PATTERN OF INHERITANCE OF A SELF-FERTILITY GENE IN AN AUTOTETRAPLOID PERENNIAL RYEGRASS (*Lolium perenne* L.) POPULATION

Javier Do Canto[a,1,*], Bruno Studer[b], Ursula Frei[a] and Thomas Lübberstedt[a]

[a] Department of Agronomy, Iowa State University, 2104 Agronomy Hall, Ames, IA 50011-1010, USA. [b] Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, 8092, Zurich, Switzerland.

[1] Present address: Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA Tacuarembó, Ruta 5 km 386, Tacuarembó, Uruguay.

[*]Corresponding author, E-mail: jdocanto@inia.org.uy

Running title: Self-fertility in tetraploid ryegrass

Abstract

A mutation causing self-fertility (SF) in perennial ryegrass (*Lolium perenne* L.) was studied at the tetraploid level. The aim of this work was to determine a) whether SF remains functional in a tetraploid population; and b) if the SF mutation expresses dominance in heterozygous pollen grains. A tetraploidized plant carrying SF alleles was self-pollinated to create a segregating F_2 population. In the F_2 individuals, pollen compatibility ranged between 38% and 84% showing that SF remained functional. The *SF* locus genotype was the main determinant of pollen compatibility explaining 78% of the variation. The observed segregation was significantly different from the expected under both SF being dominant or recessive models ($P_{(\chi 2)} \leq 0.001$), and tended to be intermediate between them, indicating partial

dominance or additive gene action. The frequency of the different genotypes suggested that pollen grains homozygous for the mutation have a competitive advantage over heterozygous pollen and that pollen compatibility is affected by the interaction with additional loci. The implications of our results for breeding polyploid grasses are discussed.

Key words: perennial ryegrass – tetraploid - self-fertility - segregation

Introduction

Self-incompatibility (SI) in grasses is controlled gametophytically by at least two multiallelic and independent loci, *S* and *Z*. The incompatibility phenotype of the pollen grain is determined by its haploid genome and depends upon the combination of *S* and *Z* alleles in the pollen grain. A pollen grain is incompatible when the same *S* and *Z* alleles carried by pollen are present in the pistil (Lundqvist 1954; Yang et al. 2008). In some gametophytic SI systems, the increase in ploidy level has been associated with the breakdown of SI. This is the case of the S-RNase mechanism present in the Solanaceae, Rosaceae, and Fabaceae families among others, where the competition among alleles in the diploid pollen has been reported as the cause of the SI breakdown (Lewis and Modlibowska 1942; Brewbacker 1954; de Netancourt 2001; Stone 2002; Robertson et al. 2011). In contrast, SI in grasses keeps its functionality at higher ploidy levels (Lundqvist, 1957, 1962, 1968; Fearon et al. 1984) and such allele competition has not been reported. The *S* and *Z* locus mediated SI mechanism remains functional with no allele dosage effects, where the pollen tube growth is inhibited, if just a single allele at both the *S* and *Z* locus in diploid pollen have a counterpart in the female genotype (Lundqvist 1957; Fearon et al. 1984; Arias-Aguirre et al. 2014).

At least one additional locus involved in SI, independent of *S* and *Z*, has been identified in perennial ryegrass (*Lolium perenne* L.) (Thorogood et al. 2005), *Phalaris coerulescens* Desf. (Hayman and Richter 1992) and in rye (*Secale cereale* L.) (Voylokov et al. 1998). Mutations at this locus can mask the SI mechanism and pollen grains carrying the mutation are able to grow in the stigma in self-pollinations independently of the *S* and *Z* alleles present. In perennial ryegrass, this self-fertility (*SF*) locus is located in linkage group 5 (LG5) (Thorogood et al. 2005;

Arias-Aguirre et al. 2013).The inheritance and mode of action of the *SF* locus is known at the diploid level but no attempts have been made to study the gene at higher ploidy levels. Thus, it is not known how it segregates, or whether plants carrying the mutation would remain self-fertile. For instance, the effect of the mutation could be suppressed or compensated by a larger gene copy of incompatibility alleles restoring SI. Also, in a heterozygous pollen grain it is unknown whether self-compatibility or SI prevails, which affects the expected genotypic frequencies in the offspring.

In autotetraploids, genotypic segregation at a particular locus depends on the gamete frequencies resulting from the prevailing type of inheritance. In strict autotetraploids quadrivalents are formed (multisomic inheritance) and chromosomes segregate randomly (Muller 1914). Crossing-over can occur between chromatids of any pair of chromosomes making a total of 28 possible gamete combinations for a given locus (Haldane 1930). The frequency of these gametes are affected by the frequency of crossing-over, which in turn is dependent on the distance of the locus to the centromere. In addition, sister chromatids may end up in the same gamete in a process known as double-reduction, increasing the expected fraction of homozygous gametes (Haldane 1930; Mather 1935, 1936). However, meiotic configuration varies even within the same species (Ahloowalia 1967), and while some chromosomes join in quadrivalents, others will pair forming bivalents. This condition reduces the number of chiasmata compared to quadrivalents and no double reduction occurs. As a consequence, gamete frequencies in autopolyploids will vary depending not only on the distance of the locus to the centromere, but also in the type of configuration the chromosomes form in meiosis I (Ahloowalia 1967; Macefield and Evans 1976).

The relevance of studying this gene at higher ploidy levels relies in the fact that many of the agronomically important perennial grass species are polyploid and self-incompatible (Vogel and Pedersen 1993; Posselt 2010). In addition, genome doubling by artificial means is one of the tools employed by breeders to create improved varieties, and cultivars of artificially produced tetraploids are commercialized (Posselt 2010; Humphreys et al. 2010). In such species, SF would enable inbred line development, purging deleterious alleles or increasing the frequency of favorable ones by self-pollination.

The overall objective of this study was to improve the understanding of SF in perennial ryegrass at the tetraploid level. Segregation of the genotypes at the *SF* locus in a tetraploid F_2 population was used to infer dominance in diploid pollen as well as gamete frequencies. Specific objectives were to determine a) whether SF remains functional in a tetraploid perennial ryegrass population; and b) if the SF mutation expresses dominance in heterozygous pollen grains.

Materials and methods

Plant materials and genome doubling

The F_1 plant that produced the *SF* mapping population of Arias-Aguirre et al. (2013) and Do Canto et al. (2018) was selected for the experiment. This is a diploid self-fertile plant heterozygous for the *SF* locus on LG5 and will be referred from now on as foundational diploid F_1 plant, FDF_1. The genotype of FDF_1 for the *SF* locus is SF_{IF}, (SF_I is the allele causing SI and SF_F is the self-fertile allele), and is also heterozygous for *S* and *Z*.

A total of ten individual young vegetative tillers with their roots were excised from FDF_1 and placed in water overnight. Tillers were treated by immersing roots and meristem in a 0.4 % aqueous colchicine solution for 72 hours at 25° C. Tillers were then washed with distilled water, transplanted to pots and grown under greenhouse conditions. After 10 weeks, 5 new shoots from each of the treated tillers were transplanted to individual pots totaling 50 individuals with potentially doubled chromosome number.

The ploidy level of the new shoots from the treated tillers was determined by flow cytometry analysis following the method described by Dolezel et al. (1989). Mechanical isolation of cell nuclei was performed by chopping a 5 cm fresh leaf sample with a razor blade in 1.95 ml of LB01 lysis buffer (15 mM Tris (hydroxymethyl) aminomethane, 2 mM Na_2EDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 15 mM mercaptoethanol and 0.1% (v/v) Triton X-100). The suspension was filtered using a 50 µm nylon mesh and centrifuged at 3000 rpm for 3 minutes. The supernatant was discarded and the pellet re-suspended in 225 µl of LB01 buffer. The solution was transferred to a flow cytometry tube with 25 µl of propidium iodide fluorescent dye (1mg/mL in LB01 buffer). DNA content of the isolated nuclei was measured by flow cytometry at the Flow Cytometry Facility at Iowa State University using a BD FACSAria™ III instrument (BD Biosciences, San Jose, CA, USA). A diploid and a tetraploid control were used and the relative ploidy was determined using the ratio of nuclei with different DNA content level.

A resulting tetraploid shoot (tetraploid F_1 plant: FTF_1) (genotype SF_{IIFF}) was allowed to grow and then vernalized in a growth chamber for six weeks at 5°C under short-day photoperiod conditions (8 hours light/16 hours dark) to induce flowering. After vernalization

this plant was placed back in the greenhouse under long-day photoperiod conditions and 23°C to induce flowering. Before anthesis, spikes were covered with paper bags to produce self-pollinated seed. The F_2 seed (tetraploid F_2) was planted in trays and seedlings transplanted to individual pots. The tetraploid F_2 population was composed of 77 plants.

DNA extraction and genotyping

A CTAB (cetyl trimethylammonium bromide) based DNA extraction protocol was followed to obtain DNA from the tetraploid F_2 population, the FTF_1 plant and from diploid F_2 plants of known genotypes used as controls. Ground leaf samples were suspended in CTAB buffer and incubated for 1 hour at 65°C. A chloroform washing step and RNase treatment were used for purification. Isopropanol was used for nucleic acid precipitation and pelleted by centrifugation followed by two washing steps with 75% ethanol. The DNA pellet was then re-suspended in sterile distilled water.

The tetraploid F_2 population was genotyped with a marker linked to the *SF* locus (Table 1) using High Resolution Melting analysis (HRM) (Studer et al. 2009). PCR reaction mix containing 0.2 mM of dNTPs, 25 mM of magnesium chloride, 20 µM of each forward and reverse primers, 1x LCGreen dye (BioFire Diagnostics, Inc., Salt Lake City, UT, USA), and 20 ng of DNA were employed, plus 20 µl of mineral oil to prevent evaporation during light scanning exposure. PCR amplification was conducted using a BIO RAD T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) based on the protocol of Studer et al. (2009) but the annealing time was increased to 1 min and the number of cycles increased to 44 to improve specificity. HRM analysis was done in a LightScanner™ Instrument and LightScanner® Analysis

Software with Call-IT® 2.0 software modules (Idaho Technology Inc., Salt Lake City, UT, USA), with melting temperature range from 60°C to 98°C.

To assign different HRM melting curves to specific genotypic classes, controls with known marker genotypes were used. A diploid plant homozygous at the *SF* locus (genotype SF_{FF}) was used as control for the quadruplex genotype (SF_{FFFF}), and a heterozygous diploid genotype (SF_{IF}) was used as control for the duplex (SF_{IIFF}) since they produce similar curves in HRM. For the triplex, a control was created by mixing DNA of the diploid genotypes SF_{FF} and SF_{IF} in a 1:1 ratio. However, no homozygous SF_{II} plant was available, thus, there was no control for the SF_{IIII} genotype, and a control for the SF_{IIIF} genotype could not be created.

Phenotyping

The tetraploid F_2 population was vernalized in a growth chamber and then allowed to flower in a greenhouse under similar conditions described previously for the FTF_1 plant. Plants that flowered had their spikes covered with paper bags to avoid pollen contamination and produce self-pollinated seeds. The level of pollen compatibility upon self-pollination was determined using an *in vitro* pollination test (Lundqvist 1961) and fluorescence microscopy (Kho and Baer 1968) as described in Do Canto et al. (2018). Pollinated stigmas were analyzed using a Zeiss Axioplan II UV light microscope (Carl Zeiss, Göttingen, Germany) at the Microscopy and NanoImaging Facility of Iowa State University. Incompatible pollen grains exhibit inhibited growth, while in compatible pollen grains, pollen tubes could be observed growing towards the style. Pollen grains were counted and the percentage of compatible and incompatible pollen grains was determined.

Hypothesis testing

Based on possible dominance relationships between alleles in diploid pollen at the *SF* locus, different models for allele relationship were proposed (Table 2): a) SF_F recessive: the SF_I allele is dominant over the SF_F allele, thus a heterozygous pollen grain (SF_{IF}) is self-incompatible; b) SF_F dominant: the SF_F allele is dominant over the SF_I allele, thus a heterozygous pollen grain (SF_{IF}) is self-compatible; and c) incomplete dominance: partial dominance results in either delayed or partial inhibition of pollen tubes. Such a pollen grain may be able to reach the ovary depending on its distance to the ovary as well as how much competition with other pollen grains it has.

Gamete frequencies under tetrasomic inheritance were estimated based on maximum equational segregation (ME) (Mather 1936), which takes double-reduction into account. It assumes that quadrivalents are formed and crossing over occurs, but all sister chromatids separate equationally at meiosis I. Thus sister chromatids will appear in the same interphase nucleus 1/3 of the times and in the same gametes in 1/6 of the tetrads. Double-reduction is symbolized by α, and when maximum equational segregation occurs α = 1/6. Thus, the expected gamete frequencies are calculated as follows:

$$AA = (1 + 2\alpha)/6$$

$$Aa = (2 - 2\alpha)/3$$

$$aa = (1 + 2\alpha)/6$$

Based on the gamete frequencies expected under ME segregation (Table 3) and pollen compatibility under the different dominance models, the expected genotype frequencies in the resulting tetraploid F_2 population were derived (Table 4).

Statistical analysis

Observed and expected segregation ratios were compared under the different models and tested using chi-square tests of goodness of fit at a 95% probability level to determine whether the data fit any of the models.

To analyze the effect of the genotype at the *SF* locus on the level of pollen compatibility, the MIXED procedure in SAS® software version 9.4 (SAS Institute Inc., Cary, NC.) was used to handle unequal repetitions (individual plants with the same *SF* locus genotype). The *repeated/group* option was used to account for unequal variances between the different genotypes and to generate heterogeneous errors. The effective degrees of freedom was calculated based on Satterthwaite's approximation and used for the T-test between all pairwise comparisons. Sum of squares were obtained by calling the *method=Type 3* option for both the full and the reduced models. The residuals sum of squares from both models were used to estimate the percent reduction in variability due to the model.

Results

From the 50 clones analyzed by flow cytometry, three samples showed a peak in number of cells with a relative propidium iodide fluorescence indicative of tetraploidy (Supplemental Figure S1). Individual tillers of those plants were labeled and reanalyzed to

prevent having mixoploids, which is a common phenomenon in colchicine-treated individuals. A single tetraploid tiller, resulting into the F_1 tetraploid plant (FTF_1), was selected for producing an F_2 population.

Selfing of the tetraploid F_1 plant FTF_1 yielded 92 F_2 seeds. From those, 80 germinated (87 % germination), and 3 seedlings died resulting in a population of 77 plants that were used for this experiment.

The F_2 population was succesfully genotyped with the marker linked to the *SF* locus (Supplemental Table S2) and four genotype classes appeared (Figure 1). Three of them, the quadruplex, triplex and duplex, could be identified according to the available controls. Since the SF_{IIII} genotype was not expected based on the diploid behavior, it was assumed that the fourth genotype corresponded to the simplex. Aditionally, the double-peak shape of the fluorescence (not shown) corresponded better to a heterozygous than a homozygous type and was different from the triplex and duplex. Consequently, 18 plants were identified as SF_{FFFF}, 25 as SF_{IFFF}, 31 as SF_{IIFF}, and 3 as SF_{IIIF}.

A comparison between the observed *SF* genotype segregation and the expected distribution under the different models is shown in Figure 2. In the SF_F dominant model the most frequent genotype is SF_{IIFF}. In the recessive model the most abundant genotype is SF_{IFFF} while the SF_{IIIF} genotype is not expected. The observed number of SF_{FFFF} is similar to the expectation for a SF_F recessive model. In contrast, the most abundant observed genotype was SF_{IIFF} and the percentage of SF_{IFFF} plants was closer to the SF_F dominant model, although the number of SF_{IIIF} genotypes was lower than expected for SF_F dominance.

The Chi-square test of goodness of fit for different allele relationship models showed that, with a 95% probability, the observed segregation was significantly different from both SF_F dominant and recessive models (Table 5).

From the 77 plants only 32 were phenotyped since the rest did not flower. Pollen compatibility in these plants ranged from 18 % to 96 % (Supplemental Table S2). The least squared means for compatibility per genotype and 95% confidence interval are given in Table 6. The effect of the genotype at the *SF* locus was highly significant (Pr > F <0.0001). Least square mean estimates were 69.9, 38.6, 45.7 and 84.3% for genotypes SF_{IIIF}, SF_{IIFF}, SF_{IFFF} and SF_{FFFF} respectively. In all pairwise comparisons, the difference between the genotypes least square means were significant (Pr > |t| = 0.01), that is, the level of pollen compatibility produced by a particular *SF* locus genotype was significantly different from the rest. The genotype at the *SF* locus explained 78% of the variation.

Discussion

Our results show that SF stays functional in autotetraploid perennial ryegrass. It was possible not only to obtain selfed progeny but also the SF_F allele was transmitted to the offspring, which in turn showed variable levels of pollen compatibility.

Hayman and Richter (1992), speculated that in *Phalaris coerulescens* Desf. a diploid pollen grain heterozygous for a *SF* mutation would be self-incompatible. In our work, the presence of SF_{IIIF} individuals favors the SF_F dominant model, since such a genotype can only be generated from a SF_{II} ovule fertilized by a SF_{IF} pollen grain. The frequency of the SF_{IFFF} genotype is very close to the expected in the dominant model, and the SF_{IIFF} genotype is the most

abundant, also favoring a dominant inheritance pattern. However, the observed number of SF_{FFFF} genotypes would only be possible, if SF_{IF} pollen grains were incompatible. In fact, the segregation of the SF_F allele did not fit any of the proposed models but tended to be intermediate between the hypothesis of SF_F being dominant and recessive, indicating incomplete dominance or additive inheritance.

The presence of 3 SF_{IIIF} genotypes and the excess of SF_{IIFF} compared to the expected number under the recessive model indicates that SF_{IF} pollen grains were not fully inhibited and were able to reach the ovary under competition with SF_{FF} pollen grains. However, the frequency of these two genotypes is lower than the expected under the dominance model suggesting that SF_{FF} pollen grains have a competitive advantage over the heterozygous and are more likely to fertilize the egg. Such competitive advantage was previously noticed in autotetraploid *O. organensis*, where compatible crosses with pollen heteroallelic for the *S* locus produced less progeny than crosses with homoallelic pollen (Lewis 1947). The incomplete inhibition of heterozygous pollen grains in our experiment resembles the delayed incompatibility response observed in the grass *Alopecurus pratensis* L. (2n = 4x = 28), which exhibited a weak SI response. Pollen tubes grow a considerable distance before being arrested, while some of them are able to reach the ovary (Heslop-Harrison 1979).

The specific type of tetrasomic inheritance affecting this particular locus may have a slight effect on our results, although a very large population size would be required for testing segregation types (Bailey, 1961). A more descriptive statistic in autopolyploids is the index α that characterizes to which extent double reduction is affecting the data (Mather 1936). Unfortunately, the absence of the SF_{IIII} in our population complicates the estimation of double

reduction. The α index would be easier to estimate in a cross between SF_{FFFF} as female and SF_{IIIF} as the pollen donor where only the SF_{IF} male gamete is compatible and only the SF_{IFFF} genotype is expected in the progeny. The presence of SF_{FFFF} individuals would be an indication of double reduction and its extent could be estimated by dividing its frequency by 4 (Fisher and Mather 1943; Doyle 1973). However, the competitive advantage of SF_{FF} over SF_{IF} pollen grains would inflate the frequency of the SF_{FFFF} genotype, confounding both effects. The reciprocal cross eliminates the competition effect and assuming the rate of double reduction is the same in both gametophytes, this would enable to calculate the true double reduction value. The difference between the indexes of both crosses could be attributed to the competitive advantage of the SF_{FF} pollen grain.

Another explanation for the observed genotypic distribution is that a proportion of the pollen grains that would be compatible based on the SF_F dominant model, are rendered incompatible as a result of complex interactions between the *SF* locus and the genetic background. This is supported by the observed phenotypes of the different *SF* genotypes: the most surprising observation is that the SF_{FFFF} genotype is not 100% compatible as was expected and there is no longer a discrete distribution of phenotypic classes as observed in diploids. While in the diploids the *SF* locus has a clear qualitative mode of action, in the tetraploids it explained 78% of the observed variability. In sour cherry (*Prunus cerasus* L.), a tetraploid species, progeny with similar S-haplotypes differed in compatibility phenotypes suggesting that background genes were modulating the interaction between pollen and stigma S-genes (Hauck et al. 2002). Similarly, allele dosage at other loci of quantitative effect including the *S* and *Z* loci, are probably interacting to modify the level of pollen compatibility in our population.

Our results have implications for breeding polyploid grasses. As in diploids, selfing combined with targeted selection could play a role in eliminating deleterious alleles or increase the dosage of the favorable ones. Simulation studies showed that the mutation load can be higher in tetraploids, but decreases with increasing selfing rates, demonstrating that purging also occurs in tetraploids (Ronfort 1999). In contrast, increase in inbreeding depression with selfing generation found by Ozimec and Husband (2011) supports delayed purging in tetraploids. Whether purging efficiency is higher or lower than in diploids, the frequency of deleterious mutations always decreases with increasing selfing rate. Understanding the mode of gene action of *SF* mutants is essential for introgressing this trait in breeding populations for such purposes. Additionally, in allopolyploid grasses, inbred line development can lead to hybrid breeding. The *SF* locus could be introgressed from perennial ryegrass to other related polyploid grasses within the *Lolium-Festuca* complex by interspecific hybridization (Thomas and Humphreys 1991; Zwierzykowski 1996; Thomas et al. 2003; Humphreys et al. 2003), either by direct crossing or using third species as a bridge (Thomas et al. 1999).

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Declarations

Contribution of authors: JD contributed to the experimental design, conducted the phenotypic assays, genotyped the population, analyzed the data, and was the primary writer of the manuscript. BS contributed with molecular markers essential for this experiment and critically reviewed the manuscript. UF significantly contributed with materials necessary for the experiment and assisted with laboratory related activities. TL conceived the experiment, contributed with the experimental design, assisted with the interpretation of results and critically reviewed the manuscript.

Conflict of interest: The authors declare no conflicts of interest.

Data availability: Genotypic and phenotypic data are provided as supplemental material

Supplemental information is available online

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Supporting Information

Supplemental Figure S1. Flow cytometry histograms.

Supplemental Table S2. Genotypic and phenotypic data.

Table 1. Molecular marker employed in the experiment.

Primer	Distance to *SF* locus	Forward sequence	Reverse sequence	Reference
12_23502	0.8 cM	AACCAGGAATCTGCTCATCC	GGAAACGGGTCACCAAGAA	Manzanares 2013; Do Canto et al. 2018

Table 2. Pollen from the FTF_1 plant (SF_{IIFF}). Expected compatibility for the different pollen genotypes.

	SF_{FF}	SF_{IF}	SF_{II}
SF_F recessive	compatible	incompatible	incompatible
SF_F dominant	compatible	compatible	incompatible
Incomplete dominance	compatible	partially compatible	incompatible

Table 3. Expected gametic frequencies for a given locus according to maximum equational segregation (ME) (Mather 1936).

AA	*Aa*	*aa*
0.222	0.556	0.222

Table 4. Expected *SF* locus genotypic proportions in the F_2 generation according to different models.

Dominance model	SF_{FFFF}	SF_{IFFF}	SF_{IIFF}	SF_{IIIF}
SF_F recessive	22.2	55.6	22.2	0
SF_F dominant	6.3	31.7	46.0	15.9
Incomplete dominance	> 6.3; < 22.2	> 31.7; < 55.6	> 22.2; < 46.0	< 15.9

Table 5. Chi-square test of goodness of fit for different allele relationship models at the *SF* locus.

SF_F allele	χ^2	Degrees of Freedom	P-value
recessive	19.4	2	0.00006
dominant	42.7	3	< 0.00001

Table 6. Pollen compatibility least square means and 95% confidence interval limits for the different *SF* genotypes. The number of plants of each genotype is given in brackets.

	SF_{FFFF}	SF_{IFFF}	SF_{IIFF}	SF_{IIIF}
% compatibility	84.3 ±4.6 (5)	45.7 ±3.2 (12)	38.6 ±2.7 (13)	69.9 ±7.5 (2)

Titles and legends to figures

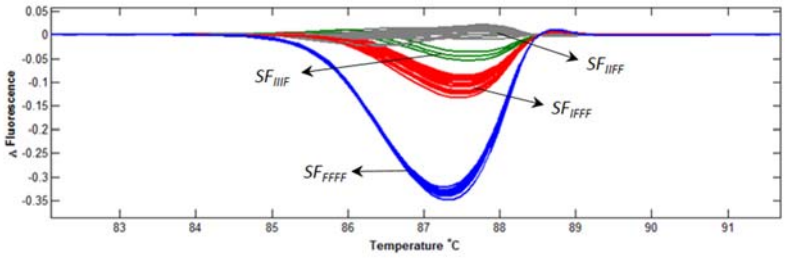

Figure 1 Difference fluorescence curves for *SF* locus marker. Four different curves were obtained, each one corresponds to a different genotype class. The arrows indicate the genotype of each curve.

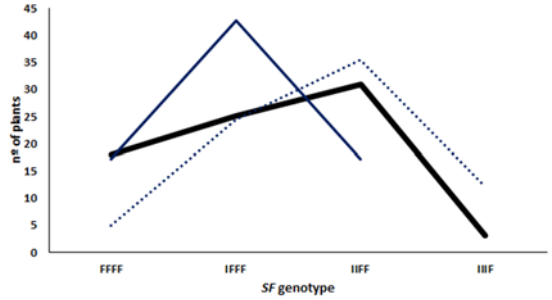

Figure 2 Number of observed and expected *SF* genotypes under different models. Thick solid line: observed; thinner solid line: SF_F allele recessive; doted line: SF_F allele dominant.