High-resolution mapping of the *S* and *Z* loci of *Phalaris coerulescens*

X.-Y. Bian, A. Friedrich, J.-R. Bai, U. Baumann, D.L. Hayman, S.J. Barker, and P. Langridge

Abstract: Self incompatibility (SI) in *Phalaris coerulescens* is gametophytically determined by two unlinked multiallelic loci (*S* and *Z*). Neither the *S* nor *Z* genes have yet been cloned. As part of a map-based cloning strategy, high-resolution maps of the *S* and *Z* regions were generated from distorted segregating populations using RFLP probes from wheat, barley, oat, and *Phalaris*. The *S* locus was delimited to 0.26 cM with two boundary markers (*Xwg811* and *Xpsr168*) and cosegregated with *Xbm2* and *Xbcd762*. *Xbcd266* was the closest marker linked to *Z* (0.9 cM). A high level of colinearity in the *S* and *Z* regions was found in both self-incompatible and -compatible species. The *S* locus was localized to the subcentromere region of chromosome 1 and the *Z* locus to the long arm end of chromosome 2. Several rice BAC clones orthologous to the *S* and *Z* locus regions were identified. This opens the possibility of using the rice genome sequence data to generate more closely linked markers and identify SI candidate genes. These results add further support to the conservation of gene order in the *S* and *Z* regions of the grass genomes.

Key words: Phalaris coerulescens, self-incompatibility, distorted segregation, mapping, map-based cloning, synteny mapping.

Résumé : L'auto-incompatibilité (SI) chez le *Phalaris coerulescens* est gamétophytique et elle est déterminée par deux locus multialléliques non-liés (*S* et *Z*). Aucun de deux gènes n'a encore été cloné. Dans le cadre d'une stratégique de clonage positionnel, des cartes génétiques à haute résolution des régions *S* et *Z* ont été produites à l'aide de populations montrant une distorsion de la ségrégation et des sondes RFLP provenant du blé, de l'orge, de l'avoine et du *Phalaris*. Le locus *S* a été localisé au sein d'un intervalle de 0,26 cM bordé par les marqueurs *Xwg811* et *Xpsr168*. Les marqueurs *Xbm2* et *Xbcd762* montraient une co-ségrégation avec ce locus. Le marqueur *Xbcd266* était le plus proche du locus *Z* (à 0,9 cM). Un fort degré de co-linéarité des régions *S* et *Z* a été observé tant chez les espèces auto-incompatibles qu'auto-compatibles. Le locus *S* est situé dans la région sub-centromérique du chromosome 1 tandis que le locus *Z* se trouve à l'extrémité du bras long du chromosome 2. Plusieurs clones BAC du riz contenant des régions orthologues ont été identifiés. Ceci ouvre la voie à l'utilisation de la séquence génomique du riz afin de produire des marqueurs génétiques encore plus fortement liés et d'identifier des gènes candidats. Ces résultats appuient davantage la conservation de l'ordre des gènes au sein des régions *S* et *Z* chez les génomes des graminées.

Mots clés : Phalaris coerulescens, auto-incompatibilité, distorsion de la ségrégation, cartographie, clonage positionnel, cartographie de la synthénie.

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Introduction

Self incompatibility (SI) is not only an important genetic mechanism to have evolved in flowering plants to promote gene diversity, it also provides an excellent system for studying cell-to-cell recognition and signal transduction. Several different SI systems have evolved. SI is normally controlled by a multiallelic locus (Franklin et al. 1995). Recently, exciting progress has been made in understanding SI molecular mechanisms in single-locus systems (Golz et al. 2000; Jordan et al. 2000; Nasrallah 2000). SI in grasses is under the control of two unlinked, multiallelic loci, *S* and *Z*, which interact complementarily (Hayman 1992; Lundqvist 1962; Wricke and Wehling 1985). A pollen grain is incompatible

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when both the *S* and *Z* alleles are matched by the alleles in the recipient pistil. Identification of genes from grasses is the essential step to further understanding the molecular mechanisms of self-incompatibility in grasses.

Several strategies have been tried to clone the *S* genes from grasses, but none of them have been successful. Wehling and his coworkers (1994) explored the possibility of cloning homologous sequences using degenerate primers derived from the *Brassica SLG* (S-linked glycoprotein) sequences. However, no further progress has been made since then. Li et al. (1994) chose a differential screening strategy to clone SI genes from *Phalaris*, but later evidence proved this effort failed to find any candidate for *Z* and just identified an *S*-linked gene (Baumann et al. 2000; Langridge et al. 1999). An attempt to correlate the stylar protein patterns with the *S* and *Z* alleles failed for *Phalaris coerulescens* (Tan and Jackson 1988).

Map-based cloning allows cloning of genes whose products have not been identified (Paterson and Wing 1993; Wing et al. 1994). It consists of mapping a trait in a large segregating population, constructing a genomic contig that covers the target region, and characterizing candidate genes (Tanksley et al. 1995). The first requirement of map-based cloning is the precise mapping of a target gene and the determination that the molecular markers flanking the gene are not only genetically linked, but are also physically close to the target gene. Colinearity among the grass genomes permits transferring markers between species and allows for cross species gene isolation, i.e., of genes that have been mapped precisely on the genetic maps in large genome species by map-based cloning in a smaller genome model species like rice (Bennetzen and Freeling 1997; Sasaki 1997). Progress made in rice genome sequencing would greatly help map-based cloning of SI genes from the grasses.

Colinear genetic organization around the *S* and *Z* loci would greatly help the strategy of map-based cloning. Cornish and coworkers (1980) found a linkage between *S* and *PGI-2* (phosphoglycoisomerase) in *Lolium perenne*. This linkage was also found in *P. coerulescens*, *Festuca pratensis*, and *Secale cereale* (Leach and Hayman 1987). The *S* locus was located on chromosome 6 in *Lolium* and 1R in rye by linkage to *PGI-2* and *Prx-7* (a leaf peroxidase) (Lewis et al. 1980; Wricke and Wehling 1985). For *Z*, a linkage was found with the beta-glucosidase locus on chromosome 2 in rye, with a recombination value of 14.4% (Gertz and Wricke 1989). For *Lolium*, another linkage was indicated between the *Z* locus and the isozyme glutamate oxalacetatetransaminase, *GOT-3*, located on chromosome 2 (Lewis et al. 1980; Thorogood and Hayward 1991). Voylokov and his coworkers (1998) reported that *S* was closely linked to *Xpsr634* on 1R and *Z* to *Xbcd266* on 2R in rye.

Phalaris coerulescens is a model system for studying self incompatibility in the grasses. It is a perennial grass and can be clonally propagated, thereby simplifying genotype identification and maintenance (Hayman 1956). A comprehensive collection of different genotypes and mutants is available. Compared with the other characterized self-incompatible species in the grasses, such as *Secale*, *Lolium*, and *Hordeum*, the size of *Phalaris* genome, which has a set of seven pairs of chromosomes, is relatively small, and has a generic mean 2*C* DNA value of about 2.8 to 7.8 pg, which is just about three times that of the rice genome (Watson 1990). This puts *Phalaris* in an advantageous position for the study of SI. The aim of this research was to construct fine maps of the *S* and *Z* regions using RFLP probes from wheat, barley, oat, and rye genetic maps in the regions around *S* or *Z*, and to explore the possibility of using rice genome sequence information to facilitate cloning of SI genes from *Phalaris*.

Materials and methods

Plant materials and mapping populations

Genotypes of *P. coerulescens* used in this study were derived from a collection in the Department of Genetics, The University of Adelaide (Hayman 1956). The operation of the gametophytic self-incompatibility system leads to distorted segregation ratios for genes linked to self-incompatibility locus and (or) loci in a partially compatible cross. The distorted segregation ratios can be used to estimate the recombination frequencies between *S* and closely linked markers (Leach and Hayman 1987; Wricke and Wehling 1985). Two distorted segregating populations were generated for mapping the *S* or *Z* locus. One cross ($S_1S_1Z_1Z_2 \times S_1S_2Z_1Z_1$) was called the *S* tester population. In the cross, only S_2 pollen grains are compatible and the S_1 pollen grains are incompatible (Fig. 1). Thus, all individuals in the progeny should have the identical genotype (S_1S_2) at *S*. The genotypes of the *Z* locus were either Z_1Z_2 or Z_1Z_1. All of the progeny (862) in the *S* tester population were named STA and numbered from STA1. The other cross ($S_1S_2Z_2Z_2 \times S_2S_2Z_1Z_2$) was called the *Z* tester population, which gave a population with an identical genotype (Z_1Z_2) at *Z* and either an S_2S_2 or S_1S_2 genotypeat *S*. All of the progeny (213) in the *Z* tester population were named ZTB and numbered from ZTB1.

Leach (1988) reported the methods on how to calculate the distance between a marker and *S* using partially compatible populations. If there is a recombination between a marker (M) and *S*, M is homozygous and *S* is heterozygous in the *S* tester population (Fig. 1). The number of homozygous individuals at M in the population determines the recombination frequency (r) between *S* and M in the population. The recombination frequency (r) equals homozygous individuals divided by total number of individuals (n) in the population. An approximately 95% confidence intervalis equal to

$$r \pm 1.96 \times \sqrt{r(1-r)/n}$$

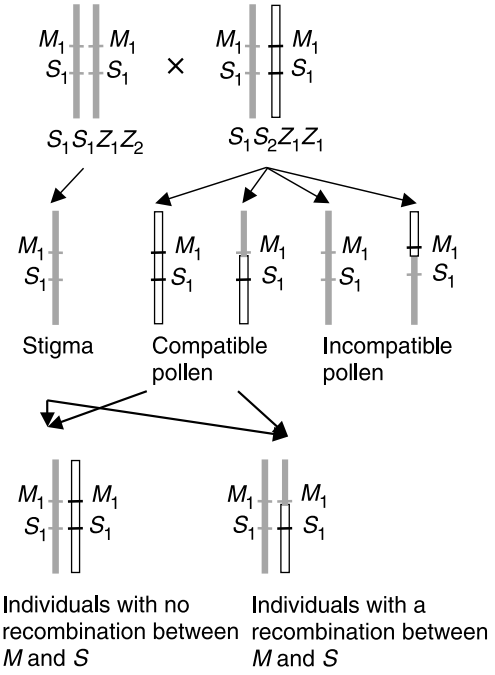
The chromosomes in the tester populations behave like those in reverse backcrosses. So, the backcross model with codominant markers and unique paternal genotype was selected in the Map Manager QTXb08/QTB29ppc program (Manly et al. 2001) to analyse the populations. Kosambi's mapping function was used for calculation of genetic distances (X) (Kosambi 1944) (X) as follows:

$$X = ¼ \text{ Ln}[(1 + 2r)/(1 - 2r)].$$

Parental screening and progeny segregation analysis

RFLP probes (97) mapped on chromosomes 1 and 2 of wheat, barley, and rye in the region around *S* and *Z* (Borner and Korzun 1998; Langridge et al. 1995; Nelson et al. 1995; Qi et al. 1996) were tested for their cross hybridization with

Fig. 1. The behaviours of *S* and a linked marker, M, in the construction of the *S* tester population. In this cross, the maternal parent ($S_1S_1Z_1Z_2$) produces gametes of a single genotype (S_1M_1) and the paternal parent ($S_1S_2Z_1Z_1$) produces four different genotypes of gametes, since recombination occurred during meiosis. However, only the pollen grains with the S_2 genotype can germinate on the stigma (S_1), so all progeny in the population are heterozygous at *S* (S_1S_2). The individual with no recombination between *S* and M is heterozygous at M (M_1M_2). The individual with a recombination between *S* and M is homozygous at M (M_1M_1). The grey bars represent the chromosome carrying allele 1 and white bars represent the chromosome carrying allele 2.



Phalaris DNA and their ability to detect polymorphisms. The origins of these probes are listed in Table 1. The methods for genomic DNA extraction, DNA gel blotting, and hybridization were detailed by Pallotta and coworkers (2000). The genomic DNAs of the parents ($S_1S_1Z_1Z_2$, $S_1S_2Z_1Z_1$, $S_1S_2Z_2Z_2$, and $S_2S_2Z_1Z_2$) were digested by *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, or *Xba*I. The parents for both the *S* and *Z* tester populations were used in parental screening to help distinguish the alleles. The probes showing clear polymorphisms were used for mapping.

Individuals showing the maternal genotype for all markers were not included in the calculation, as the small genetic regions being worked on mean that double recombinations are rare. If an individual is homozygous for the markers at one side of a self-incompatibility locus and heterozygous for the markers at the other side of the locus, it is a true recombinant; if an individual is homozygous for the markers on both sides of the target locus, it is probably a contaminant resulting from self pollination. To distinguish the important recombinants from contaminants, genotyping by pollination was carried out. Unpollinated stigmas were dissected out and "planted" on medium (2% agar, 10% sucrose, and 0.0001% boric acid). After pollination, the stigmas were kept in a moist environment at 25 °C overnight, stained with a drop of pollen stain solution (100 mg cotton blue in 100 mL lactic phenol) on a slide, and germinated and ungerminated grains of pollen were counted under a microscope after 24 h (Watkins 1925).

Identification of AFLP markers closely linked to *Z*

DNA samples with different SI genotypes were bulked according to *Z*, thus three bulks (Z_1Z_1, Z_1Z_2, and Z_2Z_2) were produced. Each bulk included nine DNA samples and the *S* genotypes were S_1S_1, S_1S_2, or S_2S_2 randomly within each bulk. AFLP reactions were done according to Vos and his coworkers (1995). PCR samples (3.5 μL) were separated on a 6% polyacrylamide sequencing gel (containing 7.5 M urea) at 40 W in 1× TBE buffer (90 mM Tris base, 90 mM boric acid, and 2 mM Na_2EDTA) for 2.5 h. The gel was dried on a piece of Whatman paper for half an hour and exposed to X-ray film for 1 or 2 days at –20 °C.

BLAST search of NCBI databases

To identify rice contigs orthologous to the *S* or *Z* regions, the sequences of the RFLP probes linked to *S* or *Z* were used to search databases at NCBI using BLASTN and TBLASTX algorithms (Altschul et al. 1997). The sequences of the probes PSR168, PSR544, and PSR653 were provided by M. Gale (John Innes Centre, UK). The *Bm2* cDNA sequence was described in chapter 3 of Bian (2001). CDO1173, BCD762, BCD207, BCD921, BCD266, and CDO680 cDNA sequences were obtained from GrainGenes (http://wheat.pw.usda.gov/). The annotation of the identified rice contigs was done using Rice Genome Automated Annotation System (RiceGAAS; http://rgp.dna.affrc.go.jp/Analysis.html).

Results

Parental screening and segregation analysis

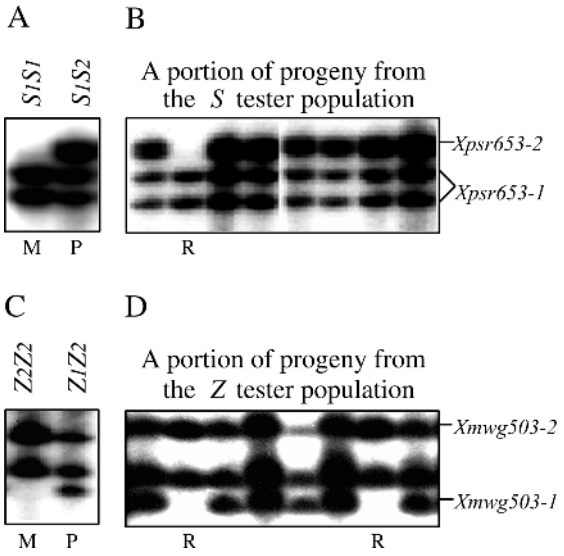
To identify polymorphic RFLP markers linked to *S* or *Z*, total genomic DNA from the parents ($S_1S_1Z_1Z_2$, $S_1S_2Z_1Z_1$, $S_1S_2Z_2Z_2$, and $S_2S_2Z_1Z_2$) of each tester population were hybridized with RFLP probes mapped on chromosome 1 and 2 of wheat, barley, and rye in the region around *S* and *Z*. Figures 2A and 2B show the *Bgl* II RFLPs detected by PSR653 in the parents and randomly selected individuals from the *S* tester population. The individual indicated by "R" was homozygous at *Xpsr653*. Figures 2C and 2D show the *Dra*I RFLPs detected by MWG503 in the parents and randomly selected individuals from the *Z* tester population. Two individuals indicated by "R" were homozygous at *Xmwg503*. The closer a marker is to *S* or *Z*, the less chance there is of recombination. These results indicated that the two tester populations fit the requirement for mapping.

A total of 97 probes identifying markers on Triticeae chromosomes 1 and 2 (see Materials and methods) were tested for cross hybridization to *Phalaris* genomic DNA and for their abilities to detect polymorphisms between the parents of tester populations (Table 2). Oat and wheat cDNA probes gave more polymorphisms than cDNA probes from barley. In total, 66% of tested cDNA clones showed strong cross hybridization and detected polymorphisms between parents. Compared with tested cDNA probes, the genomic probes from wheat and barley gave a lower percentage (55%) of RFLPs.

Table 1. Sources of RFLP probes used in this study.

Clones	Clone types	Sources
ABC	American barley cDNA	A. Kleinhofs, Washington State University, USA
ABG	American barley genomic	A. Kleinhofs, Washington State University, USA
B4e	*Phalaris* cDNA	P. Langridge, Adelaide University, Australia
BCD	Barley cDNA	M. Sorrells, Cornell University, USA
Bm2	*Phalaris* cDNA	P. Langridge, Adelaide University, Australia
CDO	Oat cDNA	M. Sorrells, Cornell University USA
cMWG	Barley cDNA	A. Graner, IPK, Germany
IAG	Rye genomic	P. Wehling, IAC, Germany
KSU	*T. tauschii* genomic	B. Gill, Kansas State University, USA
MWG	Barley genomic	A. Graner, IPK, Germany
PSR (1–200)	Wheat cDNA	M. Gale, John Innes Centre, UK
PSR (201–)	Wheat genomic	M. Gale, John Innes Centre, UK
WG	Wheat genomic	M Sorrells, Cornell University, USA

Fig. 2. Parental and progeny segregation RFLP analysis. Genomic DNAs (~10 μg) from the indicated parents and progeny of the tester populations were digested with *Bgl*II (A and B) or *Dra*I C and D). The gel blots were hybridized with [α-^{32}P]-labelled PSR653 (A and B) or MWG503 C and D). M and P represent the maternal and paternal parents of the tester populations, respectively. R denotes recombinants identified in the population. Restriction fragments marked with *Xpsr653-1* correspond to the *Xpsr653* allele found in the S_1S_1 parental line and the restriction fragments marked with *Xpsr653-2* correspond to the *Xpsr653* allele in the S_2S_2 parental line. Restriction fragments marked with *Xmwg503-1* correspond to the *Xmwg503* allele found in the Z_1Z_1 parental line and the restriction fragments marked with *Xmwg503-2* correspond to the *Xmwg503* allele in the Z_2Z_2 parental line.



Segregation analysis and map construction for the *S* and *Z* loci

To obtain a linkage map of the *S* locus region, 9 probes revealing clear polymorphisms between parents were used to analyse a subset of 369 individuals from the *S* tester population. Individuals (17) showing maternal genotypes for all 9 markers were to be S_1S_1 (data not shown). These results indicate that these 17 individuals might be produced by self-pollination and could not be included in the map construction.

Table 3 shows the segregation data of the 9 markers in the 352 individuals from the *S* tester population. The LODs for all 9 markers were over 79, which indicates that all of these markers are closely linked. The genetic distances between the nine markers ranged from 0 to 9.38 cM. The paternal genotypes were the majority in the population and maternal genotypes were minor. These results met our expectation that the progeny with maternal genotypes carried the recombinations between *S* and the markers. The total number of recombinations in the studied region was 60 in a map distance of 17.5 cM. The recombination frequency in this region was 3.43 recombinations/cM.

A partial linkage map (Fig. 3B) was drawn with the map distances listed in Table 3. *Xbm2* still cosegregated with *S*. *Xpsr634* was the most closely linked marker to *S* from one side and *Xpsr937*, *Xcdo1173*, and *Xcdo99* were also closely linked to *S* on the same side. On the other side, *Xpsr544* and *Xbcd207* were the closest linked markers to *S*, *Xpsr653*, and *Xb4e* with the distances of 1.14 and 10.52 cM away from *S*, respectively.

The genetic map of *S* was compared with Triticeae consensus (Van Deynze et al. 1995) and rye genetic maps (Korzun et al. 2001) to obtain more information about the *S* locus region. Figure 3 shows the colinear relations of the *S* genetic map with Triticeae consensus chromosome 1 and rye chromosome 1R. *Xcdo1073* and *Xbcd207* were identified in the centromere region on Triticeae consensus chromosome 1 (Van Deynze et al. 1995). This region was homologous to segments from chromosomes 5 and 10 of rice. *Xpsr937*, *Xpsr634*, *S*, and *Xbm2* were identified on rye chromosome 1R. It is interesting that all of these markers are also located in the centromere region. These results indicate that the *S* locus is located at the centromeric region of *Phalaris* chromosome 1.

To obtain a linkage map of the *Z* locus region, 10 probes revealing clear polymorphisms between parents were used to analyse 213 individuals from the *Z* tester population. Individuals (2) showing maternal genotypes for all 10 markers were found to be Z_1Z_1 by pollination (data not shown). These results indicate that these two individuals might be

Table 2. Cross-hybridization and polymorphisms of RFLP probes.

The prefix of probes	Clone type	No. of probes tested	No. of probes showing polymorphisms	% of probes detecting polymorphisms
ABC	Barley cDNA	6	1	17
BCD	Barley cDNA	18	8	44
cMWG	Barley cDNA	3	2	67
CDO	Oat cDNA	23	20	87
PSR (1–200)	Wheat cDNA	9	8	89
Subtotal		59	39	66
MWG	Barley genomic DNA	5	3	60
KSU	*T. tauschii* genomic DNA	12	7	58
PSR (201–)	Wheat genomic DNA	16	9	56
WG	Wheat genomic DNA	5	2	40
Subtotal		38	21	55

Table 3. Segregation data for markers linked to *S* in the *S* tester population with 352 progeny.

	No. of maternal genotypes	No. of paternal genotypes	No. of recombinations	Total readable no.	Map distance (cM)	Standard error*	95% confidence interval		LOD
Xcdo99	16	320							
			11	332	3.31	0.98	1.7	5.9	79
Xcdo1173	13	333							
			11	345	3.19	0.95	1.6	5.7	82.7
Xpsr634	2	349							
			2	351	0.57	0.4	0.1	2.1	100.3
S	0	352							
			0	352	0	0	0	0.9*	106
Xbm2	0	352							
			1	351	0.29	0.28	0	1.6	102.7
Xpsr544	1	350							
			3	351	0.85	0.49	0.2	2.5	98.2
Xpsr653	4	347							
			32	341	9.38	1.58	6.3	12.5	56.5
Xb4e	34	308							

Note: *Xpsr937* and *Xbcd207* are not shown in the table, as they had the same segregation data as *Xcdo1173* and *Xpsr544*, respectively.
*Calculated by the method of Stevens (1942), as there is no recombination between *Xbm2* and *S*.

produced by self pollination and could not be included in the map construction. Segregation data of the 10 RFLP markers in a total of 211 progeny from the *Z* tester population were analysed with the aid of Map Manager (Table 4). The LODs for all 10 markers ranged from 24.2 to 62.9, which indicate that these markers are closely linked to each other. The map distances between markers ranged from 0.9 to 12.2 cM. The numbers of paternal genotypes were the majority in the population and those of maternal genotypes were minor. The total number of recombinations in the studied region was 55 in a map distance of 31.2 cM. The recombination frequency in this region was 1.76 recombinations/cM.

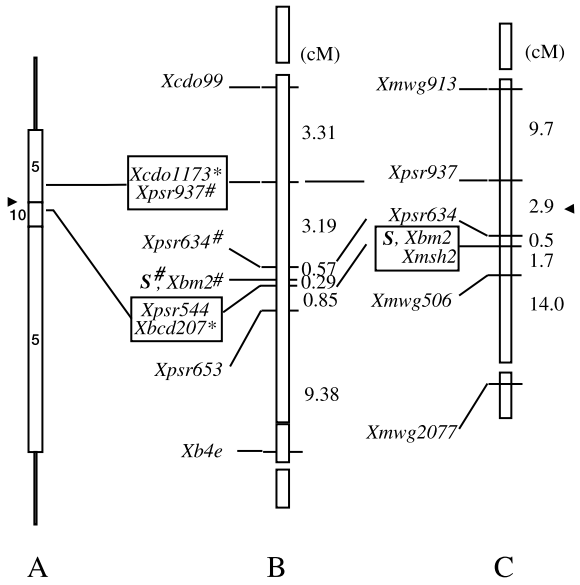
A partial linkage map (Fig. 4B) was drawn with the map distances listed in Table 4. No cosegregating markers were identified for *Z*. The most closely linked marker was *Xbcd266*, which was about 0.9 cM away from *Z*. *Xcdo680* was on the same side as *Xbcd266* and mapped 6.3 cM away from *Z*. On the other side, a big gap of 12.2 cM between *Z* and the closest markers (*Xmwg503*, *Xksuf11*, and *Xksu2*) could not be filled. The other five markers segregated further away from *Z* on this side.

The genetic map around *Z* was compared with Triticeae consensus (Van Deynze et al. 1995) and rye genetic maps (Korzun et al. 2001) to obtain more information about the *Z* locus region. Figure 4 shows the colinear relations of the *Z* genetic map with Triticeae consensus chromosome 2 and rye chromosome 2R. *Xbcd680* was found in the middle of the long arm of Triticeae chromosome 2. This region is colinear to a segment from rice chromosome 4. *Z* and *Xbcd266* were found in the long arm of rye chromosome 2R. These data indicate that *Z* might be located on the long arm of *Phalaris* chromosome 2. Altogether, the segregation analysis and map construction indicates that the *S* and *Z* regions are conserved in both self-compatible and -incompatible species.

Fine mapping of the *S* locus and estimation of physical: genetic distance

To find more markers tightly linked to *S*, RFLP probes detecting loci in the *S* locus homologous regions of wheat and barley were hybridized with genomic DNA digested with enzymes (*Bgl*II, *Dra*I, *Eco*RV, and *Xba*I) from parents and four recombinants from the *S* tester population (Fig. 5). These

Fig. 3. Comparison of *Phalaris* genetic map of *S* with Triticeae consensus and rye genetic maps. (A) Simplified Triticeae consensus map 1 with conserved segments from homologous rice chromosomes superimposed. Numbers identify rice chromosomes. (Van Deynze et al. 1995). (B) *Phalaris* genetic map of *S*. The map was constructed with 352 individuals of the *S* tester population (detailed data in Table 3) (C) 1R partial genetic map of *Secale cereale* (Korzun et al. 2001). Genetic distances are given in centimorgans (cM). Asterisk (*) indicates markers found on the Triticeae consensus chromosome 1 and pound sign (#) indicates markers found on rye chromosome 1R. Arrowhead indicates the centromere locations and lines between chromosomes indicate the location of markers found on both chromosomes.



four recombinants contain recombinations not only on both sides of *S*, but also the closest recombination points to *S* (Fig. 5A). These screenings allowed identification of probes that both showed polymorphisms and were closely linked to *S*. Figure 5B shows *Dra*I RFLPs detected by BCD921. This probe is polymorphic between parents, heterozygous in the three recombinants and homozygous in STA54. Therefore, BCD921 detected a marker between *Xpsr544* and *Xpsr653*. Figure 5C shows *Bgl*II RFLPs detected by PSR168. This probe is polymorphic between parents, but heterozygous in the four recombinants. PSR168 thus detected a marker that cosegregated with *S*. By this method, we further identified *Xpsr168*, *Xbcd762*, and *Xwg811* cosegregating with *S* in the *S* tester population of 352 individuals.

To identify further recombinants and increase resolution of the map, the remaining 493 individuals from the *S* tester population were screened using probes PSR634 and PSR653. A total of five individuals with breakpoints between *Xpsr634* and *S* and six individuals (STA982, showing the maternal genotype for all markers, was proven to be a contaminant using pollination tests) carried breakpoints between *S* and *Xpsr653* (Fig. 6). Three recombinants with breakpoints between *Xcdo1173* and *Xpsr634* and two recombinants with breakpoints between *Xpsr653* and *Xbcd22* were also included as controls (Fig. 6). All the recombinants were used to separate *Xbm2*, *Xbcd762*, *Xpsr168*, and *Xwg811*, which cosegregated with *S* in the subset (352 progeny) of the *S* tester population. *Xpsr168* detected one recombinant (in STA525) out of the six between *S* and *Xpsr653* (Fig. 6A). *Xwg811* detected a different recombinant (in STA306) out of the five between *Xpsr634* and *S* (Fig. 6B). *XBm2* and *Xbcd762* (data not shown) could not detect any recombinant (Fig. 6C). These results enabled us to construct a fine map of the *S* locus region (Fig. 7B) with a resolution of 0.13 cM (one recombinant from a population of 844 individuals).

The relationship between physical and genetic distance in the target region is the critical parameter for map-based cloning. The fine map of the *S* locus region was compared with the group 1 consensus physical map of wheat (Boyko et al. 1999). *Xbcd762* (cosegregating with *S*) and *Xcdo1173* (about 4 cM away from *S*) were separated by a small physical region on the short arm of wheat consensus physical map 1 (Fig. 7A). However, they were separated from *Xpsr544* by a large physical fragment containing the centromere. *Xpsr544* was closely linked to *Xbcd762* in the *Phalaris* genetic map. These results suggest that the *S* locus is located at a position close to the centromere and on the short arm of *Phalaris* chromosome 1, and that the ratio of physical and genetic distance might be large compared with that of other regions.

The sequences of seven markers (*Xpsr168*, *Xpsr544*, *Xpsr653*, *Xcdo1173*, *Xbcd762*, *Xbcd207*, and *Xbm2*) of the *S* linkage map were used to search the NCBI databases using BLASTN and TBLASTX algorithms to detect rice clones orthologous to the *S* region. However, only Bm2 and CDO1173 detected rice clones with significant *E* values. One rice BAC clone (accession No. AC084817, clone No. P0419C04) was detected with Bm2 with an *E* value of 8×10^{-22} and positives of 94% (120/127). The other rice BAC clone (accession No. AC079357, clone No. P0033D06) was detected with CDO1173 with an *E* value of 3×10^{-24}.

These two rice BAC clones are located on rice chromosome 5 and are 14 cM apart (Fig. 7C; http://genome.sinica.edu.tw/). P0419C04 on chromosome 5 is at the genetic position of 31 cM and P0033D06 is at 17 cM. The physical distance between these two clones in rice is about 2420 kb (Saji et al. 2001), or 173 kb/cM. Based on the genetic distance between *Xbm2* and *Xcdo1173* being 4 cM, 605 kb of the rice orthologous region would cover about 1 cM genetic distance in *Phalaris*. This result indicates that the recombination rate in the *S* locus region in *Phalaris* is suppressed.

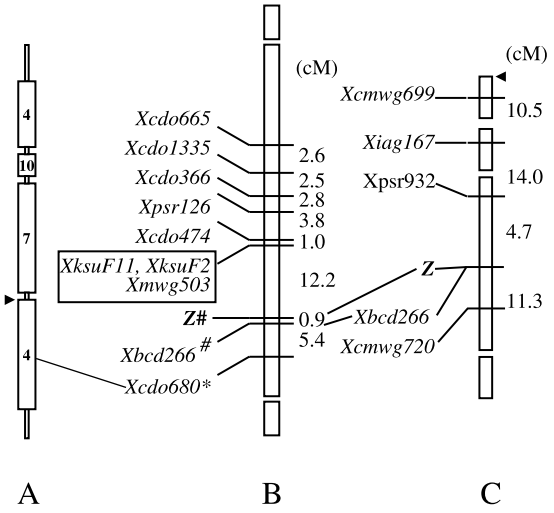
As *Xbm2* cosegregated with *S* in *Phalaris*, the rice clone (P0419C04) detected by Bm2 was analysed further with the aid of RiceGAAS. The clone had not been fully sequenced and there were four gaps at the time of this study. The order of the pieces is believed to be correct as given. However, the sizes of the gaps between them are not known. A total of 19 putative genes was predicted (data not shown) on this rice BAC clone (139.9 kb in length). The gene density was about 7 kb/gene. Predicted genes included a putative phosphate translocator (*Oryza sativa*), a receptor protein kinase (*Arabidopsis thaliana*), and a monosaccharide transporter (*Arabidopsis thaliana*). Each of those might be involved in pollen germination. These results indicate that this rice clone is useful for identifying more markers linked to *S* and candidate *S* genes.

Table 4. Segregation data for markers linked to *Z* in the *Z* tester population.

Marker	No. of maternal genotypes	No. of paternal genotypes	No. of recombinations	Total readable no.	Map distance (cM)	Standard error	95% confidence interval		LOD
Xcdo665	19	98							
			3	115	2.6	1.49	0.5	7.6	28.6
Xcdo1335	34	173							
			5	199	2.5	1.11	0.8	5.9	49.8
Xcdo366	29	172							
			3	107	2.8	1.6	0.6	8.2	26.3
Xpsr126	18	94							
			4	105	3.8	1.87	1	9.8	24.2
Xcdo474	27	175							
			2	202	1	0.7	0.1	3.6	55.9
XksuF15	25	184							
			0	209	0	0	0	1.4	62.9
*XksuF2**	25	184							
			25	209	12.2	2.24	7.6	16	29.7
Z	0	211							
			2	211	0.9	0.67	0.1	3.4	58.6
Xbcd266	2	209							
			11	205	5.4	1.57	2.7	9.6	43.1
Xcdo680	13	192							

**Xmwg503* is not shown in the table, as it had the same segregation data as *XksuF2*.

Fig. 4. Comparison of the *Phalaris* genetic map around the *Z* locus with Triticeae consensus and rye genetic maps. (A) Simplified Triticeae consensus map 2 with conserved segments from homologous rice chromosomes superimposed. Numbers identify rice chromosomes. (Van Deynze et al. 1995). (B) *Phalaris* genetic map of *Z*. The map was constructed with 211 individuals of the *Z* tester population (detailed data in Table 4). (C) 2R partial genetic map of *Secale cereale* (Korzun et al. 2001). Asterisk (*) indicates markers found on the Triticeae consensus chromosome 2 and pound sign (#) indicates markers found on rye chromosome 2R. Arrowhead indicates the centromere locations and lines between chromosomes indicate the location of markers found on both chromosomes.



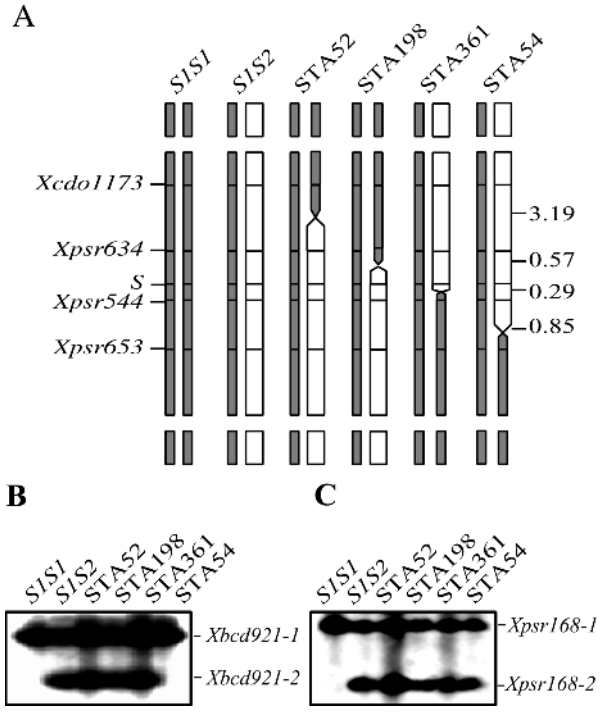
Fine mapping of the *Z* locus and estimating physical: genetic distance

The strategy described above was also used to identify additional RFLP markers linked to *Z*, but was not successful. This might be caused by *Z* being located in a gene-poor region. To identify more markers closely linked to *Z*, AFLP was used. A total of 128 primer combinations was screened by bulked segregant analysis (BSA) and 12 primer combinations showed potential polymorphisms (data not shown). We used the recombinants from the *Z* tester population to map the 12 AFLP primer pairs. Figure 8A shows the breakpoint locations in the recombinants used. ZTB177 and ZTB168 carried the breakpoints between *Z* and *Xbcd266*. ZTB60, ZTB114, and ZTB139 carried breakpoints between *Xmwg503* and *Z*. The two parents (Z_2Z_2 and Z_1Z_2) of the *Z* tester population and a line with the Z_1Z_1 genotype were included as well. The 12 potential primer combinations were analysed on the parents of the *Z* tester population and recombinants with break points around *Z*. Unfortunately, only one primer combination (*Pst*I ACA – *Mse*I CCT) gave the expected pattern that would indicate a recombination between *Xbcd266* and *Xcdo680* (Fig. 8B). This suggests that *Xbcd266* is more closely linked to *Z* than the AFLP marker.

The *Z* genetic map was compared with consensus physical map 2 of wheat (Boyko et al. 1999). *XksuF2*, *XksuF11*, and *Xbcd266* were located in a small physical region at the end of the long arm of wheat chromosome 2 (Fig. 9A). These results indicate that *Z* might be located at the end of *Phalaris* chromosome 2.

The sequences of *Xcdo680* and *Xbcd266* of the *Z* linkage group were used to search the NCBI databases using BLASTN and TBLASTX algorithms. A rice BAC clone (H0212B02, accession No. AL442007) was identified by BCD266 with an *E* value of 5×10^{-11}. CDO680 identified a

Fig. 5. Identification of RFLP markers tightly linked to the *S* locus. (A) Schematic representation of the genotypes of the parents and recombinants used in the screening. The grey bar represents the *S1* allele and the white bar represents the *S2* allele. The recombination is represented by an X-shaped change between grey and white bars. Markers are labelled at the left of the picture and lines on each chromosome. Numbers at right indicate the genetic distances between markers. (B and C) Genomic DNA from the indicated parents and recombinant lines were digested with restriction enzymes and hybridized with [α-^{32}P]-labelled probes. (B) RFLP analysis of BCD921 with *Dra*I digestion. (C) RFLP analysis of PSR168 with *Bgl*II digestion. The restriction fragments corresponding to the alleles in the parental lines detected by probes were labelled at the right of the pictures.



rice clone (H0421H08, accession No. AL442117) with an *E* value of 10^{-13}. The clone (H0212B02) detected with BCD266 was completely sequenced. The gene density was 7.4 kb/gene. This BAC clone is located at 114 cM of rice chromosome 4 (Zhao et al. 2002), which has a total length of 129 cM (http://www.ncgr.ac.cn/rice/chr4/indica.htm). The nearby BAC clones such as H0721B11 (AL732335) and H0315F07 (AL732338) might be useful for finding markers more closely linked to *Z*.

Discussion

Map-based cloning *Phalaris* SI genes with the aid of rice genome sequencing information

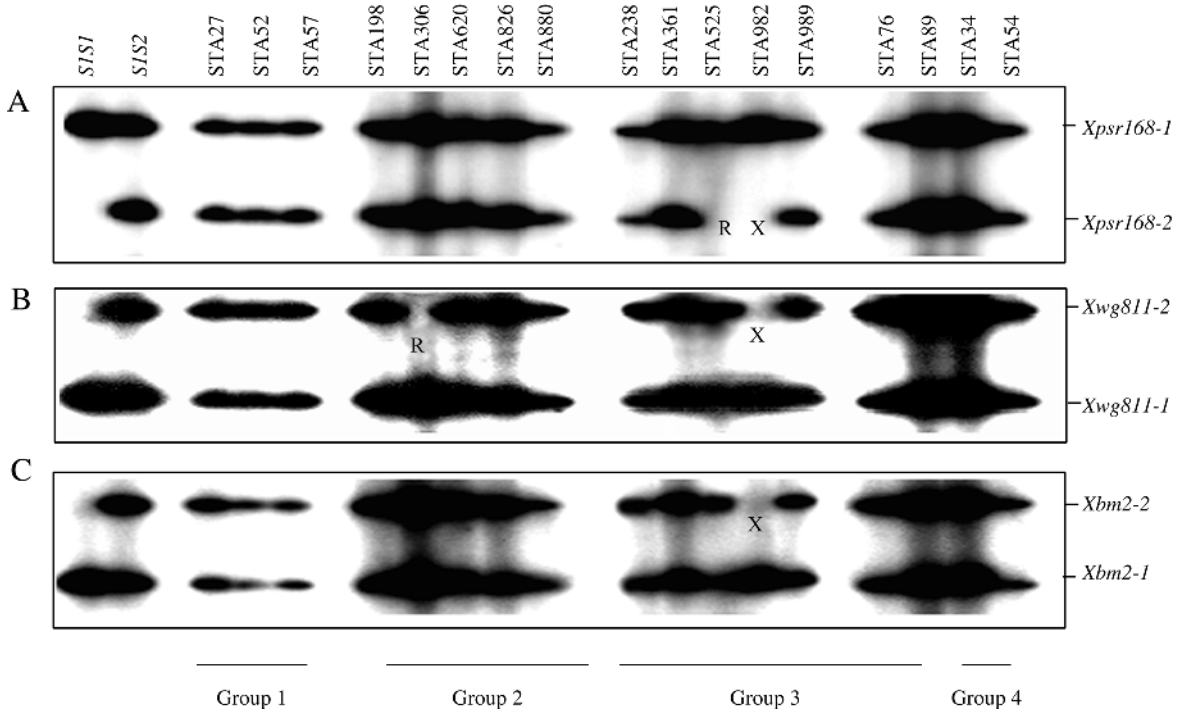
This study of markers linked to the *Phalaris S* and *Z* loci is part of a map-based cloning strategy to identify the *S* and *Z* genes from grasses. Using RFLP probes developed from wheat, barley, oat, and *Phalaris*, high-resolution maps of the regions surrounding *S* and *Z* were constructed. The *S* locus was delimited to 0.26 cM with two boundary markers. *Xbm2* and *Xbcd762* cosegregated with *S* in the *S*-tester population with 844 individuals. At a 95% confidence interval, the maximum genetic distance from *Bm2* to *S* was about 0.35 cM. *Z* was delimited to 0.9 cM from one side by *BCD266*. The feasibility of cloning the *Phalaris* SI genes by the map-based cloning strategy can be estimated from a comparison of the size of the *Phalaris* genome in base pairs and in map units. The genome size of the *Phalaris* genus is relatively small, with a mean 2*C* DNA value between 2.8 and 7.8 pg (Watson 1990). Viinikka and Kallio (1993) examined three species of *Phalaris* and found that the chromosomes of *P. coerulescens* were the smallest, with a total haploid C-banded length of 31 µm. So, one can assume that the genomic size of *P. coerulescens* is close to 2.8 pg/2*C*, which can be translated into 1400 Mb/*C* (1 pg is about 1000 Mb) (Bennett and Smith 1976). According to Kosambi's mapping function (Kosambi 1944), the maximum distance per chromosome is about 150 cM. The maximum total genetic length of *Phalaris* chromosomes is about 1050 cM and the average physical : genetic distance ratio is therefore about 1.33 Mb/cM.

Reduced recombination is characteristic of the pericentric region of the large Triticeae chromosomes (Kunzel et al. 2000; Pedersen et al. 1995). The *Phalaris S* locus is located in the subcentromere region. Provided that there is about four-fold suppression in this region (Kunzel et al. 2000), the physical and genetic ratio at the *S* locus might be 5.6 Mb/cM. *Bm2* cosegregated with the *S* locus in a population with 844 individuals. In this population, there is still only a 57% chance of resolving markers 0.1 cM apart. At a 95% confidence interval, the maximum genetic distance of *Bm2* from *S* is about 0.35 cM (Stevens 1942), which might cover about a 1.86-Mb region. As for *Z*, the situation is no better. The *Z* locus is located in the middle region of the long arm of chromosome 2. If one assumes there is no recombination suppression in this region, the physical and genetic ration is about 1.33 Mb/cM. Provided a marker cosegregating with *Z* in a population with only about 211 individuals (211 meiotic events), the probability of resolving markers within 0.1 cM is about 19%. The 95% confidence interval is about 1.4 cM, so the distance between *Z* and the closest marker might be about 1.96 Mb. This distance needs at least 20 steps of chromosome walking to cover the whole *S* or *Z* region using *Phalaris* BAC clones.

Identification of rice contigs orthologous to the *Phalaris S* and *Z* regions opened the possibility of calculating the ratio of physical to genetic distances in these regions and to seek candidate self-incompatibility genes. The sequences of markers on the *S* and *Z* linkage maps were used to search NCBI databases. Bm2 and CDO1173 hit two linked rice clones, which are located in rice chromosome 5. BCD266 identified a rice BAC clone located in rice chromosome 4. The identification of rice regions orthologous to the *S* and *Z* regions provide a starting point for map-based cloning of SI genes via the aid of rice genome sequence information.

It was found that a rice contig of 600 kb could cover 1 cM genetic distance in the *S* region of *Phalaris*. When the rice genomic sequencing project is completed, it will be feasible to use rice contigs to cover the entire *S* and *Z* regions. At a 95% confidence interval, the maximum genetic distance of *Bm2* from *S* is about 0.35 cM. If the microcolinearity is conserved, the distance between *Bm2* and *S* would be within

Fig. 6. Fine mapping of the *S* locus region by recombinants. S_1S_1 and S_1S_2 were the parents used to construct the *S* tester population. The recombinants are labelled on the top of the pictures. Group 1 includes the selected recombinants (STA27, STA52, and STA57) carrying breakpoints between *Xcdo1173* and *Xpsr634*. Group 2 includes all the recombinants (STA198, STA306, STA620, STA826, and STA880) identified between *Xpsr634* and *S*. Group 3 includes all recombinants (STA238, STA361, STA525, STA982, STA989, STA76, and STA89) identified between *S* and *Xpsr653*. Group 4 includes the selected recombinants (STA34 and STA54) between *Xpsr653* and *Xbcd22*. Each lane contains genomic DNA (10 μg) that was digested with a restriction enzyme and hybridized with [α-^{32}P]-labelled probes. The restriction fragments detected by the probes were labelled on the right of pictures to indicate alleles. X represents a contaminant from self-pollination and R represents the recombination identified. (A) *Dra*I digestion of the genomic DNA probed with PSR168; (B) *Dra*I digestion of the genomic DNA probed with WG811; (C) *Bgl*II digestion of genomic DNA probed with Bm2.



210 kb of rice physical fragments. Other clones, which overlap with Bm2-positive BAC clones, would be needed. The predicted genes on the outmost ends of this contig could be used to delimit the rice physical region orthologous to the *S* region. One could also use the rice contig sequences to predict genes. Homologs of these genes in *Phalaris* could be identified, and study of the expression pattern of these genes would shed light on the functions of these genes.

Alternatively, the identified rice BAC clones could be used as probes to screen the *Phalaris* stigma and pollen cDNA libraries. The expression patterns of identified cDNA clones should give some clues about their function. This method has been used to search for expressed sequences in a 76-kb *SLG/SRK* region of the *S-9* haplotype of *Brassica campestris* and to successfully predict the candidates for the pollen *S* gene (Suzuki et al. 1999).

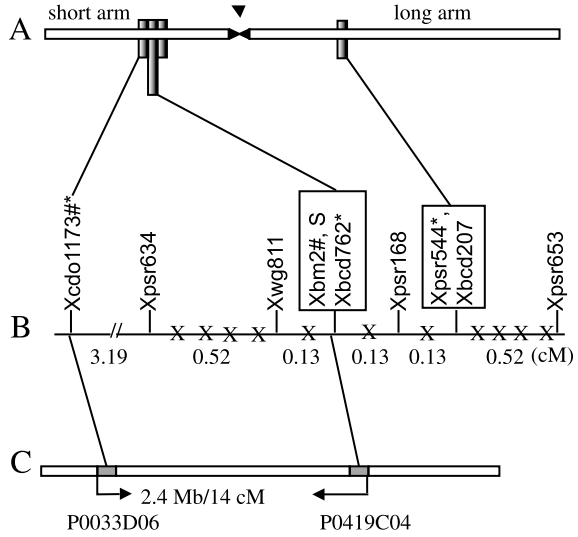
The limitation of using rice contigs to seek candidate *S* genes is that the homologs of SI genes might not be present in the rice genome. Although there may be SI in wild rice (Nayar 1967; Oka and Morishima 1967), the genetics of the wild rice system have not been determined. On the other hand, cultivated rice varieties are self compatible. In the Brassicaceae, the deletion of SI genes has been proposed as a mechanism for the evolution of autogamy in *Arabidopsis* (Conner et al. 1998). A similar process may have led to the loss of SI in cultivated rice. If a large deletion has caused SI loss, then the markers developed from rice sequences may not be sufficiently close to allow identification of SI genes from *Phalaris*. Another problem may be that microsynteny may break down in the region of the SI genes. Kusaba and his coworkers (2001) reported that the *S* locus of *Arabidopsis lyrata* occupies a different chromosomal location than the *Brassica S* locus. Recent studies at the gene level have demonstrated that microcolinearity of genes is less conserved than originally predicted (Keller and Feuillet 2000). Small-scale rearrangements and deletions complicate the microcolinearity between closely related species, such as rice and other grasses.

One limitation of this calculation is that a large rice physical distance was used. The ratios of physical to genetic distances are not even along the genome, even in small physical regions. It was found that the relationship between physical and genetic distance can deviate by one or two orders of magnitude in the different regions of the barley genome (Buschges et al. 1997). A similar situation was seen in a 550-kb region of the apple genome, in which the physical to genetic ratios varied largely (Patocchi et al. 1999).

Conservation of the *S* and *Z* locus regions

It is believed that an SI system is common within any given plant family (Conner et al. 1998; ten Hoopen et al. 1998). The colocalization of CP100 (a cDNA marker linked

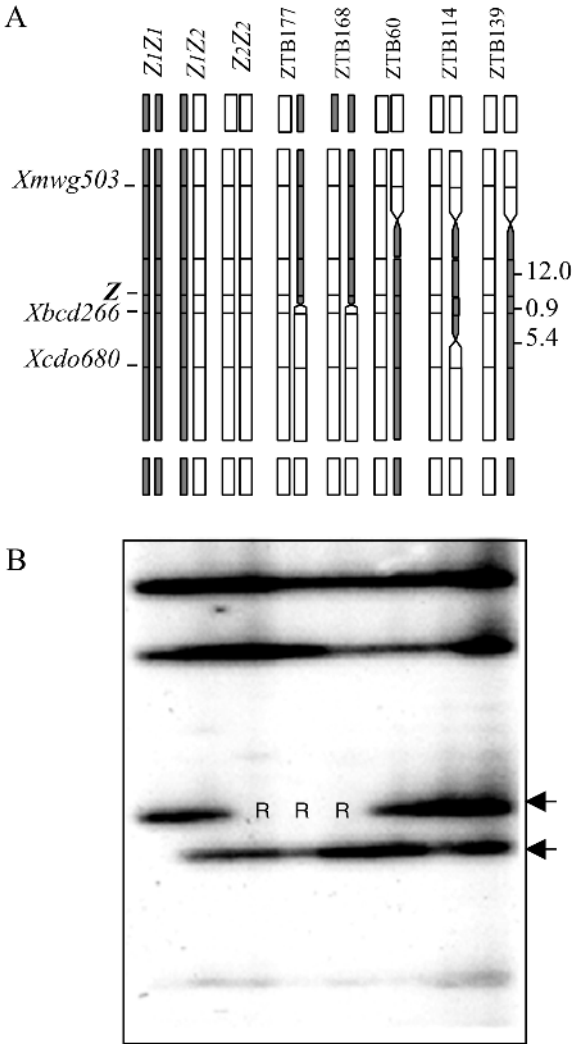
Fig 7. Comparison of the fine map of *S* with wheat and rice physical maps. (A) Consensus physical map 1 of *Triticum aestivum* (Boyko et al. 1999). The vertical bars represent the deletion portion of the wheat chromosome 1. (B) The fine map of *S*. The map was drawn by the data generated from Fig. 6. X indicates the breakpoints identified in each region. Asterisk (*) indicates markers found on wheat consensus physical map 1 and pound sign (#) indicates markers identifying rice BAC clones. (C) Simplified physical map of rice chromosome 5 (Saji et al. 2001). The shaded bars represent the rice BAC clones identified by the markers indicated.



to the *S* locus of potato), peroxidase, and the *S* locus in *Petunia* revealed synteny around the *S* locus between four members of the Solanaceae (ten Hoopen et al. 1998). A high degree of colinearity at the submegabase scale between the two homeologous regions of the *S* locus has been found in the crucifer family(Conner et al. 1998). We found that the linkage groups around the *S* and *Z* loci in the Pooideae, a subfamily of the Poaceae, are conserved. In this paper, the conservation around the *S* and *Z* locus regions between *Phalaris* (Aveneae) and rye (Triticeae) was very high. Recently, Thorogood and coworkers (2002) reported conservation around the *S* and *Z* locus regions between rye and *Lolium* (Poeae). These findings support the hypothesis that the *S*–*Z* self-incompatibility system might be common in the Poaceae, or at least in the Pooideae. These results support the conclusion that self-incompatibility arose early in the diversification of plants and a single self-incompatibility system exists within a family.

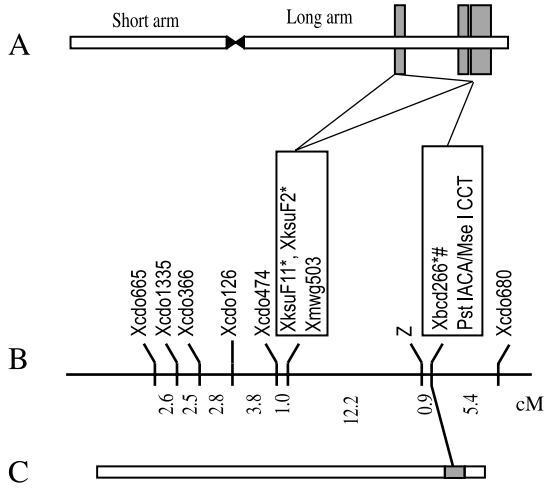
It is assumed that self-incompatibility genes were recruited from ancestral gene families during the early stages of angiosperm evolution and, since that time, new alleles have arisen at a low rate (Xue et al. 1996). Wang et al. (2001) proposed that intragenic recombination contributed to the generation of the allelic diversity of the *S* RNAse gene and mutations have been found to generate new alleles in a self-incompatible population (Matton et al. 1999). Therefore, there must have been a period of high recombination when the *S* (and *Z*) allele diversity was generated. However, these genes then appear to have been fixed and shielded from further diversification.

Fig. 8. Identification of AFLP markers closely linked to *Z*. (A) Schematic representation of the genotypes of parents and recombinants used in the recombinant mapping. The grey bar represents the Z_1 allele and the white bar represents the Z_2 allele. Recombination is represented by an X-shaped change between grey and white bars. Markers are at the left of the picture and positions are indicated by lines on each chromosome. Numbers at right indicate the genetic distances between markers. (B) The *Pst*I ACA – *Mse*I CCT primer combination shows polymorphisms among Z_1Z_1, Z_2Z_2, and Z_1Z_2. Five critical recombinants were used to map the AFLP marker. Arrows indicate the polymorphic bands, which suggest that the marker detected by this primer combination was located at the same position as *Xbcd266*. R represents the recombinations identified.



The *S* locus was located on the short arm of chromosome 1 close to the centromere and the *Z* locus was located at the end of the long arm of chromosome 2, where the recombination rates are reduced compared with more distal regions (Pedersen et al. 1995). The location of SI genes in a conserved region of the genome might be helpful to maintain intactness through minimizing further allele generation and keeping the pollen and stigma *S* genes closely linked. ten Hoopen et al. (1998) argued that a subcentromeric localization of the *S* locus in *Petunia* was helpful in maintaining a

Fig. 9. Comparison of *Phalaris* fine map of *Z* with wheat and rice physical maps. (A) Consensus physical map 2 of *Triticum aestivum* (Boyko et al. 1999). The vertical bars represent deletion portion of the chromosome. (B) *Phalaris* fine map of *Z*. Asterisk (*) indicates markers found on the wheat chromosome and pound sign (#) indicates markers found on the rice chromosome. (C) Simplified physical map of rice chromosome 4 (Zhao et al. 2002). The shaded bar represent the rice BAC clone identified by the marker indicated.



functional *S* allele by preventing separation of pollen–*S* from the stigma *S* RNase.

Segregation distortion mapping

Segregation distortion associated with *S* or *Z* has been used previously to identify markers linked to the self-incompatibility loci (Leach and Hayman 1987). Leach (1988) reported an extensive exploration of the types of crosses, methods of linkage estimation, progeny size, and controls needed for accurate analysis of distorted segregation ratios. The work reported in this chapter represents the mapping of oat, wheat, barley, and rye RFLP probes on *Phalaris* distorted segregating populations. This led to the construction of the high-resolution maps of *S* and *Z* regions. The *S* locus was delimited to within 0.25 cM with two flanking markers, and the *Z* locus to within 1.0 cM from one direction only. This opens the way for map-based cloning of the *S* and *Z* genes.

The advantages of using such populations for mapping *S* and *Z* are significant. The first advantage is that it saves a huge amount of time that would be needed to genotype all individuals in large populations. When the population reaches a certain size, such as 1000 individuals, it is extremely difficult to genotype all individuals in the population. The second advantage is that this kind of population enables detection of contaminating individuals from cross pollination. This is important, as even a few contaminants would cause major inaccuracies when fine mapping. The contaminating levels of different batches of lines used in this study ranged from 0.2% to 5.1% for *S*, and from 0% to 1% for *Z*. These might be caused by the environmental conditions when the crosses were made. It is a common observation that in many self-incompatible grass species a few seeds are obtained after "self pollination" (Hayman 1992). The normal seed set of self pollination is about 1% and could be increased to 25% at temperatures of 30 to 35 °C. The highest selfing seed set was obtained for rye when exposed to the high temperatures together with 60%–80% relative humidity 2–4 days before anthesis (Wricke 1978).

The type of populations constructed enables one to map the target locus and readily distinguish recombinants from contaminants. At the initial mapping stage, it was thought that there might be two recombinants between *Bm2* and *S* in the 96 individuals of the *S* tester population. These two recombinants proved to be contaminants by pollination tests and assessment of other markers linked to *S*. In rye, P. Wehling (Federal Centre for Breeding Research of Cultivated Plants, Gross Luesewitz, Germany) also identified three putative recombinants between *Bm2* and *S* in a population of 107 individuals. Unfortunately, the testing and confirmation of such putative recombinants is very difficult to achieve in rye, an annual, relative to the perennial, *Phalaris*. Therefore, the apparent recombination between *Bm2* and *S* in rye cannot be given great weight.

Because of the high level of self-pollinated contaminants identified in the populations used in this work, the real double recombinants on both sides of *S* and *Z* might have been lost. However, the limitation is probably trivial. The recombination rate between *Xcdo1173* and *Xpsr653* is 6.4%, and the map distance is 6.4 cM calculated by Kosambi's mapping function. The rate of double recombination is about 0.035%, which means only 1 double recombination in 2869 individuals. The two flanking markers of *Z* showed a recombination rate of about 12.9%. The distance between these two markers is 13.1 cM. Thus the double recombination rate in this region is only 0.2%, which means there could be only about 1 double recombinant in 500 individuals of the *Z* tester population. The loss of one double recombinant would not make a large difference in the predicted genetic distances.

In conclusion, construction of fine maps of the *S* and *Z* region using distorted segregating populations provided information on the genetic locations of *S* and *Z*, shed light on the evolution of SI in the grasses and set a starting point for the map-based cloning of these genes. Identification of the rice genome regions orthologous to the *S* and *Z* regions opens the possibility of using the rice genome sequence to identify SI candidate genes and generate new markers closely linked to *S* or *Z*.

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