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The *S* locus receptor kinase gene encodes a soluble glycoprotein corresponding to the SRK extracellular domain in *Brassica oleracea*.

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Summary

Self-incompatibility in *Brassica* is controlled by the *S* locus which contains at least two genes. *SLG* encodes a secreted *S* locus glycoprotein whilst *SRK* encodes a putative *S* locus receptor kinase which consists of three domains: an extracellular domain sharing extensive sequence identity with SLG, a transmembrane region, and a cytoplasmic domain exhibiting a serine/threonine protein kinase activity. Here, we report the existence of truncated forms of the SRK protein corresponding to the extracellular domain of the putative receptor. These proteins were detected by an antibody which recognizes the N-terminus of SRK₃ and, in an F₂ progeny segregating for the *S*₃ haplotype, were only expressed in plants possessing the *S*₃ haplotype. The truncated SRK proteins were expressed specifically in stigmas but, unlike the membrane-spanning SRK₃ protein, were soluble and occurred as four different glycoforms sharing the same amino acid backbone as shown by deglycosylation experiments. Several *SRK*₃ transcripts that may code for these truncated SRK₃ proteins have been identified by RACE

PCR, stigma cDNA library screening and RNA blot analysis. These transcripts are apparently generated by a combination of alternative splicing and the use of alternative polyadenylation signals. The existence of truncated forms of the *S* locus receptor kinase highlights some similarities between plant and animal receptor kinases. In animals, soluble extracellular domains of receptors have been described and, in some cases, have been shown to play a role in the modulation of signal transduction. By analogy, the soluble, truncated SRK proteins may play a similar role in the self-incompatibility response.

Introduction

In the Brassicaceae, the self-incompatibility response which prevents self-fertilization is characterized by the rejection of self-pollen grains on the papillar cells of the stigma. This response is controlled genetically by a single multiallelic locus, the *S* locus, which exists as more than 50 allelic variants (Ockendon, 1974, 1982). Rejection of self-pollen occurs when the same *S* allele is expressed both in pollen and stigma. Numerous molecular studies performed in the genus *Brassica* have revealed the complexity of the *S* locus, which consists of at least two physically linked genes (Boyes and Nasrallah, 1993): the *S* Locus Glycoprotein (*SLG*) gene which encodes an abundantly secreted glycoprotein (Nasrallah *et al.*, 1985, 1987) and the *S* locus Receptor Kinase (*SRK*) gene which encodes a membrane-spanning protein kinase with an extracellular domain sharing extensive sequence identity (around 90%) with the *SLG* gene (Stein *et al.*, 1991). Because of the presence of at least two transcriptional units at the *S* locus, the *S* allelic variants have been referred to as *S* haplotypes by Boyes and Nasrallah (1993). Two major classes of *S* haplotypes have been defined according to criteria based on polymorphism among *SLG* sequences and on the dominance or recessive interactions occurring between *S* haplotypes in heterozygous plants (Nasrallah *et al.*, 1991). Class I *S* haplotypes occupy a high position in the dominance series and exhibit a strong self-incompatibility phenotype. Class II is composed of the three pollen recessive haplotypes *S2*, *S5* and *S15*, which exhibit a weak self-incompatibility phenotype. In *Brassica oleracea*, sequence information is

available for nine *SLG* alleles, and in four cases the corresponding *SRK* gene has also been characterized (the S_3 , S_6 and S_{29} haplotypes of class I, Delorme *et al.*, 1995; Kumar and Trick, 1994; Stein *et al.*, 1991 and the S_2 haplotype of class II, Stein *et al.*, 1991). From these molecular investigations some features can be identified as being common to *S* locus genes of both classes. All *SLG* alleles reported to date but one, SLG_2 from the S_2 haplotype, encode a single 1.6 kb transcript (Delorme *et al.*, 1995; Gaude *et al.*, 1993; Goring *et al.*, 1992a,b; Nasrallah *et al.*, 1987, 1988; Trick and Flavell, 1989). More detailed characterization of the SLG_2 gene revealed that it contains an intron and produces two transcripts. One transcript of 1.6 kb encodes the expected secreted glycoprotein, while the second transcript of 1.8 kb codes for a membrane-anchored SLG protein (Tantikanjana *et al.*, 1993). A 1.8 kb transcript was not found in the class I S_6 haplotype (Tantikanjana *et al.*, 1993). The structure of the *SRK* gene is more complex and consists of seven exons interspaced with six introns (Delorme *et al.*, 1995; Kumar and Trick, 1994; Stein *et al.*, 1991). Several sizes of *SRK* transcript have been detected in stigma tissues including three transcripts of 4.1, 3.0 and 2.3 kb which are thought to correspond to an unspliced *SRK* transcript, a fully spliced *SRK* transcript and an alternatively spliced transcript, respectively. Interestingly, all alleles of the *SRK* gene analysed to date possess an in-frame stop codon directly following the conserved GT motif at the 5' end of the first intron (Delorme *et al.*, 1995; Kumar and Trick, 1994; Stein *et al.*, 1991). This stop codon could be retained during alternative splicing to generate transcripts encoding a truncated form of the SRK protein consisting of only the extracellular domain (Stein *et al.*, 1991). Both *SLG* and *SRK* genes have been shown to be expressed in anthers and stigmas by RNA gel blot analysis and amplification of transcripts by PCR (Delorme *et al.*, 1995; Goring and Rothstein, 1992; Guilluy *et al.*, 1991; Stein *et al.*, 1991). However, the level of expression of the *S* locus genes in anthers is much lower than in stigmas and the role that may be played by male or female *SLG/SRK* products in the

self-incompatibility response is still unclear. Nevertheless, there is evidence that the SRK protein is essential for the operation of self-incompatibility. Mutations of the *SRK* gene, which result in either a non-functional protein kinase or in the absence of *SRK* expression, were associated with a self-compatible phenotype (Goring *et al.*, 1993; Nasrallah *et al.*, 1994). This finding supports the model of self-incompatibility proposed by Stein *et al.* (1991) in which SRK, perhaps in combination with SLG, would bind to a putative *S* locus pollen ligand to trigger the recognition event through a signal transduction pathway involving a cytoplasmic phosphorylation cascade that leads to pollen rejection.

To better understand the molecular basis of the male/female recognition event involved in the self-incompatibility response, it is important to identify the *S* locus gene products present at the pollen-stigma interface. Recently, we have characterized the SLG and SRK products of the class I *S*₃ haplotype of *Brassica oleracea* and demonstrated that the SRK₃ glycoprotein is located in the membrane (Delorme *et al.*, 1995). In this report, using a combination of protein and mRNA analysis, we show that the *SRK*₃ gene produces an alternative transcript that encodes a soluble, truncated SRK₃ protein which corresponds to the extracellular domain of the receptor kinase. This finding, together with the identification of a membrane-anchored form of the SLG₂ protein (Tantikanjana *et al.*, 1993), indicates that the papillar cells of the stigma express a complex mixture of SLG/SRK proteins which may interact in different ways with the putative SRK ligand.

Results

Identification in stigmas of soluble glycoproteins antigenically related to SRK

We have recently characterized the *SLG* and *SRK* genes of the *S*₃ haplotype and have shown that the N-terminus of both proteins was sufficiently divergent to allow their identification by specific anti-peptide antibodies directed against the first 10 amino acid residues of each sequence (Delorme *et al.*, 1995). An antibody specific for the N-terminal end of SRK₃ (MAb 157-35-50, see experimental procedures) was used to look for the presence of putative truncated forms of the SRK protein in extracts from a range of tissues. In the stigma extract, this antibody detected a 120 kDa protein which has been shown to correspond to SRK₃ (Delorme *et al.*, 1995) and a cluster of four additional proteins, two strongly cross-reacting at 62.8 and 59.4 kDa and two weaker bands corresponding to 65.2 and 56.7 kDa (Figure 1a). These latter four proteins were candidates for truncated forms of SRK₃. To determine whether these proteins corresponded to secreted or membrane-anchored forms of the extracellular domain of SRK, soluble stigma proteins were extracted in a detergent-free buffer and any

contaminating membrane vesicles were removed by an ultracentrifugation step. The four proteins were found in the soluble fraction (Figure 1b, lane 1) but were not detected in the pelleted membrane vesicles even when extract equivalent to 20 times as much tissue was loaded on the gel (Figure 1b, lane 2). These data indicated that the four proteins correspond to soluble forms of the extracellular domain of SRK₃. This was supported by the fact that they did not cross-react with an antibody (anti-SRK₃C-dom, Delorme *et al.*, 1995) raised against the cytoplasmic C-terminal domain of SRK₃ (data not shown). The pelleted membrane vesicles contained SRK₃ and another membrane-associated product of 67 kDa which was only weakly recognized by MAb 157-35-50 (indicated by an arrowhead in Figure 1b, lane 2).

The extracellular domain of SRK₃ shares extensive sequence identity (around 90%) with SLG₃, it was therefore important to verify that the four proteins of 65.2, 62.8, 59.4 and 56.7 kDa were not encoded by *SLG₃*. Three glycoforms of SLG₃, of 60.0, 61.5 and 63.5 kDa have been previously identified by SDS-PAGE electrophoresis and immunodetection on protein blots using an antibody (anti-SLG₃N-ter) raised against the N-terminal amino acid sequence of SLG₃ (Delorme *et al.*, 1995). Because of the close similarity in molecular mass observed between the SLG₃ glycoforms and the four proteins detected by MAb 157-35-50, we chose to separate soluble stigma proteins according to their electric charge by IEF. Proteins were then electrotransferred onto a nitrocellulose membrane and the same lane was immunostained first with anti-SLG₃N-ter (to detect SLG₃) and secondly with MAb 157-35-50 (to detect SRK₃ related proteins). Figure 2a shows that the proteins detected by MAb 157-35-50, of pI 7.13 and 7.16, respectively, did not cross-react with anti-SLG₃N-ter indicating that these proteins are not encoded by the *SLG₃* gene. The fact that both antibodies detected multiple bands probably reflects the presence of different glycoforms of each gene product (Umbach *et al.*, 1990; and see below). We verified that the proteins detected by MAb 157-35-50 after IEF separation corresponded to those identified in Figure 1b, lane 1 by a selective two-dimensional electrophoresis analysis. Stigma extracts were separated by IEF and proteins focusing between pI 7.13 to 7.16 were recovered and run an SDS polyacrylamide gel alongside total stigma proteins as a control. Two proteins were recognized by MAb 157-35-50 and these corresponded to two of the four soluble proteins detected previously (Figure 2b). It was not clear why the two larger proteins of 65.2 and 62.8 kDa were not detected after this procedure. It is possible that the difference may have been due to the different treatments received by the two extracts. The native conditions used to prepare stigma extracts and to separate proteins by IEF (see experimental procedures) may have allowed some proteolytic and/or glycolytic degradation.

SRK₃ has been shown to be glycosylated (Delorme *et al.*, 1995). It was therefore likely that soluble, truncated forms of SRK₃ were also glycosylated. To test

this prediction, soluble stigma proteins were deglycosylated and immunoblotted with antibody MAb 157-35-50. Figure 3 shows that after deglycosylation only a single protein of 46.7 kDa was immunostained. This result is consistent with the predicted molecular mass of a soluble, truncated SRK₃ polypeptide (46.5 kDa) corresponding to the extracellular domain of SRK₃ as calculated from the deduced amino acid sequence. Taken together, the above data indicate that the four proteins of 65.2, 62.8, 59.4 and 56.7 kDa are glycoforms of a soluble, truncated form of SRK₃. To estimate the relative abundance of the soluble, truncated forms of the SRK₃ protein, stigmatic extracts were separated by IEF and stained with Coomassie Blue for quantitative densitometry. The data obtained (not shown) revealed that the soluble, truncated forms of SRK₃ were at least twenty five times less abundant than the SLG₃ protein.

Densitometric analysis of the SDS-PAGE immunoblot presented Figure 1a showed that soluble, truncated forms of SRK₃ were about 1.85 times more abundant than SRK₃, assuming that MAb 157-35-50 has a similar affinity for these proteins.

Expression of soluble, truncated forms of SRK₃ in an F₂ population corresponds with the presence of the S₃ locus.

The monoclonal antibody MAb 157-35-50 recognizes the SRK protein of the S₃ haplotype but not that of the S₂₅ haplotype. An F₂ population segregating for the S₃ and S₂₅ haplotypes was therefore used to determine whether the presence of soluble, truncated forms of SRK₃ corresponded with the presence of the S₃ haplotype (as would be predicted if these proteins were encoded by SRK₃). Figure 4 shows the results obtained for two parental plants, an F₁ hybrid and seven F₂ progeny which are representative of the 22 plants analysed. Antibody MAb 157-35-50 only detected the soluble, truncated SRK₃ proteins in plants which had been shown to be either homozygous or heterozygous for the S₃ haplotype indicating that these molecules are encoded by a gene linked to the S locus.

Identification of transcripts encoding a soluble, truncated SRK₃ protein

The presence of a conserved in-frame stop codon situated at the 5' end of the first intron of the SRK gene suggests that transcripts which fail to splice out the first intron would encode a soluble, truncated form of SRK consisting only of the extracellular domain

(Stein *et al.*, 1991). To determine whether the soluble, truncated forms of SRK_3 were produced as a result of such an alternative splicing process, we looked for transcripts which contained intron 1 sequence in stigma mRNA. For this purpose, we used a probe corresponding to the 5' end of intron 1 (nucleotides 1342 to 1648 of SRK_3 (Delorme *et al.*, 1995; see Figure 5d). This probe detected a major transcript of 1.5 kb and three minor transcripts of 4.1, 2.9 and 2.2 kb (Figure 5a). No SRK_3 mRNA was detected in anther or root tissue using this probe although a transcript hybridizing to an SRK_3 kinase domain probe has been shown to be expressed in anthers (Delorme *et al.*, 1995). A 4.1 kb transcript has also been shown to hybridize to a probe derived from the kinase domain of SRK_3 (Delorme *et al.*, 1995) indicating that this band corresponds to a full-length SRK_3 transcript which retains intron 1.

In order to estimate the relative abundance of the SRK_3 transcripts, the same RNA blot was re-probed with the 3' untranslated region of SLG_3 . This probe detected a single, stigma-specific transcript of 1.5 kb (Figure 5c). Analysis of the autoradiographs by densitometry revealed that the SRK_3 1.5 kb transcript was about half as abundant as the SLG_3 mRNA (Figures 5b and c). This difference in mRNA abundance is significantly lower than the difference in protein abundance estimated above (SLG_3 being at least 25 times as abundant as the soluble, truncated forms of SRK_3) and suggests differences in post-transcriptional regulation of expression of the two genes.

Rapid Amplification of cDNA Ends (RACE) PCR was used to identify the polyadenylation site of the SRK_3 1.5 kb transcript. A single, 1.48 kbp PCR product was obtained from stigma cDNA after 30 PCR amplification cycles (data not shown). No PCR amplification was obtained when a mock cDNA synthesis carried out in the absence of reverse transcriptase was used as a template. DNA sequencing of the RACE-PCR product identified a polyadenylation site at a position corresponding to nucleotide 178 of intron 1, 20 base pairs downstream of a consensus polyadenylation signal (Figure 5d). Analysis of 4 cDNA clones corresponding to the 1.5 kb transcript also revealed polyadenylation sites corresponding to sites in the 5' end of intron 1 (Figure 5d). A similar variability was observed for full-length transcripts of SRK_3 (Delorme *et al.*, 1995). The 1.5 kb transcript therefore contains intron 1 sequence and possesses an open reading frame which is predicted to end at the in-frame TAG termination codon near the 5' end of intron 1 (Figure 5d).

In order to confirm that the 1.5 kb transcript was derived from SRK_3 and not from one of the other members of the S gene family present in the *B. oleracea* genome, one of the 1.5 kbp cDNAs was completely sequenced and shown to be 100% identical to the corresponding region of SRK_3 . In addition, the intron 1 probe described above consistently hybridized to a single fragment in S_3S_3 genomic DNA digested with a range of restriction enzymes and did not hybridize to $S_{25}S_{25}$ genomic DNA. Analysis of genomic clones carrying the SRK_3 gene ruled out the possibility of a duplicated form of

SRK₃ in close proximity to the *SRK₃* gene itself (data not shown).

Discussion

The SRK₃ gene encodes multiple transcripts

RNA blot analysis has shown that expression of *SRK₃* in stigmas involves the generation of a complex set of alternative transcripts. Four transcripts were identified with a probe corresponding to the 5' end of intron 1 and two additional transcripts have been identified using a kinase domain probe (Delorme *et al.*, 1995). Although the exact structure of all these transcripts is not known, there is evidence that they are generated as a result of both incomplete splicing of introns and by the use of alternative polyadenylation sites (although other processes such as the use of alternative promoters may also be involved). The 1.5 kb transcript identified with the intron 1 probe (Figure 5a,b) was shown to be the result of the use of alternative polyadenylation sites on transcripts which retained part of intron 1 (Figure 5d). The 4.1 kb transcript (Figure 5a), on the other hand, probably represented a full-length transcript which had retained intron 1 due to incomplete splicing. A similar transcript of 4.1 kb is encoded by the *SRK₆* gene (Stein *et al.*, 1991) and transcripts which retain introns have been identified with other members of the *S* gene family in *Brassica* (Kumar and Trick, 1994 and M. Pastuglia, unpublished results). It is not known at present whether the partially spliced transcripts are functional or whether they accumulate in the nucleus, however, the 4.1 kb transcript, for example, is apparently polyadenylated and is abundant relative to the fully spliced *SRK₃* transcript (Delorme *et al.*, 1995).

Expression of a soluble, truncated form of the SRK₃ receptor kinase

The *SRK₃* transcripts which retain intron 1 sequence are predicted to encode a soluble, truncated protein which we designate eSRK₃ (extracellular domain of *SRK₃*). Analysis of the *SRK₃* gene sequence suggested that eSRK₃ should be a soluble glycoprotein with a polypeptide backbone of 46.5 kDa, expressed specifically in stigmas of plants carrying the *S₃* haplotype. We have identified a protein which corresponds to this description (Figures 1, 2, 3 and 4). Therefore, like many animal receptor kinase genes (Johnson *et al.*, 1991; Petch *et al.*, 1990), *SRK₃* expresses a soluble form of the receptor as a result of alternative splicing. Examples exist of alternative polyadenylation signals within introns being used to produce truncated receptors in animals (Baumbach *et al.*, 1989; Flickinger

et al., 1992; Johnson *et al.*, 1991) although truncated receptors are more commonly produced by splicing out an exon or by creation of a new exon. In animals, truncated receptors have also been shown to be generated by proteolysis (Downing *et al.*, 1989). The presence of a conserved triplet of basic residues (K₄₂₄RRK) on the N-terminal side of the transmembrane domain could form part of a protease processing site. However, it is unlikely that eSRK₃ is generated proteolytically from SRK₃ for two reasons. Firstly, the abundance of transcripts containing intron 1 (and hence coding for eSRK₃) in stigmas indicates that most, if not all of the eSRK₃ protein is synthesized independently of SRK₃. Secondly, SRK₃ exists as a single glycoform, the deglycosylation of which results in a shift in migration equivalent to 26 kDa (Delorme *et al.*, 1995). On the other hand, four glycoforms of eSRK₃ were detected, deglycosylation of which resulted in shifts of between 18.5 and 10 kDa. It is unlikely, therefore, that these glycoforms were derived from the SRK₃ protein. However, we cannot rule out the possibility that the eSRK₃ glycoforms are derived from partially glycosylated precursors of SRK₃ or that they are the result of a combination of protease and glycosylase activity.

In addition to SRK₃ and the four soluble, truncated eSRK₃ proteins, monoclonal antibody MAb 157-35-50 also weakly detected a 67 kDa protein associated with the membrane fraction of a stigma extract (Figure 1b, lane 2). The size of this protein and its presence in the membrane fraction are consistent with it being a truncated, membrane-anchored form of SRK₃ which retains the S and transmembrane domains but which lacks the intracellular kinase domain. The mouse receptor kinase gene *trkB* has also been shown to encode a truncated protein which lacks a kinase domain but retains the extracellular and transmembrane domains (Klein *et al.*, 1990). However, in the case of *trkB*, the truncated protein was expressed in tissues other than those expressing *trkB*. Efforts to identify a mRNA capable of encoding a membrane-anchored, truncated protein form of SRK₃ have so far been unsuccessful, although the wide range of SRK₃ transcript sizes present in stigma tissues (Delorme *et al.*, 1995) indicates that such a mRNA could exist. Nevertheless, we cannot rule out the possibility that the 67 kDa protein is a degradation product of SRK₃ either present *in vivo* or produced during extraction. It is also possible that the 67 kDa protein is encoded by an, as yet, uncharacterized gene related to SRK₃.

The eSRK₃ protein closely resembles SLG₃. These two proteins share 82.6 % similarity and both are soluble glycoproteins which are predicted to be secreted from the cells of the stigma. It will be interesting to determine whether they serve the same function and, if so to explain why both proteins are present in the same tissue. One possibility is that, before the *SLG* gene existed, the level of eSRK expression was limiting and that, during evolution, this led to the selection of a gene duplication which put the eSRK coding sequence and promoter under the control of an enhancer to create *SLG*. Although the exact function of eSRK remains to be determined, several possible roles which have been proposed for SLG (Goring and Rothstein, 1992; Stein *et al.*, 1991) can also be suggested for eSRK. In animal systems, soluble, truncated forms of both the platelet derived growth factor receptor (Duan *et al.*, 1991) and the epidermal growth factor receptor (Flickinger *et al.*, 1992) have been shown to act as antagonists to the full-length membrane-bound protein. Alternatively, it has been suggested that a soluble form of the growth hormone receptor may act as a reservoir of growth hormone in serum (Baumann *et al.*, 1987). It is likely that eSRK interacts with SRK either as part of a ligand binding complex or indirectly by binding to a common ligand. The level of eSRK protein detected in stigmas (the eSRK protein was approximately twice as abundant as SRK) would at first view tend to support the hypothesis that eSRK interacts directly with SRK rather than indirectly by competition. However, if SLG has evolved to supplement the level of eSRK, as suggested above, then the high abundance of eSRK / SLG in the stigma might indicate an indirect interaction, with these proteins perhaps being involved either in recruiting ligand molecules for the SRK receptor or alternatively in binding ligand to prevent it diffusing away from the site of pollen-papillar cell contact. This latter hypothesis might help explain the observation of Sarker *et al.* (1988) that a single papillar cell can respond simultaneously to a self-incompatible and a self-compatible pollen, the latter hydrating but the former failing to hydrate.

The overall picture of *S* locus gene expression is becoming increasingly complex with the identification of eSRK in the class I *S*₃ haplotype and of a membrane-anchored form of SLG (mSLG₂) associated with the class II *S*₂ haplotype (Tantikanjana *et al.*, 1993) expressed in addition to the SLG and SRK proteins themselves (Figure 6). To our knowledge, the data presented here constitutes the first evidence of soluble, truncated forms of a plant receptor kinase. This observation adds to the accumulating evidence that strong homologies exist between the mechanisms of signal transduction used by animal and plant cells.

Experimental procedures

Plant material, genetic crosses and determination of incompatibility phenotype

The *Brassica oleracea* var. *acephala* *S*₃ *S*₃ homozygous line and an *F*₂ population obtained by self-fertilization of an *S*₃ *S*₂₅ *F*₁ hybrid have been described (Delorme *et al.*, 1995). The incompatibility phenotype of the *F*₂ progeny was determined by self-

pollination and by crosses to tester plants (Delorme *et al.*, 1995).

Protein extraction, electrophoretic analysis and deglycosylation of proteins

Protein extraction, separation of proteins by isoelectric focusing (IEF) or SDS-PAGE, electrotransfer onto nitrocellulose membranes and detection of antigen with antibodies was as described previously (Gaude *et al.*, 1991, 1993). For the identification of soluble proteins, protein was extracted from stigmas in TBS buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and the extract was centrifuged at 150 000 g for 1 h at 4°C before loading on an SDS polyacrylamide gel as described (Delorme *et al.*, 1995). The pellet was washed with extraction buffer to eliminate any contaminating soluble proteins and ultracentrifuged again. The washed pellet was loaded on an SDS polyacrylamide gel. For selective two-dimensional electrophoresis, stigma extracts were run on an IEF gel alongside pI markers. The marker lane was cut off and stained with Coomassie Blue for 2 h during which time the lanes containing stigma protein were stored at 2°C. Staining of pI markers allowed the determination of the region of the gel encompassing pI 7.13 to 7.16. This region, which contained the stigma proteins of interest, was cut away and heat-denatured in two-fold concentrated SDS-PAGE loading buffer at 100°C for 5 min. The sample was centrifuged at 5000 g for 1 min at room temperature and loaded on an SDS polyacrylamide gel.

In order to estimate the relative amounts of SLG₃ and soluble, truncated SRK₃ protein in stigmas, 40 mg of stigma protein was loaded on an IEF gel, the Coomassie Blue-stained gel was scanned using a Personal Densitometer™ (Molecular Dynamics Inc, France) and the data analysed with ImageQuaNT™ software. The relative amounts of SRK₃ and soluble, truncated SRK₃ were estimated similarly on an immunoblot of stigma proteins (20 mg) separated by SDS-PAGE and probed with MAb 157-35-50 using the ECL detection kit reagents (Amersham, Les Ulis, France).

Deglycosylation of proteins was as described (Delorme *et al.*, 1995).

Antibody production and immunodetection on protein blots

Antibody anti-SLG₃N-ter has been described (Delorme *et al.*, 1995). Monoclonal antibody MAb 157-35-50 was derived from the same hybridoma fusion as the anti-SRK₃ antibody MAb 85-36-71 (Delorme *et al.*, 1995; Gaude *et al.*, 1993). The clone MAb 157-35-50 was used in this study because it cross-reacts more strongly with the truncated SRK₃ proteins. Antigen/antibody complexes were visualized using goat anti-mouse or goat anti-rabbit antibody conjugated either to alkaline phosphatase (Promega, Madison, USA) or horseradish peroxidase (Amersham) as secondary antibodies. For alkaline phosphatase development, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as colour development substrates. For horseradish peroxidase, ECL detection kit reagents (Amersham) were used.

RNA gel blot analysis

For RNA gel blots, total RNA was extracted by the method of Jackson and Larkins (1976). Roots were harvested from 3 week old seedlings, all other tissues were from mature, flowering plants. The developmental stage of anthers was determined by fluorescence microscopy observation of DAPI-stained microspores. Poly(A)⁺ RNA was purified from 100 µg batches of total RNA by hybridization to magnetic beads carrying oligo(dT)₂₅ (Dynal AS, Oslo, Norway).

RNA was separated on formaldehyde gels and transferred to nylon filters for hybridization. DNA probes were prepared using a random priming DNA labelling kit (Boehringer Mannheim, Meylan, France). Hybridizations were carried out under standard conditions and filters were washed at high stringency (Sambrook *et al.*, 1989). Autoradiographs were scanned for densitometry using an Arcus scanner (Agfa, St Quentin-en-Yvelines, France) and the data analysed with Scan Analysis software (Biosoft, Cambridge, UK).

Rapid Amplification of cDNA Ends (RACE) PCR, cDNA cloning and DNA sequencing

For RACE-PCR, poly(A)⁺ RNA was extracted directly (Jakobsen *et al.*, 1990) from a single mature stigma and purified by hybridization to magnetic beads carrying oligo(dT)₂₅ (Dynal AS). A DNase digestion was carried out to remove any contaminating

DNA and the poly(A)⁺ RNA was reverse transcribed (First strand cDNA synthesis kit; Stratagene, LaJolla, CA). RACE-PCR was carried out essentially as described by Frohman *et al.* (1988) except that the PCR amplification was carried out in one rather than two stages using oligonucleotide A (5'-GAAAGGATCCACCATGCAAGGTGTACGATACATCT-3') which spans the ATG start codon of *SRK₃* and an oligo(dT) adapter. PCR amplification was carried out for 30 cycles of denaturation at 94° C for 30 sec, annealing at 55° C for 30 sec and extension at 72° C for 3 min with a final extension of 10 min. PCR products were cloned into pCRscript (Stratagene) and two clones were sequenced from both ends.

For cDNA cloning, cDNA was synthesized from poly(A)⁺ RNA of mature stigma of an *S₃S₃* homozygous plant and cloned in lambda Zap II (Stratagene). One million pfu of the primary library were screened with a probe corresponding to nucleotides -180 to 170 of the *SRK₃* gene (numbering relative to the first base of the ATG initiation codon, Delorme *et al.*, 1995). Four clones, out of 600 which hybridized to the probe, were further analysed by restriction mapping and sequencing. One clone was completely sequenced and the other three partially sequenced at the 3' end. Sequencing was carried out by the dideoxy-nucleotide chain termination method (Sanger *et al.*, 1977) using the Sequenase system (version 2.0; U.S. Biochemicals) and custom-synthesized oligonucleotides. Sequence data was analysed using Lasergene sequence analysis software (DNASTAR, London, UK).

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Figure legends

Figure 1. Identification of soluble truncated forms of SRK₃ which are expressed specifically in stigmas.

(a) Immunoblot detection of SRK₃ encoded proteins in a range of tissues. Total proteins

(20 mg per well) extracted from stigma (St), anthers containing unicellular (U) or bicellular (B) microspores and root (R) were separated by SDS-PAGE before immunoblotting using MAb 157-35-50 as a primary antibody.

(b) Soluble stigmatic proteins (lane 1) or proteins from pelleted stigma membrane vesicles (lane 2) were separated by SDS-PAGE and immunoblotted as in (a). The amount of protein extract loaded was equivalent to 2 stigmas in lanes 1 and 40 stigmas in lane 2. The membrane-anchored 67 kDa protein is indicated by an arrowhead. Molecular masses of proteins, calculated with reference to molecular mass markers, are shown in kilodalton (kDa).

Figure 2. The soluble, truncated forms of SRK₃ are antigenically distinct from SLG₃.

(a) Comparison of the proteins detected with an anti-SLG₃ antibody (anti-SLG₃N-ter, lane 1) with those detected by an anti-SRK₃ antibody (MAb 157-35-50, lane 2). Soluble protein (10 mg per deposit) was separated by IEF and immunoblotted.

(b) The proteins detected by MAb 157-35-50 in (a), lane 2 were recovered from an IEF gel and run on SDS-PAGE (lane 2) alongside an extract of total stigma protein (lane 1). The amount of stigma protein deposited on the IEF gel was 150 mg whereas 10 mg of total stigma protein was loaded on the SDS-PAGE gel (lane 1). Truncated SRK₃ proteins were detected with antibody MAb 157-35-50. Molecular masses, calculated with reference to molecular mass markers, are shown in kilodaltons (kDa).

Figure 3. Deglycosylation of the soluble, truncated SRK₃ proteins.

Soluble stigma protein was either untreated (lane 1) or deglycosylated (lane 2) and separated by SDS-PAGE before immunoblotting. The amount of protein loaded on the gel was 10 mg per well. The primary antibody was MAb 157-35-50.

Figure 4. Expression of soluble, truncated SRK₃ proteins in an F₂ population segregating for the S₃ and S₂₅ haplotypes.

(a) Detection of soluble, truncated SRK₃ antigen in stigma protein (10 mg total protein per well) separated by SDS-PAGE and immunoblotted using MAb 157-35-50.

(b) S locus phenotypes of the plants analysed in (a) as determined by genetic crosses. The presence of the S₃ and/or the S₂₅ haplotype is indicated by 3 and 25 respectively.

Figure 5. Identification of SRK₃ transcripts which retain intron 1 in stigma mRNA.

(a, b and c) RNA blot analysis of SRK₃ (a and b) and SLG₃ (c) transcripts in a range of tissues. Each lane was loaded with 2 to 4 µg of poly(A)⁺ RNA. The DNA probes were derived from intron 1 of SRK₃ (a and b, see below) or from the 3' untranslated region of SLG₃ (c). (b) is the same as (a), lane St except that the exposure time was shortened to give an exposure equivalent to that of (c). St: stigma; U and B: anthers containing unicellular or bicellular microspores, respectively; R: root. The sizes of transcripts, calculated with reference to RNA size markers, are shown in kilobases (kb).

(d) Diagrammatic representation of the exon 1 / intron 1 junction in SRK₃. Exon 1 sequence is shown in bold type, intron 1 sequence in standard type. The 3' end of the eSRK₃ open reading frame is shown and the TAG termination codon is marked with an asterisk. The underlined sequence corresponds to the probe used in (a) and (b).

Also shown are polyadenylation sites identified by 3' RACE PCR (arrowhead) or by

sequencing of cDNA clones (I) and a putative polyadenylation signal (in italics).

Figure 6. Schematic representation of secreted and membrane-anchored proteins encoded by the S_3 (class I) and the S_2 (class II) haplotypes.

The soluble, truncated SRK₃ protein is represented as eSRK₃, the membrane-anchored form of SLG₂ by mSLG₂. SLG proteins are cross-hatched. The extracellular domain of SRK is shown as a white box. Kinase and transmembrane domains are shown as black and stippled boxes respectively. Within the *S* domains, the cysteine rich domain is represented by a box and putative glycosylation sites by branched side chains.