Theor Appl Genet (1999) 99:391–397

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Centromeric localization of an *S-RNase* gene in *Petunia hybrida* Vilm.

Received: 3 December 1998 / Accepted: 8 December 1998

Abstract *S*-RNase has been identified to be an *S*-allele-specific stylar determinant contributing to the self-incompatibility response in Solanaceae. In order to examine the physical location of the *S-RNase* gene, multi-color fluorescence *in situ* hybridization (FISH) using the S^{B1}*-RNase* cDNA probe and ribosomal RNA gene (rDNA) probe was performed on an $S^{B1}S^{B2}$ heterozygote of *Petunia hybrida*. The S^{B1}*-RNase* gene was detected as a doublet signal close to the centromere of chromosome III. Next, we performed FISH using a large genome probe prepared from a λSB1–311 clone (20 kb) which contains the S^{B1}*-RNase* gene and its 3′ flanking region. This probe hybridized to the centromeric regions of all *P. hybrida* chromosomes. Sequence analysis of the λSB1–311 clone revealed the presence of a repetitive sequence consisting of a novel 666 bp unit sequence. A subclone (pBS-SB1B5) containing this unit sequence also hybridized to all of the centromeric regions, confirming that this unit is the centromeric specific repetitive sequence. These data suggested that the S^{B1}*-RNase* gene is located very close to (within a distance of 12 kb from) the centromeric-specific repetitive sequence. Likewise, the pBS-SB1B5 probe hybridized to the centromeric regions of all chromosomes in *P. littoralis*, another *Petunia* species. However, the probe did not hybridize to the centromere of the chromosomes from other species in Solanaceae. These results suggested that this centromeric repetitive sequence might be a genus-specific one.

Communicated by F. Mechelke

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Key words Centromeric specific repetitive sequence · FISH · *Petunia hybrida* · Self-incompatibility · *S-RNase* gene

Introduction

Self-incompatibility (SI) is a major mechanism which prevents self-fertilization and promotes cross-fertilization in angiosperms (de Nettancourt 1977). SI in Solanaceae is of the gametophytic type and controlled by a single multiallelic locus called the *S*-locus (Newbigin and Clarke 1993). When the *S*-allele of a pollen matches one of the two *S*-alleles of a style, pollen-tube growth will be arrested in the transmitting tract of the style. One of the *S*-allele products is a glycoprotein with RNase activity that is expressed in the transmitting tract of the style; this protein is called *S*-RNase (McClure et al. 1989). The involvement of *S*-RNase in SI response has been demonstrated by a loss- and gain-of-function transformation experiments (Lee et al. 1994; Murffet et al. 1994). Although the stylar *S*-determinant, *S*-RNase, has been analyzed in depth, no pollen *S*-determinant in the SI response has been identified. As the pollen *S*-determinant gene should be located close to the *S-RNase* gene (Lewis 1960), it is important to clarify the location of the *S-RNase* gene on the chromosome and analyze its flanking region.

Petunia is a genus of Solanaceae, and its SI has been well studied at the molecular level. However, the cytological character of the *S-RNase* gene region is still unknown. Our purpose in the study presented here is to determine the location of the *S-RNase* gene on the chromosome and to characterize the flanking region of the *S-RNase* gene in one *S* line of *Petunia hybrida*. First, we performed FISH in *P. hybrida* using the cDNA of *S-RNase* as a probe to detect the *S-RNase* gene on a chromosome. Second, we performed multi-color FISH using the cDNA of *S-RNase* and rDNA as probes to identify the chromosome on which the *S-RNase* gene is located. Third, we performed FISH using a large genomic clone

(20 kb) that includes the *S-RNase* gene at its 5´ terminal region as a probe, which to analyze the flanking region of the *S-RNase* gene. In this paper, we describe the exact localization of the *S-RNase* gene in *P. hybrida* and the finding of a novel centromeric repetitive sequence in the flanking region of the *S-RNase* gene.

Materials and Methods

Plant material

Petunia hybrida Vilm. PB line, *P. littoralis*, *Solanum sanitowongei* L. and *Nicotiana tabacum* were grown in a greenhouse at 25°C. *P. hybrida* PB is a newly isolated self-incompatible diploid line (Entani et al. submitted). This line is an $S^{B1}S^{B2}$ heterozygote, and the sequences of its *S-RNases* (S^{B1}- and S^{B2}-*RNase*) will appear in DDBJ, EMBL and Gen Bank Nucleotide Sequence Databases under the accession number AB016522 and AB016523, respectively.

Genomic DNA gel blot analysis

The genomic DNA (2.5 µg) of *P. hybrida* PB was extracted according to Murray and Thompson (1980), digested with *Eco*RI or *Hin*dIII and then electrophoresed on 0.7% (w/v) agarose gel. The fractionated DNAs were blotted onto a Hybond N^+ membrane (Amersham Pharmacia). Probe labeling, hybridization and detection of hybridized DNA were carried out using AlkPhos Direct (Amersham Pharmacia) following the manufacturer's instructions. Stringent washing was performed at 65°C.

Isolation of genomic clone

The genomic DNA was partially digested by *Sau*3AI and cloned into λEMBL3 (Stratagene). Digoxigenin-labeled S^{B1}-*RNase* cDNA was used as the probe to screen the genomic DNA library.

Polymerase chain reaction (PCR)

PCR was carried out using LA *Taq* DNA polymerase (Takara) following the manufacturer's instructions. A solution of isolated λ clone was used as the template. For amplifying the λ insert, we used the left-arm primer of λEMBL3 (CTTGCAGACAAACTGCGCAACTCGTGAAAG) and the right-arm primer of λEMBL3 (GAACACTCGTCCGAGAATAACGAGTGGATC). For confirming the location of the S^{B1}-*RNase* gene in the λSB1–311, wqe used the S^{B1} sense primer (GATCACAGCTCACGTCAGTCATCTTCGTTC), S^{B1} antisense primer (TGGAACCCTTGAGTAATTGCCTGACAGTGTTAT) and the above-mentioned λ primers.

Subcloning and sequencing

Insert DNA of isolated clones was amplified by PCR with LA *Taq* DNA polymerase (Takara). The insert DNA was partially digested by deoxyribonuclease I and electrophoresed. Digested fragments of ranging from 0.7 kbp to 3.0 kbp were purified from the agarose gel using Gene Clean II (Bio 101), then the ends of the fragments were blunted by DNA blunting kit (Takara) and cloned into pBluescript IIKS- (Stratagene). Approximately 200 clones were sequenced (Model 377, Applied Biosystems).

Chromosome preparation and staining

Either young flower buds (less than 1.5 mm in length) of *P. hybrida* and *P. littoralis* or root tips of *S. sanitowongei* and *N.tabacum* were excised and fixed with, ethanol: acetic acid (3:1) at 4°C overnight. Chromosome preparation was carried out using the method described by Fukui et al. (1996) with some modifications. The samples were washed with distilled water and then macerated with an enzymatic mixture containing 4% cellulase Onozuka RS (Yakult, Tokyo, Japan), 2% Macerozyme R-200 (Yakult), Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) and 1 m*M* EDTA for 90 min at 37°C. After the samples had been washed with distilled water, each bud was chopped into small pieces with fine forceps in a few drops of the fixative on a glass slide. The samples were then pretreated with RNase A (100 µg/ml) at 37°C for 1 h. After washing with 2×SSC, the sample slides were incubated with 0.001% pepsin in 0.2 *N* HCl at 37°C for 20 min. The sample slides were subsequently washed with PBS with 50 m*M* $MgCl_2$, fixed with 4% paraformaldehyde in the same buffer and then washed again with PBS. The sample slides were dehydrated in an ethanol series of 70%, 90% and 99% and then air-dried.

Fluorescence in sitz hybridization (FISH)

In situ hybridization was performed following the method described previously (Ohmido and Fukui 1997) with some modifications.

For the detection of the S^{B1}-*RNase* gene, cDNA encoding S^{B1}-RNase was biotin-labeled by PCR. The hybridization mixture consisted of 50% formamide, 4% sodium dextransulphate, 2×SSC, 100 ng labeled probe and 2 µg salmon sperm DNA (16 µl per slide). The mixture was layered on the slides, covered with a cover slip, denatured at 80°C for 5 min and hybridized overnight. After stringent washing at 55°C, the biotin-labeled probe was detected using a Gene Point Kit (DAKO, Calif.) and streptavidine-Cy3 conjugate (Amersham Pharmacia). The chromosomes were counterstained with DAPI (4,6,-diamidino-2-phenylindole).

Multi-color FISH was performed to detect the S^{B1}-*RNase* gene and the 17s-5.8s-25 s ribosomal RNA gene (rDNA) (Sano and Sano 1990), the latter being a marker for chromosomes II and III (Frantz et al. 1996). The S^{B1}-*RNase* and rDNA were biotin- and digoxigenin-labeled, respectively, by the random primer labeling method. Two probes were simultaneously hybridized to the chromosomes. After stringent washing at 55°C, the biotin-labeled probe was detected with Gene Point Kit and avidine-FITC (fluorescein isothiocyanate). The digoxigenin-labeled probe was detected with anti-digoxigenin-rhodamine (Boehringer Mannheim), and the signal was enhanced with anti-Sheep-Texas red (Vector Lab.). The chromosomes were counterstained with DAPI.

Detection of the S^{B1}-*RNase* gene by FISH using the λSB1–311 clone was performed in *P. hybrida*, *P. littoralis*, *S. sanitowongei* L. and *N. tabacum*. The λSB1–311 was labeled with Spectrum Green-dUTP (Vysis) by the nick-translation labeling method. After high-stringent washing in 50% formamide/2×SSC at 55°C or low stringent washing in 30% formamide/2×SSC at 37°C, the chromosomes were counterstained with DAPI.

For the detection of the subclone pBS-SB1B5 in the λSB1–311 clone by FISH, the probe was biotin-labeled by PCR. After stringent washing at 55°C, the biotin-labeled probe was detected by an avidin-FITC conjugate and enhanced by the secondary immunological reaction of the biotinylated anti-avidin and fluorescein-avidin (Vector Lab.). The chromosomes were counterstained with DAPI.

The fluorescent signals of FITC (green), Spectrum Green (green), Cy3 (red), rhodamine (red) and DAPI (blue) were detected by fluorescence microscopy (DM RB, Leica) with an automatic filter wheel (Micro Mover-W, Photometrics). The different fluorescent images were acquired separately in the IP Lab-PVCAM system through a cooled CCD camera (Photometrics). Then they were merged into single composite images and pseudo-colored based on the original fluorescent colors. The composite images were printed out using a color printer (Pictrography, Fuji).

Results

Genomic DNA gel blot analysis

We carried out DNA blot analysis using a cDNA of *S*^{B1}-*RNase* as a probe in order to confirm that this probe hybridizes only with the *S*^{B1}-*RNase* gene in the *S*^{B1}*S*^{B2} heterozygote. The *S*^{B1}-*RNase* cDNA probe was hybridized to the genomic DNA fragments of the *S*^{B1}*S*^{B2} heterozygote digested with *Eco*RI and *Hin*dIII (Fig. 1A). Only one band was detected in each digest, indicating that this cDNA probe specifically hybridized with the *S*^{B1}-*RNase* gene in the genome of this heterozygote. On the other hand the *S*^{B2}-*RNase* cDNA probe specifically hybridized with the *S*^{B2}-*RNase* gene (Fig. 1B). The *S*^{B1}- and *S*^{B2}-*RNase* genes did not cross-hybridize with each other under these experimental conditions. These results seem to be reasonable given that the nucleotide sequences between the *S*^{B1}- and *S*^{B2}-*RNase* have only 60% homology.

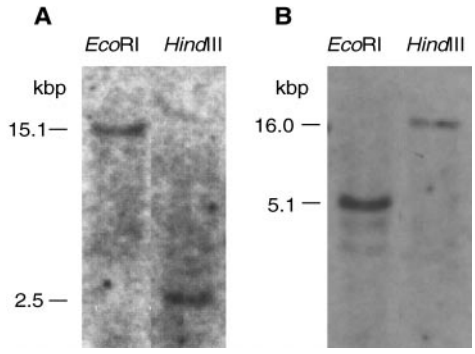

Fig. 1A, B Genomic DNA gel blot analysis of the *P. hybrida* PB line (*S*^{B1}*S*^{B2}). *S*^{B1}-*RNase* cDNA (**A**) or *S*^{B2}-*RNase* cDNA (**B**) was used as the probe. Restriction endonucleases used for digestion are indicated at the top of each lane. The sizes of the hybridized fragments are shown to the *left* of each gel blot

FISH using the *S*^{B1}-*RNase* cDNA probe

In order to detect the physical location of the *S-RNase* gene in the chromosomes of *P. hybrida*, we performed FISH using biotin-labeled *S*^{B1}-*RNase* cDNA as a probe. On the metaphase chromosomes (2n=14), the *S*^{B1}-*RNase* gene was detected as a red doublet fluorescent signal, which has been composed with the image of the DAPI-stained chromosomes (Fig. 2A). These doublet signals were located at the subcentromeric region of a single chromosome. As several repetitive experiments showed the same doublet signals at the identical position, we concluded that this must be the locus of *S*^{B1}-*RNase* gene. Based on the chromosome length and the position of the centromere, that the chromosome on which the doublet signal was detected was chromosome III.

Multi-color FISH using the *S*^{B1}-*RNase* gene and rDNA as the probes

The karyotype of *P. hybrida* has been examined (Smith et al. 1973; Fransz et al. 1996). rDNA is a marker of chromosome II and III. We therefore performed multi-color FISH using the *S*^{B1}-*RNase* gene and rDNA as probes in order to identify the chromosome on which the *S*^{B1}-*RNase* gene is located. Figure 2B is a composite image of the DAPI stained blue chromosomal image, the green signal image of the *S*^{B1}-*RNase* gene and the red signal image of rDNA. There were three red signals and one dispersed red signal. As chromosome II is a satellite chromosome, the rDNA signal is strong and sometimes dispersed. Therefore, the dispersed signal here is due to the existence of the satellites being apart from its harboring chromosome, II (arrow). The green signal of the *S*^{B1}-*RNase* gene was observed as a doublet signal close to the centromere of a single chromosome which had a red doublet signal of rDNA gene at the end of its short arm. These results clearly indicate that the chromosome on which the *S*^{B1}-*RNase* gene is located is the chromosome III.

FISH using a large genomic clone, λSB1–311

In order to characterize the genomic organization of the *S*-locus, we obtained several genomic clones containing the flanking region of the *S-RNase* gene. Of these, we paid the most attention to the λSB1–311 clone, which had the longest insert (20 kb) containing the *S-RNase* gene.

The λSB1–311 insert could be amplified by PCR using λ left- and right-arm primers (Fig. 3A). The location of the *S*^{B1}-*RNase* gene in the λSB1–311 insert was determined by PCR as follows. The PCR fragment using the λ left-arm primer and *S*^{B1}-*RNase* antisense primer was 700 bp in size, and PCR fragments using the λ right-arm primer and *S*^{B1}-*RNase* sense primer were similar in size to the whole insert (Fig. 3A). Therefore, the *S*^{B1}-*RNase* gene localizes near the left arm of the clone (Fig. 3B).

We performed FISH analysis using this λSB1–311 clone as a probe. Figure 2C is a composite image of DAPI-stained chromosome images and the image of the green signals from the λSB1–311 clone. The strong green signals were detected at the centromeric regions of all the chromosomes in *P. hybrida*, and the intensity of these signals visually demonstrated that the λSB1–311 clone contains some kind of repetitive sequences. To examine whether the repetitive sequenses occur commonly in the family Solanaceae, another *Petunia* species (*P. littoralis*) and a species from Solanaceae (*S. melongena* and *N. tabacum*) were examined by FISH using the λSB1–311 clone as a probe. Signals were observed at all the centromeric regions of the chromosomes in *P. littoralis*, as shown in Fig. 2D, as well as in *P. hybrida* (Fig. 2C); no signal could be observed in *S. sanitowongei* and *N. tabacum.*

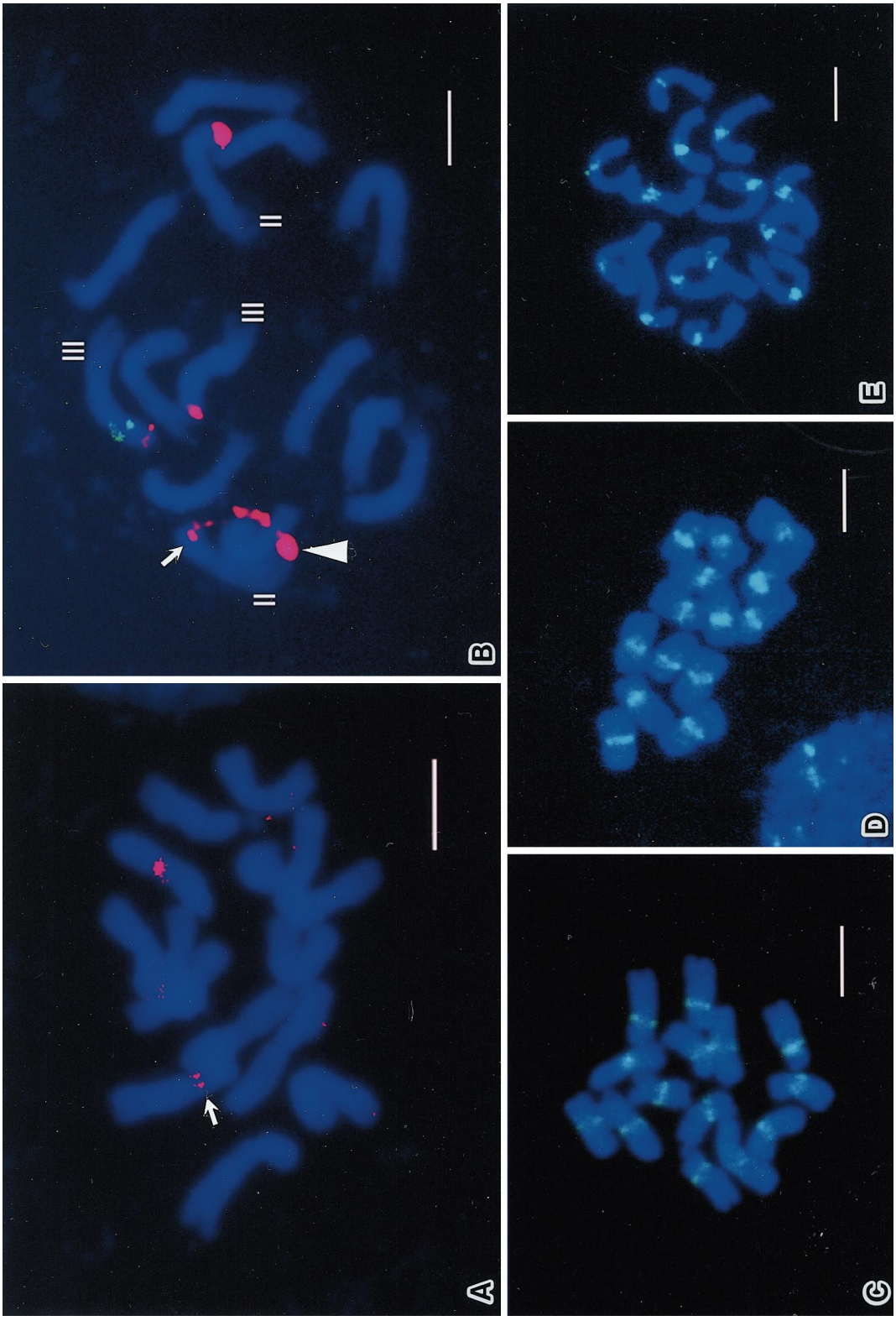

Fig. 2A–E FISH analysis of *P. hybrida* and *P. littoralis*. **A** FISH of *P. hybrida* using S^{B1}*-RNase* cDNA as a probe; a doublet signal (*arrow*) is detected on a subcentromeric region of a single chromosome, **B** Multi-color FISH of *P. hybrida* using S^{B1}*-RNase* cDNA and rDNA as probes. *II* Satellite chromosome II, *III* chromosome III. One satellite (*arrowhead*) is apart from its harboring chromosome, III (*arrow*). **C** FISH of *P. hybrida* using the λSB1–311 clone containing the S^{B1}*-RNase* gene as a probe, **D** FISH of *P. littoralis* using the λSB1–311 clone as a probe, (**E**) FISH of *P. hybrida* using the pBS-SB1B5 subclone as a probe. *Bar* 5 μm.

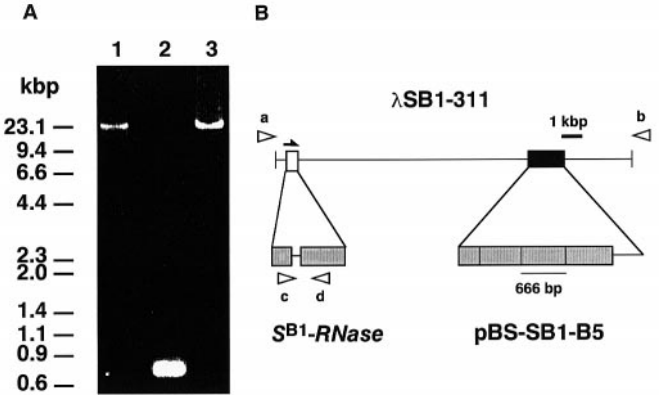

Fig. 3 A DNA fragments amplified by PCR using the λSB1–311 clone as a template. *Lane 1* PCR products using EMBL left- and right-arm primers, lane *2* PCR products using the EMBL right-arm and S^{B1}-*RNase* antisense primers, lane *3* PCR products using the EMBL left-arm and S^{B1}-*RNase* sense primers. The molecular weight markers are indicated on the left. **B** Schematic representation of the λSB1–311 clone. *Open box* represents S^{B1}-*RNase* gene, *filled box* the pBS-SB1B5 subclone. Arrowheads indicade primers: *a* EMBL right-arm primer, *b* EMBL left-arm primer, *c* S^{B1}-*RNase* sense primer, *d* S^{B1}-*RNase* antisense primer.

FISH using a subclone pBS-SB1B5 from λSB1–311

To characterize the above-mentioned centromeric repetitive sequence we randomly subcloned and sequenced the insert of the λSB1–311 close. Among these subclones, a subclone pBS-SB1B5, contained three repeats of a 666-bp unit sequence (Fig. 4). The position of the pBS-SB1B5 subclone was mapped at a distance of about 12 kb from S^{B1}-*RNase* (Fig. 3B). We could not find any special characteristics in the sequence which have been reported in other related repetitive DNA families.

FISH was performed using the pBS-SB1B5 subclone as the probe. Figure 2E is a composite image of DAPI-stained chromosome images and the image of the green signals from the pBS-SB1B5 sequence. Green signals were observed at the centromeric regions of all the chromosomes, suggesting that this repetitive sequence is the centromeric repetitive one.

Discussion

Chromosomes in *P. hybrida* (2n = 14) have previously been identified on the basis of chromosome size, position of centromere and the localization of the rRNA gene cluster. In addition, single-copy genes such as chalcone synthase A (*chsA*) have been mapped (Frantz et al. 1996; Hoopen et al. 1996). In the present study, the analysis by FISH using the S^{B1}-*RNase* cDNA and rDNA as probes demonstrated clearly that the *S*-locus occurs close to the centromere of chromosome III. Furthermore, we sequenced and performed FISH analyses of genomic clone λSB1–311, which contains the *S-RNase* gene. The λSB1–311 probe hybridized to the centromeric regions of all chromosomes. A subclone pBS-SB1B5 containing three repeats of a 666-bp unit sequence of λSB1–311 also hybridized to the centromeric regions of all chromosomes. We therefore concluded that this 666-bp unit in pBS-SB1B5 is a centromeric specific repetitive sequence. Sequencing analysis clarified that the *S-RNase* gene is located at a distance of 12 kb from a centromeric specific repetitive sequence. Robbins et al. (1998) determined that the *S*-locus is located at a subcentromeric region by analysing the locus of T-DNA closely tagged to the *S*-locus. In the present study, we could precisely show that the *S*-locus is located at the centromeric region of chromosome III, adjacent to a novel centromeric specific repetitive sequence.

In *Lycopersicon*, the *S*-locus has been shown to be located close to the centromere of chromosome I (Bernatzky 1993; Bernacchi and Tanksley 1997). If the *S*-locus in Solanaceae is commonly located close to the centromere, the centromeric localization would be important in relation to the structure and function of the *S*-locus. A centromere, which is distinguished morphologically as a primary construction, has an important function in the segregation of chromosomes. In general, the centromeric region is condensed throughout the cell cycle. Thus, there is a slight possibility of recombination in the region around the centromere. In *Petunia*, a heterochromatic region where little T-DNA integration occurs is found around the centromere (Dietrich et al. 1981; Hoopen et al. 1996). Therefore, the *S*-locus in Solanaceae might be said to exist in a region of high genetic stability.

In Solanaceae, many cDNAs of *S-RNases* have been identified and characterized. Comparisons of their sequences have clarified the sequence diversity of *S-RNase* (Tsai et al. 1992; Coleman and Kao 1992). It has been speculated that the allelic specificity of *S-RNase* was generated from the accumulation of point mutations and not from the intragenetic recombination (Coleman and Kao 1992; Tsai et al. 1992). The centromeric location of the *S*-locus may suppress recombination and preserve the sequential uniqueness of each *S*-locus.

Coleman and Kao (1992) suggested the presence of DNA repeats in the flanking region of the *S-RNase* gene in *Petunia inflata* by DNA blot analyses. The analysis of subclone pBS-SB1B5 in the present study clarified that the novel centromeric specific repetitive sequence is present at the flanking region of the *S-RNase* gene.

The pBS-SB1B5 probe also hybridized to all the centromeric regions of chromosomes in *P. littoralis*, another *Petunia* species. The probes, however, did not hybridize to the centromere of the chromosomes from other species in Solanaceae. These results suggest that the centromeric repetitive sequence of *P. hybrida* found in this study might be a genus-specific. In dicotyledoneae, repetitive sequences at the centromere have also been identified in *Arabidopsis* (Murata et al. 1994; Kamm et al. 1995), *Brassica* (Iwabuchi et al. 1991), *Lycopersicon*

Fig. 4 An alignment of the repetitive sequences in the pBS-SB1 B5 subclone. Identical nucleotides are indicated by *asterisks.*

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1                                                                                80
TGCACATATTATATTATGTGCAGAGGAACCAAGCATATCCTTGTGGAGATAGTTACACATAATATAATAGGTGTACAAAT
TGCACATATTATATTATGTGCAGAGGAACCAAGCATATCATTGTACAAATAGTTACACAGAATATAATAGGTGTACACAT
TGCACATATTATATTATGTGCAGAGGAACCAAGCATATCATTGTACAAATAGTTACACAGAATATAATAGGTGTACACAT
TGCACATATTATATTATGTGCAGAGGAACCAAGCATATCATTGTGCAAATAGTTACTCATAATAAAATAG-TGTACACAT
************************************* ****  * ******** ** **** ***** ****** **

81                                                                              160
TATATTATATGCGTGCACATATTATAATATGTGCACACATAATGTTGTATTTTCCAAATCTTAGAAATAGAGAATGATCC
AATATTATATGCGTGCACATATTATAATATGTGCACCCATAGTGTTGTTTTTTCTAAATCTTAGAAATGGAGAATAATCC
AATATTATATGCGTGCACATATTATAATATGTGCACCCATAGTGTTGTTTTTTCTAAATCTTAGAAATGGAGAATAATCC
AATATCATATGTGTGCACATATTATAATATGTGCACACATAATATTGTATTTTCCAAATCTTAAAAATAGAGAATGATCC
 **** ***** ************************ **** * **** ***** ******** **** ****** ****

161                                                                             240
CACTATTTCTTGCACTGAATTATACTGAATAGG-CGTAATTTT-GTGGTATATATGCTCTTATTGTATCCATCTTGGCCT
CACTATTGCTTGTACTGAATTATAGTGAATAGGGCGTAATTTTTGCGGTATATGTGTTCTTATTGTATCCATCTTGGCCT
CACTATTGCTTGTACTGAATTATAGTGAATAGGGCGTAATTTTTGCGGTATATGTGTTCTTATTGTATCCATCTTGGCCT
CACTATTGCTTGCACTGAATTATAGT
******* **** *********** ******** ********* * ******* ** **********************

241                                                                             320
ATTTGATCGTCCTTTGTTGATACTGCATGAAAACCTTGGGTTGAAGGCTGAATTCAAACAAAGTGTTACATTATTAGTTT
ATTTGATCGTCCTTTGTTGATACTGCAAGTAAACCTTGGGTTGAAGGCTGAATTCAAACAAAGTGTTACATTATTAGTTT
ATTTGATCGTCCTTTGTTGATACTGCAAGTAAACCTTGGGTTGAAGGCTGAATTCAAACAAAGTGTTACATTATTAGTTT
*************************** * ************************************************

321                                                                             400
TGGATAATGATGATAATAGTGTAAAAGACACGAACCAAGTGCTTTGGTGCATATTTTAATCGTTTCACATTGAAATGCAC
TGGATAATGATGATACTAGTGTAAAAGACACGAACCAAGTGCTTTGGTGCATATTTTTATCGTTTCACATTGAAATGCAC
TGGATAATGATGATACTAGTGTAAAAGACACGAACCAAGTGCTTTGGTGCATATTTTTATCGTTTCACATTGAAATGCAC
*************** ****************************************** **********************

401                                                                             480
TATAAGTATATCAATTTGTGTGGATTGTTGAGATTTCTCACTCATTATCAAGTCTAGACTTGATGAGTACCAAAGAACAC
TATAAGTATATCAATTTGTGTGGATTGTTGAGATTTCTCACTCATTATCAAGTCTAGACTTGATGAGTACCAAAGAACAC
TATAAGTATATCAATTTGTGTGGATTGTTGAGATTTCTCACTCATTATAAAGTCTAGACTTGATGAGTACCAAAGAACAC
********************************************** ******************************

481                                                                             560
ATAGAAAAATGCTTGAATGAGTTTTGATTAGACCAAAGCAACATATTATATATGAAAATATTACCAAAACTACGTGTACC
ATAGAAAAATGCTTGAATGAGTTTTGATTAGACCAAAGCAACATATTATATATGAAAATATTACCAAAACTACGTGTACC
ATAGAAAAATGCTTGAATGAGTTTTGATTAGACCAAAGCAACATATTATATATGAAAATATTACCAAAACTACGTGTACC
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561                                                                             640
GTAAAATAAATGGACAGCTAACGCATTGCAAAGTACATAACTAAGATATGCATGGTTCCACAGCACATCATACAATATTT
GTAAAATAAATGGACAGCTAACGCATTGCAAAGTACATAACTAAGATATGCATGGTTCCACAGCACATCATACAATATTT
GTAAAATAAATGGACAGCTAACGCATTGCAAAGTACATAACTAAGATATGCATGGTTCCACTGCACATCTCACAATATGT
************************************************************ *******  ******* *

641                   666
GTAGACATCATTTAATATGTGCACCG
GTAGACATCATTTAATATGTGCACCG
GTACACATCATTTAATATGTGCACCG
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(Ganal et al. 1988), and *Vigna* (Galasso et al. 1995). However, these sequences were all species-specific or genome-specific. Therefore, the centromeric-specific repetitive sequences of the present study is the first genus-specific sequence reported in dicotyledoneae.

In the present study we determined the centromeric location of *S-RNase* in *P. hybrida*. The results suggest that the gene encoding the unknown *S*-specific pollen determinant should also be located close to the centromeric region of chromosome III. In *Lycopersicon*, the *S*-locus controls not only SI but also a number of floral characteristics affecting reproductive behavior (Bernacchi and Tanksley 1997). An extensive analysis of the region close to the centromere of chromosome III will elucidate not only the pollen *S*-determinant but also gene function in the reproductive process in *Petunia*.

Recently, stable artificial chromosomes in humans have been constructed with the ingredients of centromere and telomere (Harrington et al. 1997; Ikeno et al. 1998). Such artificial chromosomes may become a suitable vector for the functional analysis of large complex genes. Further characterization of the centromeric region around the *S-RN*ase gene in *P. hybrida* might provide useful information for the construction of plant artificial chromosomes.

Acknowlegements We are grateful to Tokiko Nakanishi and Keiko Fujii for technical assistance, and Akiko Arai for supporting to cultivate plant materials. This work was supported in a part by Grants-in-Aid for Special Research on Priority Areas (nos. 07281102 and 17281103; Genetic Dissection of Sexual Differentiation and Pollination Process in Higher Plants) from the Ministry of Education, Science, Culture and Sports, Japan.

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