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Identification and mode of action of self-compatibility loci in *Lolium perenne* L.

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The two-locus gametophytic incompatibility system in perennial ryegrass (*Lolium perenne* L.) is not always fully effective: obligate selfing of plants sieves self-compatible pollen mutants, and self-fertility becomes fixed in subsequent generations. Self-compatibility (SC) was investigated in an F_2 family. *In vitro* self-pollinations were analysed and recorded and plants were classified as being either partially or fully compatible. Distorted segregation ratios of markers on linkage group (LG) 5 were found, which indicate the possible presence of a gametophytic SC locus. Interval linkage analysis of pollen compatibility after selfing confirmed that this distortion was due to a locus (*T*) analogous to the *S5* locus of rye. However, even though markers in this region were, on average, less than 1 cM apart, the minimum number of plants possessing the unfavoured allele was never less than 6% for any marker locus. We proved that this was because of the presence of another SC locus, exhibiting gametophytic selection, segregating in this population and identified by interval mapping analysis of compatibility classes of *in vitro* self-pollinations. This locus was located on LG1, and probably corresponds to the *S* locus. We show that the *T* locus, a relic of a multilocus system, functions through interaction with the *S* locus: F_2 segregation of incompatibility phenotypes and linked markers demonstrated that the *S*/*t* pollen genotype combination, expected to be compatible on selfing, was sometimes incompatible. Further evidence is presented to show that this interaction must be dependent on yet another locus located on LG2. A prime candidate would be the *Z* incompatibility locus.
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Introduction

The outcrossing ryegrasses (*Lolium perenne* L. and *L. multiflorum* Lam.) commonly occur throughout the temperate world. The breeding of improved cultivars of both species and their hybrids is largely based on the functioning of a two-locus (*SZ*), multiallelic, gametophytic incompatibility system (Cornish *et al*, 1979; Fearon *et al*, 1983) that prevents selfing, enabling the production of genetically variable but stable synthetic populations exhibiting good combining ability. Breeding has been partly responsible for a significant improvement in the productivity and quality of marginal forage grasslands, the aesthetics of landscaped grass areas and the playability of natural grass sports surfaces.

Seed setting on selfing in *Lolium* does occur at relatively low levels, and was observed as long ago as 1924 (Jenkin, 1924). Self-fertility in ryegrass has attracted interest (Jones and Jenabzadeh, 1981), as the ability to produce self-fertile inbred lines could enable the production of F_1 hybrid cultivars (Hayward, 1988; Hayward *et al*, 1991). Low seed set suggested to Jenkin (1931) that self-fertility was under polygenic control. In perennial ryegrass, a self-compatibility (SC) locus independent of the *S* and *Z* incompatibility loci has been shown to segregate in F_2 and F_3 populations derived from a cross between two inbred lines (Thorogood and Hayward, 1991). In the closely related self-fertile species, *Lolium temulentum*, a self-fertile allelic variant of one of the incompatibility loci was identified in backcrosses to perennial and Italian ryegrass (Thorogood and Hayward, 1992). This locus showed apparent joint segregation with the isozyme *GOT/3*. Although the *GOT/3* locus maps to linkage group (LG) 3 (Jones *et al*, 2002), interaction of a locus or loci on LG3 with the *S* locus or a locus or loci closely linked with *S* on LG1 will cause genetic associations with markers of LG1 and LG3 (Thorogood *et al*, 2002). Thorogood and Hayward (1992) observed joint segregation of the *GOT/3* locus with self-fertility in their backcross families and significant distortion of the *GOT/3* locus was observed in the progeny from selfing half-self-compatible plants from these families (Thorogood, 1991). As it was known that GOT/3 was not linked to the *S* locus, it was assumed that linkage to the *Z* locus was responsible for these observations. The more recent findings by Thorogood *et al* (2002) of genetic interaction between loci on LG3 and LG1, which strongly imply the interaction of the *S* locus with a locus on LG3, linked to the *GOT/3* locus, led us to suspect that the SC mutation occurred at the *S* and not the *Z* locus in this population.

At least three SC mutations, at the *S* and *Z* loci and at least one additional locus, designated *T*, have also been reported in the grass species *Phalaris coerulescens* (Hayman and Richter, 1992) and, in Rye (*Secale cereale* L.), three incompatibility loci, *S*, *Z* and *S5*, have been

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mapped on LGs 1, 2 and 5 respectively (Fuong *et al*, 1993; Voylokov *et al*, 1993). Although these loci have been mapped in the *Gramineae*, there is no published evidence to indicate any functional relationship between the *S* and *Z* loci and this additional locus, for which the only significant form appears to be a nonfunctional self-fertility allele.

A genetic analysis of *in vitro* self-pollinations of plants of an F_2 population, derived from a cross between two perennial ryegrass plants of separate contrasting third-generation inbred lines, was made with the aim of mapping and quantifying the loci responsible for the high levels of self-fertility generally observed in this population. Such information is essential for future studies of evolution of the grass incompatibility system and also an essential step towards a full molecular understanding of what appears to be a more complex system of self-incompatibility than the original two-locus system first proposed around 50 years ago in Rye, *S. cereale* L. (Lundqvist, 1954, 1956), *Festuca pratensis* L. (Lundqvist, 1955) and *P. coerulescens* Desf. (Hayman, 1956).

Information on the interaction of unlinked SC and self-incompatibility genes would also be useful in a practical sense to plant breeders attempting to manipulate traits, by marker-assisted selection, controlled by genes linked to incompatibility loci. Such interaction could lead to unconscious selection for other deleterious traits unlinked to the trait of interest.

Materials and methods

The family used was an F_2 population of 188 plants from a selfed F_1 plant derived, in turn, from a cross between two unrelated plants that had been inbred by obligate selfing over three generations (Figure 1). Both parents and especially the F_1 derivative were self-fertile as indicated by mean numbers of seed obtained on selfing.

The map used in this study was developed further from the framework map for this F_2 family published by Armstead *et al* (2002) and extended by Armstead *et al* (2004), and had a genome coverage of 672 cM. Additional markers are simple sequence repeats (SSRs) mainly prefixed rv but also 17ca1, 25ca1, 83ca1, 08ga1, 14ga1, 22taga1, 55taga1 and 59taga1. Genotype data were provided by Kieran Elborough of Vialactia, New Zealand and had been produced using their methods. The heterologous restriction fragment length polymorphism (RFLP) probe cdo580 on the previous maps has been replaced with a sequence tagged site (STS) (iacdo580), which maps exactly to the original RFLP. This was analysed on an ABI3100 sequencer, using DNA extracted with the QIAGEN DNEasy Plant Mini Kit (QIAGEN, Crawley, UK). In all, 75 RFLPs, 54 amplified fragment length polymorphisms (AFLPs), 90 SSRs, three invertase gene probes, 13 STSs and three isozymes were mapped onto seven LGs (presumed to represent the haploid set of seven chromosomes of *L. perenne*) (Armstead *et al*, 2002) using JoinMap 2.0 (Stam and van Ooijen, 1995). Regions of the genome with consistent distorted marker segregation ratios, favouring one of the homozygous genotypes over the heterozygotes, but maintaining a 1:1 ratio of homozygotes to heterozygotes, can be associated with loci segregating for SC. Regions with distorted segregation ratios were analysed to assess the likelihood that these distortions were due to such associations in our population.

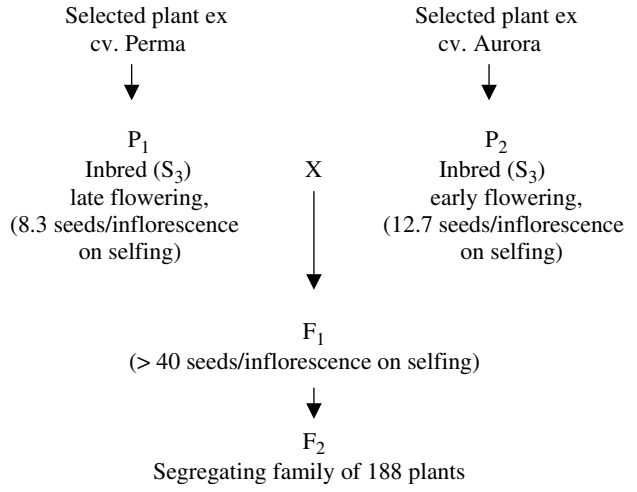

Figure 1 Pedigree of F_2 mapping family.

Ramets of each of the F_2 family individuals were grown in 15 cm diameter pots in Humax John Innes No3 with wetting agent. Plants were vernalised (short days, low temperature) naturally in an unheated, unlit glasshouse over winter. The plants were then allowed to flower naturally.

In vitro self-pollinations were made using the technique of Lundqvist (1961) and self-pollinated stigmas were stained in decolourised aniline blue (Martin, 1959) and observed microscopically at low power ($\times 10$) under fluorescent light. Details are also given by Thorogood *et al* (2002). In total, 77 self-pollinations were successfully completed and scored. Pollinations fell into two classes: those estimated to be about half-self-compatible where half of the pollen grains germinated, their tubes penetrating deep into the stigma tissue and the other half having their tubes arrested at or near the stigmatic surface soon after germination; and those classified as fully self-compatible with all viable pollen grains producing long pollen tubes. SC loci were identified by interval mapping the two class SC data using MapQTL 4.0 (van Ooijen *et al*, 2000). Quantitative trait loci (QTL) for SC classes, scored as proportions of self-compatible pollen grains observed after *in vitro* self-pollination, were identified in genomic positions that gave a logarithmic odds ratio (LOD score) of linkage with molecular markers greater than three units. The programme was also used to generate a nonparametric (Kruskall–Wallis) test for association of markers with SC. This was deemed a necessary confirmatory test when the LOD score from the interval analysis was less than three but still produced a significant peak in a specific region of the genome.

Partial incompatibility found in gametophytic incompatibility systems is associated with segregation distortion of linked markers where maximum distortion occurs at the self-incompatibility locus (Leach, 1988). By identifying the position of maximum distortion, the incompatibility loci have been mapped in rye (Wricke and Wehling, 1985; Fuong *et al*, 1993; Voylokov *et al*, 1993). We also looked at areas of marker segregation

distortion in our ryegrass mapping family and compared map positions of markers with maximum distortion and the positions of segregating SC loci as identified by standard interval linkage analysis. The map positions with the greatest marker distortion were estimated by plotting the observed percentage (after angular transformation) of recombinants against the map distance for each marker. A quadratic equation was fitted to the curve and bootstrap estimates, from 100 bootstrap samples and the minimum value of the curve (minimum recombination frequency) and the point (map distance) at which this occurred were obtained using Genstat 6th Edition (Payne, 2000).

Results

Of the 77 pollinations completed, 39 were found to be approximately half- and 38 fully self-compatible.

Distorted marker segregation

In our ryegrass population, a major concentration of markers on LG5 showing distorted segregations occurred within the 11–38 cM region (Armstead *et al*, 2004). Distortion of codominant markers favoured one of the homozygote classes over the other, whereas the heterozygote class, on the whole, remained at the expected frequency (Table 1). There was never a case where the unfavoured homozygote was completely absent and the region with greatest distortion was estimated to occur at a distance of 19.9 cM (between 7.99 and 24.21 cM at the 95% confidence interval) where the unfavoured allele was transmitted at a minimum estimated frequency of 6.7% (between 4.8 and 8.1% at the 95% confidence interval). The closest markers to this location are the SSR marker rv0184 (19.8 cM) and the RFLP marker RZ206 (20.9 cM). The only other region of extensive distorted marker segregation was that on LG7 where the majority of the co-dominant marker segregations were characterised by significant distortion (Armstead *et al*, 2004) with a deficiency of one of the homozygote classes at the expense of the other (Table 2). Greatest distortion occurred at 45.9 cM (between 33.8 and 52.3 cM at 95% confidence interval) with an estimated minimum of 9.0% (between 7.9 and 10.5% at 95% confidence interval) of the plants representing the deficient homozygous class.

Table 1 Segregation ratios of codominant markers on LG5

Marker locus	*Map distance*	*Marker genotype*			$\chi^2_{[2]}$	$\chi^2_{[1]}$
		aa	ab	bb	*1:2:1* aa:ab:bb	*1:1 hom:het*
rv0757	11.5	10	31	46	36.98***	7.18**
GSY60.2	13.6	14	82	59	26.65***	0.52^{NS}
rv0814	16.4	6	44	40	25.73***	0.04^{NS}
F29-1	17.0	10	70	55	30.19***	0.19^{NS}
CDO127	17.0	10	87	64	37.27***	0.52^{NS}
CDO1380.2	17.0	10	90	67	39.92***	1.01^{NS}
rv0184	19.8	6	44	38	23.27***	0.00^{NS}
RZ206	20.9	11	81	65	37.31***	0.16^{NS}
BCD1087	21.5	10	52	49	27.85***	0.44^{NS}
rv0495	23.7	6	49	36	20.32***	0.54^{NS}
PSR574	24.7	11	89	50	25.51***	5.23*
rv0082	28.7	6	49	35	19.40***	0.71^{NS}
rv0950	28.7	5	45	37	23.64***	0.10^{NS}
rv1258	30.7	8	44	34	15.77***	0.05^{NS}
CDO412	32.2	16	20	30	16.18***	10.24**
rv1112	36.0	11	33	34	15.41***	1.85^{NS}
rv0340	37.8	10	47	34	12.76**	0.05^{NS}
R2710	66.5	35	81	40	0.55^{NS}	0.23^{NS}

NS: ratio not significantly different from expected; ratio significantly different at *$P<0.05$, **$P<0.01$, ***$P<0.001$.

QTL analysis

Segregation distortion may arise through one of several mechanisms operating pre- or postzygotically and cannot be attributed to a specific cause such as gametophytic incompatibility. So a QTL analysis was made to confirm or otherwise that these distortions were due to the presence of a segregating SC locus. The analysis of the SC phenotypes was made with two classes of SC, half-compatible and fully-compatible, and revealed two QTL, one on LG5 with a maximum LOD score of 6.08 obtained for a marker at 19.8 cM and another on LG1 with a maximum LOD score of 3.39 at 13.2 cM (Table 3). The QTL on LG5 was associated with distorted segregation of linked markers but that on LG1 was not.

Marker analysis

Genotypes of the closest codominant markers to the SC QTL (iacdo580 for the QTL on LG1 and RZ206 for that on LG5) were assigned to the 77 plants that were classified for their SC response (Table 4). All plants with iacdo580

Table 2 Segregation ratios of codominant markers on LG7

Marker locus	*Map distance*	*Marker genotype*			$\chi^2_{[2]}$	$\chi^2_{[1]}$
		aa	Ab	bb	*1:2:1* aa:ab:bb	*1:1 hom:het*
R2869	7.7	28	67	39	1.81^{NS}	0.00^{NS}
CDO545	11.9	25	77	55	11.52**	0.06^{NS}
OSW	19.3	28	97	37	7.32*	6.32*
rv1284	25.1	13	51	25	5.13^{NS}	1.90^{NS}
C764	27.1	15	65	42	12.48**	0.52^{NS}
rv1411	28.6	10	52	28	9.38**	2.18^{NS}
rv0479	30.7	4	52	26	17.71***	5.90*
08ga1	31.8	12	47	31	8.20*	0.71^{NS}
RZ144	32.7	19	86	60	20.67***	0.30^{NS}
rv0134	33.3	9	52	30	11.55**	1.86^{NS}
rv0711	34.2	7	48	30	13.87***	1.42^{NS}
59taga1	37.6	7	54	28	13.97***	4.06*
rv0020_1	38.7	7	54	28	13.97***	4.06*
rv0293	39.5	7	57	25	14.30***	7.02**
rv0440	40.5	7	54	26	13.37**	5.07*
GSY60.1	41.0	13	91	59	28.18***	2.21^{NS}
rv0459	42.2	8	57	25	12.82**	6.40*
PSR690	43.5	11	61	57	33.19***	0.38^{NS}
C390	45.5	8	81	52	30.59***	3.13^{NS}
rv1254	46.3	5	52	27	16.29***	4.76*
rv0397	47.4	6	57	28	16.45***	5.81*
RZ952	47.4	10	91	52	28.56***	5.50*
rv0264	52.5	9	54	28	11.11**	3.18^{NS}
LtCOa	55	16	92	49	18.52***	4.64*
LtCOb	58.3	26	78	50	7.51*	0.03^{NS}
rv0817	72.4	13	53	21	5.62^{NS}	4.15*
ACP	80.3	25	94	51	9.86**	1.91^{NS}
rv0663	89.7	20	48	22	0.49^{NS}	0.40^{NS}
rv0005	95.9	21	47	22	0.20^{NS}	0.18^{NS}
CAT	102.2	12	39	20	2.49^{NS}	0.69^{NS}

NS: ratio not significantly different from expected; ratio significantly different at *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 3 LOD scores produced on interval mapping for pollen compatibility estimates on *in vitro* self-pollination for genomic regions on LGs 1 and 5, linked markers and their relative map positions

LG1				*LG5*			
Map distance	*Locus*	*LOD score*	*% variance explained*	*Map distance*	*Locus*	*LOD score*	*% variance explained*
1.7	PGI	2.17	11.3	0.0	E39M4909	1.49	9.2
7.5	iacdo580	3.21	15.7	7.1	H11G05	3.15	18.0
11.6	E41M5709	3.19	16.1	11.5	rv0757	3.99	22.1
13.2	rv0659	3.39	16.3	13.6	GSY60.2	3.57	18.8
13.2	K10F08	3.39	16.3	16.4	rv0814	5.64	28.0
14.7	E38M4709	2.48	12.7	16.4	E51174S	5.18	26.1
16.0	rv0785	2.30	11.8	17.0	F29-1	5.85	28.1
16.0	E36M5503	2.30	11.8	17.0	CDO127	5.71	27.6
16.8	E36M5508	2.17	11.3	17.0	CDO1380.2	5.71	27.6
17.5	E42M3308	2.58	12.9	19.8	rv0184	6.08	29.5
17.5	K04D01	2.57	12.9	20.9	RZ206	5.68	27.5
19.0	rv1447	1.74	9.3	21.5	BCD1087	5.46	26.6
20.7	rv0165_1	1.21	6.6	23.7	rv0495	3.72	19.1
23.2	BCD1072	1.06	5.8	24.7	K03B03	3.15	15.6
24.5	M16Bb	0.86	4.6	24.7	PSR574	3.31	16.3
24.5	rv0182	1.06	5.7	28.7	rv0082	1.81	10.1
26.4	OSE	0.53	3.0	28.7	rv0950	1.81	10.1
28.9	B6101	0.45	2.5	30.7	rv1258	2.37	13.3
29.5	rv0624	0.57	3.2	32.2	CDO412	3.12	18.2
30.6	83ca1	0.56	3.1	36.0	rv1112	1.08	6.6
32.5	55taga1	0.46	2.7	37.8	rv0340	0.71	4.6
34.5	E42M3309	0.41	2.4	41.5	E40M5907	0.48	3.2
35.2	BCD738	0.64	3.6	44.7	B6103	0.54	3.5

Table 4 Numbers of plants observed with each combination of iacdo580 (LG1) linked to *S* and RZ206 (LG5) linked to *T* genotypes that were approximately 50 and 100% self-compatible

Marker		*No. of plants*		
iacdo580 (LG1)	*RZ206 (LG5)*	*50%*	*100%*	*Total*
aa (*SS*)	*aa* (*tt*)	1[a]	1	2
aa (*SS*)	*ab* (*Tt*)	0	10[b]	10
aa (*SS*)	*bb* (*TT*)	0	5[c]	5
ab (*Ss*)	*aa* (*tt*)	3	0	3
ab (*Ss*)	*ab* (*Tt*)	24[b]	3[a,c]	27
ab (*Ss*)	*bb* (*TT*)	2[c,d]	15[c]	17
bb (*ss*)	*aa* (*tt*)	0	0	0
bb (*ss*)	*ab* (*Tt*)	9[c]	0	9
bb (*ss*)	*bb* (*TT*)	0	4	4
		39	38	77

[a]Recombination in SC QTL region at LG1.
[c]Includes one RZ206 genotype predicted from flanking markers.
[b]Includes three RZ206 genotypes predicted from flanking markers.
[d]One plant with recombination in SC QTL at LG5, and one plant with predicted RZ206 genotype with no recombinants identified associated with either SC QTL (misclassified?).

genotype *aa* except one, and all plants with RZ206 genotype *bb* except two, were 100% self-compatible. Plants heterozygous for both loci were approximately 50% self-compatible apart from three plants that were fully self-compatible. All iacdo580 *ab*, RZ206 *aa* plants and all RZ206 *ab*, iacdo580 *bb* plants were approximately 50% self-compatible. No iacdo580 *bb*/RZ206 *aa* genotypes were found. Only 77 plants were classified for their SC reaction mainly because many plants of this population were virtually male-sterile producing nondehiscent anthers and very low % pollen viability scores (D Thorogood, unpublished). However, many of these plants were classified for their iacdo580 and RZ206 genotypes giving a total of 167 plants. The frequencies of the genotypes of these plants are given in Table 5. The segregation ratios of these two markers for the 167 plants did not differ from the ratios obtained for the subset of 77 plants that were classified for self-incompatibility score (data not shown). Therefore, sterility had no effect on the segregation of these markers and consequent marker analyses can be based on the whole set of plants rather than the smaller subset.

Interpretation of results

The overall 1:1 segregation of F_2 plants into half-self-compatible and fully self-compatible is indicative of a single gametophytic locus segregating in the F_1. Only pollen with the SC allele from the heterozygous plant is able to effect fertilisation, giving rise to either half-self-compatible or fully-self-compatible plants. The SC status of the plant would depend on whether pollen fertilises a female gamete with the SC allele or one with the normal functioning self-incompatibility allele. However, it is unlikely that a hybrid between two highly self-compatible inbred plants from diverse sources, if segregating at all, would be segregating for less than two SC factors, that is, at least one derived from each parent. The situation is remarkably similar to that recounted by Thorogood and Hayward (1991) working on a completely unrelated F_2 family. Then, we identified a single SC gene independent of both *S* and *Z* presumably originating from one of the original inbred parents. It was then necessary to suggest that the other inbred line must have been obtained through pseudo-SC, influenced by weak incompatibility alleles or polygenic modifiers of the incompatibility system. We are now in a stronger position to investigate this apparent anomaly in our current F_2 family because of the existence of an extensive set of DNA markers that map the *L. perenne* genome, and

Table 5 Total number of plants observed for each iacdo580 and RZ206 genotype combination compared to expected numbers based on two expected segregation ratios (see text for details of derivation of models)

Marker		*Expected ratio of plants (model 1)*	*Expected ratio of plants (model 2), 36% transmission*	*Expected ratio of plants (model 2), 50% transmission*	*No. of plants observed*	*Expected nos. (model 1)*	*Expected nos. (model 2) 36%*	*Expected nos. (model 2) 50%*
iacdo580 (LG1)	*RZ206 (LG5)*							
aa (*SS*)	*aa* (*tt*)	1	0.4	1	6	13.8	6.3	8.1
aa (*SS*)	*ab* (*Tt*)	2	1.4	3	21[a]	27.7	23.9	24.7
aa (*SS*)	*bb* (*TT*)	1	1.1	2	11	13.8	17.6	16.6
ab (*Ss*)	*aa* (*tt*)	1	0.4	1	4	13.8	6.3	8.1
ab (*Ss*)	*ab* (*Tt*)	3	2.5	2.5	48[b]	41.4	41.4	41.4
ab (*Ss*)	*bb* (*TT*)	2	2.1	2	38[c]	27.7	35.2	33.0
bb (*ss*)	*aa* (*tt*)	—	—	—	1[d]	—	—	—
bb (*ss*)	*ab* (*Tt*)	1	1.1	1	19[e]	13.8	17.6	16.6
bb (*ss*)	*bb* (*TT*)	1	1.1	1	19	13.8	17.6	16.6
					$\chi^2_{[7]}$	22.32**	5.18[NS]	7.60[NS]

[a]Includes three RZ206 genotypes predicted from flanking markers.
[b]Includes four RZ206 genotypes predicted from flanking markers.
[c]Includes two RZ206 genotypes predicted from flanking markers.
[d]The existence of this plant can be explained because it has been derived from a recombination event at LG5 in the region of the SC locus. It has been left out of the analysis. The marker genotypes of all other plants are used, which are assumed to generate very similar segregation ratios to those expected for *S* and *S5* genotypes. Errors associated with this assumption are small as shown by the fact that only 6.5% (five of 77) of plants (Table 4) were found to have been derived after a recombination event in the region of the *S* or *S5* locus.
[e]Includes one RZ206 genotype predicted from flanking markers.
**Significant deviation of observed ratios from expected at the $P<0.01$ level.
[NS]Observed ratios not significantly deviating from expected.
Note that a heterogeneity χ^2 test was made between the total observed ratios of plants in each genotype and the subset of plants that were screened for *in vitro* self-pollination reaction and this was not significant (data not shown).

software to analyse linkage associations between markers and trait (in this case SC) loci.

Distorted marker segregation

Significant and consistent distorted marker segregation ratios can be used to identify markers associated with SC mutations of incompatibility loci. Such distortions have been identified in *P. coerulescens* (Hayman and Richter, 1992) and used to map the *S*, *Z* and *S5* loci heterozygous for SC alleles in rye (Fuong *et al*, 1993; Voylokov *et al*, 1993). In our ryegrass population, large regions of marker segregation distortion occurred on LGs 5 and 7 (see also Armstead *et al*, 2004) where one of the homozygous classes was favoured over the other. This is a typical symptom of the activity of a gametophytic SC locus but could be due to other causes such as linkage to lethal and sublethal genes operating at any stage of the plant development process. The LG5 region of marker distortion is particularly attractive as a location for an SC locus, as a locus (*S5*) with a similar location has been identified in rye (Fuong *et al*, 1993; Voylokov *et al*, 1993), which is highly syntenic with ryegrass. Unlike in the case of Rye, in our perennial ryegrass F_2 family, there was never a case where the unfavoured homozygote was completely absent (Table 1), the lowest frequency calculated from the allele frequencies of marker loci in this region being 6.7% at a map position of 19.9. This indicates that there must be another factor, either allelic with this locus or at a separate locus, that determines a degree of SC.

The region of distorted segregation of markers on LG7 (Table 2) may be a candidate for this extra SC locus, although there is no record in any grass species that this region may contain such a locus. Again, in this region on LG7, the unfavoured homozygote class is never completely absent, the lowest frequency being estimated at 9.0% at a map position of 45.9. At this stage, confirmatory evidence that these regions contain SC loci is still required.

QTL analysis

The QTL analysis confirms that the observed marker distortions were due to the presence of a segregating SC locus on LG5 (designated *T*). No QTL was observed on LG7 and it is more likely that the distortion at LG7 (Table 2) has another, unknown, cause. The SC QTL on LG1 is not associated with any significant distortion but maps closely to the RFLP-derived STS marker, iacdo580, that was found by Thorogood *et al* (2002) to map just 9.2 cM away from the *S* locus in an unrelated mapping family. It is highly likely that the SC locus identified by our QTL analysis and the *S* locus are one and the same.

Marker analysis (models to explain action of *S* and *T* loci)

The data show that both iacdo580 and RZ206 markers are closely linked to gametophytic SC loci. Iacdo580 *aa* genotypes and RZ206 *bb* genotypes were in most cases 100% self-compatible, and *ab*/*aa* iacdo580/RZ206, *bb*/*ab* iacdo580/RZ206 and *ab*/*ab* iacdo580/RZ206 genotypes were approximately 50% self-compatible and *bb*/*aa* genotypes iacdo580/RZ206 were not recovered Table 4). We know that the RZ206 *b* allele linked to the SC allele *T* derives from P1, 'Perma' (P1 genotype for RZ206 marker = *bb*), and iacdo580 *a* allele linked to the SC allele *S* derives from P2, 'Aurora' (P2 genotype for iacdo580 marker = *aa*), and therefore the two different SC variants have arisen separately during the development of each of the inbred parental lines. The six plants that did not fit

the pattern could, bar one, be explained by recombination events occurring in one or the other of the two regions (Table 6), which, we would have to assume, occurred between the SC locus and the marker. Thus plant 4/7 could reasonably be classified as *Ss/tt*, 9/7, 11/2 and 22/5 as *SS/Tt* and 3/3 as *Ss/Tt*, thus explaining these plants' SC statuses. Only in one plant (9/9) were we unable to identify a recombination event in either the LG1 or LG5 region, and it must be assumed that this plant has been misclassified.

We are now in a position to develop models to explain the behaviour of these two SC loci in our F2 population.

Model 1: The findings are consistent with the hypothesis that two independent SC loci are segregating where only pollen possessing at least one of the SC alleles from either locus will be compatible on selfing and will produce F_2 progeny. We will refer to this hypothesis as model 1 (Figure 2). The data however fail to fit the model in two major respects:

(1) This model requires the double-heterozygote F_2 plants, like the F_1, to be 75% self-compatible. We assessed our double heterozygotes to be approximately 50% self-compatible but acknowledge that it is often difficult to distinguish classes of partial compatible reactions. This difficulty may also be compounded by the existence of polygenic modifiers of SC, which have been suggested to exist in *Lolium* (Jenkin, 1931; Jones and Jenabzadeh, 1981).

(2) The segregation ratios of the F2 plants at iacdo580 and RZ206 do not agree with those expected in model 1. All plants that had been genotyped for iacdo580 and RZ206, plus 10 plants from which the genotypes of iacdo580 and RZ206 could be deduced from the genotypes of markers flanking either side, were used to analyse the ratios observed. We used the total number of molecularly characterised plants rather than limiting our analysis just to those characterised for both linked markers and SC, as the marker ratios of these plants were not significantly different from those of the subset of plants characterised for SC, as indicated by a nonsignificant heterogeneity χ^2 test (data not shown). Complete linkage between these markers and the SC loci was assumed and the small number of recombinants, which undoubtedly occurred, would not overly affect the ratios obtained. The ratio of the eight genotypes on selfing the F_1 deviated significantly from the model 1 ratio (Table 5) and it is clear that segregations at the two loci differ from each other (Table 7). In particular, the iacdo580/RZ206 genotypes *aa/aa* (*SS/tt*) and *ab/aa* (*Ss/tt*) were deficient (Table 5). These genotypes can only be formed by fertilisation by *S/t* pollen (see Figure 2), which strongly suggests that this pollen genotype is partially selected against.

Model 2: A second model based on this differential transmission can be developed. A total of 10 *aa/aa* (*SS/tt*) and *ab/aa* (*Ss/tt*) genotypes were recovered in the F_2 when 28 would have been expected according to model 1, which gives an *S/t* pollen transmission rate of 36%. Based on a 36% transmission rate of *S/t* pollen, the observed number of genotypes in the F_2 then agrees with the ratio expected (Table 5). However, this is only an estimate of the actual transmission rate based on a limited number of plants. We are unable to attach

Table 6 Genotypes of markers on LG1 and LG5 for plants with apparently aberrant SC scores and marker associations

	Marker	*Map distance (cM)*	*Plant number*					
			4/7	*9/7*	*11/2*	*22/5*	*3/3*	*9/9*
LG1	rv0913	0	—	a	a	—	h	h
	PGI	1.7	h	h	h	a	h	h
	iacdo580	**7.5**	**a**	**h**	**h**	**h**	**h**	**h**
	E41M5709	11.6	a	—	—	—	—	—
	rv0659	13.2	a	h	h	—	h	h
	E38M4709	14.7	a	—	—	—	—	—
	rv0785	16	a	h	h	—	h	h
	E36M5503	16	a	—	—	—	—	—
	E36M5508	16.8	a	—	—	—	—	—
	E42M3308	17.5	a	—	—	—	—	—
LG5	E51174S	7.1	—	—	—	—	—	b
	F29-1	11.5	—	h	h	h	h	—
	CDO127	13.6	a	h	—	h	h	b
	CDO1380.2	16.4	a	h	h	h	h	b
	rv0184	16.4	a	h	h	—	b	b
	RZ206	**17**	**a**	**h**	**h**	—	**b**	—
	BCD1087	17	a	—	—	—	h	—
	rv0495	17	a	h	h	—	b	b
	PSR574	20.9	a	h	—	h	b	b
	rv0082	21.5	a	h	h	—	b	b
	rv0950	23.7	a	h	h	—	b	b
	rv1258	24.7	a	h	h	—	b	b
	CDO412	24.7	a	—	—	—	b	—

a: *aa* genotype; b: *bb* genotype; h: *'ab'* genotype.
Shaded areas show regions where a recombination event has occurred that can explain the anomaly between SC and marker genotype statuses of apparently aberrant plants. In one plant (9/9), the discrepancy cannot be attributed to a recombination event and it is assumed that the plant has been misclassified for either marker genotype or SC phenotype.
Markers and marker genotypes in bold are those closest to the SC QTL.

♀ \ ♂	S/T	S/t	s/T	s/t
S/T	SS/TT	SS/Tt	Ss/TT	X
S/t	SS/Tt	SS/tt	Ss/Tt	X
s/T	Ss/TT	Ss/Tt	ss/TT	X
s/t	Ss/Tt	Ss/tt	ss/Tt	X

Figure 2 Eight genotypes expected on selfing F_1 plant heterozygous for two independent gametophytic SC loci (model 1).

Table 7 Test of heterogeneity between segregation ratios at iacdo580 and RZ206 loci when compared to ratios expected in model 1 (1:3:2)

	Genotype			*df*	χ^2
	aa	ab	bb		
Iacdo580	38	90	38	2	9.83**
RZ206	10	88	68	2	14.53***
Total	48	178	106	2	2.03^{NS}
Sum				2	24.36***
Heterogeneity				2	22.33***

NS: nonsignificant; ***$P<0.001$; **$P<0.01$.

Table 8 Observed % SC for the F_2 *S/T* genotypes and those expected on models 1 and 2 (reduced transmission of *S/t* pollen in *S* heterozygotes at 36 and 50%)

Iacdo580/RZ206 genotype	S/T *locus genotype*	*SC score*			
		Observed	*Model 1*	*Model 2 (36% transmission)*	*Model 2 (50% transmission)*
aa/aa	*SS/tt*	100	100	100	100
aa/ab	*SS/Tt*	100	100	100	100
aa/bb	*SS/TT*	100	100	100	100
ab/aa	*Ss/tt*	50	50	18	25
ab/ab	*Ss/Tt*	50	75	59	62.5
ab/bb	*Ss/TT*	100	100	100	100
bb/aa	*ss/tt*	—	—	—	—
bb/ab	*ss/Tt*	50	50	50	50
bb/bb	*ss/TT*	100	100	100	100

confidence limits to this observed transmission, but the simplest model would be based on a transmission rate of 50% because this would suggest that an extra single segregating gene is responsible for the differential response.

If *S/t* pollen is sometimes arrested at the stigma surface (ie the interaction is integral to the gametophytic incompatibility process), this would lead to reduced SC of all plants that possess *S* and *t* alleles. This would explain the reduced SC of *Ss/Tt* from the 75% expected in model 1 (Figure 2). The 50% transmission of *S/t* would result in 62.5% SC (Table 8), which is practically indistinguishable from the 50% SC recorded for the double-heterozygote F_2 plants. *Ss/tt* genotypes would also show reduced SC scores and at 50% transmission only 25% of pollen grains would be expected to be compatible on selfing. This is somewhat lower than the 50% estimated for the three *Ss/tt* plants scored but not inconceivably so.

The major anomaly with the reduced *S/t* allele transmission model at this stage is that the two genotypes *SS/tt* (one plant) and *SS/Tt* (10 plants) were unequivocally 100% self-compatible. Clearly, *S/t* pollen appears to be always compatible on selfing in genotypes that are homozygous for the self-fertility allele, *S*. The percentage compatibility of the eight F2 genotypes expected on selfing the plants under the conventional model 1, and model 2 with 36 and 50% transmission is given in Table 8. The reason for the differential response of *S/t* pollen, depending on the allelic composition of the *S* locus, is unclear although it would appear that a functional allele, *s*, in the style is required to elicit an incompatible response to *S/t* pollen. The fact that the *S/t* pollen is not fully excluded from the fertilisation process, as indicated by the fact that *SS/tt* and *Ss/tt* F_2 genotypes are formed at all, indicates the involvement of a third locus.

We reclassified our pollinations so that they agreed with the pollinations expected on model 2 with 50% transmission. This meant changing our 50% SC scores of genotypes *Ss/tt* to 25% and those of genotypes *Ss/Tt* to 62.5% (Table 8). All other SC reactions remained as for model 1. Interval and nonparametric (Kruskall–Wallis; see Lehman, 1975) analyses on the reclassified data revealed QTL at the same two regions on LG1 (*S* locus) and LG5 (*T* locus) in accordance with the original data

Table 9 Interval and Kruskall–Wallis analyses (Lehmann, 1975) results based on *in vitro* self-pollination with SC scores adjusted to fit model 2 (50% transmission of *S/t*) for a genomic region on LG2

Map distance	*Locus*	*LOD score*	*% variance explained*	*Kruskall–Wallis* K *value*
0.0	M4136	1.61	10.2	7.21**
5.0		1.17	8.7	
10.0		0.59	3.7	
10.3	CDO38_1/3	0.56	3.5	3.57
15.3		0.88	6.4	
20.3		1.09	7.4	
23.8	FpAFPD	1.12	6.7	4.48
24.4	FpAFPH	1.16	6.8	2.73
25.5	E39M5805	1.26	7.5	3.44*
28.6	BCD855	1.50	8.8	4.11
29.5	R3349	1.16	6.9	5.70*
30.4	Fp12.1	1.52	8.9	4.44
31.4	Fp12.2	1.46	8.6	4.24
31.4	R2395_3	1.63	9.5	2.86*
34.3	CDO365	1.54	9.0	7.27**
34.3	ARH1A07	1.17	6.9	7.38**
35.1	C451	1.88	10.8	11.43****
35.1	rv0116	1.96	11.2	4.89*
35.1	CDO395.2	1.35	7.9	5.56**
35.8	CDO59	1.73	10.0	4.03
35.8	CDO385.2	1.65	9.7	6.09**
35.8	rv0062	1.40	8.2	5.17*
37.0	22taga1	1.55	9.4	4.47
37.0	E40M5910	2.02	12.0	7.25***
38.3	E42M3310	1.74	10.2	3.71*
38.8	17cal	1.42	8.5	4.14
40.2	Rv0037	1.02	6.4	4.74*
42.4	B6106	0.26	1.7	4.48**
42.4	E41M5713	0.11	0.7	1.76
43.1	Rv0981	0.07	0.4	6.87**
43.1	C145	0.28	1.7	1.41

*$P<0.1$; **$P<0.05$; ***$P<0.01$; ****$P<0.005$.

plus an additional locus, previously undetected, on LG2 (Table 9). This extra locus could well correspond to the *Z* locus known to be located on LG2 in perennial ryegrass (Thorogood *et al*, 2002), which, of course, is known to complement the *S* locus to elicit a self-incompatible response. It therefore appears likely that the *S*, *Z* and *T* loci interact to determine pollen–stigma compatibility.

Conclusions

The identification of a locus (*T*) in *Lolium*, which is nonallelic to either the *S* or *Z* locus and which clearly has an interactive role with these loci, to determine compatibility status reinforces the possibility raised by Hayman and Richter (1992) that the two-locus grass system has evolved from a multilocus system such as that found in *Ranunculus* species and *Beta vulgaris* (Osterbye, 1975; Larsen, 1977; Lundqvist, 1990). This locus adds to the one identified by Thorogood *et al* (2002) on LG3, which interacts with the *S* locus and thus also has a role to play in the incompatibility response in *Lolium*.

In a practical sense, the observed interaction between the incompatibility loci is important for marker-assisted selection procedures for accelerated breeding: if traits are selected for that are linked to *S*, *T* or *Z*, then there is a distinct possibility that this selection will not only determine frequencies of linked alleles but also alleles linked to the other loci. The fact that such gene interaction was observed between the *S* locus and a

locus on LG3 in *L. perenne* as well (Thorogood *et al*, 2002) shows that selection at or near any of these loci on LGs 1, 2, 3 or 5 is likely to have profound effects on allele frequencies in a substantial proportion of the *Lolium* genome.

References

Armstead IP, Turner LB, Farrell M, Skot L, Gomez T, Montoya T *et al* (2004). Synteny between a major heading-date QTL in perennial ryegrass (*Lolium perenne* L.) and the *Hd3* heading-date locus in rice. *Theor Appl Genet* **108**: 822–828.

Armstead IP, Turner LB, King IP, Cairns AJ, Humphreys MO (2002). Comparison and integration of genetic maps generated from F2 and BC1-type mapping populations in perennial ryegrass (*Lolium perenne* L.). *Plant Breed* **121**: 501–507.

Cornish MA, Hayward MD, Lawrence MJ (1979). Self-incompatibility in ryegrass. I. Genetic control in diploid *Lolium perenne* L. *Heredity* **43**: 95–106.

Fearon CH, Hayward MD, Lawrence MJ (1983). Self-incompatibility in ryegrass. 5. Genetic control, linkage and seed set in diploid *Lolium multiflorum* Lam. *Heredity* **50**: 35–45.

Fuong FT, Voylokov AV, Smirnov VG (1993). Genetic studies of self-fertility in rye (*Secale cereale* L.). 2. The search for molecular marker genes linked to self-incompatibility loci. *Theor Appl Genet* **87**: 619–623.

Hayman DL (1956). The genetic control of incompatibility in *Phalaris coerulescens* Desf. *Aust J Biol Sci* **9**: 321–331.

Hayman DL, Richter J (1992). Mutations affecting self-incompatibility in *Phalaris coerulescens* Desf. (Poaceae). *Heredity* **68**: 495–503.

Hayward MD (1988). Exploitation of the incompatibility mechanism for the production of F1 hybrid forage grasses. *Euphytica* **39**: 33–37.

Hayward MD, Thorogood D, Jones JG (1991). Manipulation and exploitation of the reproductive system in forage breeding. In: den Nijs APM, Elgersma A (eds) *Fodder Crops Breeding: Achievements, Novel Strategies and Biotechnology. Proceedings of the 16th Meeting of the Fodder Crops Section of Eucarpia*. Centre for Agricultural Publishing and Documentation (PUDOC): Wageningen, The Netherlands, pp 89–94.

Jenkin TJ (1924). The artificial hybridization of grasses. *Welsh Plant Breed Station Bull H* **2**: 2–18.

Jenkin TJ (1931). Self-fertility in perennial ryegrass (*Lolium perenne* L.). *Welsh Plant Breed Station Bull H* **12**: 100–119.

Jones ES, Mahoney NL, Hayward MD, Armstead IP, Jones JG, Humphreys MO *et al* (2002). An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. *Genome* **45**: 282–295.

Jones RN, Jenabzadeh P (1981). Variation in self-fertility, flowering time and inflorescence production in inbred *Lolium perenne* L. *J Agric Sci* **96**: 521–537.

Larsen K (1977). Self-incompatibility in *Beta vulgaris* L. I. Four complementary *S*-loci in sugar beet. *Hereditas* **85**: 227–248.

Leach CR (1988). Detection and estimation of linkage for a co-dominant structural gene locus linked to a gametophytic self-incompatibility locus. *Theor Appl Genet* **75**: 882–888.

Lehman EL (1975). *Nonparametrics*. McGraw-Hill: New York.

Lundqvist A (1954). Studies on self-sterility in rye, *Secale cereale* L. *Hereditas* **40**: 278–294.

Lundqvist A (1955). Genetics of self-incompatibility in *Festuca pratnsis* Huds. *Hereditas* **41**: 518–520.

Lundqvist A (1956). Self-incompatibility in rye I. Genetic control in the diploid. *Hereditas* **42**: 293–348.

Lundqvist A (1961). A rapid method for the analysis of incompatibility in the grasses. *Hereditas* **47**: 705–707.

Lundqvist A (1990). The complex *S*-gene system for control of self-incompatibility in the buttercup *Ranunculus*. *Hereditas* **113**: 29–46.

Martin FW (1959). Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technol* **34**: 125–128.

Osterbye U (1975). Self0incompatibility in *Ranunculus acris*. I. Genetic interpretation and evolutionary aspects. *Hereditas* **104**: 61–73.

Payne RW (2000). *The Guide to GenStat, Part 2: Statistics*. VSN International: Oxford. 782pp.

Stam P, van Ooijen JW (1995). *JOINMAP™ version 2.0. Software for the calculation of genetic linkage maps*. CPRO-DLO, Wageningen.

Thorogood D (1991). *Self- and Cross Fertility in Lolium*. PhD Thesis, University of Wales.

Thorogood D, Hayward MD (1991). The genetic control of self-compatibility in an inbred line of *Lolium perenne* L. *Heredity* **67**: 175–181.

Thorogood D, Hayward MD (1992). Self-compatibility in *Lolium temulentum* L.: its genetic control and transfer into *L. perenne* L. and *L. multiflorum* Lam. *Heredity* **68**: 71–78.

Thorogood D, Kaiser WJ, Jones JG, Armstead IP (2002). Self incompatibility in ryegrass 12. Genotyping and mapping the *S* and *Z* loci. *Heredity* **88**: 385–390.

Van Ooijen JW, Boer MP, Jansen RC, Maliepaard C (2000). *MapQTL 4.0: software for the calculation of QTL positions on genetic maps (user manual)* University of Wageningen, The Netherlands.

Voylokov AV, Fuong FT, Smirnov VG (1993). Genetic studies of self-fertility in rye (*Secale cereale* L.). 1. The identification of genotypes of self-fertile lines for the Sf alleles of self-incompatibility genes. *Theor Appl Genet* **87**: 616–618.

Wricke G, Wehling P (1985). Linkage between an incompatibility locus and a peroxidase isozyme locus (prx-7) in rye. *Theor Appl Genet* **71**: 289–291.