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ORIGINAL ARTICLE

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Isolation of S-RNase binding proteins from *Solanum chacoense*: identification of an SBP1 (RING finger protein) orthologue

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Abstract Currently, the most attractive working model of gametophytic self-incompatibility (SI) involving S-RNases postulates the presence of an inhibitor protein or complex expressed in pollen tubes that would counteract the cytotoxic effect of the ribonuclease activity of the S-RNase. Since it has been previously shown that allele-specific recognition is mediated through the hypervariable domain sequence of the S-RNase, we have targeted this region to isolate pollen-expressed interacting proteins in the yeast two-hybrid system. One of the isolated proteins corresponds to a RING finger protein highly similar to the previously isolated SBP1 protein from *Petunia hybrida*. This protein is postulated to be part of the RING finger E3 ligase family. The *ScSBP1* gene is expressed in almost all tissues tested, suggesting a more general role than only being involved in SI. Although the *ScSBP1* gene is polymorphic, linkage analysis showed that it was unlinked to the S-locus. The isolation of this S-RNase-binding protein in two different species and with four different S-RNase sequences as bait, strengthens its putative involvement in the SI response. Furthermore, comparison of the bait sequences used suggests that the SBP1 protein interacts with conserved sequences located between the HVa and HVb domains.

Keywords S-RNase binding protein · Gametophytic self-incompatibility · RING finger protein · *Solanum chacoense*

Genbank accession numbers: *ScSBP1*, AY545464

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Introduction

Self-incompatibility (SI) constitutes an important mechanism for preventing inbreeding through specific pollen recognition and rejection. In gametophytic self-incompatibility (GSI), the haploid pollen is rejected when the S-allele it expresses matches either of the two S-alleles expressed in the sporophytic tissue of the pistil (de Nettancourt 1997). In the Solanaceae, the Rosaceae, and the Scrophulariaceae, the GSI phenotype is specified by a highly multiallelic S-locus involving the expression of stylar ribonucleases. Pollen recognition occurs in the transmitting tissue of the style, where S allele-specific ribonucleases (S-RNases) trigger pollen tube RNA degradation (McClure et al. 1989). Gain of function experiments in various solanaceous species have shown that expression of a catalytically active S-RNase transgene is sufficient to alter the SI phenotype of the style without affecting the recognition properties of the pollen (Huang et al. 1994; Matton et al. 1997; Murfett et al. 1994). Few other genes affecting the SI phenotype have been identified and characterized at the molecular level. In fact, although the genetic background of the plant has clearly been shown to be involved in the SI reaction (Bernatzky et al. 1995; Kondo et al. 2002; Murfett et al. 1996), only one other gene, the stylar-expressed HT modifier, has been isolated and shown to be involved in the SI phenotype in both *Nicotiana* (McClure et al. 1999) and *Solanum* species (Kondo et al. 2002; O'Brien et al. 2002). Like the S-RNase gene, the *HT* gene affects only the SI phenotype of the style, reinforcing the hypothesis that Pollen-S is encoded by a different gene. Since S-RNases enter the pollen tube in both self-compatible (SC) and SI situations (Luu et al. 2000), the most attractive working model of SI postulates the presence of an inhibitor protein expressed in pollen. This would counteract the cytotoxic effect of the ribonuclease activity of the S-RNase on pollen tube RNAs. The inhibitor model is supported by recent evidence obtained from pollen-part mutants in *Nicotiana alata* (Golz et al. 2001), and from diploid S-heteroallelic pollen grains in *Solanum chacoense* (Luu et al. 2001). Although

the identity of the Pollen-S gene remains unknown, recently an F-box protein has been identified in the S locus of many GSI species (Entani et al. 2003; Lai et al. 2002; Ushijima et al. 2003; Yamane et al. 2003; Zhou et al. 2003). This gene, called *SLF* (S-locus F-box), fulfills all the genetic requirement to be a candidate Pollen-S gene: it is tightly linked to the S-RNase at the S-locus; it is gametophytically expressed in pollen; and it is also highly polymorphic, like the S-RNase gene. Furthermore, it has recently been shown to physically interact with S-RNases (Qiao et al. 2004). Since no gain or loss of function experiments have proven that this polymorphic F-box protein is the Pollen-S gene, the function of this gene remains debatable. Nevertheless, F-box proteins have been shown to be involved in the degradation of specific protein substrates (del Pozo and Estelle 2000; Kipreos and Pagano 2000), and the SLF protein would fit well into the inhibitor model, where it could target S-RNases for degradation through the ubiquitin-proteasome pathway. This, in conjunction with the identification of a RING-finger domain protein as an S-RNase-binding protein (*Petunia* SBP1 protein; Sims and Ordanic 2001), support a view where the outcome of a given SI or SC reaction would be determined by the protein degradation machinery.

In order to determine if other genes are involved in SI, we have initiated a two-hybrid screen to isolate pollen proteins that can interact with the S-RNase. Since we have previously demonstrated the role of the hypervariable regions in the allele-specific recognition and rejection process (Matton et al. 1997, 1999), in this paper we focus on proteins that interact specifically with the two hypervariable regions in S-RNases, and particularly, on the characterization of a *S. chacoense* SBP1 homologue.

Materials and methods

Plant material

The diploid, SI, wild potato *Solanum chacoense* Bitt. (2*n*=2×=24) was grown in a greenhouse with 14–16 h light per day. The genotypes used were IP 230582 (S-alleles S13 and S14) as female progenitor and IP 458314 (S-alleles S11 and S12) as pollen donor for the segregating population analysis. G4 genotype (S-alleles S12 and S14) was used as a female progenitor with V22 (S-alleles S11 and S13) as pollen donor for all other analyses. Pollen tube messenger RNA was obtained from in-vitro-grown pollen tubes by spreading V22 pollen grains on sterile nitrocellulose membranes laid over sterile Petri dishes containing 0.1 mM calcium chloride, 0.2 mM boric acid, 10% sucrose and 1% agar, and incubating for 24 h at room temperature. All other RNA used for RNA gel blot analyses was extracted from G4 plants.

Two-hybrid cDNA library construction

Total RNA from pollen tubes grown in vitro was isolated as described previously (Jones et al. 1985). Poly(A)+ RNA was purified from total RNA by affinity chromatography using oligo dT agarose beads type 7 (Amersham Pharmacia, Montréal, Québec, Canada). The library was constructed in the Hybri-Zap two-hybrid vector following the manufacturer's instruction (Stratagene, La Jolla, Calif.). Approximately 1 million clones were mass-excised from this library and plasmid DNA was isolated by the alkaline lysis method for subsequent yeast two-hybrid screening (Sambrook et al. 1989).

Construction of Gal4-BD bait plasmids

In order to amplify the hypervariable domains of the endogenous S-RNases, mRNA from *S. chacoense* styles of the V22 genotype (S-alleles S11S13) was extracted with an RNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario, Canada). RNA (1 µg) was reverse transcribed with 2 µM oligo dT20 and 120 U MMLV reverse transcriptase following the manufacturer's instruction (Gibco BRL, Burlington, Ontario, Canada). PCR amplification was carried out with *PWO* polymerase (Roche, Laval, Québec, Canada) and gene specific primers flanking the HVa and HVb hypervariable regions of the S11 and S13 RNases. The two specific primers were designed to amplify both S11 and S13 RNases (upstream primer 5′-GAGA-GAATTCGAGGGACCACAGCTGTTG-3′; downstream primer 5′-GAGAGTCGACGGATCCATGCTTTAGATATTG-3′) with added *Eco*RI and *Sal*I restriction sites (underlined) respectively for cloning in the pBD-Gal4 plasmid. The HV region was amplified with 25 ng reverse transcription product as DNA template. Thermocycling conditions were as follows: 35 cycles with a denaturing step of 30 s at 94°C, an annealing step at 52.5°C for 30 s (with a 0.2°C increment for each following cycle), and a 2-min polymerization step at 72°C. PCR products were digested with 10 U *Eco*RI and *Sal*I restriction enzymes following the conditions recommended by the manufacturer (New England Biolabs, Mississauga, Ontario, Canada). Digested PCR products were ligated with T4 DNA ligase (Gibco BRL) in a predigested pBD plasmid to create an in-frame chimeric GAL4-binding domain construct. Sorting of S11 and S13 S-RNase alleles was carried out by sequencing. One clone for each allele (pBD-S11HVab and pBD-S13HVab) was used to transform yeast strain PJ69-4A using the TRAFO yeast transformation procedure (Gietz and Woods 2002).

Yeast two-hybrid screening

Two-hybrid screenings were carried out with *Saccharomyces cerevisiae* PJ69-4A strain (MATa, *trp 1-901*, *leu2-3*, *112 ura3-52*, *his3-200*, *gal4*, *gal 80*, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ) previously transformed with either the pBD-S11HVab or the pBD-S13HVab bait constructs. False positives, through auto-activation of the His reporter genes by the bait construct alone, were prevented by the addition of 1 mM 3-amino-1,2,4-triazole (Sigma-Aldrich, Oakville, Ontario, Canada) to the solid medium. cDNA (100 µg) derived from the mass-excised pollen tube cDNA library was used to transform the bait-containing PJ69-4A strain. Screening was performed according to the TRAFO procedure. Positive clones were considered only if they successfully passed all three selection criteria: (1) growth on medium lacking histidine; (2) growth on medium lacking adenine; (3) expression of β-galactosidase activity. Positively scored clones were validated by segregation analysis to eliminate auto-activating prey protein and sequenced using an Applied Biosystems ABI Prism 3100 automated sequencer.

Isolation and gel blot analysis of RNA and DNA

RNA extraction was conducted as described previously (Jones et al. 1985). For each tissue tested, 10 µg RNA was separated in a formaldehyde/MOPS gel, then transferred to Hybond N+ membranes (Amersham Pharmacia), and fixed by UV crosslinking (120 mJ/cm^2). To confirm equal loading between RNA samples, a 1 kb fragment of *S. chacoense* 18S RNA was PCR amplified and used as a control probe. Prehybridization was performed at 45°C for 3 h in 50% formamide solution (50% deionized formamide, 6× SCC, 5× Denhardt's solution, 0.5% SDS and 200 µg/µl denatured

salmon DNA). Hybridization of the membranes was performed overnight at 45°C in the same solution. Genomic DNA isolation was performed by a modified CTAB extraction method (Reiter et al. 1992). DNA (10 µg) was digested completely overnight with 20 U of either *Eco*RI, *Eco*RV or *Hin*dIII as recommended by the supplier (Roche). DNA gel blot analyses were performed as described by Sambrook et al. (1989) and DNA was transferred to Hybond N+ membranes prior to UV crosslinking. Prehybridization and hybridization were performed for 3 h at 65°C in 50% phosphate solution (50% of 0.5 M Na_2PO_4 pH 8.0, 1% BSA, 7% SDS, 1 mM EDTA).

Probes used for the detection of the *ScSBP1* gene and transcripts were synthesized from a full-length cDNA by random labeling using the Strip-EZ DNA labeling kit (Ambion, Austin, Tex.) in the presence of α-^{32}P dATP (ICN Biochemicals, Irvine, Calif.). Following hybridization, membranes were washed 30 min at 25°C and 30 min at 35°C in 2× SSC/0.1% SDS; 30 min at 45°C and 30 min at 55°C in 1× SSC/0.1% SDS; and finally 10 min at 55°C in 0.1× SSC/0.1% SDS. Prior to the 18S cRNA probe hybridization, blots were stripped following the manufacturer's instructions (Ambion). Autoradiography was performed at −86°C on Kodak Biomax MR film (Interscience, Markham, Ontario, Canada).

In vitro transcription and translation

Coupled in vitro transcription/translation was performed in the presence of ^{35}S-labeled methionine in the TNT coupled wheat germ extract system (Promega, Madison, Wis.) following the manufacturer's instructions. Protein separation was performed on a 10% SDS-PAGE gel and estimation of the size of the translation products was determined using Kaleidoscope Prestained MW protein standards (Bio-Rad, Mississauga, Ontario, Canada).

Results and discussion

Yeast two-hybrid screening

To isolate pollen proteins that could interact specifically with the S-RNases, a two-hybrid screen was performed using the region covering the two hypervariable domains (HVa and HVb) of the *S. chacoense* S11 and S13 RNases (Matton et al. 1997). Several lines of evidence suggest that the HV regions are involved in allele-specific recognition of the pollen-S protein. Firstly, the HV regions have been shown by site-directed mutagenesis to be sufficient for S-allele recognition and rejection (Matton et al. 1997, 1999, 2000). Secondly, it has been shown by 3-D protein modeling that the HV regions adopt a helix-turn-helix conformation that could be involved in protein/protein interactions (Parry et al. 1998). Thirdly, a crystal structure of the SF11 RNase from *N. alata* has shown that the HV regions are exposed on the molecule's surface and are clearly separate from the catalytic domain (Ida et al. 2001). The region covering S11 HV domains was thus PCR-amplified, inserted into the yeast two-hybrid pBD-Gal4 vector, and used to screen a prey library constructed from mRNAs isolated from in-vitro-grown pollen tubes (from a S11S13 parent genotype). Six million cotransformed yeast cells were plated and, at the end of the stringent selection process, five yeast colonies survived on media lacking histidine and adenine. Furthermore, these five colonies also expressed significant β-galactosidase activity (data not shown). DNA sequencing analysis revealed four different clones: a phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)—a gene involved in late stages of purine biosynthesis; a secretory peroxidase; two peroxisomal targeting signal 1 receptor (PEX5)-related proteins; and a RING-finger protein highly similar to the *SBP1* gene from *Petunia hybrida* (Fig. 1) (Sims and Ordanic 2001). Since the PEX5 protein has been found in other yeast two-hybrid screens with unrelated bait proteins, it was considered a false positive and discarded. The *Petunia* S-RNase binding protein 1 was originally isolated from an anther library in a yeast two-hybrid screening with the N-terminal portion of S1-RNase as a bait (Sims and Ordanic 2001). Hence, we named our S11 HV domain interacting protein ScSBP1 for *Solanum chacoense* SBP1, which will be described hereafter.

ScSBP1 protein interaction was further analyzed with the HV domain of S13-RNase. Binding properties were similar to those of the HV domain of S11-RNase (Fig. 1C, S11 HVab and S13 HVab) with both constructs showing

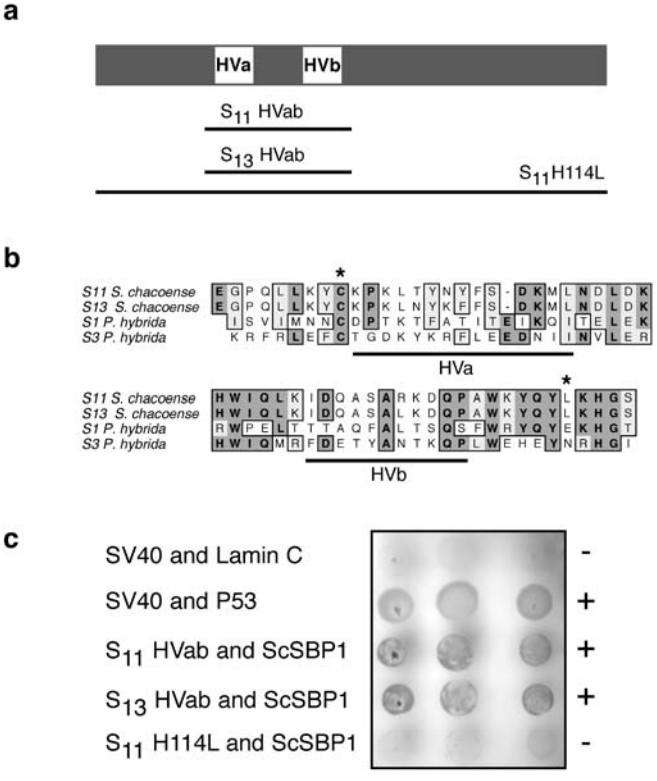

Fig. 1a–c Yeast two-hybrid screening of S-RNase hypervariable (HV) domain interacting proteins. **a** Schematic representation of S-RNase protein regions (*underlined*) used for yeast two-hybrid bait constructs. S11RNaseH114L is a mutated S11-RNase that has a leucine instead of a histidine in its catalytic domain (O'Brien et al. 2002). **b** Amino acid alignment of the S11-HVab and S13-HVab bait constructs used for the two-hybrid screen. The S-RNase HV domains are underlined. *Asterisks* delimit the shortest bait constructs used in *Petunia hybrida* by Sims and Ordanic (2001). **c** Yeast growth on selective medium. Co-transformed yeast cells were transferred to medium lacking adenine after initial histidine selection. SV40 and Lamin C, and SV40 and p53 represent negative and positive controls, respectively, in the HybriZap two-hybrid system used. + Protein-protein interaction between bait and prey proteins, −absence of interaction with only background level yeast growth

similar yeast growth on selective media. Although quite similar at the amino acid level, these two S-RNases behaved differently in crossing experiments (Matton et al. 1997, 1999), suggesting that the ScSBP1 protein binds S-RNases without allele specificity. This was also observed with the S1 and S3-RNase alleles of *P. hybrida* (Sims and Ordanic 2001). Since using quite divergent HV domains in four S-RNases from two species all resulted in the isolation of the SBP1 protein, this interaction is most probably biologically relevant for the SI process. Furthermore, this also suggests that HV domain interaction with the SBP1 protein might be mediated by the amino acid region between the HVa and HVb domains, which are more conserved between the S-RNases (Fig. 1B). Although the smallest HV constructs used by Sims and Ordanic (2001) also show some overlap with the C-terminal part of the HVb region used in this study (the S1-HV and S3-HV constructs encompass the region delineated by asterisks in Fig. 1B), it is improbable that this region is involved in SBP1 binding since it is quite divergent in the Petunia S3-RNase. When a full-length S11-RNase construct was used for direct protein interaction, ScSBP1 binding could not be detected (Fig. 1C, S11H114L). This had also been observed in *P. hybrida*, where the SBP1 protein could not interact with full-length S1-RNase (Sims and Ordanic 2001). One possibility suggested by the authors is that the full-length S-RNase is lethal to the yeast cell due to its intrinsic ribonuclease activity. As such, only improperly folded fusion protein would be stable in the yeast cell and hence limit protein/protein interactions with SBP1. In this study we used a mutated S11-RNase where histidine 114 has been replaced by a leucine (O'Brien et al. 2002). The catalytic activity of this fusion protein has been lost, but still no binding with the full length S-RNase could be obtained (Fig. 1C, S11H114L), suggesting that lack of protein-protein interaction between SBP1 and the full-length S-RNases is probably due to improper folding, irrespective of the catalytic activity of the S-RNase. Since the S-RNase is normally secreted, and possesses multiple disulfide bridges, it is most probable that, in the absence of these disulfide bridges, no properly folded S-RNases can be produced intracellularly in yeast cells.

SBP1 sequence analysis

Since the ScSBP1 protein isolated in the two-hybrid screen was not full-length, we screened our internal *S. chacoense* EST database for a longer clone. One clone perfectly matched the original *ScSBP1* clone. This longer *ScSBP1* cDNA (accession number AY545464) was 1,253 bp long (excluding the polyA tail) and corresponded to the size of the mRNA, as determined by RNA gel blot

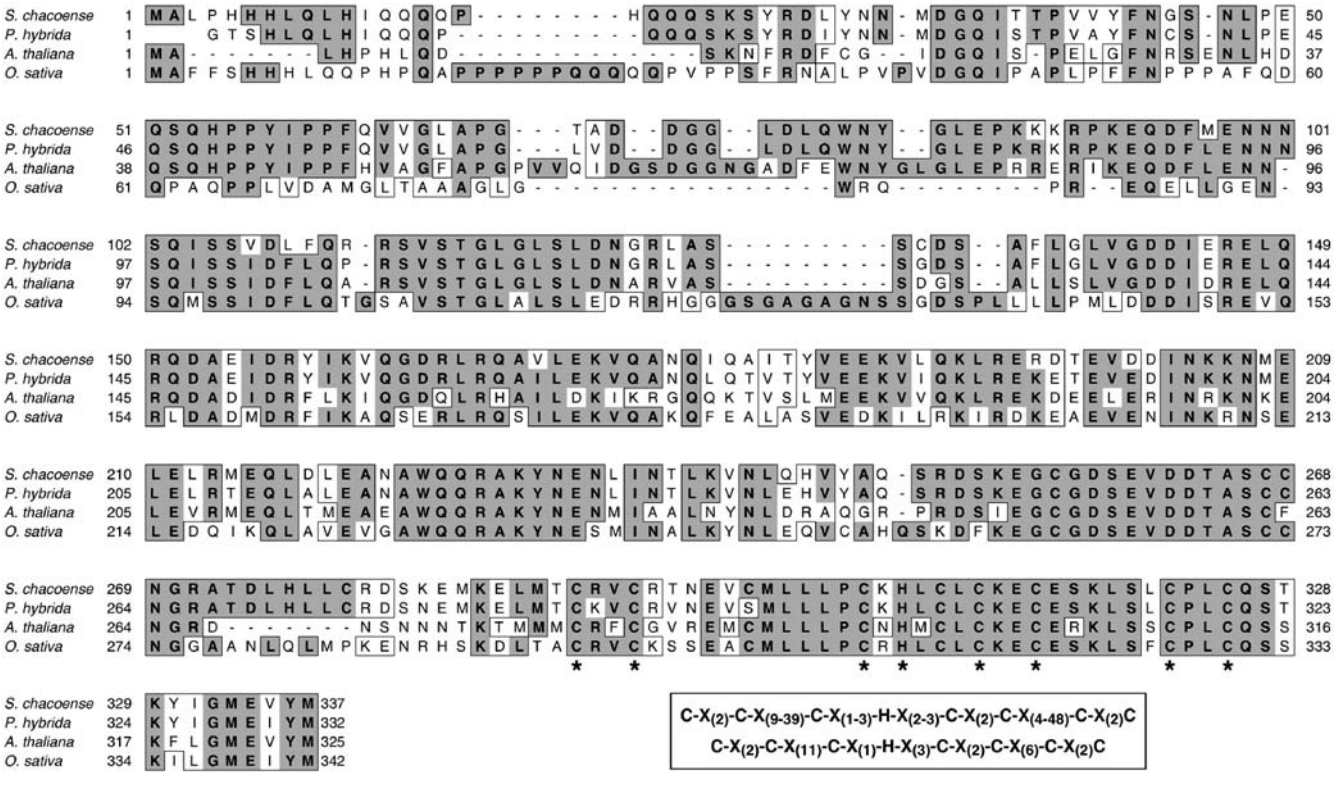

Fig. 2 Alignment of the deduced protein sequences of *Solanum chacoense* SBP1 (ScSBP1) with SBP1-like proteins from *P. hybrida* (accession number AAF28357), and similar proteins from *Arabidopsis thaliana* (accession number At1g45976), and rice (accession number NP_912418). Dark and light gray shading represents amino acid identity and similarity, respectively. Asterisks represent the cysteine-histidine signature typical of the RING-HC finger motif. The alignment was constructed with ClustalW in MacVector 7.2; gaps were introduced manually in the N-terminal region to correctly align the sequences. *Inset* Characteristic spacing of conserved cysteine and histidine residues in RING finger domains (above) and the exact spacing found in the SBP1-like proteins (below)

analysis (~1.3 kb, Fig. 3), suggesting that the *ScSBP1* cDNA is full-length or near full-length. It has two putative initiation codons separated by 30 amino acids, encoding open reading frames (ORFs) of 337 and 306 amino acids, respectively (Fig. 2). Both initiation codons are in optimal ATG context (Joshi et al. 1997; Lukaszewicz et al. 2000), and no stop codon could be found upstream of the first methionine corresponding to the longest ORF. The estimated MWs of the two ORFs are 38,5 kDa and 34,7 kDa. The shortest ORF corresponds to the previously published *P. hybrida* SBP1 protein (also 306 amino acids long). In order to determine if the first or second ATG encountered was used preferentially, a coupled in vitro transcription/translation reaction was performed in a wheat germ extract. Only one protein band of 39 kDa could be detected on the SDS-PAGE gel (data not shown), suggesting that only the first ATG encountered is used, at least in a plant in vitro translation system.

The deduced ScSBP1 protein showed strongest amino acid sequence identity with another *S. chacoense* SBP1-like cDNA (AY390600) recently deposited in GenBank (96% identity); with the deduced SBP1 protein from *P. hybrida* (90% identity, 95% similarity); with a hypothetical protein from *Arabidopsis thaliana* (59% identity, 73% similarity), and with an uncharacterized protein from rice with 46% identity (72% similarity). An amino acid alignment of these proteins is shown in Fig. 2, except for the deduced AY390600 protein sequence since it is most probably an allelic variant of ScSBP1. All SBP1-like proteins have a RING finger protein domain in their C-terminus, spanning amino acids 140–337 in the ScSBP1 protein (Freemont 2000). Conserved cysteine residues are present in the four SBP1-like proteins within a C-X-X-C-X(9–39)-C-X(1–3)-H-X(2–3)-C-X-X-C-X(4–48)-C-X-X-C context (asterisks in Fig. 2, and inset), which characterize this group of protein as RING-HC proteins. RING finger proteins mediate protein-protein interactions, and a growing body of evidence defines them as E3 ubiquitin protein ligases (Freemont 2000).

ScSBP1 mRNA expression analysis

The *ScSBP1* expression pattern was determined by RNA gel blot analysis (Fig. 3, upper panel) with various vegetative (petals, stems, leaves, roots and tubers) and reproductive (stamens, pollen, pollen tubes, fertilized ovules 2, 3, 4, 5 and 6 days after pollination, and pollinated styles and ovaries 0 and 48 h post-pollination) tissues. Equal loading of mRNA sample in each lane was verified with an 18S cRNA probe (lower panels). *ScSBP1* mRNAs could be detected in all tissues tested except tubers, and displayed an expression pattern similar to that determined for the *P. hybrida SBP1* gene in common tissues tested (Sims and Ordanic 2001), although strict comparison is not possible since we used RNA gel blot analyses, and *Petunia SBP1* expression was analyzed by RT-PCR. Although the *ScSBP1* gene has been identified in a yeast two-hybrid screen from a cDNA library constructed from pollen tube mRNAs, it is expressed in virtually all tissues tested. This suggests that it has a more general function than only being involved in the SI response. The possibility that the SBP1 protein acts as a ubiquitin ligase, as suggested by the presence of a RING-HC domain (Freemont 2000), would suggest that it could be involved in general protein degradation, specificity being determined by another ancillary protein, similarly to the SCF ubiquitin ligase complex involved in auxin regulated responses (Gray et al. 1999; Hellmann and Estelle 2002).

ScSBP1 copy number and linkage to the S-locus

ScSBP1 gene copy number was determined by digestion of *S. chacoense* genomic DNA followed by DNA gel blot analysis of the restriction fragments obtained. A labeled full-length *ScSBP1* cDNA was used to probe the membrane (Fig. 4A). Genomic digestion with *Hin*dIII revealed two restriction fragments (0.8 kb and 4 kb), while digestion with either *Eco*RI or *Eco*RV revealed a single DNA band of approximately 1.6 kb and 3 kb, respectively. The two bands resulting from *Hin*dIII digestion could be explained by the presence of a restriction site at position 980–985, and only one band was expected in the *Eco*RI digest since no restriction site was detected in the cDNA sequence. Oddly, only one fragment for *Eco*RV was detected in the DNA gel blot analysis (Fig. 4A), despite the fact that an *Eco*RV restriction site could be detected in the *ScSBP1* cDNA sequence (at position 632–637). Because our *ScSBP1* cDNA was obtained from a different genotype than the one used for DNA gel blot analysis, this could be explained by the presence of polymorphic alleles for the *ScSBP1* gene. A recently submitted sequence of an *SBP-1* like gene (AY390600), also from *S. chacoense* and 96% identical at the amino acid level to ScSBP1, supports this hypothesis. A similar situation was previously observed for the *ScHT* SI modifier gene, which showed polymorphism, although being unlinked to the S-locus (O'Brien et al. 2002). This led us to assess if the *ScSBP1* gene was polymorphic and linked to the S-locus. A population of individuals segregating for the S-locus was derived from a cross between S11S12 and S13S14 parents.

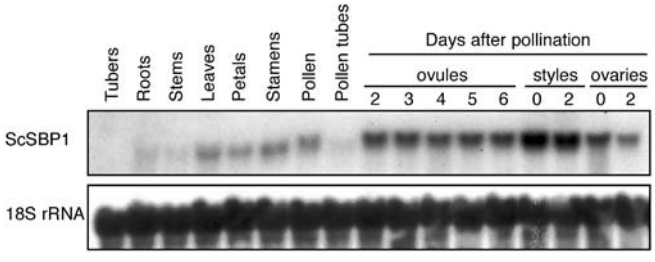

Fig. 3 RNA expression analysis of the *ScSBP1* gene in various *S. chacoense* tissues. *ScSBP1* mRNA accumulation was determined in vegetative and reproductive tissues as well as in female reproductive tissues after pollination. Total RNA (10 μg) from each tissue was probed with the *ScSBP1* cDNA insert, then stripped and reprobed with the 18S cRNA gene as a loading control

Genomic DNA from these individuals was digested with *Eco*RV (Fig. 4B, upper panel) or *Hin*dIII (Fig. 4B, middle panel). In both cases the *ScSBP1* appeared polymorphic. Some individuals showed two bands for the *Eco*RV enzyme as predicted from the cDNA sequence, while other individuals showed only one band (Fig. 4B, upper panel). The *Hin*dIII enzyme digestion resulted in double or triple band patterns. Only the higher molecular weight bands are shown (Fig. 4B, middle panel). A lower molecular weight band detected in all individuals corresponded to the 0.8 kb *Hin*dIII fragment shown in Fig. 4A (not shown in Fig. 4B). This polymorphism was not detected for the *P. hybrida SBP1* gene (Sims and Ordanic 2001). In order to determine linkage to the S-locus, the DNA gel blots were then probed with S14-RNase. Figure 4B (lower panel) revealed that the *ScSBP1* gene is not linked to the S-locus, since no strict correlation could be made between the different banding patterns shown in Fig. 4B (upper and middle panels) and the S14-RNase segregation pattern (Fig. 4B, lower panel).

Conclusion

Using a two-hybrid strategy to isolate proteins that could interact specifically with the hypervariable domains of the S-RNases, we have retrieved four different proteins, one of them corresponding to a previously isolated S-RNase-binding protein from *P. hybrida* (Sims and Ordanic 2001). In this study, we have extended our current knowledge of this protein, which could be involved in the SI response. The fact that two independent screens in two different SI species recovered the same protein strongly suggests that this protein-protein interaction is biologically relevant to the SI response. Furthermore, by comparing the constructs used to isolate the *P. hybrida* and *S. chacoense* SBP1 proteins, we propose, based on the high sequence divergence between the HV domains of the S-RNase used in this study and in the study of Sims and Ordanic (2001), that the conserved region between the HVa and HVb region is the domain most probably interacting with the SBP1 protein. Alternatively, a particular structural feature, like the helix-turn-helix motif predicted for the HVa-HVb region (Ida et al. 2001; Parry et al. 1998) could be recognized. The absence of an allele-specific interaction in the two studies supports these two possibilities, and implies that another protein provides the specificity factor needed for allele-specific recognition and SI rejection response.

Recently, an F-box protein has been characterized in two different plant families that share a GSI system based on S-RNases: the *Scrophulariaceae* (Lai et al. 2002) and the *Rosaceae* (Entani et al. 2003). F-box proteins have been characterized as components of the SCF complexes that specifically bind substrates for ubiquitin-mediated proteolysis by the 26S proteosome (Kipreos and Pagano 2000). This S-locus F-box gene shows the typical characteristics of a putative Pollen-S gene, being highly polymorphic and tightly linked to S-RNase at the S-locus. Furthermore, physical interaction between S-RNases and the *Antirrhinum* AhSLF-S2 F-Box protein has been recently demonstrated, as well as interaction of this F-box protein with Skp1- and Cullin-like proteins, suggesting the formation of an SCF-type complex. Ubiquitination of S-RNases during compatible pollinations has also been demonstrated (Qiao et al. 2004). As mentioned by the latter authors, this suggests that the AhSLF-S2 F-Box protein could be a general inhibitor of S-RNases, targeting them to the ubiquitin-proteasome pathway. The absence, or reduction in the total amount, of S-RNases would enable pollen tube growth in compatible pollination. Conversely, inhibition of S-RNase destruction, either through a competitive interaction with another F-Box protein (specificity factor) or a modified AhSLF-S2 protein, would lead to pollen tube growth arrest mediated by the cytotoxic activity of the S-RNases. Although in most SCF-ubiquitin ligase complexes, substrate targeting is determined by the modular F-box protein, the RING finger protein has also been shown to be involved in controlling ubiquitination specificity (Noureddine et al. 2002). Thus, it is possible that the SBP1 protein could be the RING finger protein part of the E3-ubiquitin ligase complex that targets the S-RNases for degradation. This RING finger protein could contribute to ubiquitination complex formation specificity, as suggested by its interaction with S-RNases. Furthermore, prior to ubiquitination, proteins bound for degradation by the 26S proteasome are often tagged by a posttranslational modification like phosphorylation (Bachmair et al. 2001). Indeed, previous data described the non-allele specific phosphorylation of S-RNases by a calcium-dependent protein

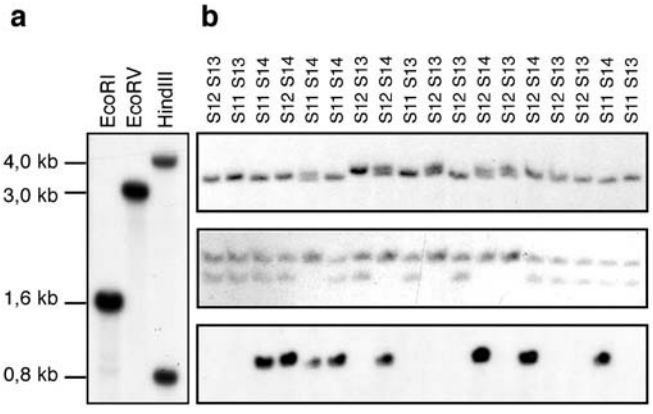

Fig. 4a,b DNA gel blot analysis of the *ScSBP1* gene and linkage analysis with the S-locus. **a** *ScSBP1* gene copy number analysis. Genomic DNA (10 µg) isolated from *S. chacoense* leaves was digested with *Eco*RI, *Eco*RV or *Hin*dIII restriction enzymes, and probed with the complete *ScSBP1* cDNA insert. Molecular weights of the fragments appear on the left. **b** Linkage analysis of the *ScSBP1* gene with the S-locus. Genomic DNA (10 µg) from *S. chacoense* leaves isolated from an F1 population (S11S12 × S13S14) segregating for four S-alleles was digested with either *Eco*RV (*upper panel*) or *Hin*dIII (*middle panel*). Identical DNA gel blots had been previously probed with the corresponding S-RNase cDNAs to determine the genotype of the plants (labeled above each lane) and tested by crossings. One S-allele hybridization is shown for the S14-RNase (*lower panel*, *Eco*RV digestion)

kinase in the pollen tube of *N. alata* (Kunz et al. 1996). Confirmation of the role of the SBP1 proteins and of the different proteins involved in the SCF complex, awaits transgenic experiments to determine the role of protein degradation and the nature of the specificity factor in S-RNase-mediated SI systems.

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