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A genetic map of the *Nicotiana alata S* locus that includes three pollen-expressed genes

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Abstract The *S* locus of solanaceous plants includes separate genes that control the self-incompatibility phenotype of the pistil and of the pollen. The gene controlling the self-incompatibility phenotype of the pistil encodes an extracellular ribonuclease, the S-RNase. The gene(s) controlling the self-incompatibility phenotype of pollen (the *pollen-S* gene) has yet to be identified. As part of a long-term strategy to clone the *pollen-S* gene by chromosome walking, a detailed map of the region near the *S* locus of *Nicotiana alata* was generated using a total of 251 F_2 plants. The map spans an interval of approximately 2.6 cM and contains five markers as well as the S-RNase gene. Two markers were detected with heterologous probes that also detect sequences linked to the *S* locus of *Solanum tuberosum* and the homologous region of the *Lycopersicon* genome. Three markers were identified by differential display using *N. alata* pollen RNA as a template. One of these markers is a pollen-expressed sequence, *48A*, which detects a polymorphic marker no more than 0.5 cM from the *S* locus. RNA blot analysis indicates that the *48A* gene is expressed primarily during pollen development after the completion of meiosis and is therefore a candidate for the *pollen-S* gene. The utility of these markers and the possible involvement of *48A* in the molecular mechanism of self-incompatibility are discussed.

Key words Gametophytic self-incompatibility · *S* locus · Differential display · Pollen-expressed genes · Restriction fragment length polymorphisms (RFLPs)

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Introduction

Gametophytic self-incompatibility in plants from the Solanaceae is controlled by a single, multiallelic *S* locus (de Nettancourt 1977). The pistils of self-incompatible plants from this family reject pollen when both pistil and pollen express the same *S* allele. No rejection occurs when they express different *S* alleles, and the pollen tube continues to grow through the style towards the ovary where it may fertilise an ovule. The only identified product of the solanaceous *S* locus is an allelic series of extracellular glycoproteins with ribonuclease activity called S-RNases (Anderson et al. 1986). These are abundantly expressed in the pistils of self-incompatible plants but are also present at a low level in developing pollen (Anderson et al. 1986; Dodds et al. 1993). Although it is possible that S-RNases control the self-incompatibility phenotype of both pollen and pistil, available evidence indicates that they only determine the self-incompatibility phenotype of the pistil (Dodds et al. 1999; Lee et al. 1994; Murfett et al. 1994). This suggests that a separate gene (or genes) within the *S* locus controls the self-incompatibility phenotype of pollen. The identities of the *pollen-S* gene and its product are essential pieces of knowledge to further understanding of the molecular basis of self-incompatibility.

Several strategies to identify the *pollen-S* gene have been devised (Kao and McCubbin 1996; Matton et al. 1994). Map-based cloning is one option, but this requires the development of a well-resolved genetic map of an *S*

locus. Although our studies of self-incompatibility in *Nicotiana alata* have indicated this is a suitable species in which to attempt map-based cloning, there are currently no genetic maps of any *Nicotiana* species and, consequently, no source of already mapped probes. Unmapped genomes can be studied using heterologous probes from related species (Helentjaris 1993). Genetic maps incorporating both molecular and phenotypic markers are available for two solanaceous species, tomato (a self-compatible species; Tanksley et al. 1988, 1992) and potato (a self-incompatible species; Gebhardt et al. 1991), and tomato and potato probes can hybridise to DNA from other solanaceous species. For example, tomato probes have been used to construct a genetic map of pepper (Tanksley et al. 1988, 1992; Prince et al. 1993), and ten Hoopen et al. (1998) recently showed that a potato probe (CP100) that detects a marker-linked *S* locus also detects a marker linked to the *Petunia S* locus. The approach of using heterologous probes from potato and tomato to identify markers near the *N. alata S* locus is therefore considered to have a high probability of success.

A second approach to identifying markers is to use a method based on the polymerase chain reaction (PCR) such as differential display (Liang and Pardee 1992). This technique is routinely used to isolate genes expressed differentially in different tissues or in the same tissue in response to different physiological conditions. When used in conjunction with bulked segregant analysis (Michelmore et al. 1991), differential display can also be used to either identify markers linked to a gene of interest or to identify the gene of interest itself. For example Ikeda et al. (1997) used a bulked segregant approach and differential display to identify a stigma transcript produced by the *Brassica campestris MOD* locus. Mutations in *MOD* alter stigmatic function in the *Brassica* self-incompatibility system and cause a self-compatible phenotype.

The object of the study detailed here was to identify markers linked to the *N. alata S* locus as a part of the groundwork necessary to cloning the *pollen-S* gene using a map-based strategy. We used both heterologous probes and differential display to identify linked markers. For heterologous probes, we used markers linked to the *S* locus of potato (Gebhardt et al. 1991), and the homologous region of the tomato genome (Tanksley et al. 1992), that cross-hybridized with *N. alata* DNA. Using differential display, we compared pollen transcripts from *N. alata* plants bearing different *S* alleles. Using these heterologous and differential display markers, we prepared a map of the *S* locus using large families of F_2 plants.

Materials and methods

Plant material and pollination assays

N. alata lines homozygous for the S_2, S_3, S_6 and S_7 alleles are described in Jahnen et al. (1989). The F_1 family was produced by crossing an S_2S_3 plant and an S_6S_7 plant (see Fig. 1). An S_2S_6 plant from this family was selected and self-pollinated at the green bud stage. Seeds from three selfed capsules were surface-sterilised and germinated on water agar. Seedlings were transferred to soil and grown to maturity. The F_3 family was produced by bud self–pollinating an S_2S_6 individual from the F_2 family. Self-pollinating an S_2S_6 plant that resulted from an S_2S_2 and S_6S_6 cross produced the second F_2 family.

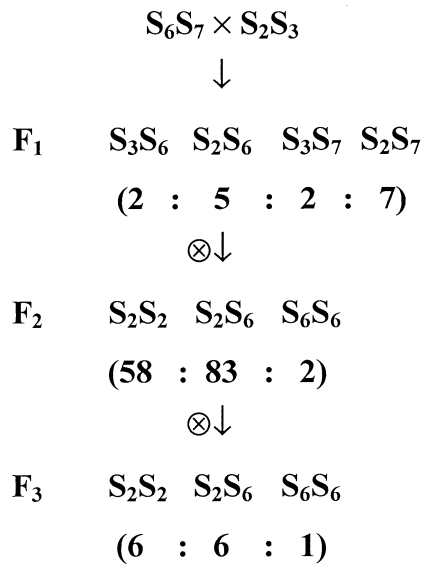

Fig. 1 Scheme showing development of the F_2 mapping family and the inbred F_3 family. The F_2 and F_3 families were produced by bud self-pollinating an individual S_2S_6 plant from the preceding generation. The *numbers in parentheses* refer to the number of plants in each genotypic class

S phenotypes were determined by a series of pollinations. Immature floral buds were emasculated soon after petal opening, and pollen from a dehiscent anther taken from a plant with known S alleles was spread over the stigmas of four or more flowers. The flowers were bagged and scored 7–10 days later. A pollination was compatible if a large capsule developed and incompatible if the flower had abscised.

Polymerase chain reaction (PCR)

S genotype was determined by PCR using total leaf DNA as template. Two pairs of primers that amplify either the S_2- or S_6-RNase gene were used. pV1.6 (5′–GGTAAAGAAGATGACTATAACAT) and pV2.6 (5′–TTCATACAATCAGCTTTCTCTC) amplify the region between bases 202 and 423 of the S_6-RNase gene, and pV1.2 (5′-TTACTGCGATCGCTCCAAACC) and pC3 (5′-ACAACACGTGCCATGCCTT) amplify the region between bases 195 and 446 of the S_2-RNase gene (Dodds et al. 1993). Leaf DNA (250 ng to 2 µg) was placed in a 25 µl reaction vessel containing 10 m*M* TRIS-Cl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1 m*M* each dNTP, 1 µ*M* of each primer, and 0.5 u *Taq* polymerase (Perkin Elmer Cetus). Amplifications were done in a Perkin-Elmer 9600 Gene-amp system over 40 cycles (1 min at 96°C, 1 min at 55°C, 2 min at 72°C) followed by 10 min at 72°C. Products were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining.

Pollen collection, RNA extraction and blotting

Pollen was collected from F_3 individuals as described by Dodds et al. (1993). Approximately equal amounts of pollen were pooled from plants with the same *S* genotype. The different stages of anther development in *N. alata* were defined by floral bud length as

described by Dodds et al. (1993). RNA was prepared from pollen, developing anthers and other *N. alata* tissues by the method of McClure et al. (1990). RNA samples were separated on a denaturing agarose gel, blotted onto a nylon membrane and hybridised with a radiolabeled 48A probe by the method described in Dodds et al. (1993).

Preparation of pollen cDNA and differential display

Pollen cDNA was prepared from total RNA as described by Song et al. (1995) except that the oligonucleotides MK1–3 (MK1: 5´TTTTTTTTTTTT(A/C/G)A 3´; MK2: 5´–TTTTTTTTTTTT-(A/C/G)C 3´; MK3: 5´ TTTTTTTTTTTT(A/C/G)G 3´) were used to prime the reverse transcription reaction. Pollen cDNA was amplified by PCR using the same MK primer (end-labeled with γ-[^{32}P]-ATP) and an arbitrary decamer primer from the UBC 1–200 series (University of British Columbia, Vancouver, Canada). PCR was done in a volume of 12.5 µl containing pollen cDNA (4 µl), 1.25 µl 10×*Taq* buffer II (Perkin-Elmer), 2.5 µ*M* $MgCl_2$, 10 µ*M* dNTPs, 1 µ*M* UBC primer and 3 µ*M* [^{32}P]-labeled MK primer. DNA was amplified over 40 cycles of the following thermal programme: 94°C for 30 s, 44°C for 1 min and 72°C for 30 s. The programme concluded with a 5-min extension at 72°C. Reaction products were analysed on a non-denaturing 5% acrylamide gel as described by Song et al. (1995) except that 1×TBE (20×TBE is 1 *M* TRIS base, 20 mM EDTA and 1 *M* boric acid) was used as the running buffer. After electrophoresis, the gels were dried and exposed to film at –70°C for 1–2 days. Amplified fragments were recovered, reamplified and cloned into pGEM-T vector (Promega) as described by Song et al. (1995).

Restriction fragment length polymorphism (RFLP) analysis

Total leaf DNA (10 µg) was extracted as previously described (Golz et al. 1999) and digested with 30–40 U of restriction endonuclease (Promega) for 16 h. Digested DNA was separated on a 0.8% agarose gel, blotted onto a Hybond N-filter (Amersham) and crosslinked to the filter using a UV-crosslinker (Hoefer).

Potato and tomato probes were supplied by Dr. Ch. Gebhardt (Max Planck Institute for Plant Breeding Research, Cologne, Germany) and Dr. S. Tanksley (Department of Plant Breeding and Biometry, Cornell University, Ithaca, USA), respectively. Inserts were isolated from plasmids following restriction digestion with appropriate enzymes and electrophoresis through an agarose gel. Inserts were labeled with [^{32}P]-dCTP using a random primer labeling kit (Promega). DNA fragments obtained by differential display and the *N. alata* S_2- and S_6-RNase cDNAs (Anderson et al. 1986, 1989) were labeled in the same way.

Filters probed with DNA fragments from potato and tomato were prehybridised at 65°C for 2 h in a buffer containing 6×SSPE (20× is 3 *M* NaCl, 0.2 *M* Na_2HPO_4 and 0.02 *M* EDTA), 0.5% SDS, 5× Denhardt´s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA) and 50 µg/ml herring sperm DNA (Sambrook et al. 1989). Hybridisation was done in prehybridization buffer at 55°C for 16 h, and the filters were washed three times (20 min each wash) in 2×SSC (20× is 3 *M* NaCl, 0.3 *M* trisodium citrate), 0.1% SDS at 55°C.

Filters probed with the differential display fragments and the S_2- and S_6-RNase cDNAs were prehybridised at 42°C for 2 h in a buffer containing 5×SSPE, 100 µg/ml herring sperm DNA and 50% formamide. Hybridisation was done using the conditions used for prehybridisation, and the final wash for the filters was at 42°C in 0.2×SSC, 0.1% SDS. Filters were exposed to Kodak X-AR film for at least 4 days at –70°.

Linkage analysis

MAPMAKER 3.0 was used to analyse the linkage relationship of markers (Lander et al. 1987). Data were entered using the "F_2 intercross" format and analysed using the Haldane mapping function and a log of likelihood ratio (LOD) threshold of 3.0.

Results

Genetic material used in this study and the inheritance of *S* alleles

Before searching for genes linked to the *S* locus, we used the random amplified polymorphic DNA (RAPD) technique (Williams et al. 1990) to assess the level of DNA variability in our laboratory stocks of *N. alata* plants. These plants were originally provided to us as seed by researchers in New Zealand (S_1S_3 and S_2S_3) and the Netherlands (S_6S_7; see Anderson et al. 1986). Vigorous plants, selected from seedlings grown from these seeds, were bud self-pollinated and representative plants homozygous for each *S* allele were identified in the resulting families. These plants have subsequently been propagated vegetatively (Anderson et al. 1986; Jahnen et al. 1989). Given the pedigree of our homozygous plants, we therefore expected to find few differences within the S_1S_1, S_2S_2 and S_3S_3 group of plants and within the S_6S_6 and S_7S_7 group of plants. This indeed proved to be the case (N. Nass, N. Treloar and E. Newbigin, unpublished results). However, when we compared the DNA amplified from the S_2S_2 and S_6S_6 plants, we found 16% of the scoreable bands were only present in S_2S_2 plants and 21% were only present in S_6S_6 plants. This indicated that there was enough variation between the S_2S_2 and S_6S_6 plants to anticipate a successful search for polymorphic genes.

Figure 1 shows how the F_2 and F_3 families used in this study were produced from an F_1 individual (genotype S_2S_6). The 143 plants in the F_2 family were used to map genes relative to the *S* locus and the 13 plants in the F_3 family were used as a source of pollen RNA for differential display. A second F_2 family of 109 plants was also used in the mapping experiment. This family was produced by bud self-pollinating an S_2S_6 plant that had been produced by a compatible cross between an S_2S_2 and an S_6S_6 plant.

We determined the S phenotype of all plants in the F_1 and F_3 families and about two-thirds of the 251 F_2 plants by pollinations with pollen from plants with known S alleles. We determined the *S* genotype of all plants in this study either by PCR with primers specific to the S_2- or S_6-RNase gene (Fig. 2A) or by probing DNA blots with radiolabeled S_2- and S_6-RNase cDNAs (Fig. 2B). Both techniques gave the same *S* genotype when used on DNA from the same plant and, except for a few S_2S_2 and S_2S_6 plants that sometimes formed small capsules when pollinated with S_2 pollen, the *S* genotype and S phenotype of each plant was the same.

The observed segregation of *S* alleles in some families differed from expectation. One F_2 family contained 58 S_2S_2 plants, 83 S_2S_6 plants and only 2 S_6S_6 plants. The small number of S_6S_6 plants explains why the observed

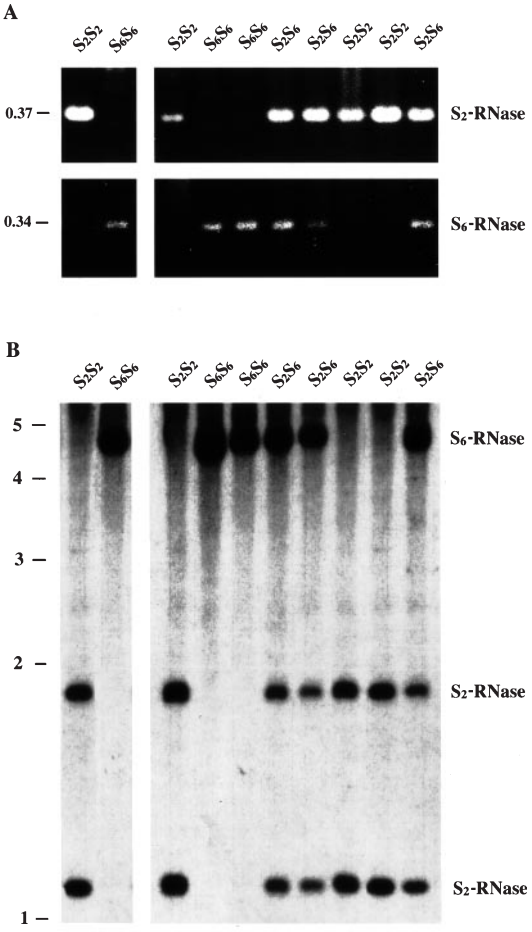

Fig. 2A, B *S*-genotype determination of *N. alata* plants. **A** Determination of *S* genotype by the PCR. The *upper panel* shows amplification of part of the S_2-RNase gene by PCR with primers pV1.2 and pC3. Genomic DNA from control S_2S_2 and S_6S_6 plants (labeled S_2S_2 and S_6S_6) and selected F_2 individuals (labeled according to S phenotype) was used as template. The *lower panel* shows amplification from the same samples of part of the S_6-RNase gene with primers pV1.6 and pV2.6. *Numbers* on the *left* of the figure indicate the size (in kb) of the amplified products. **B** Determination of *S* genotype by DNA blot analysis. Each lane contains genomic DNA (10 µg) from control S_2S_2 and S_6S_6 plants and selected F_2 individuals (labeled according to S phenotype). DNA was digested with *Hin*dIII and the blot was hybridised with [^{32}P]-labeled S_2-RNase and S_6-RNase cDNAs. The identity of the S-RNase hybridising bands is indicated at the *right* of the figure. The size of molecular-weight markers (in kilobases) is indicated at the *left* of the figure

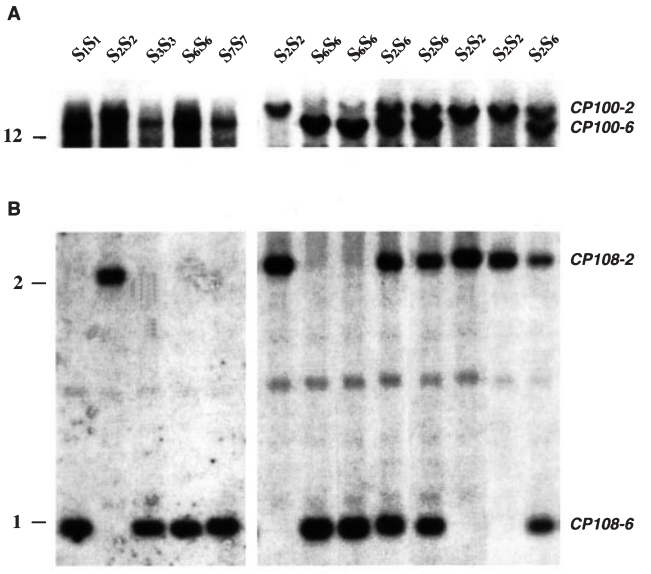

Fig. 3A, B RFLP analysis of markers detected by potato probes. **A** RFLP analysis of CP100. Each lane contains genomic DNA (10 µg) from the indicated homozygous plant and selected F_2 individuals (labeled according to S phenotype). DNA was digested with *Xba*I and hybridised with a [^{32}P]-labeled CP100 cDNA. The identity of the CP100 hybridizing bands is indicated at the *right* of the figure. **B** RFLP analysis of CP108. The DNA samples used in **A** were digested with *Hin*dIII, and the blot was hybridised with a [^{32}P]-labeled CP108 cDNA. The identity of the CP108 hybridizing bands is indicated at the *right* of the figure. The size of the molecular weight markers (in kilobases) is indicated at the *left* of *A* and **B**

segregation ratio (0.41:0.58:0.01; S_2S_2:S_2S_6:S_6S_6) and the expected ratio (0.25:0.5:0.25) are significantly different (P<0.005). The distortion may be due to either a pre-zygotic barrier that selectively prevents S_6 pollen from reaching the ovules or a post-zygotic barrier that selectively prevents S_6S_6 embryos from developing. A pre-zygotic barrier would lead to an F_2 family with roughly equal numbers of S_2S_6 and S_2S_2 plants; a post-zygotic barrier to a family with twice as many S_2S_6 plants as S_2S_2 plants. The observed ratio of S_2S_6 to S_2S_2 plants (1.43:1) does not rule out either hypothesis, and the reason for the distorted segregation ratio is not known. A skewed ratio was also seen in the F_3 family (6 S_2S_2 plants, 6 S_2S_6 plants and 1 S_6S_6 plant) but not in the other F_2 family (17 S_2S_2 plants, 64 S_2S_6 plants and 28 S_6S_6 plants).

RFLP analysis of the potato markers CP100 and CP108

Nineteen probes that identify markers linked to the *S* locus of potato (*Solanum tuberosum*; Gebhardt et al. 1991) or the homologous region of the tomato (*Lycopersicon*) genome (Tanksley et al. 1992) were tested for cross-hybridisation with *N. alata* DNA. Blots of digested DNA from plants homozygous for either the S_2 or S_6 allele were hybridized at moderate stringency to each labeled probe (see Materials and methods). Eleven of the probes were genomic clones (GP128, GP184, GP206, TG21, TG24, TG59, TG70, TG125, TG184, TG192 and TG310), and eight were cDNAs (CP45, CP100, CP108, CD15, CT62, CT122, CT151 and CT229). Six genomic probes did not hybridise to *N. alata* DNA or hybridized non-specifically; 2 genomic probes and four cDNA probes gave only a weak hybridisation signal; and three cDNAs and 4 genomic clones hybridised strongly. Five of these either did not detect a restriction fragment length polymorphism (RFLP), detected an RFLP that was too weak to be reliably scored, or detected an RFLP that was unlinked to the *S* locus (N. Nass and E. Newbigin, data not shown). The remaining 2 probes (CP100 and CP108) detected RFLPs between the S_2S_2 and S_6S_6 plants.

Table 1 Summary of data showing linkage between the *S* locus and markers detected by differential display probes (48A, 133G and 167A) and potato probes (CP100 and CP108)

Number of recombinant plants (total number of plants)				
Probe	S_2S_2	S_2S_6	S_6S_6	Total
48A	0 (59)	0 (126)	0 (30)	0 (215)
133G	2 (49)	4 (115)	2 (30)	8 (194)
167A	1 (36)	2 (91)	0 (29)	3 (156)
CP100	0 (36)	0 (89)	0 (29)	0 (154)
CP108	0 (54)	0 (116)	0 (28)	0 (198)

Figure 3A shows the *Xba*I RFLP detected by CP100. However, most restriction enzymes did not yield RFLPs for CP100, indicating that this genomic region has little DNA variability. We refer to the CP100 RFLPs in the S_2S_2 plant and S_6S_6 plant as *CP100–2* and *CP100–6*, respectively. Figure 3A also shows segregation of the *CP100* polymorphism in 8 of the 154 F_2 plants that were analysed. No recombinants between the *S* locus and *CP100* were identified (Table 1), which placed *CP100* less than 0.7 cM from the *S* locus. Similarly, Figure 3B shows the *Hin*dIII RFLP detected by CP108 and its segregation in 8 F_2 plants. No recombinants between the CP108 RFLPs (*CP108–2* and *CP108–6*) and the *S* locus were identified in 198 plants (Table 1). This indicates that the two loci are separated by less than 0.6 cM. As there were no recombinants, the method of Stevens (1942) was used to estimate that the maximum distance separating *CP100* and *CP108* from the *S* locus was 0.75 cM and 1 cM, respectively (confidence limit=95%).

Differential display of pollen-expressed genes

The F_3 family was produced by two generations of inbreeding (Fig. 1). The overall level of genetic variation in this family was therefore expected to be 25% of that in the F_1 family. To reduce the effect of unlinked polymorphisms further, we collected pollen from the 6 S_2S_2 plants and 6 S_2S_6 plants and separately pooled them. RNA extracted from the pooled samples was then used as template for differential display. Three hundred and seventy-two pairs of primers (produced by combining 1 of 124 available arbitrary primers with one of the three MK primers) were tested and approximately 11,000 bands were scored for polymorphisms between the S_2S_2 and S_2S_6 RNA pools. Six bands were present in the S_2S_6 sample and not in the S_2S_2 sample. The DNA present in these bands was reamplified and cloned. The cloned fragments were short (<300 bp), and their sequences indicated that they were derived from the 3′ untranslated region of different transcripts (data not shown). When used to probe blots of digested DNA from each of our homozygous lines, the cloned sequences hybridised to either a single fragment or to a small number of fragments, and each probe detected a different pattern of fragments. Two probes detected genomic fragments that were not polymorphic among the homozygous lines, and one probe detected an RFLP that was not linked to the *S* locus (J.H. Li and E. Newbigin, data not shown). The three remaining probes, which were named 48A, 133G and 167A, all detected RFLPs between our *S*-homozygous plants.

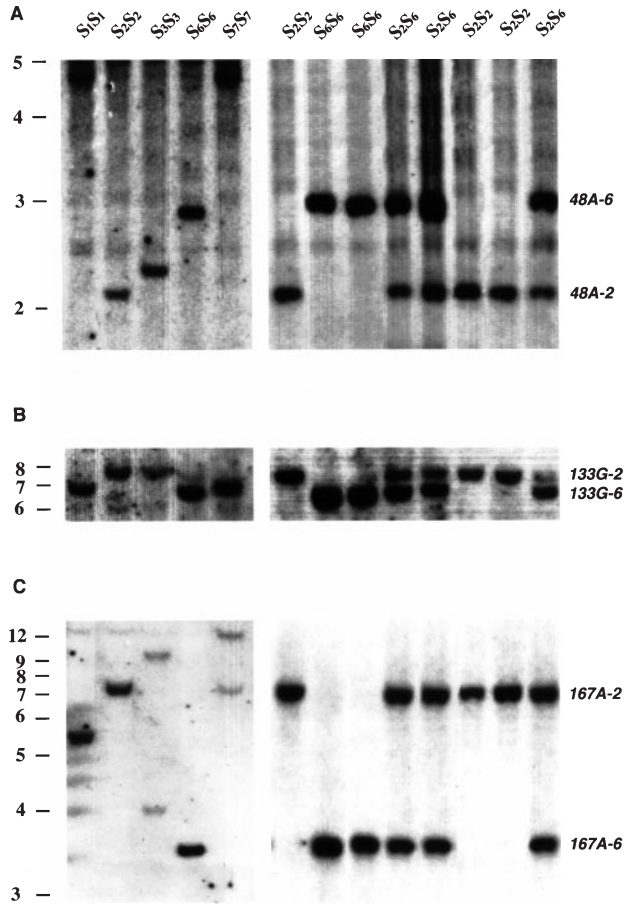

Fig. 4A–C RFLP analysis of probes obtained by differential display of pollen RNA. **A** RFLP analysis of 48A. Each lane contains genomic DNA (10 µg) from the indicated homozygous plant and selected F_2 individuals (labeled according to S phenotype). DNA was digested with *Hin*dIII and hybridised with a [^{32}P]-labeled 48A cDNA. The identity of the 48A hybridizing bands in the F_2 individuals is indicated at the *right* of the figure. **B** RFLP analysis of 133G. The DNA blots used in **A** were stripped and hybridised with a [^{32}P]-labeled 133G cDNA. The identity of the 133G hybridizing bands is indicated at the *right* of the figure. **C** RFLP analysis of 167 A. The DNA samples used in **A** were digested with *Eco*RI, and the blot was hybridised with a [^{32}P]-labeled 133G cDNA. The identity of the 133G hybridizing bands in the F_2 individuals is indicated at the *right* of the figure. The size of molecular-weight markers (in kilobases) is indicated at the *left* of **A**, **B** and **C**

RFLP analysis of 48A, 133G and 167A

Figure 4A shows the *Hin*dIII RFLPs detected by probe 48 A. Each *S*-homozygous plant had a different 48A RFLP, and every restriction enzyme we tested produced a similar RFLP series. The RFLPs in the S_2S_2 and S_6S_6

plants were named *48A-2* and *48A-6*, respectively. Figure 4A also shows segregation of the 48A RFLP in 8 F_2 plants. We analysed a total of 215 F_2 plants with this probe and found no evidence of recombination between *48A* and the *S* locus (Table 1). This indicated that *48A* and the *S* locus are separated by less than 0.5 cM, with the calculated 95% confidence limit for the distance between the two loci being 0.7 cM.

Figure 4B shows a similar analysis of the probe 133G. This probe hybridised to a single *Hin*dIII fragment, and only 2 133G RFLPs were found in our *S*-homozygous lines. The 133G RFLP shared by the S_2S_2 and S_3S_3 plants was called *133G-2*, and the 133G RFLP shared by the S_1S_1, S_6S_6 and S_7S_7 plants was called *133G-6*. Figure 4B shows segregation of the 133G RFLPs in representatives from one of the F_2 families. Overall, eight *133G* recombinants were identified in 194 F_2 plants (Table 1). In 2 plants *133G-6* was associated with the S_2 allele, and in 6 plants *133G-2* was associated with the S_6 allele. *133G* and the *S* locus are therefore separated by between 2 and 2.3 cM.

Figure 4C shows the *Eco*RI RFLPs identified by probe 167A. As with 48A, each of our *S*-homozygous plants had a different 167A RFLP, and a comparable series of RFLPs were produced by all the restriction enzymes we tested. The RFLPs in the S_2S_2 and S_6S_6 plants were named *167A-2* and *167A-6*, respectively. Figure 4 C shows the segregation of these RFLPs in representatives from an F_2 family. Table 1 shows three recombinants for *167A* were found in 156 plants. In 1 plant, *167A-6* was associated with the S_2 allele and in the other 2 plants, *167A-2* was associated with the S_6 allele. This indicated that *167A* and the *S* locus are separated by between 0.6 and 1.3 cM.

Figure 5 shows the order of markers inferred by combining the segregation data for CP100, CP108 and the three differential display markers. The total length of the map is 2.6 cM. Because no recombination was found between the *S* locus and three of the markers (*CP100*, *CP108* and *48A*), the order of these markers with respect to each other could not be determined. This finding is consistent with earlier reports suggesting that recombination at the *S* locus is suppressed (Clark and Kao 1991; Coleman and Kao 1992).

Expression analysis of 48A

The probe 48A detected a polymorphic gene less than 0.5 cM from the *S* locus. Because the differential display probes were amplified from pollen RNA, the gene encoding the 48A sequence is a candidate for *pollen-S*. As pollen in a gametophytic self-incompatibility system displays its own haploid *S* genotype, expression of the *pollen-S* gene is expected to occur at some stage after the completion of microspore meiosis. RNA blot analysis was used to see if the expression of *48A* during *N. alata* pollen development conforms to this expectation.

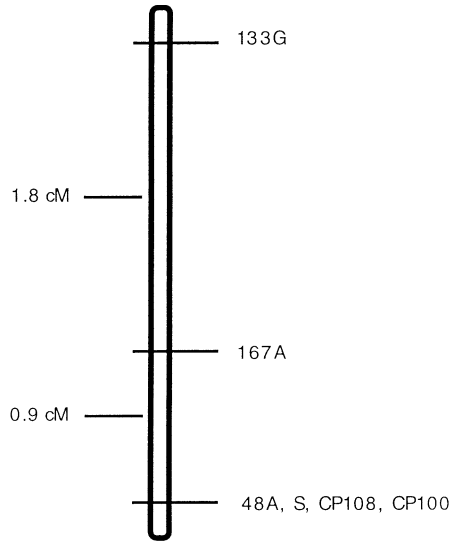

Fig. 5 Integrated map of markers linked to the *N. alata S* locus. Distances between markers are in centiMorgans (cM). The order of markers *48A*, *S*, *CP108* and *CP100* is arbitrary

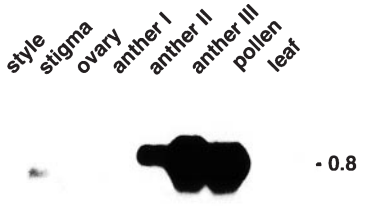

Fig. 6 RNA gel blot analysis of the *48A* gene. RNA samples from mature style, stigma, ovary, pollen and leaf and anthers at three developmental stages (anther I-III) were separated on a 2% agarose gel containing 7% formaldehyde, blotted onto a nylon membrane and hybridised to a [^{32}P]-labeled 48A probe. Lanes contain 10 µg of total RNA, and the blot was exposed to film for 72 h at –70°C. The size of the *48A* transcript (in kilobases) is indicated at the *right* of the figure

RNA was extracted from anthers at three stages of development defined by the length of the floral bud (see Dodds et al. 1993). Stage I anthers (present in 5- to 10-mm-long floral buds) contain pollen in late meiosis and post-meiotic, pre-vacuolate pollen; stage-II anthers (20- to 30-mm-long floral buds) contain mainly vacuolate pollen in which the premeiotic RNA has been degraded and the synthesis of gametophytic RNA has begun; stage-III anthers (50- and 70-mm-long floral buds) contain nearly mature pollen packed with RNA synthesised after meiosis. The 48A probe detected a transcript of approximately 0.8 kb that was abundant in mature pollen and stage-III anthers (Fig. 6). A lower level of expression was found in stage-II anthers and in mature stigmas. No expression was detected in stage-I anthers, other *N. alata* floral tissues or leaves.

Discussion

Our study of markers linked to the *N. alata S* locus is part of a broader approach aimed at identifying the *pollen-S* gene. Earlier studies of *S*-linked genes in the Solanaceae identified a cathodic peroxidase 3 cM from the *N. alata S* locus (Labroche et al. 1983) and an anodic peroxidase 9.9 cM from the *Lycopersicon peruvianum S* locus (Tanksley and Loaiza-Figueroa 1985). The number of *S*-linked markers has recently increased with the mapping of the tomato and potato genomes (Tanksley et al. 1992; Gebhardt et al. 1991). Markers from these plants have been widely used to study the inheritance of self-incompatibility and other reproductive barriers in either wild tomatoes or in crosses between cultivated tomato and its wild relatives (Bernacchi and Tanksley 1997; Bernatzky 1993; Chetelat and DeVerna 1991). Co-segregation of a potato marker (CP100) with the petunia *S* locus has also been used as evidence of synteny around the solanaceous *S* locus (ten Hoopen et al. 1998). Because these studies had other aims, none attempted to produce a high-resolution map of the solanaceous *S* locus. We have now produced a high-resolution map, which is prerequisite to the positional cloning of the *pollen-S* gene.

The feasibility of cloning the *pollen-S* gene by this approach can be estimated from a comparison of the size of the *N. alata* genome in base pairs (2,350 mbp/1 C; Narayan 1987) and in map units. Data from other solanaceous species indicates that a higher content of DNA does not result in a proportional increase in recombination (Prince et al. 1993). Thus, even though the *N. alata* genome contains twice as much DNA as the tomato genome, it is likely that the size of the linkage maps of the two species will be approximately the same (1,300 cM; Tanksley et al. 1992). From this, we estimate that 1 cM in *N. alata* corresponds on average to 1.8 mbp. Three markers (*CP100*, *CP108* and *48A*) are separated from the *S* locus by no more than 0.5–0.7 cM, and a fourth marker (*167A*) is separated by between 0.6 and 1.3 cM. Deviations from estimates of the relationship between physical distance and recombinational distance in different genomes are commonly reported; nonetheless, the availability of a high-density map allows us to identify large genomic fragments that include two or more of the *S*-linked markers.

The lack of recombination between the *S* locus and three of the markers is potentially a serious impediment to cloning the *pollen-S* gene using a map-based approach. However, we have generated a set of *N. alata* plant lines with mutations that affect the expression of the self-incompatibility phenotype through the pollen component (Golz et al. 1999). These plants are a valuable resource in our search to identify the *pollen-S* gene. The mutant phenotype in these plants (which are called pollen-part mutants) is associated with the presence of an extra *S* allele in some pollen grains (Pandey 1965). In most of the lines, the extra *S* allele is located on a short additional chromosome called a centric fragment, but in a few lines, no additional chromosome is present and the extra *S* allele is presumed to be part of another chromosome. Pandey (1967) considered that centric fragments and translocated *S* alleles were produced by fragmentation of an *S*-bearing chromosome. This suggests that the extra *S* alleles in the mutant lines are still associated with markers that would normally flank the *S* locus. The number of markers associated with the extra *S* allele is likely to be different because each line is derived from a separate mutational event. It should therefore be possible to order *S*-linked markers by determining which markers are present in each pollen-part mutant line. This approach to mapping does not in principle differ from the classical use of primary trisomics to locate a gene to a particular chromosome or linkage group (see Reyes et al. 1998, for one recent example) or the use of monotelotrisomics to determine the order of genes on a chromosome arm (Khush and Rick 1968). The pollen-part mutant lines should therefore not only help us resolve ambiguities in the existing *N. alata* map but also allow accurate placement of the *pollen-S* gene with respect to surrounding markers.

Several features make *48A* a candidate for *pollen-S*. These include its close linkage to the *S* locus, its expression in haploid pollen grains and, of particular interest, the presence of a different *48A* RFLP in each of our *S* homozygous lines. Although these features are suggestive of an involvement in self-incompatibility, they are not proof that *48A* is *pollen-S*. Polymorphism at *48A* could indeed be a result of the same type of frequency-dependent selection that maintains the S-RNase polymorphisms. This would imply some role for the *48A* gene in self-incompatibility. However, it is also possible that polymorphism is a consequence of the close proximity of *48A* to the *S* locus, in which case this gene may play no direct role in self-incompatibility.

48A alleles (to use the term in its broadest sense) may be neutral variants that ”hitch-hike” on the *S* locus and accumulate mutations gradually over evolutionary time. This phenomenon was first reported at the *Adh* locus of *Drosophila melanogaster*, where balancing selection for the F/S nonsynonymous substitution also maintained polymorphisms at closely linked sites (Kreitman and Hudson 1991). The *167A* locus is also polymorphic and may be a second example of a gene hitchhiking on the *S* locus. The two possible mechanisms for maintaining *48A* polymorphisms (selection and hitchhiking) can be distinguished from each other using appropriate statistical tests (see for example Clark and Kao 1991), but this would require complete *48A* sequences from several different *S* alleles.

The *48A*, *133G* and *167A* genes were identified by just one differential display band each. This suggests that a continued search for pollen-expressed genes linked to the *S* locus may be productive. An indicator that the survey included all the genes in this region would be the identification of two or more differential display bands from the same gene. Early studies on the kinetics of DNA/RNA hybridisation point to the transcription of large numbers of genes in pollen (Mascarenhas 1990).

On the basis of published estimates of 20,000–24,000 different pollen-expressed genes, each centiMorgan of the *N. alata* genome would contain, on average, 15–18 genes. As we identified only three genes in a region of approximately 3 cM, it is likely that other pollen-expressed genes are near the *S* locus. Apart from self-incompatibility, several other traits related to either pollination biology or reproductive behaviour are known to be in this region (Bernacchi and Tanksley 1997). The strategy of map-based cloning to identify the *pollen-S* gene could also help unravel the nature and sequence of this complex of tightly linked and co-evolved genes. This study reports a significant step in that direction.

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