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Abstract
Lolium perenne (ryegrass) is a species of major economic importance due to its multiple uses such as forage, turf, and cover crop and its potential use as a feedstock for bioenergy. Ryegrass occurs naturally as a diploid (2n=14). However, several autotretraploid cultivars were obtained via colchicination. Ryegrass displays a two-locus gametophytic self-incompatibility system (SI) that remains intact even at the tetraploid level. There are two plausible models for SI at the tetraploid level, Model I in which only one different allele in the pollen grain from the female at either S or Z locus makes pollen compatible. Model II states that any S-Z combination in the pollen grain matching the female would make pollen incompatible. The goals of this study are i) to evaluate which of the two models better explains SI in our bi-parental autotetraploid ryegrass population; ii) to provide evidence that SI maintenance was not specific to genotypes used in previous studies; and iii) to evaluate the sensitivity and efficacy of HRM genotyping in polyploid species. The present study used 43 progeny of a cross between two autotetraploids and characterized them with three HRM-based markers that were reported to co-segregate with Z. The confirmation of the underlying model for SI in autotetraploids in combination with the use of markers as well as automated software for pollen compatibility estimation such as PollenCALC can be of great use to perform controlled crosses in tetraploid ryegrass.

Disciplines
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Validation of two models for self-incompatibility in autotetraploid ryegrass using High Resolution Melting-based markers

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ABSTRACT

Lolium perenne (ryegrass) is a species of major economic importance due to its multiple uses such as forage, turf, and cover crop and its potential use as a feedstock for bioenergy. Ryegrass occurs naturally as a diploid (2n=14). However, several autotretraploid cultivars were obtained via colchicination. Ryegrass displays a two-locus gametophytic self-incompatibility system (SI) that remains intact even at the tetraploid level. There are two plausible models for SI at the tetraploid level, Model I in which only one different allele in the pollen grain from the female at either S or Z locus makes pollen compatible. Model II states that any S-Z combination in the pollen grain matching the female would make pollen incompatible. The goals of this study are i) to evaluate which of the two models better explains SI in our bi-parental autotetraploid ryegrass population; ii) to provide evidence that SI maintenance was not specific to genotypes used in previous studies; and iii) to evaluate the sensitivity and efficacy of HRM genotyping in polyploid species. The present study used 43 progeny of a cross between two autotetraploids and characterized them with three HRM-based markers that were reported to co-segregate with Z. The confirmation of the underlying model for SI in autotetraploids in combination with the use

of markers as well as automated software for pollen compatibility estimation such as PollenCALC can be of great use to perform controlled crosses in tetraploid ryegrass

INTRODUCTION

Lolium perenne (ryegrass) is an important crop species due to its multiple uses such as forage, turf, cover crop and its potential use as a feedstock for bioenergy (Arias Aguirre et al., 2011; Donnison, 2009; Studer B et al., 2010). Ryegrass occurs naturally as a diploid species (2n=14). However, autotretraploid cultivars were obtained via colchicination and released for forage production. Both diploid and autotetraploid genotypes display a gametophytic self-incompatibility system (SI) (Baumann et al., 2000; Fearon et al., 1984a; Thorogood et al., 2002). This mechanism is determined by two independent loci named S and Z, located in linkage groups 1 and 2, respectively (Thorogood et al., 2002). Both loci are involved in preventing self-pollination.

The consensus model for gametophytic SI in diploid grasses suggests that fifty percent of the pollen produced by a pollen donor is compatible to a pollen receptor, if the pollen donor genotype has one different allele at either S or Z compared to the receptor genotype (Klaas et al., 2011; Thorogood et al., 2002; Yang, 2009; Yang et al., 2008). Additionally, if the pollen donor has precisely one different allele at each locus, S and Z, then 75 % of the pollen is compatible with the pollen receptor genotype. If the number of different alleles at either locus of the pollen donor increases, pollen is 100% compatible.

In autotetraploid species the interaction between pollen and stigma alleles is more complicated due to the presence of four alleles at a locus and sixteen possible pollen haplotypes for both loci. In contrast to gametophytic SI in several dicots, where polyploidy causes breakdown of self-incompatibility, the monocot SI system remains functional in polyploids (Fearon et al., 1984a).

Two plausible models for SI have been proposed by Arias et al. (2012) and are in agreement with those tested by Fearon and Hayward (1984): Model I: if any allele at either S or Z in the pollen differs from the alleles for S or Z in the pistil, pollen is compatible (Arias Aguirre et al., 2012); Model II: if at least one allele at S and one allele at Z locus present in the pollen grain matches the pistil, then pollen is incompatible (Arias Aguirre et al., 2012).

Initial studies on the appropriate genetic model for SI in autotetraploids was addressed by in vitro pollination tests (Fearon et al., 1984a; Fearon et al., 1984b). Fearon & Hayward (1984) used pollen from diploid plants as testers against their tetraploid counterparts, and found evidence supporting Model II. However, some key questions remain to be answered. First, does the model proposed hold true when tetraploid plants are pollinated with their own diploid pollen? Does the number of alleles at each locus affect the proposed models or is there dominance? Moreover, percentage of compatibility is confounded between ploidy and the effect of SI loci since the pollen used came from a diploid parent. Finally, the accuracy of determining SI genotypes by using in vitro pollination tests is limited. Pollen viability, media, and staining can affect the assessment of in vitro pollen germination and, therefore, SI genotype inference. Besides, it is challenging to differentiate pollen compatibilities of 30% and 58%, which were expected frequencies in the cited study, and could result in miss-classification of genotypes. Thus, application of molecular markers linked to SI loci help to increase accuracy of classification and determination of compatibility percentages, and ultimately the correct model for SI in autotetraploids.

Manzanares, et al. (2011) showed that HRM-based markers were able to discriminate between different Z haplotypes in ryegrass. The markers tested in this study were developed within 200

kb in the Z region and enabled discrimination of a total of 13 haplotypes among different populations of selected half-sibs that trace back to nine founders (Manzanares et al., 2011).

The goals of this study were i) to evaluate the efficiency of HRM genotyping for discriminating different iso-allelic genotypes in autopolyploid species; and ii) to evaluate which of the two models better explains SI in our bi-parental autotetraploid ryegrass population.

Materials and Methods

Plant materials

The genomes of the two parents, F1-30 and F1-39, of the VrnA population (Jensen et al., 2005) with defined S and Z genotypes were doubled using colchicine. The tetraploid individuals were crossed to obtain a segregating population (Figure 1). In this population, a total of two alleles are segregating for the S locus that are represented by letters A and B, and three alleles for the Z locus represented by numbers 1, 2, and 3. A total of 50 plants were used for this study, grown in the Iowa State University Agronomy greenhouses in Ames, IA, for 3 months. Three month old plants were vernalized in a growth chamber for five weeks at 5C with a 10 h light per day photoperiod. After vernalization, plants were grown until flowering at a 16 h day length at 20C in the Iowa State University Horticulture greenhouses in Ames, IA. We used the progeny of this cross, retrospectively, to infer which model best describes SI in our autotetraploid population.

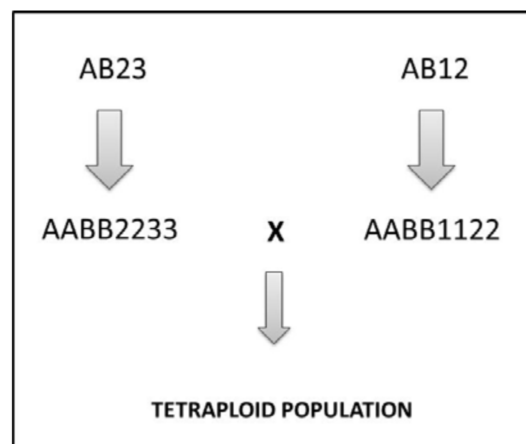

Figure 1. Scheme of autotetraploid population development using colchicine doubling. Alleles from S are assigned as letters and alleles for Z are assigned numbers

The estimation of expected frequencies under the two possible models was performed, using the pollen compatibility software PollenCALC (Arias Aguirre et al., 2012). This software predicts pollen compatibility between two tetraploid genotypes. The software predicts genotypic classes, gamete frequencies, and progeny genotypes. Expected frequencies were only calculated for the AABB 2233 (female) x AABB 1122 (male) cross, because seed was only harvested on the AABB2233 genotype. Even though there are two different alleles for Z in F1-39, they show a similar phenotype (Studer, unpublished data). Since S genotypes were identical in both parents, alleles for S were not expected to contribute to compatibility in the parental cross. Therefore, we concentrated our efforts on the segregating Z locus. A male with a Z-1122 produces three pollen genotypes but not all three genotypes are compatible to the female AABB 2233 (Table 1).

Table.1 Possible pollen genotypes from Z locus produced by male AABB1122 and compatibility to the AABB 2233 under each model

	Compatibility to female AABB 2233	
Pollen genotype	**Under Model I**	**Under Model II**
11	Yes	Yes

12	Yes	No
22	No	No

We used PollenCALC to estimate the expected haplotype frequencies of Z in the gametes of both the male and female genotype under Model I and Model II (Table 2). Female gamete haplotypes for Z were not expected to differ under either model. Under Model I, the male AABB 1122 is 84% compatible with the female AABB2233 under the assumption of normal chromosome segregation. This means that only pollen grains with a Z-11 and Z-12 are expected to be compatible with the AABB 2233 female.

Table 2. Expected genotypic frequencies for Z locus for all female gametes and compatible male gametes for the doubled F1-30 and F1-39 parents under Model I and Model II

Female gametes		**Compatible male gametes Model I***		**Compatible male gametes Model II****	
Genotype	*Expected frequency*	*Genotype*	*Expected frequency*	*Genotype*	*Expected frequency*
22	0.16	**12**	0.672	**11**	0.168
23	0.68	**11**	0.168		
33	0.16				

* Pollen gametes carrying 22 would be incompatible under Model I
** Pollen gametes carrying 12 and 22 would be incompatible under Model II

For Model II, the expectation is that only pollen carrying the Z-11 genotype are compatible with the AABB 2233 female, thus only q 16.8% of the pollen from the AABB1122 male is expected to pollinate the female.

Since it is difficult to genotype gametes we relied on the progeny, where only compatible pollen genotypes were expected to contribute. For Model I, six genotypic classes are expected for the Z locus in the progeny resulting from two compatible pollen genotypes with three possible female gametic genotypes (Table 3). In contrast, under Model II only pollen carrying a Z-11 genotype is compatible with the female and, therefore, only three genotypic classes for the Z locus are expected in the progeny (Table 4). All calculations were performed under the assumption that all compatible pollen grains are viable and equally able to pollinate the female and that the female gamete is not inhibited after an incompatible reaction. Therefore, the frequencies of male gametes used in Table 2 and 3 were recalculated, weighing expected compatible pollen frequencies by the pollen compatibility under each model. For instance, for Model I the frequency of male pollen genotype Z-12 is 0.672/0.84 =0.8 (Table 2).

Table 3. Expected genotypic classes and frequencies for the Z locus tetraploid population progeny under Model I using weighed frequencies

Male Gametes		**Female Gametes**		**Progeny**	
Weighed Frequency	Genotype	Weighed Frequency	Genotype	Genotypic Class	Expected Frequency
0.8	12	0.16	22	1222	0.13
	12	0.68	23	1223	0.55
	12	0.16	33	1233	0.13
0.2	11	0.16	22	1122	0.03
	11	0.68	23	1123	0.13
	11	0.16	33	1133	0.03

Table 4. Expected genotypic classes and frequencies for the Z locus tetraploid population progeny under Model II using weighed frequencies

Male Gametes		Female Gametes		Progeny	
Weighed Frequency	Genotype	Weighed Frequency	Genotype	Genotypic Class	Expected Frequency
1	11	0.16	22	1122	0.16
	11	0.68	23	1123	0.68
	11	0.16	33	1133	0.16

Ploidy level analysis

Plant tissue was collected and kept cool until chopping. Chopping was performed with a razor blade and LB01 buffer (Dolezel et al., 1989). The resulting solution was poured through a nylon mesh into 2 ml Eppendorf tubes and centrifuged at low speed for 3 minutes. Supernatant was discarded and the pellet was resuspended using the LB01 lysis buffer. Finally, 25 µl propidium iodide stock was added to the solution, which was transferred to a flow cytometry tube. Fifty plants were analyzed by flow cytometry in the Flow Cytometry Facility at Iowa State University. A diploid and a tetraploid control were used to determine ploidy levels of the 50 plants.

Phenotypic assays

For 43 plants, flowering tillers were bagged with white Lawson bags for four weeks. Additional tillers were used for in vitro pollination tests in 10 plants to confirm self-incompatibility. Stigmas were collected on 2% agar containing 10% sucrose and 100 ppm Boric acid. Petri dishes containing stigma were incubated at 25C for 24 h. Stigmas were pollinated with pollen from the same plant ("self-pollinated") and placed back in an incubator for 4 hours. Ovaries were cut using razor blade and placed on microscope slides. A drop of a staining solution, 0.2% aniline

blue in 2% K_3PO_4, was placed over pollinated stigmas and covered with a cover slide. Pollinated stigmas were inspected under a Zeiss Axioplan 2 Imaging UV light microscope and images were analyzed with the Axiovision software (Zeiss, Germany) at the Microscope and Imaging Facility at Iowa State University.

Z haplotype genotyping

After confirming that plants in the population were self-incompatible, molecular analysis by high resolution melting (HRM) genotyping was performed (Studer et al., 2009). PCR reactions contained 1x LCGreen dye (Idaho Technology, Inc., UT, USA), 0.1 mM of dNTPs, 10 µM of each forward and reverse primer, 20 mM of magnesium chloride, and 20 ng of DNA. In addition, 15 µL of mineral oil was added to the PCR mix to avoid evaporation. PCR amplification was conducted in a PTC-200 Thermal Cycler (MJ Research Inc., Watertown, MA, USA) following the protocol of Studer et al. (2009). A final cycle of 30 s at 94C and 30 s at 25C was added for heteroduplex formation. The LightScanner Instrument (96-well plate format) was used for HRM analysis and the LightScanner software Version 2 (Idaho Technology, Inc., Utah) was used for melting analysis. Melting temperatures ranged from 68-98C in an auto exposure setting. A total of three markers (Studer et al. 2012) were used to determine the segregation of Z in 43 tetraploid progeny as well as the male parent.

Quantitative and Qualitative Discrimination of the two models

The main difference between Model I and II is the number of Z genotypes expected in the progeny. Model I, which is a more permissive model would result in six genotypic classes in the progeny compared to the three expected under Model II. Unfortunately, the difference between those expected genotypes is not due to presence/absence of alleles, but rather their number.

Under Model I the six expected genotypes are 1222, 1223, 1233, 1122, 1123 and 1133 (Table 3). From those genotypes only 1122, 1123 and 1133 are expected under Model II and at different frequencies (Table 4). HRM genotyping can be used as co-dominant marker system in diploid species (Studer et al., 2009), but it was not clear, whether different isoallelic autotetraploid genotypes such as 1122 and 1222 can be discriminated. To solve this problem, we added a 1:1 mixture of the diploid parents F1-30 and F1-39 with defined haplotypes for Z-12 and Z-23, respectively, which together were expected to represent one of the most frequent genotypic classes under Model I. We used a Z-12 genotype to represent the 1122 tetraploid parental class, which is expected to be the most frequent under Model II. Finally, we included a Z-13 genotype and all its pair-wise combinations to the two parental classes to have controls for all possible genotypes expected under Model I. We were able to represent all possible genotype classes, except for 1222. The purpose of these controls was to ask, whether genotypes expected under Model I but not Model II could be identified (qualitative analysis).

Independent of this qualitative analysis, we addressed the question, whether segregation among progeny genotypes was consistent with one of the two models. Once haplotypes were assigned to the corresponding expected genotypic classes, we used X^2 tests, to compare observed with expected genotypic frequencies. The analysis for Model I was done with five degrees of freedom and with two degrees of freedom for Model II.

Results

Phenotypic assays and flow cytometry

Out of the 50 plants grown for this study, only 43 were confirmed to be tetraploids after flow cytometry. These 43 plants produced normal tillers and florets after vernalization. Florets and

stigmas had a larger size compared to diploid plants. The average number of tillers and florets was not significantly different among plants (data not shown). None of the individuals produced seed after selfing, and no pollen tubes grew after using stigmas and self-pollen in in-vitro pollination tests.

Z haplotype genotyping

The three markers used for Z genotyping were polymorphic in our population. The 43 individuals showed different curves or marker genotypes under high resolution melting. No single marker was able to assign a genotype to the corresponding genotypic class. For instance, markers ZB1 and ZB2 classified all individuals into only two classes (Figures 2 & 3), whereas ZB3 discriminated three different genotypes (Figure 4). We found a total of four haplotypes for the Z locus among the 43 samples, which supports Model I (Table 5), if haplotype frequencies are not taken into consideration.

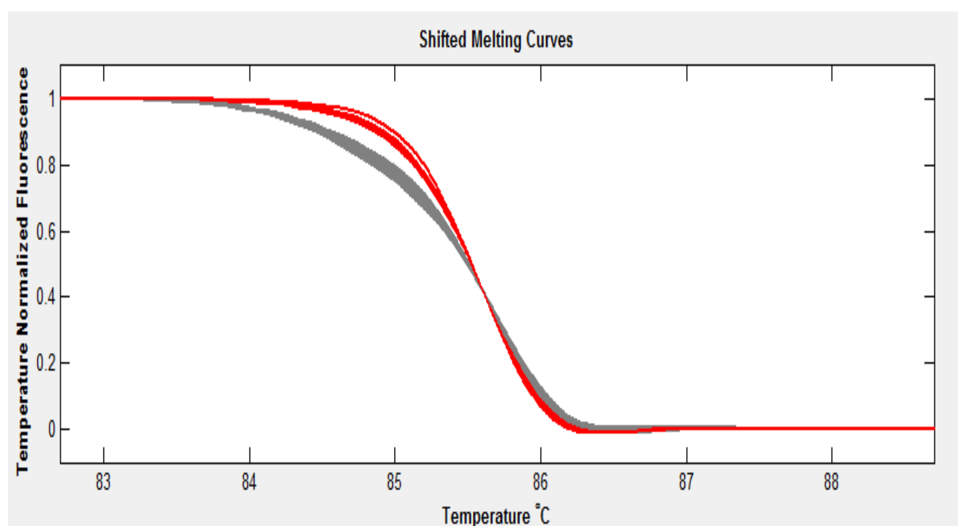

Figure 2. Shifted melting curves for primer ZB1 shows a clear segregation of the 43 genotypes into two categories

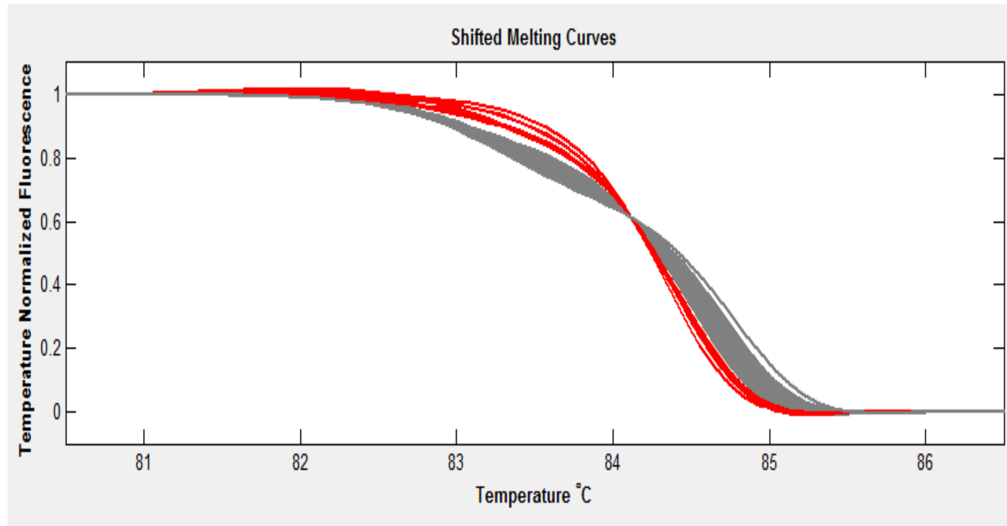

Figure 3. Shifted melting curves for primer ZB2 that similarly to ZB1 show a segregation of the population into two groups

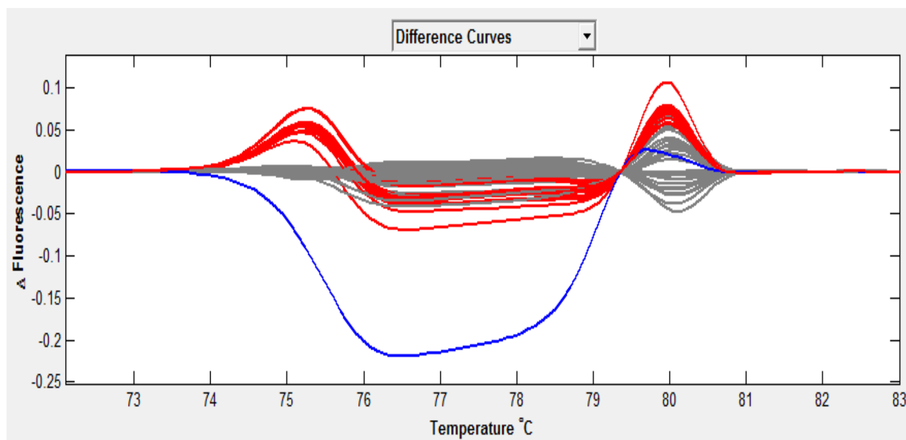

Figure 4. Difference curves for primer ZB3 showed up to three different curves for the 43 genotypes in the population.

Table 5 Melting curves calls for markers ZB1, ZB2 and ZB3 and the respective haplotype designation

Corresponding DNA control	call_ZB1	call_ZB2	call_ZB3	Number of individuals in this category
1122	A	A	A	10
1133	A	A	D	8
1123	B	A	D	24
1223	B	B	D	1
1233	B	B	A	0
			TOTAL	43

Quantitative and Qualitative Discrimination of genotypic classes:

We performed a qualitative discrimination of the genotypic classes for which we used a 1:1 mix between the diploids F1-30 and F1-39 and a Z-13 sample and were able to assign all observations to corresponding genotypic classes in both models. We were able obtain DNA controls for all expected genotypic classes under Model II and all expected genotypic classes under Model I except for 1222. Using these controls, we were able to assign the 42 progeny to genotypic classes 1122, 1133, and 1123. We also found one individual in the progeny corresponding to the 1223 genotypic class. The other two genotypic classes expected under Model I were missing. Table 6 shows that observed segregation ratios differed significantly

(p=0.001) from expected segregation under Model I, whereas the phenotypic data did not show a significant deviation from the expected frequencies under Model II.

Table 6. Chi Square test for hypothesis testing of Model I and Model II.

			MODEL I			
Z Genotype	**Expected Frequency**	**Expected counts**	**Observed counts**	**Obs - Exp**	**Squared**	**Squared/expected**
1222	0.13	5.6	0	-5.6	31.36	5.6
1223	0.55	23.7	1	-22.7	515.29	21.7
1233	0.13	5.6	0	-5.6	31.36	5.6
1122	0.03	1.3	10	8.7	75.69	58.2
1123	0.13	5.6	24	18.4	338.56	60.5
1133	0.03	1.3	8	6.7	44.89	34.5
					SUM	186.2
					P Value	<0.001
			MODEL II*			
Z Haplotype	**Expected Frequency**	**Expected counts**	**Observed counts**	**Obs- Exp**	**Squared**	**Squared/expected**
1122	0.16	6.72	10	3.28	10.7584	1.60
1123	0.68	28.56	24	-4.56	20.7936	0.73
1133	0.16	6.72	8	1.28	1.6384	0.24
					SUM	2.57
					PVALUE	0.463

*Only 42 individuals were use in this test because the 43th has a genotype which in not consistent with any other of genotypic classes expected under this model

DISCUSSION

The use of HRM-based markers is a rapid and sensitive essay for genotyping. The present study adds further proof to the data presented by Manzanares et al. (2011) and provides evidence that HRM genotyping can be reliably performed in tetraploid ryegrass. It is important to remark that when, used in tetraploids, diploid DNA mixes are needed to assign melting curves to the

corresponding genotypic classes. Once melting curves are assigned to specific genotypes, tetraploids themselves can be used as controls for genotypic classes.

Both the absence of seed upon self-pollination and the lack of pollen tube growth upon selfing in in vitro pollination test demonstrate that SI prevails at the tetraploid level in the population used in this study. The autotetraploid parents of the population used in this study are different in their genetic background from the materials used by Fearon and Haywards (1984). Our study provides thus additional evidence that SI is consistent across different induced tetraploid populations.

The male gametic frequency correction made by weighting the expected frequencies by the pollen compatibility helped to determine the expected genotypic frequencies more accurately. Two main conclusions can be drawn from our study: (1) the female is not losing receptivity after encountering an incompatible pollen grain, and (2) even minute amounts of compatible pollen are sufficient to pollinate virtually all female gametes.

In this study, the number of expected haplotypes found was misleading in terms of model testing and an additional step was required to determine the best model to explain the observed segregation. For example, the presence of four genotypic classes would be an indication of the validity of Model I, because under Model II only three classes are possible. However, to further confirm this hypothesis, discrimination between genotypic classes was needed. In autotetraploids the assignment of observed genotypes to expected genotypic classes is not as simple as in diploids. At this ploidy level, the presence of a single allele in more than one copy increases the uncertainty of assigning the correct genotypic classes. This problem is worse in case of distorted segregation, such as for markers linked to SI. The addition of an equal mixture of diploid parents DNA as well as a Z-13 genotype and all possible combinations between the three helped us to assign observed genotypes to the corresponding genotypic classes. These DNA mixture

previously used in tetraploid potato to replicate five different biallelic combinations (De Koeyer, 2010), however, there are no reports of a successful implementation to distinguish genotypic classes with different combinations of more than two alleles. The present study provides evidence of the possibility of discriminating between genotypic classes and suggests that HRM can be used for genotyping polyploids efficiently. We were not able to discriminate genotypes 1222 and 1122, because we did not have respective diploid DNA to reconstruct genotype 1222. However, we were able to discriminate other isoallelic genotypes, such as 1223 and 1233. Even though both genotypes can form the same type of duplexes after re-annealing, the frequency of those duplexes is different between 1223 and 1233. Therefore, they are expected to show different melting curves. DNA mixtures for genotypic classes 1223 and 1233 were clearly discriminated by one of the markers (Figure 3), where individual 1223 is represented by a blue line whereas the 1233 DNA control falls into the grey category. Therefore, we are confident that HRM would also have discriminated the 1122 and 1222 genotype classes. Absence of a 6th distinct genotype from the five genotypes that we were able to reconstruct within the segregating autotetraploid population was interpreted as evidence for absence of a 1222 genotype.

Our data support Model II in which a combination of at least a single allele at S and Z in the pollen grain that matches the pistil can make pollen incompatible. Moreover, we provided direct evidence that the number of alleles in pollen does not affect the efficiency of the self-incompatibility system. The one deviating genotype in this population (1223) might have occurred by pollen contamination. Alternatively, it may be due to a leaky SI, which allows germination of incompatible pollen and further fertilization at low frequency. In tetraploids, chromatid segregation might be another possibility for occurrence of unexpected marker genotypes. However, in the case of this specific population chromatid segregation would not

result in any different pollen haplotype since all possible pollen genotypes haplotypes produced by chromatid segregation are already present under normal chromosome segregation (Z-11, and Z-22). Therefore, the only difference would have been in the frequency and not the genotypes. Another explanation may be aneuploidy in our population. However, we discard this possibility due to screening of those 43 genotypes using flow cytometry. With all this information, the most possible explanation is that an event occur between the marker and the Z allele which caused the appearance of the unexpected genotype in the progeny.

Model I is more restrictive than Model II, from an evolutionary perspective, Model II in which a single allele differing at either S or Z cause pollen compatibility would mean more pollen compatibility in the longer run. Therefore more inbreeding would be possible. Out of the four alleles in the pollen (two at S and two at Z) only one needs to be different from the female to be make pollen compatible under Model II. Instead, under Model I pollen grains need to have one different allele at both loci in order to be compatible. Therefore, Model I ensures outcrossing and is more successful at minimizing inbreeding depression(Arias Aguirre et al., 2011).

In the series of papers published in the 1980ies (Fearon et al., 1984a; Fearon et al., 1984b; Fearon et al., 1984c), the authors demonstrated the validity of the S-Z combination model using pollination tests (which we call Model I). In the first manuscript of this series, the authors used pollen from a diploid plant to pollinate a related autotetraploid. In a following manuscript they used pollen from a tetraploid plant to pollinate a related diploid. In both manuscripts they used in vitro pollination tests to make inferences about parental genotypes and pollen compatibility. There are several biases introduced during in vitro pollination tests, one is the mixture of living and dead pollen, another is the time of pollen collection and finally the possibility of an unknown lethality rate due to the staining process. In addition, there is the difficulty of counting successful

pollinations in pollen grains that may be in clusters. In our research we used progeny to retrospectively make inferences about the parents by assessing the inheritance of polymorphic markers linked to the Z region. Another difference of the present study is that we used gametes from tetraploid plants to assess compatibility and we were, therefore, able to rule out dosage effects and dominance of the alleles present in the population. Although we confirm a similar model as the one described by Fearon (1984), we avoided the confounding effects of fertilizing gametes that come from plants with different ploidy levels. We were thus able to show that no dominance effects occur in male or female gametes.

Tetraploid ryegrass cultivars are part of the current germplasm pool and the understanding offered by studies in the 1980s was of great importance for cultivar development at this ploidy level. In this study, we were able to provide further supporting evidence of the S-Z model for self-incompatibility in autotetraploid ryegrass by use of linked markers. Genotyping of both SI loci is of great of interest for grass breeding programs.

In order to use SI for hybrid seed production in tetraploid ryegrass, it is important to maintain sufficient diversity at S and Z to maximize seed production in a cross of two genotypes (Arias Aguirre et al., 2012). Alleles segregating for S and Z need to be as different as possible among heterotic groups to maximize seed set. HRM-based genotyping combined with the bioinformatic tools such as PollenCALC can be used for parent selection and to create or improve existing heterotic pools based on S-Z allele differences to maximize seed set in hybrid seed production programs

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