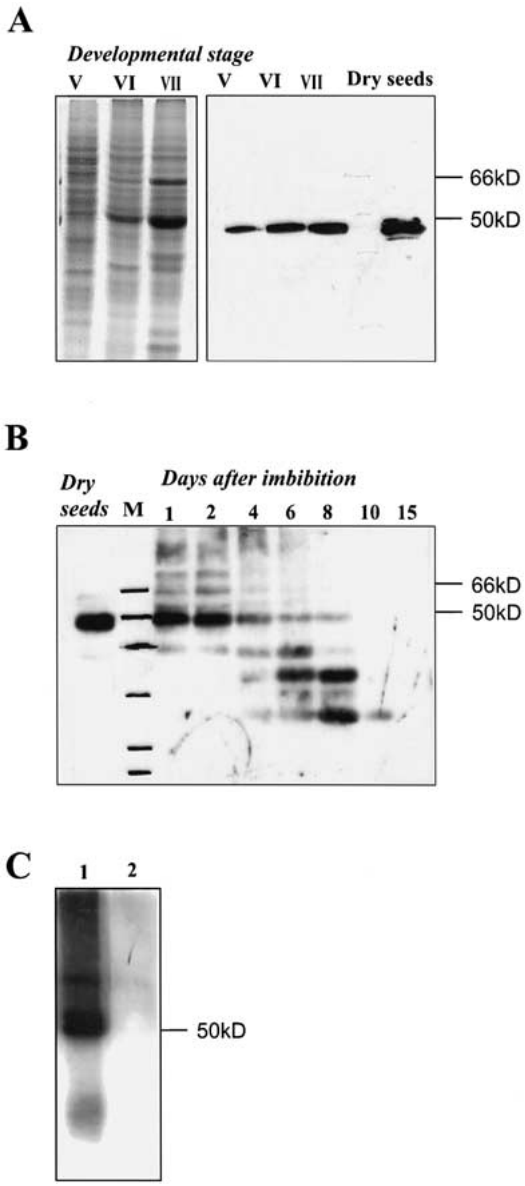

Figure 6. Detection of VfSBPL protein expression levels during seed development and germination. A. Total protein was isolated from developing seeds of stages V, VI and VII and separated on two SDS-PAGE gels in parallel, one used for Coomassie staining (left 3 lanes) and the other one for blotting (right part). 50 kDa vicilin is running together with VfSBPL. B. Similarly, protein was isolated from seeds 1, 2, 4, 6, 8, 10 and 15 days after imbibition. In each lane 50 μg protein extract was separated by SDS-PAGE and immunoprobed with affinity-purified anti-VfSBPL antibody at 1:5000 dilution. Marker protein positions are outlined. C. 1 μg each of an enriched vicilin preparation was separated in two lanes of a SDS-PAGE gel. The gel was blotted and lane 1 reacted for 1 h with a vicilin antiserum diluted 1:1000, lane 2 with the SBPL antiserum under the same conditions. After additional incubation for 1 h with an anti-rabbit IgI-peroxidase conjugate the stain was detected with the ECL kit of Amersham.

presses the H^+-sucrose symporter gene *PmSUC2* from *Plantago major,* was performed to screen for a possible decrease in the high sucrose influx rates. This might be expected, if a passive transporter catalyses the efflux of sucrose that has been imported by an active H^+-sucrose symporter. However, again no difference was observed between the strains expressing *VfSBPL* cDNA in sense (MGY20) or antisense (MGY21) orientation (data not shown).

Discussion

Plant seeds as typical sink organs are characterized by their storage function for mainly carbohydrates, proteins and/or oils. The carbon skeletons necessary for building these compounds are predominantly derived from sucrose. During seed development sucrose is imported via the phloem and distributed as such or after cleavage into hexoses and/or hexose phosphates mainly symplastically within tissues and exclusively apoplastically between maternal and filial tissues (for recent reviews, see Patrick, 1997; Weber *et al*., 1998). Active and passive transport processes are facilitated and regulated by a range of active transporters and passive facilitators (see Sauer and Tanner, 1993; Tanner and Caspari, 1996). Thus, transporters may play an important role in determining sink strength and storage product accumulation. Surprisingly, one protein group, the so-called sucrose-binding proteins or SBP's, have been reported to be involved in sucrose transport but are at the sequence level closely related to the typical 7S vicilin-type storage protein globulins and could thus fulfil a dual function.

'Sucrose-binding proteins' and storage protein vicilins are members of a single protein family

In an attempt to understand the relationship between metabolism and development we became interested in isolating a faba bean homologue of the soybean GmSBP. By using a PCR approach we isolated the gene sequence described coding for a 54 kDa protein but numerous attempts failed to identify another family member closer in size to the soybean gene. Also Southern blotting experiments (Figure 2) provided no indication for other related genes.

Braun *et al*. (1996) and Grimes and Overvoorde (1996) described independently that GmSBP is at the sequence level clearly related to plant 7S storage proteins collectively called vicilins. This is also

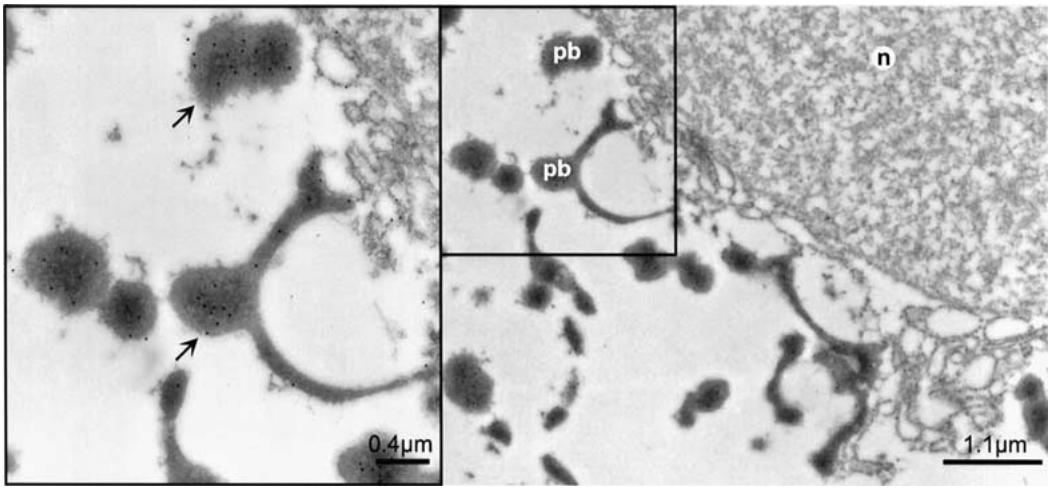

Figure 7. Electron micrograph of a *Vicia faba* cotyledon stage VII cell and immunogold localization of VfSBPL in the protein storage vacuole compartment. The indicated insert is enlarged and the arrows point to immunogold-labelled protein clumps. n, nucleus.

true for VfSBPL. It is evident from Figure 1B that according to the available data vicilin-like SBP proteins and vicilin proteins separated early during dicot evolution since they form together a group clearly separated from the monocot vicilin-like proteins. Interestingly, all these proteins together with all the 12S legumins share several structural characteristics and form a large superfamily, which may have radiated from a fern vicilin-like precursor gene (Shutov *et al.*, 1995, 1998; Shutov and Bäumlein, 1999). At least the well-characterized higher-plant storage proteins of this class are stored in membrane-lined protein bodies and serve as reserve deposit to nourish the young seedling after germination. No clear data in this respect are available for members of two offshoot groups of the vicilin branch, one of which formed by SBPs and a Forssman antigen-binding lectin from velvet bean (*Mucuna derringiana*) seeds (Shutov and Bäumlein, 1999). The lectin is of special interest since it recognizes and binds specific carbohydrate structures.

Presently, several members of a SBP family have been described only from legumes at either the cDNA and/or protein level, the soybean 'founder' 62 kDa GmSBP (Grimes *et al.*, 1992), another soybean SBP of 64 kDa with 91% homology at the cDNA level (Pedra *et al.*, 2000), a pea SBP with a deduced molecular mass of 54 kDa (Castillo *et al.*, 2000) and the faba bean 52 kDa VfSBPL (unprocessed molecular mass 54 kDa) described here which is 85% homologous to the pea protein. We found some sequence variation at the cDNA and promoter sequence level but this cannot be clearly attributed to different genes. Bcause of

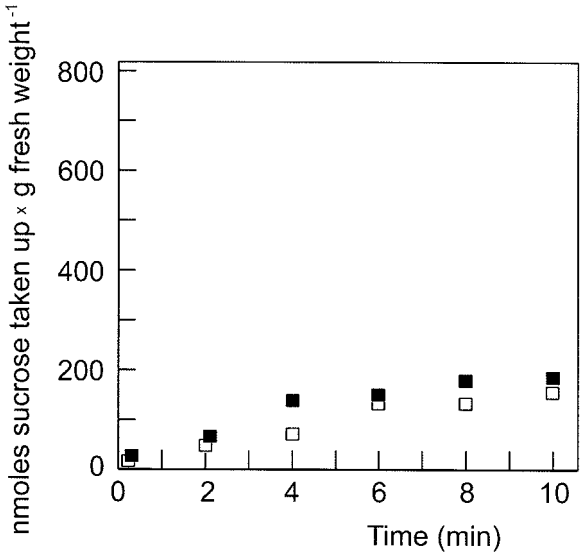

Figure 8. Analysis of the sucrose transport capacity of yeast cells expressing the *VfSBPL* cDNA in sense (MGY22; open squares) or antisense orientation (MGY23; filled squares). Cells were grown with glucose as sole carbon source, harvested and adjusted in 50 mM sodium phosphate buffer (pH 5.5) to a final density of 10 A_{260} units per ml. Uptake was determined with ^{14}C-labelled sucrose at an initial outside concentration of 1 mM.

the length difference between soybean SBPs on the one hand and the pea/faba bean SBPs on the other, it remains questionable whether we can regard these proteins as orthologues. The two major 'deletions' responsible for the reduced length in the pea/faba bean sequences as compared to soybean are found in the N-terminal half and near the C-terminus (Figure 1A);

they contain no motifs indicative for any possible function but the C-terminal 'deletion' is in that part of the molecule that is, according to Castillo *et al*. (2000), cleaved off in pea and found in the nucleus (see below).

The structure of the *VfSBPL* gene is typical of legume vicilin storage protein genes with 6 exons and 5 introns. All exon/intron boundaries are at homologous positions as compared to those in the *Vicia faba* 50 kDa vicilin gene (Figure 1C) described by Weschke *et al*. (1988) underlining the evolutionary relatedness of the compared genes.

Experiments to reveal VfSBPL function provided contradictory results

VfSBPL cDNA was engineered into a specific yeast strain, which allows the detection of sucrose transport through the yeast cell membrane, but no such transport could be detected (Figure 8). This result differs from that reported by Overvoorde *et al*. (1996) for the GmSBP but still does not in itself disprove the ability of the protein to bind sucrose and facilitate sucrose diffusion. Therefore, as a next step in the analysis, we localized *VfSBPL* mRNA by *in situ* hybridization and the respective protein by electron microscopy with the help of polyclonal antibodies raised against a recombinant fusion protein in rabbits.

In situ hybridization revealed a puzzling situation. The expression pattern of the *VfSBPL* vicilin gene during seed development showed overall similarities to that of a 50 kDa vicilin storage protein gene: both are expressed predominantly in the endosperm and thin-walled parenchyma of the seed coat during early development and expression gradually spreads to storage parenchyma and axis cells of the embryo during seed maturation (for vicilin, see Borisjuk *et al*., 1995). However, strong labelling of the epidermis of stage V embryos (Figure 4) was never observed with storage protein gene probes but rather reminds to the pattern of the sucrose-transporter gene *VfSUT1* (Weber *et al*., 1997b), which is highly expressed at this stage. In addition, labelling of the seed coat vascular bundles has regularly been found with the *VfSBPL* probe but is untypical of both vicilin storage protein and sucrose-transporter mRNAs.

Immunolocalization work provided clear evidence for localization in electron-dense lumps of storage protein vacuoles during seed maturation (for stage VII, see Figure 7), which have long been known to contain storage proteins (Bewley and Black, 1994). At earlier stages labelling in parenchyma cells is found within the vacuolar space (stage V) or concentrated in membranous patches within that vacuoles (stage VI)(not shown). In all investigated stages we never saw discrete labelling of nuclei (see below).

To test the *VfSBPL* vicilin sequence in an *in planta* assay for a possible role related to sucrose the coding sequence was engineered behind the patatin promoter B33 (Rocha-Sosa *et al*., 1989; kindly provided by U. Sonnewald, IPK Gatersleben) and transformed into potato plants in Dr Sonnewald's group. Extensive investigations of several independent lines containing relatively high levels of *VfSBPL* mRNA as detected by northern blotting revealed no detectable changes in soluble sugars (glucose, fructose, sucrose) and starch during tuber development (Heim *et al*., unpublished results).

SBP proteins may fulfil different functions during seed development

The functional characterization of the first isolated 62 kDa-protein from soybean seeds (GmSBP) as sucrose-binding protein was based on several lines of evidence as the tight plasma membrane association and the immunolocalization in phloem companion cells and cotyledon transfer cells (see Grimes *et al*., 1992). The data published on functional characterization in yeast (Overvoorde *et al*., 1996) suggest only very limited transport into the yeast cell which could be explained by facilitating sucrose diffusion in an unknown way. Recently Pedra *et al*. (2000) reported that a soybean cDNA coding for a 64 kDa GmSBP, closely related to the 62 kDa GmSBP, affects plant growth and carbohydrate partitioning in leaves of transgenic tobacco plants when expressed in sense or antisense orientation under control of the 35S promoter. A final interpretation of the data would need the sequence of the tobacco SBP mRNA but the reported results are clearly different from our unpublished results with transgenic potato plants (see above), which failed to show any measurable changes in the carbohydrate status of tubers over-expressing *VfSBPL*.

The easiest interpretation of the discrepancies between the conclusions worked out in soybean and our data is that the *VfSBPL* gene we describe is not only different in size but also in function. It shows several characteristics of a storage protein like the structurally related vicilin storage proteins at least during the maturation stage of seed development. However, the gene has been separated from typical vicilin storage pro-

tein genes early in evolution and could, therefore, have kept or acquired additional functions. This is not impossible since, for instance, patatin beside of its storage function has still enzymatic activity (Rosahl *et al*., 1987). Seed storage globulins have their evolutionary roots in desiccation-related proteins of fungi and fern spores and, therefore, a role of SBPs in the adjustment of osmotic pressure by weak binding of sucrose would be not unlikely (Shutov *et al*., 1998).

Yet another function with respect to desiccation was recently suggested by the work of Castillo *et al*. (2000). These authors purified a 16 kDa protein (p16) from nuclei of ungerminated pea embryo axis and found the coding sequence to be the 3′-terminal part of a pea cDNA encoding a 54.4 kDa polypeptide 85% homologous to VfSBPL described here. Interestingly, p16 was localized in preliminary immunolocalization studies both to nuclei and protein bodies. At present, we have no indication of a similar p16 polypeptide in faba beans, since in western blot experiments we did not see a ca. 16 kDa band nor did we find label over nuclei in immunolocalization studies. The antibody used which may not recognize a respective epitope, could easily explain this failure. Thus, the functions of the SBP-type proteins remain enigmatic in spite of a considerable amount of data and further clarifying experiments are necessary.

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