植 物 学 报
Acta Botanica Sinica 2003, **45** (1): 47 – 52 http://www.chineseplantscience.com

The Self-incompatibility (*S*) Locus of *Antirrhinum* Resides in a Pericentromeric Region

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Abstract: The self-incompatibility (*S*) loci from the Solanaceae, Rosaceae and Scrophulariaceae encode a class of ribonucleases, known as S RNases, which have been shown to control the pistil expression of self-incompatible reaction. In the former two families, the *S* loci have been shown to be located near centromere. However, the chromosomal location of the *S* locus in *Antirrhinum*, a species of the Scrophulariaceae, is not known. To determine its chromosomal location and genomic organization, an S_2 *RNase* gene and its corresponding 63 kb BAC clone were separately used for fluorescence *in situ* hybridization (FISH) of mitotic metaphase chromosomes of a self-incompatible *Antirrhinum* line of S_2S_5. The results showed that the S_2 *RNase* detected a doublet signal near the centromere of the smallest chromosome (2n = 16). Two separate doublet signals of the tested BAC sequence were shown on both sides of the centromeres of all eight pairs of the chromosomes, suggesting that the *Antirrhinum S* locus is located in a pericentromeric region. Furthermore, a retrotransposon, named *RIS1* (retrotransposon in the *S* locus), which has not been identified yet in *Antirrhinum*, was found next to S_2 *RNase*. Taken together, the centromeric location of the *S* locus from the three S-RNase-based self-incompatible families provides a further support on a common origin of their evolution as well as suppressed recombination.

Key words: *Antirrhinum*; self-incompatibility (*S*) locus; pericentromere; retrotransposon; FISH

Self-incompatibility (SI) is an intraspecific reproductive barrier which prevents self-fertilization and promotes cross-fertilization in angiosperms. SI in the Solanaceae, Scrophulariaceae and Rosaceae is of the gametophytic type controlled by a multi-allelic locus, the *S* locus (de Nettancort, 2001). A known product encoded by the *S* locus is a class of ribonucleases, called S RNases, which have been shown to be the style determinant in SI response by loss- and gain-of-function transformation experiments in the solanaceous species (Clarke and Newbigin, 1993; McCubbin *et al*, 2000a). However, the identity of a pollen S-determinant or *Sp* is still elusive although it is genetically linked to the *S RNase* gene (McCubbin and Kao, 2000). Li *et al* (2000) obtained three cDNA markers linked to the *S* locus in *Nicotiana alata* and found that one cDNA, *48A*, is specifically expressed in anther. McCubbin *et al* (2000b) also obtained several pollen cDNA makers linked to the *S* locus in *Petunia inflata*. Lai *et al* (2002) identified an F-box gene, *Ah-SLF-*S_2, which was located 9 kb downstream of S_2 RNase in *Antirrhinum* and specifically expressed in pollen and tapetum. Further experiments is required to demonstrate whether any of them encodes *Sp* or simply is S-linked pollen expressed genes.

Phylogenetic analyses of the S RNase sequences from the Solanaceae, Rosaceae and Scrophulariaceae have suggested that they likely share a common origin (Xue *et al*, 1996; Igic and Kohn, 2001). This is supported by the findings that the *S* loci from the former two families are both located near the centromere (Brewbaker and Natarajan, 1960; Pandey, 1965; Ten Hoopen *et al*, 1998; Entani *et al*, 1999). The chromosomal location of the *S* locus in *Antirrhinum*, a member of the Scrophulariaceae, is unknown. In the present study, we have shown that the *Antirrhinum S* locus is also located in the pericentromeric region, providing an additional support for a common origin of the *S* locus within the S-RNase-based self-incompatible species. Furthermore, such a centromeric location is consistent with the recombination suppression observed for the *S* locus.

1 Materials and Methods

1.1 Plant material

Antirrhinum majus M^{75} (self-incompatible) and self-incompatible line derived by interspecific crosses between *A. majus* and *A. hispanicum*, as well as several *S* allele segregating populations, were described previously (Xue *et al*, 1996; Lai *et al*, 2002). The plant material used in this experiment was A_1^5 (S_2S_5).

1.2 Genomic DNA blot analysis

Genomic DNA was isolated from *Antirrhinum* leaves by using the method as described (Xue *et al*, 1996). The DNA (10 μg) was digested, separated on a 0.8% agarose gel and transferred onto hybond N^+ (Amersham) membrane. Prehybridization, hybridization, and washing of the blots were performed as recommended by the manufactures. DNA sequence analysis was performed by a dotter program (http://www.cgr.ki.se/cgr/groups/

Received: 2002-06-24 Accepted: 2002-09-02
Supported by The Chinese Academy of Sciences and the National Natural Science Foundation of China (39825103).
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sonnhammer/Dotter.html). *RIS1* (retrotransposon in the *S* locus) was part of S_2*BAC* sequence under the EMBL accession number AJ300474.

1.3 Chromosome preparation

Root tips of *Antirrhinum* A_1^5 (S_2S_5) were excised and fixed with ethanol:acetic (3:1) at 4 ℃ overnight. The fixed root tips were washed thoroughly with distilled water and 0.4 mol/L sorbitol buffer (pH 4.8), then digested in 3% cellulase (Onozuka) and 0.5% pecteolyase (Onozuka) for 1 h at 37 ℃. The enzyme was carefully washed away from the softened material and replaced with the sorbitol buffer and then with distilled water. The softened root tip was transferred to ethanol-washed glass slides and was macerated in a drop of 45% acetic acid with razor blade. The slides were air dried and stored in 4 ℃. Before hybridization, the slides were treated with an ethanol series (70%, 90% and 100% ethanol; 10 min each) and then pretreated with RNase (100 μg/mL) at 37 ℃ for 1 h after washed with 2 × SSC. The slides were incubated with 0.01% pepsin (Sigma) in 0.2 mol/L HCl at 37 ℃ for 15 min and subsequently washed with 1 × PBS with 50 mmol/L $MgCl_2$ and then 1 × PBS. The slides were dehydrated in an ethanol series of 70%, 90% and 100% and air-dried.

1.4 Probe labeling

Plasmid and BAC DNA were isolated by an alkaline-lysate method (Maniatis *et al*, 1982). Purified S_2 *RNase* (a PCR fragment covering its genomic DNA) and the BAC clone was labeled by a standard nick-translation reaction mixture containing Dig-11-dUTP.

1.5 Fluorescence *in situ* hybridization (FISH)

FISH essentially followed that of Jiang *et al* (1995). About 10 ng of labeled BAC DNA was used for each slide in a hybridization mixture with 50% formamide/10% dextran sulfate/2 × SSC/10 μg of salmon sperm DNA /1 μg of cot-1 fraction of *Antirrhinum* genomic DNA, the mixture was denatured at 80 ℃ for 10 min, centrifuged briefly, and preannealed at 37 ℃ for 1 h before applied to slide. Slide-bound chromosome DNA was denatured in a solution of 70% formamide in 2 × SSC for 1.5 min at 80 ℃ and dehydrated in a − 20 ℃ ethanol series (70%, 90% and 100% ethanol; 10 min each). Twenty microliters of a hybridization mixture was applied to each slide and sealed under a coverslip, denatured at 80 ℃ and then incubated at 37 ℃ overnight.

After the overnight incubation, the coverslips were removed in 2 × SSC at room temperature, washed twice at 42 ℃ in 20% formamide (in 2 × SSC solution) for 10 min, twice at room temperature in 2 × SSC for 10 min, and then the fluorescence antibody enhancer set for DIG Detection kit (Roche Diagnostics) was used to enhance the fluorescence signal. The slides were washed twice with 4 × SSC, and the chromosomes were conterstained with PI (phenylindole) and dehydrated with the ethanol series (70%, 90% and 100% ethanol).

1.6 Digital imaging

Images were obtained with an Olympus microscope (BX-50) coupled with a cooled CCD camera (Apogee), camera control and digital image acquisition were implemented with a computer using software Image-Pro. Fluorescence was selectively imaged with filter cubes especially prepared by filter to minimize image registration problems, and then they were merged into a single composite image. The composite images were printed out using a color printer.

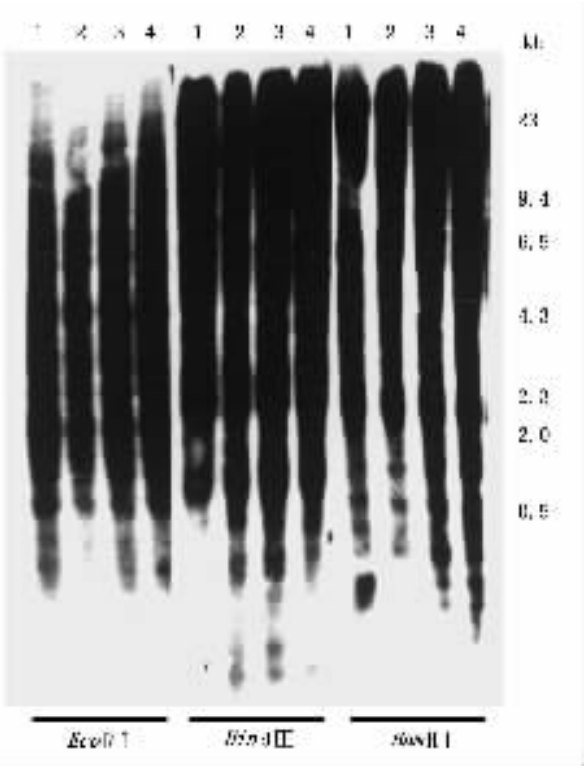

Fig.1. DNA blot analysis of *RIS1*.
Ten μg genomic DNA from various *S* allele-containing lines were restricted by *Hin*dⅢ, *Eco*RⅠ and *Bam*HⅠ respectively. After separation by agarose gel electrophoresis and blotting, the DNA was hybridized with a *RIS1* genomic sequence. Lanes 1 – 4 represent self-incompatible lines of S_1S_5, S_2S_4, S_1S_4, and S_2S_5 respectively. The molecular weight markers are indicated on the right side in kb.

2 Results

2.1 Association of the *S* locus with a nested retrotransposon

In a previous study, a genomic fragment of ca. 63 kb containing S_2 *RNase* was isolated in *Antirrhinum* and its DNA sequencing analysis revealed that four predicted ORFs (open reading frames) are homologous to retrotransposons and another five ORFs representing unknown origins (Lai *et al*, 2002). In addition, a gene, called *AhSLF*-S_2, was found to be specifically expressed in pollen and tapetum (Lai *et al*, 2002). To investigate further the nature of these predicted unknown ORFs, one ORF (*Gene8*), located besides S_2 *RNase* encoding a predicted polypeptide of 151 amino acids (Lai *et al*, 2002), was studied here. Northern blot using *Gene8* as a probe and RT-PCR (reverse transcription-polymerase chain reaction) indicated that it was not expressed in several tis-

sues tested including anther, pistil, petal, sepal and leaf (data not shown), indicating that it unlikely encodes an authentic ORF.

To determine its genomic organization, DNA blot analysis was carried out using *Gene8* as a probe. The result showed that it hybridized to multiple fragments in genomic DNA digested by several restriction enzymes, ranging from 0.1 to over 20 kb (Fig.1), indicating that it is highly repeated within the genome. To classify which type of repeats this sequence belongs to, it was further analyzed by a dot blot analysis and two nest inverted repeats, 183 bp and 196 bp long respectively, resembling LTRs (long terminal repeats) of retrotransposons (Vicient *et al*, 2001) as well as a target site duplication of 5 bp, were detected (Fig. 2), showing that it is an LTR retroelement and was referred to as *RIS1* which is 2 953 bp in length. However, no RNA expression and a divergence of LTRs of the *RIS1* suggested that it had lost its transpositional activity and was a silent copy of the RIS type retrotransposns in *Antirrhinum*. Database searches revealed that *RIS1* was only homologous to a part of an unannoted rice BAC sequence (EMBL Acc. No. AC07985.2), which is highly repeated in the rice genome based on a search against a draft rice genome sequence (http//btn.genomics.org.cn/rice/). Therefore, *RIS1* represents a repetitive sequence not previously characterized in *Antirrhinum*.

2.2 Chromosomal location of S_2 RNase gene

To investigate the chromosomal location of the *S* locus, we performed fluorescence *in situ* hybridization (FISH) of *Antirrhinum* mitotic metaphase chromosomes from a self-incompatible line of S_2S_5 using S_2 *RNase* gene as a probe. Consistent with the previous observation (Stubbe, 1966), *Antirrhinum* has a total of sixteen chromosomes (2n = 16) (Fig.3C). A doublet signal was detected with S_2 *RNase* and located near the centromere judged by the chromosome morphology (Fig.3A), similar to that in the Solanaceae and Rosaceae (Entani *et al*, 1999; Hoopen *et al*, 1998). No signal was detected on the chromosome containing S_5 allele (Fig. 3A), consistent with the sequence divergence between S_2 *RNase* and S_5 *RNase* (Xue *et al*, 1996). Although genetic and

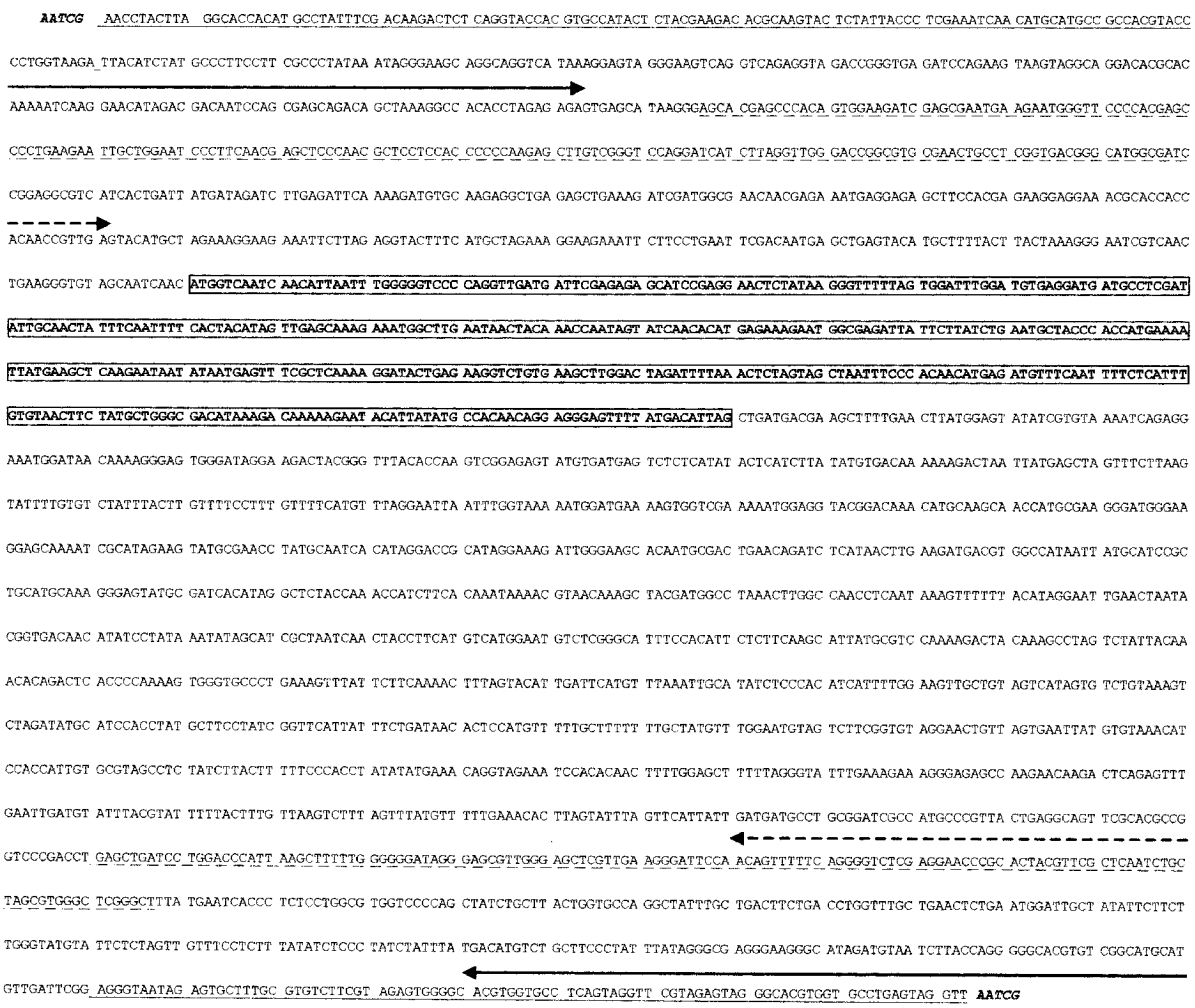

Fig.2. Recrotransposon in the *S* locus (*RIS1*) sequence.
Two nested inverted repeats are indicated by arrowed straight and dotted lines, respectively. A target site duplication is italicized and the sequence corresponding to the predicted ORF (*Gene8*) is boxed.

Fig.3. Chromosomal location of the *Antirrhinum S* locus by FISH.
A. FISH using S_2 *RNase* as probe. The arrows indicate the doublet signal detected near the centromere of the smallest chromosome. Bar = 1 μm. B. FISH using S_2*BAC* as probe. The arrows indicate two separate doublet signals on both sides of the centromere of all eight pairs of the chromosome. Bar = 1 μm. The same chromosome spread was separately used in A and B. C. *Antirrhinum* root tip at metaphase.

RFLP analysis showed that the *Antirrhinum S* locus is linked to *cyc* (*cycloidea*), it is not clear which chromosome contains *cyc* (Xue, 2000). Based on an estimation of the chromosome sizes (Fig.3), the S_2 *RNase*-containing chromosome appeared to be the smallest and therefore we tentatively conclude that the *S* locus is located on chromosome 8 in *Antirrhinum*.

2.3 The pericentromeric position of the *S* locus

To further determine in which particular portion of the centromere the *S* locus resides, the S_2*BAC* clone (Lai *et al*, 2002) was used for FISH analysis. The result showed that it hybridized to two doublet signals near the centromeres on all eight pairs of the chromosomes located on separate chromosomal arms (Fig.3B), indicating that the S_2 allele contains repetitive sequences rich on both sides of the centromeres, a region known as pericentromere (Mayer *et al*, 1999; Fransz *et al*, 2000). Due to the chromosome preparation, some chromosomes had faint and obscure signals, but the signals were clearly associated with the centromeres (Fig.3B). Taken together, the *Antirrhinum S* locus specifically resides in the pericentromeric region.

3 Discussion

We have provided the evidence that the *Antirrhinum S* locus is located within the pericentrimeric region, giving rise to further support for the notion that the S RNase-based self-incompatibility systems share a common evolutionary origin (Xue *et al*, 1996; Igic and Kohn, 2001). The pericentromeric position of the *S* locus has important implications in its origin and functional maintenance as well as further structural analysis.

The centromeric location of the *S* locus of several species in the Solanaceae and Rosaceae (Bernacchi and Tanksley 1997; Entani *et al*, 1999; Ten Hoopen *et al*, 1998) and in *Antirrhinum* (this study) clearly supports a monophyletic origin of the *S* loci from three S-RNase-based self-incompatible families (Xue *et al*, 1996; Igic and Kohn, 2001). However, it is not clear in which particular portion of the centromere the *S* locus resides. The flanking sequence analysis of an *S RNase* gene in *Petunia hybrida* showed that it has three repeats of 666 bp long which specifically hybridized to the centromere, but it is likely that the hybridization could be due to the presence of similar repeats within the centromere (Entani *et al*, 1999). In this study, we demonstrated that the *Antirrhinum S* locus is actually located in the pericentromere, indicating that the *S* loci in the S RNase-based self-incompatible families are located at the similar chromosomal region.

The sequenced pericentromeric regions in *Arabidopsis* have shown that they are repetitive in structure but different from the centromere core, consisting of numerous dispersed repeats, sometimes interspersed with functional genes (Mayer *et al*, 1999; Fransz *et al*, 2000; Haupt *et al*, 2001). The centromere core is largely composed of abundant tandem arranged 180 bp repeats and dispersed repeats, such as 106B, whereas the pericentromere contains mainly retrotransposon elements (Fransz *et al*, 2000). At present, the number of active genes is not known, but at least some active genes reside within these highly heterochromatic regions (Fransz *et al*, 2000). The

pericentromere of *Arabidopsis* chromosome 4 has a predicted gene density of less than one gene per 100 kb (Mayer *et al*, 1999). In *Antirrhinum*, two transcribed genes have been found in the *S* locus within a space of 10 kb, and sequence analysis of the *Antirrhimum* 63 kb BAC clone insert revealed that this region is rich in retrotransposons (Lai *et al*, 2002). Extensive experiments on several other predicted genes within the sequenced 63 kb region did not yield any confirmation of their expression (data not shown). The association of *RIS1* or its relatives with two expressed genes in the 63 kb region shows that the pericentromeres in *Antirrhinum* and *Arabidopsis* are similar in structure with up to about 30% transposable elements and a similar expressed gene density (Fransz *et al*, 2000; Haupt *et al*, 2001).

The association of the *S* locus with the pericentromeric region is likely closely related to its origin and functional maintenance. A centromere is a distinguished morphological primary construction of chromosome and plays an important role in the segregation of chromosome. In general, the centromeric region is condensed throughout the cell cycle, thus there is little possibility of recombination in the region around the centromere (Haupt *et al*, 2001). Therefore, the *S* locus exists in a region of high genetics stability. The allelic specificity of S RNases exemplified by their extensive divergence is likely an outcome of accumulation of point mutations rather than intragenic recombination (Coleman and Kao, 1992; Tsai *et al*, 1992). The lack of the intragenic recombination in the *S* locus appears to be related to its pericentromeric location, where recombination is severely suppressed resulting in the preservation of the sequence uniqueness of each *S* allele. The retrotransposons like *RIS1* and other repeats (Entani *et al*, 1999) identified in the *S* locus suggest that frequent transpositions occur within this region. Its implications in shaping up the *S* locus as well as its function are clearly worthy of further investigation. As suggested before (McCubbin and Kao, 2000), the abundant repeat sequences in the *S* locus will make the identification of *Sp* through a fine structural analysis of the S locus in the *S* RNase-based self-incompatible systems a difficult task. In the future, a functional complementation strategy using BIBAC (McCubbin *et al*, 2000b) or TAC clones covering the *S* locus via transformation might be useful for cloning *Sp*.

Acknowledgments: We thank CHENG Zhu-Kuan, LI Da-Yong and WU Shi-An for their help with FISH technique. MA Wen-Shi thanks the College of Life Sciences, Hebei Normal University, for support.

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金鱼草自交不亲和位点位于着丝粒的周边区

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摘要: 在蔷薇科、茄科和玄参科配子体自交不亲和中,编码花柱的 S RNase 控制花柱的自交不亲和性。在前两科植物中,自交不亲和(*S*)位点定位于着丝粒的附近,但在玄参科植物金鱼草(*Antirrhinum*)中自交不亲和位点至今未知。为了确定它在染色体上的位置和基因组结构,以基因型 S_2S_5 金鱼草根尖为材料,进行染色体的制备观察,利用地高辛标记的 S_2 *RNase* 和含有其全长的 BAC 克隆(S_2 *BAC*)为探针进行荧光染色体原位杂交(FISH),发现 S_2 *RNase* 杂交信号位于染色体的着丝粒附近,而 S_2 *BAC* 的杂交信号位于每条染色体的着丝粒的周边区,呈对称的 4 个,表明金鱼草 *S* 位点位于着丝粒的周边区。对 S_2*BAC* 预测基因的分析表明,发现一个金鱼草新的反转座子(*RIS1*)。结果显示,金鱼草 *S* 位点位于染色体着丝粒的周边区,富含转座子和反转座子,和其他两类配子体自交不亲和的位置类似,预示它们的共同起源和具有抑制重组的功能。

关键词: 金鱼草;自交不亲和位点;着丝粒周边区;反转座子;荧光染色体原位杂交

中图分类号: Q943.2 **文献标识码:** A **文章编号:** 0577-7496(2003)01-0047-06

收稿日期: 2002-06-24 **接收日期:** 2002-09-02

基金项目: 中国科学院和国家自然科学基金(39825103)。

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(责任编辑:彭 丹(实习))