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ORIGINAL PAPER

Genetic map-based location of the red clover (*Trifolium pratense* L.) gametophytic self-incompatibility locus

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Abstract Red clover is a hermaphroditic allogamous diploid ($2n = 2x = 14$) with a homomorphic gametophytic self-incompatibility (GSI) system (*Trifolium pratense* L.). Red clover GSI has long been studied, and it is thought that the genetic control of GSI constitutes a single locus. Although GSI genes have been identified in other species, the genomic location of the red clover GSI-locus remains unknown. The objective of this study was to use a mapping-based approach to identify simple sequence repeats (SSR) that were closely linked to the GSI-locus. Previously published SSR markers were used in this effort (Sato et al. in DNA Res 12:301–364, 2005). A bi-parental cross was initiated in which the parents were known to have one self-incompatibility allele (S-allele) in common. S-allele genotypes of 100 progeny were determined through test crosses and pollen compatibility. Pseudo F_1 linkage analysis isolated the GSI-locus on red clover linkage-group one within 2.5 cM of markers RCS5615, RCS0810, and RCS3161. A second 256 progeny mapping testcross population of a heterozygous self-compatible mutant revealed that this specific self-compatible mutant mapped to the same location as the GSI-locus. Finally, 82 genotypes were identified whose parents putatively shared one S-allele in common from maternal halfsib families derived from two random mating populations in which paternal identity was determined using molecular markers. Unique S-allele identity in the two random mating populations was tentatively inferred based on haplotypes of two highly allelic linkage-group one SSR (RCS0810 and RCS4956), which were closely linked to each other and the GSI-locus. Paternally derived pollen haplotype linkage analysis of RCS0810 and RCS4956 SSR and the GSI-locus again revealed tight linkage at 2.5 and 4.7 cM between the GSI-locus and RCS0810 and RCS4956, respectively. The map-based location of the GSI-locus in red clover has many immediate applications to red clover plant breeding and could be useful in helping to sequence the GSI-locus.

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Introduction

Red clover (*Trifolium pratense* L.) is an important forage legume harvested for hay, grown in pasture for grazing, and sown as a companion crop (Smith et al. 1985; Taylor and Quesenberry 1996). Red clover is a hermaphadidic allogamous diploid ($2n = 2x = 14$) with a homomorphic gametophytic self-incompatibility system (GSI) (Silow 1931; Townsend and Taylor 1985). Red clover has a very effective GSI system; in one study only 22 of 392 self pollinated plants produced seed (Williams and Silow 1933). Among the 22 self-compatible plants, only a combined 55 seeds were produced. Self-incompatibility (SI) systems have been characterized at the molecular level in Papaveraceae, Brassicacea, Poacea, Rosacea, Scrophulariacea, and Solanaceae. Brassicacea, Poacea, and Papaveraceae each have unique

homomorphic SI systems. The Rosacea, Scrophulariacea, and Solanaceae possess a similar S-RNase-mediated SI system in which glycoproteins with RNase activity degrade the pollen tube. Phylogenetic analyses of gene sequences suggest a common ancestry in all eudicots (Rosacea and Scrophulariacea included) for the S-RNase-mediated system (Allen and Hiscock 2008). The S-RNase pistil-specific genes have been cloned in various species and, more recently, the pollen-specific S-locus F-box (i.e. SLF) has been determined (McClure 2004; Zhang and Xue 2008). These two genes are so tightly linked that no recombination has been observed between them. In *Prunus* the two genes were found within 30 kb of each other (Kao and Tsukamoto 2004). In regard to red clover, the identified *Prunus* SI genes are phylogenetically the closest identified SI genes. The relationship between the *Prunus* SI genes and those of red clover is currently unknown. Based on molecular and physiological studies of various SI systems throughout the plant kingdom, Steinbachs and Holsinger (2002) estimated that SI evolved at least 21 times. They argue that strong selection pressure exists to evolve some form of SI in order to prevent inbreeding.

We do want to note that the genetic map-based location of the white clover (*Trifolium repense* L.) GSI locus is reported in this issue in a separate article (Casey et al. 2010). Based on the mapped locations reported in this article and the white clover article, cloning of the GSI locus in *Trifolium* should come to pass shortly.

Red clover's GSI system is somewhat unique. Diversity of S-alleles in red clover is unusually great. In two tested red clover populations, 41 out of 48 and 37 out of 40 S-alleles tested were unique in each population, respectively (Williams and Williams 1947). Lawrence (1996, 2000) used data from Williams and Williams (1947) to estimate there would be between 143 and 193 unique alleles per population, respectively. Such high numbers of alleles per population are an apparent anomaly for species studied outside of the *Trifolium* genus (Lawrence 2000). According to evolution and selection theory, plant species should have approximately 40 or less alleles per population (Wright 1939; Lawrence 2000; Castric and Vekemans 2004).

The objective of this study was to identify closely linked molecular genetic markers to the red clover GSI-locus.

Materials and methods

Plant material and S-allele phenotyping

Three pseudo-F_1 mapping strategies were utilized in this study (Maliepaard et al. 1997). The first strategy was creating a mapping population from a bi-parental cross with only three S-alleles present across both parents (Fig. 1).

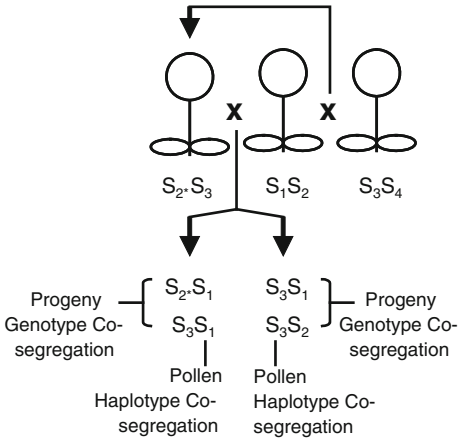

Fig. 1 Pseudo-F_1 backcross mapping population for the red clover (*Trifolium pratense* L.) gametophytic self incompatibility locus with three-S-alleles among the two mapping parents. S_{2*} is used to distinguish between the two S_2 alleles in the mapping parents. Within maternal family co-segregation of S-allele genotypes (determined through within maternal family testcrosses) with markers, map the maternally received S-alleles, while within maternal family co-segregation of pollen S-allele and markers, map the paternally received S-alleles

Briefly, a cross between two red clover plants (e.g. $S_1S_2 \times S_3S_4$) was accomplished, and one progeny from this cross was randomly chosen (e.g. S_2S_3). The three parental plants of this population were obtained from population C584 a breeding germplasm from the US Dairy Forage Research Center breeding seed stock collection. The chosen progeny was backcrossed to one of its parents (e.g. $S_1S_2 \times S_2S_3$). The progeny of this backcross constituted the mapping population. Care was taken to retain maternal identity of each mapping progeny; this allowed pseudo-F_1 mapping of both homologs of each chromosome. The key to understanding the scheme is knowledge of maternal or paternal identity of progeny even though both mapping parents were hermaphrodites. Maternal-derived S-allele identity was determined by making bi-progeny crosses among individuals sharing a mother. Due to GSI, only two progeny genotypes can exist in each maternal group (e.g. maternal-group 1: S_2S_1 or S_3S_1; and maternal-group 2: S_3S_1 or S_3S_2) if seed is produced in a within-maternal-group bi-progeny-cross, these progeny need to have different maternally derived S-alleles (Fig. 1). Utilizing a partial-diallel crossing scheme within maternal groups allowed us to identify two maternally-derived S-allele groups per maternal-group. Paternal pollen-derived S-allele identity of mapping progeny was established by knowledge of maternal identity alone. The paternal S-allele has to be the S-allele not present in the maternal plant due to GSI in a three-S-allele cross (e.g. S_1S_2 mother accepts only S_3 pollen, or S_2S_3 mother accepts only S_1 pollen) (Fig. 1). Co-segregation of molecular markers within each of the four groups was mapped (i.e. 2 maternal and 2 paternal progeny chromosome groups).

Approximately 100 progeny were utilized from this mapping population. All crosses were accomplished by hand using coffee straws cut at a 45° angle. The straw tip was inserted into a floret to trip the pistil out of the keel so that the pistil came down and touched the inside of the straw tip, collecting and depositing pollen on the surface. Three florets per parent were pollinated at once before switching to the other bi-parental-cross-parent to pollinate another three florets. This procedure was repeated until approximately 20 florets per bi-parental cross parent were pollinated. Care was taken not do damage flowers during crossing. Seed-set was determined after 28 days with seed production in either parent indicating successful cross-fertilization.

The second mapping population was a test-cross between a mutant self-compatible red clover plant and a wild-type red clover plant. The self-compatible mutant was retrieved from the US Dairy Forage Research Center (USDA-ARS) red clover breeding stocks and germplasm collections. Seed of this mutant population has been in the collection since prior to the 1970s, and may have been derived from the same self-compatible germplasm created at the University of Minnesota Agriculture Experiment Station in the late 1920s (Rinke and Johnson 1941). A self-compatible individual (e.g. $S_fS_?$) was crossed to a wild-type individual (a random genotype chosen from C584) and a randomly chosen progeny from this cross (e.g. S_fS_x, x designates an arbitrary self-incompatible S-allele) was crossed to a third wild-type individual (e.g. S_xS_x). Progeny from this later cross constituted the pseudo-F_1 mapping population. Progeny were phenotyped as self-compatible (S_fS_x) or self-incompatible (S_xS_x) by attempting to self-pollinate mapping progeny. Approximately 20 florets per plant were self-pollinated by hand using a toothpick inserted into the floret to trip the pistil out of the keel and apply a little self-pollen to the pistil without causing floral damage. If five attempted self-fertilizations yielded no seed, the plant was classified as self-incompatible. If in three attempted self-fertilizations seed set was accomplished at least once, the plant was classified as self-compatible. Co-segregation between segregating self-compatible parent SSR and progeny self-compatibility classification was accomplished to map the location of this self-compatibility mutation. 256 mapping progeny were used.

The final mapping scheme was based on two random mating breeding populations (WI21 and C584-Y-07) selected from the US Dairy Forage Research Center (USDA-ARS) forage legume breeding program. This allowed us to map the red clover GSI-locus in actual populations using insect pollinators. The crossing blocks to generate the WI21 and C584-Y-07 progeny halfsib seed were conducted at the U.S. Dairy Forage Research Dairy Farm, Prairie du Sac, Wisconsin, USA (43°21′N, 89°45′W) in a Richwood silt loam (Fine-silty, mixed, superactive, mesic Typic Argiudolls) using plants grown in the greenhouse and transplanted to the field in April 18, 2007. The crossing block design used was a modification of Riday and Krohn (2010), with 96 individuals used per cage, transplanted in three double rows of 16 plants per sub-row, 20 cm spacing between plants within 16 plant rows and 20 cm spacing between sub-rows within double rows and 30 cm spacing between double rows. Pollination and halfsib seed threshing was accomplished as described in Riday and Krohn (2010). Progeny derived from seed from 20 of the 96 WI21 halfsib families and seed from 19 of the 96 C584-Y-07 halfsib families were used for mapping.

DNA extraction

Tissue was collected from all parental and mapping progeny used. A fresh trifoliate leaf (0.1–0.2 g) from each parent or progeny was either placed in a 2-mL microcentrifuge tube and ground by hand pestle under liquid nitrogen or placed in separate wells of a 96-well PCR plate (Fisher Scientific, Pittsburgh, PA, USA) containing approximately five 1.3-mm and two 2.3-mm chrome-steel beads (BioSpec Products Inc, Barlesville, OK, USA) and ground three times at 28 Hz for 30 s in a Mixer Mill MM 301 (Retsch Inc, Newtown, PA, USA) under liquid nitrogen. Total DNA was obtained by the method of Štorchová et al. (2000) for pseudo-F_1 mapping populations or the method of Hill-Ambroz et al. (2002) for random mating breeding populations and quantified by fluorometry. Resultant DNA solutions were normalized to 5 ng μL^{-1} for subsequent PCR amplification of microsatellite loci.

Microsatellite amplification and analysis

Prior to focusing on red clover linkage group one, one or two SSR primers pairs from each linkage group (assumed based on Sato et al. (2005)) were mapped. This initial screen included RCS0810 on linkage group one. RCS0810 was obviously linked to the GSI locus in red clover. Following this initial success further linkage mapping was confined to primer pairs on linkage group one. Nine red clover primer pairs (RCS0810, RCS0907, RCS1812, RCS2393, RCS3161, RCS4956, RCS5027, RCS5615, and RCS6958) from linkage-group one developed by Sato et al. (2005) were used to identify microsatellite alleles present in parental and progeny tissues for GSI mapping purposes. An additional nine non-linkage-group one primer pairs (RCS0884, RCS1526, RCS1683, RCS2987, RCS3805, RCS4854, RCS5305, and RCS5823) were used for paternity testing in the complex mapping populations. Forward primers were synthesized (Eurofins MWG Operon, Huntsville, AL, USA) to include 5′ fluorescent dyes for loci

RCS0810 (TAMRA), RCS0907 (TAMRA), RCS1526 (6-FAM), RCS1812 (6-FAM), RCS2393 (HEX), RCS3161 (6-FAM), RCS4854 (HEX), RCS4956 (FAM), RCS5027 (HEX), RCS5305 (TAMRA), RCS5615 (FAM), and RCS6958 (FAM). PCR reactions containing between one and seven primer pairs were performed in 6 µl reaction volumes. PCR reaction final concentrations were 1× JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA), 0.2 µM of each primer, 2.2 mM $MgCl_2$, 1 M betaine (Sigma-Aldrich) and approximately 5 ng template DNA. Thermal cycling was carried out on a DNA Engine Dyad (Bio-Rad) as follows: 95°C for 1 min, 44 cycles of 95°C for 20 s; 50°C for 2 min; 72°C for 1 min, and a final incubation at 4°C for 1 min. Fragment sizes were determined on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA). The software program GeneMarker (SoftGenetics LLC, State College, PA, USA) was used to interpret the electropherograms and assign alleles to experimental data.

Data analysis

Simple pseudo-F_1 based haplotype mapping (Maliepaard et al. 1997) was conducted in Excel spreadsheets (Microsoft Co., Redmond, WA, USA) by calculating recombination fractions between molecular markers and the self-compatibility or GSI-locus depending on mapping population (Sturtevant 1913). Recombination fractions were converted to map distances using the Kosambi (1944) function. To conduct mapping in the two random mating populations, paternity of progeny was inferred using Cervus 3.0 (Kalinowski et al. 2007). Paternity was assigned to an individual if the "trio" LOD score was greater than three. Known maternity, inferred paternity, and molecular marker data were entered into SAS 9.1 (SAS Institute Inc., Cary, NC, USA). PROC FREQ, by maternal or paternal halfsib family, using the corresponding parental haplotype, was used to detect crossover events between molecular marker pairs. RCS0810 × RCS4956 two-locus haplotypes were used as proxies for putative S-alleles. Only in one case in the WI21 population were two S-alleles observed associated with one RCS0810 × RCS4956 haplotype. Based on parental RCS0810 × RCS4956 haplotypes as proxies for parental S-alleles, bi-parental combinations with scored progeny were selected in which only three putative S-alleles were found between the two parents (Fig. 2). Mapping of the pollen haplotype was conducted between closely linked SSR markers and the putative S-allele (Sturtevant 1913; Maliepaard et al. 1997). S-allele identity was confirmed by observing the expected extreme pollen haplotype segregation distortion. A SAS data management and analysis program was written to determine maternal and paternal alleles of progeny and to reveal relevant progeny of three S-allele bi-parental crosses. Chi-square tests to detect segregating alleles and LOD scores to estimate strength of linkage (Morton 1955) were performed in Excel spreadsheets. Linkage-group images were produced using MapChart 2.1 (Voorrips 2002).

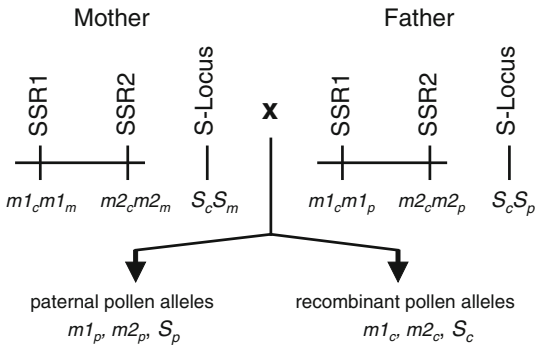

Fig. 2 Confirming the red clover (*Trifolium pratense* L.) gametophytic self-incompatibility locus map location in a random mating population by focusing on pollen haplotypes of bi-parental crosses with the S-locus and a closely linked two SSR marker haplotype (e.g. SSR1—$m1_m$, $m1_p$, and $m1_c$; SSR2—$m2_m$, $m2_p$, and $m2_c$, and inferred by tight linkage S_m—maternal, S-allele, S_p—paternal S-allele, and S_c-common S-alleles). The common S-allele (i.e. S_c) is confirmed by the closely linked common SSR loci (i.e. $m1_c$ and $m2_c$) being very rare in the pollen haplotype (i.e. recombinant pollen haplotypes)

Results and discussion

For the three-S-allele mapping population, 85 progeny were successfully genotyped in test crosses used to map the maternal S-alleles. Progeny paternal-pollen-derived-S-alleles were successfully genotyped in 100 progeny. Because identification of the maternal S-allele relied on the skill of the researcher conducting the crosses, there was a risk of maternal S-allele misdiagnosis due to human error; therefore, greater value was placed on linkage to the paternal S-allele which would not be subject to such human error. In the three-S-allele mapping population within the two maternal families, expected 1:1 segregation for the maternal S-allele was observed (i.e. 23:21, χ^2 $P = 0.76$; 21:20, χ^2 $P = 0.88$). For the self-compatible mapping population, 256 progeny were successfully genotyped. Again, expected 1:1 segregation was observed with 116 self-compatible and 140 self-incompatible or wild type (χ^2 $P = 0.13$). In most cases progeny genotyping was successful.

The publication of Sato et al. (2005) was a tremendous boon to this project as no framework linkage map was needed to be developed. To minimize resource and effort, initially one or two SSR markers per linkage group were mapped in the three-S-allele mapping population by looking for appropriate S-allele co-segregation (Fig. 1). Fortunately, in an early screen, RCS0810 was utilized, leading to

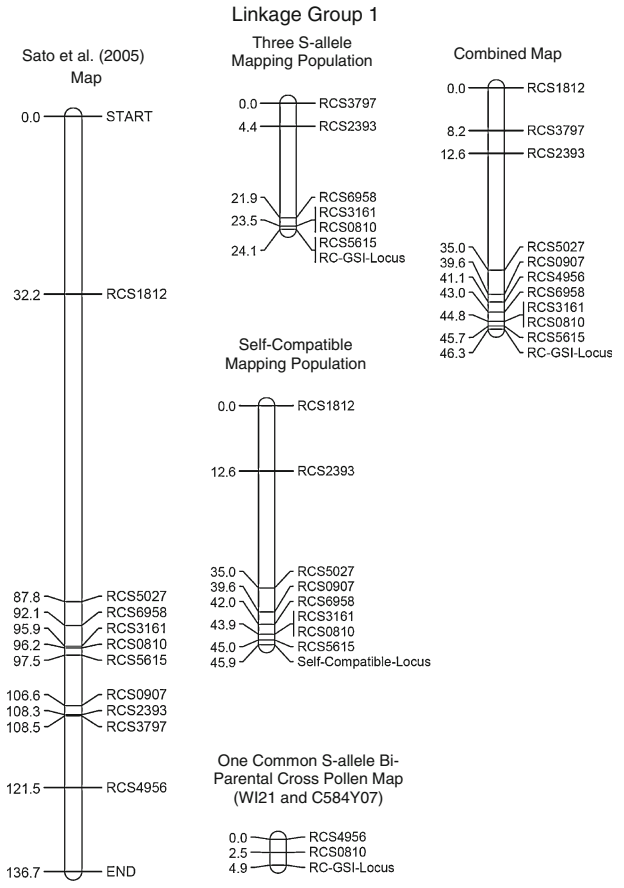

Fig. 3 Red clover (*Trifolium pratense* L.) linkage-group one for Sato et al. (2005) with all SSR markers used in this study: three-S-allele population; self-compatible mutant population; one common S-allele bi-parental-cross pollen-S-allele-linkage in random mating population (WI21 and C584-Y-07); combined linkage group for all GSI mapping populations

an almost immediate direct hit on the red clover S-locus (RCS0810-S-locus maternal 8.3 cM, paternal 1.0 cM) (Fig. 3). RCS0810 is a dinucleotide repeat that had been used in a previous paternal population testing study (Riday and Krohn 2010) and was found to be highly allelic (with imperfect repeats, noticeable stuttering, no artifacts, and easily scorable products at even very low fluorescence intensity). RCS0810 is infrequently found in a homozygous state, as was the case in the three-S-allele mapping population (with three RCS0810 alleles in the mapping population corresponding to the three S-alleles). Having three alleles per locus allowed us to simultaneously map the maternal and paternal homologs in the progeny. A similar initial screen in the self-compatible mapping population identified the location of the self-compatibility mutation on linkage-group one with linkage to RCS0810 (2 cM) and RCS1812 (54.5 cM) (Fig. 3). Based on the similar mapping location of the self-compatibility mutation to the actual S-locus, we have some confidence that we posses a mutation of the actual S-locus.

Based on this initial success, additional SSR markers from Sato et al. (2005) located close to RCS0810 were amplified. Additional SSR markers placed on the three-S-allele mapping population include RCS3797, RCS2393, RCS6958, RCS3161, and RCS5615 (Fig. 3). Additional SSR markers placed on the self-compatible map include RCS2393, RCS5027, RCS0907, RCS6958, RCS3161, and RCS5615 (Fig. 3). Based on further mapping in the three-S-allele population, it became apparent that the genotyping of the maternal S-allele likely contained a few errors, which led us to rely on the paternal S-allele segregation for fine mapping exclusively. In both mapping populations, RCS5615 mapped closest to the GSI-locus (self-compatible population 0.9 cM, no recombination observed in the three-S-allele population) (Fig. 3). Although closer to the S-locus, RCS5615 has much less allelic diversity than RCS0810 and is frequently observed in a homozygous state.

In trying to use the Sato et al. (2005) SSR markers to map the S-locus in red clover, a curious observation was made: we were unable to place an SSR marker between the GSI-locus and the telomeric region of the linkage group. This is despite the fact that many of the SSR mapped in our populations were closer to the telomeric region than RCS0810 and RCS5615 according to the Sato et al. (2005) map. Closer examination of this region on Sato et al. (2005), linkage-group one shows a noticeable marker coverage gap between RCS4235 (101.4 cM) and RCS5551 (104.1 cM). All Sato et al. (2005) SSR markers we used which mapped beyond RCS5551 (RCS0907, RCS2393, RCS3797, and RCS4956) map to locations between RCS1812 and RCS5615 on our maps (Fig. 3). We also attempted to map RCS3085 and RCS0884 onto linkage-group one, but in our mapping populations we found no linkage to other linkage-group one molecular markers or the GSI-locus.

Finally having mapped the S-locus in one bi-parental cross and one bi-parental mutant population, we wanted to attempt to map the S-locus in actual breeding populations. This would confirm the S-locus location in a broader germplasm base and demonstrate effectiveness of observing S-allele configuration and predicting and manipulating crossing outcomes. To accomplish this, two highly allelic SSR loci closely linked to the GSI-locus (RCS0810 and RCS4956) were amplified in two red clover breeding populations WI21 and C584-Y-07, both 96 parent synthetics. WI21 likely has a broader germplasm base than C584-Y-07, which was created from parents derived from four halfsib families. RCS4956 is a highly allelic, imperfect trinucleotide SSR with no artifacts, and has easily scorable amplification products under low fluorescence intensity. Based on informative halfsib haplotypes (517 maternal and 509 paternal), RCS4956 was mapped at 2.5 cM distance

(maternal 2.4 cM, paternal 2.7 cM) from RCS0810 in the WI21 and C584-Y-07 breeding populations (Fig. 3). Paternity was determined using 11 SSR loci with maternity known and all possible fathers known. Twenty-three and 20 RCS0810 × RCS4956 haplotypes were observed in WI21 and C584-Y-07 populations, respectively. Progeny haplotype homozygosity was observed for only one specific haplotype in population WI21; this haplotype was obviously associated with two separate S-alleles based on progeny haplotype configurations. We hypothesize that the number of RCS0810 × RCS4956 haplotypes observed in each population corresponds to the approximate number of S-alleles per population. Building off this hypothesis, we expect to observe severe paternal-pollen-derived haplotype distortion in progeny of crosses resulting from parents with one RCS0810 × RCS4956 parental haplotype in common (i.e. by proxy one S-allele in common) (Fig. 2). In the WI21 and C584-Y-07 populations, 82 progeny were observed whose parents shared one RCS0810 × RCS4956 haplotype in common out of 661 progeny scored that had all requisite maternal, paternal, progeny, and RCS8010 × RCS4956 haplotype information. Assuming each parental RCS0810 × RCS4956 haplotype corresponded to one S-allele, two recombinants between RCS0810 and the GSI-locus were observed (2.4 cM), and four recombinants between RCS4956 and the GSI-locus were observed (4.7 cM).

Apparently, in one paternal halfsib family, a recombination event between the RCS0810 × RCS4956 haplotype and the S-locus occurred in the paternal generation, causing the five progeny pollen RCS0810 × RCS4956 haplotypes from this father to be associated exclusively with the other S-locus possible from this father, rather than the RCS0810 × RCS4956 haplotype GSI-locus configuration that was most frequently observed in the population as a whole in the parental generation. There is a possibility that the two recombinants between the GSI-locus and the RCS0810 × RCS4956 haplotypes observed, among the 82 progeny with parents with one S-allele in common, are also due to recombination events in the parental generation. The two recombinant pollen haplotypes were from fathers contributing only one haplotype to the 82 progeny scored. Assuming a generous 2.5% recombination frequency between the RCS0810 × RCS4956 haplotype and the GSI-locus, the odds of observing two recombinations between RCS0810 × RCS4956 haplotypes and the GSI-locus in pollen haplotypes in progeny from the same father drop to 0.06%. Including only fathers that contributed two pollen haplotypes to the 82 progeny in which the parents had one S-allele in common leaves 48 progeny with zero recombination events observed between RCS0810 and the GSI-locus and one recombinant between RCS4956 and the GSI-Locus.

Based on the self-compatible, three-S-allele, WI21, and C584-Y-07 mapping progeny, a consensus map across all mapping individuals was estimated (Fig. 3). SSR marker order among maps was consistent when comparisons could be made. However, SSR marker order was not consistent with Sato et al. (2005) map in cases where the SSR marker was mapped between the hypothetical GSI-locus and the telomeric region on the Sato et al. (2005) map. Map position of RCS4956 is based on its distance to RCS0810 only (LOD = 268). RCS4956s mapped position in relation to the S-locus is based on 82 mapped homologs; we therefore have less confidence in this positioning (LOD = 20.6 and 18.3 for GSI-locus with RCS0810 and RCS4956, respectively), especially in relationship to other markers on linkage-group one.

Having markers tightly linked to the S-locus has immediate application to plant breeding and variety development. Riday and Krohn (2010) demonstrated a three-fourth population hybrid scheme based on restricting the two parental populations of the hybrids to three S-alleles each. Having SSR markers tightly linked to the GSI-locus would greatly facilitate population screening to restrict populations to desired S-alleles without having to engage in backcrossing schemes that would likely result in undesirable inbreeding. Riday (2007, 2010) pointed out the attractiveness of using paternity testing to enhance selection gains in outbred forage species breeding schemes (i.e. non-linkage-based marker assisted selection). Paternity testing efforts are enhanced by having highly allelic co-dominant markers closely linked to the GSI-locus. Since the GSI-locus is under negative frequency-dependent balancing selection (Wright 1939), alleles in general would be expected to be at lower and equal frequency, and such molecular marker information is ideal for paternity testing. Unfortunately in this study no GSI-locus linked markers were found between the S-locus and the telomeric region. We leave it to future researchers to identify such markers.

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