Genetic studies on self-fertility in perennial ryegrass (*Lolium perenne* L.) with implications for hybrid breeding in allogamous grasses

by

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ABSTRACT

Perennial grasses have diverse uses and are relevant from the agronomic and economic point of view, with main uses as forage, turf and bioenergy. In the grass family polyploidy is prevalent and both autopolyploids and allopolyploids are present. Also, within grasses there are a range of breeding systems, but hermaphrodite flower is the most frequent floral condition. Cross-pollination in species with hermaphrodite flowers is imposed by a gametophytic genetic self-incompatibility (SI). SI is controlled by two multiallelic and independent loci, *S* and *Z*. The incompatibility phenotype of the pollen grain is determined by its haploid genome. A pollen grain is incompatible when the same *S* and *Z* alleles carried by pollen are present in the pistil. This SI mechanism keeps its functionality at higher ploidy levels. Understanding the mechanisms involved in the breakdown of SI are crucial for implementing novel breeding practices. The aim of this work is to increase the knowledge of SF in outcrossing grasses for the purpose of inbred line development and making hybrid breeding possible. Mutations at S, Z and at a third locus are known to cause self-fertility (SF). In perennial ryegrass (*Lolium perenne*), a locus conferring SF is located in linkage group 5. Using segregation and linkage analysis, the *SF* locus region was reduced to 1.6 cM. This locus explained 94% of the observed variability. By aligning the flanking marker sequences to the *Brachypodium distachium* reference genome, it was found that it corresponds to an 807 Kbp region in *B. distachium*. This locus was studied at the tetraploid level and it was found that *SF* remained functional, the *SF* locus genotype was the main determinant of pollen compatibility explaining 54% of the variation, and there is incomplete

dominance between alleles at this locus in the diploid pollen grain. The prospects of migrating the *SF* locus from perennial ryegrass to other related self-incompatible were discussed. Based on the available information on hybridization between *Lolium* and *Festuca* species, different types of crosses were propose according to the particular species involved. The results and observations presented here contribute to a better understanding of the trait at both diploid and tetraploid levels and are promising as SF may readily be incorporated into breeding programs.

CHAPTER 1

GENERAL INTRODUCTION

The grass family

Perennial grasses have diverse uses and are relevant from the agronomic and economic point of view. As forage crops represent an important component of animal production systems where the success relies greatly on the efficient use of the low cost grazed pastures to provide a major proportion of the feed required (Parsons et al., 2011; Smith et al., 2014). One major use of perennial grasses is for turf and lawns. An estimation of lawn area in the USA indicated an annual increase of 155,000 hectares (Robbins and Birkenholtz, 2003) denoting the importance of turf-grass seed market. Another relatively new niche for perennial grasses is the bioenergy market. Their perennial nature is attractive especially for marginal soils because farming practices such as tillage are not required as often as for annual crops. Moreover, comparable low water and nutrient uptake, high rate of carbon fixation and the chemical composition make them effective and convenient for biomass production and conversion (Lewandowski et al., 2003; Youngs and Semerville, 2012).

The Gramineae family is composed by nearly 700 genera and 10,000 species distributed in a wide range of habitats all over the world. Polyploidy in the grass family is prevalent and both autopolyploids and allopolyploids are present. Variation in ploidy level often occurs at both the intra-genera and intra-species levels and often gene flow occurs between ploidy levels. In many cases, ploidy levels are not recognizable as distinguishable

separate taxa, and different ploidy forms are often classified as subspecies (i.e.: *Dactylis glomerata* ssp.), or morphotypes (i.e.: lowland and upland switchgrass) (Keeler, 1998). Many agronomically important grasses are polyploids and close related species are present at different ploidy levels. As a result both ploidy level and interspecific variation are often exploited in breeding programs.

Grass species have adopted a range of breeding systems, some promoting asexual propagation, some self-pollination, and some cross-pollination. Strictly, dioecious genera, having male and female flowers in different plants, are relatively few. Monoecism, the condition of having male and female flowers in the same plant, like maize, is more common. However, hermaphrodite flowers is the most frequent floral condition, followed by andromonoecism, where hermaphrodite and male flowers are present in the same plant (Connor, 1979). Hermaphrodite flowers can be either self- or cross-pollinated. Self-pollination is widespread among grasses, and most of the cereals are predominantly autogamous species. Cross-pollination in species with hermaphrodite flowers is imposed by a gametophytic genetic self-incompatibility (SI).

Self-incompatibility in grasses

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). The number of species where the

system has been found to be operative is relatively small. However, they are spread across different tribes (Poaceae, Aveneae, and Triticeae) and they occur simultaneously with self-compatible species within the same genera. Consequently, the two locus SI system is assumed to be conserved in grass species.

In contrast with other SI systems, SI in grasses keeps its functionality at higher ploidy levels. 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).

SI promotes cross-pollination and maintains high levels of heterozygosity. In these out-crossing species cultivars are developed as improved populations or synthetic varieties, where heterosis is exploited only partially, limiting genetic gain. In this sense, hybrid varieties have the potential to outperform populations and synthetic cultivars (Arias-Aguirre et al., 2011).

At least one additional locus, independent of *S* and *Z*, conferring self-fertility (SF) was identified in 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). A proper understanding of the segregation and mode of action as well as the isolation and cloning of this gene will enable the efficient production of inbred lines in species that are normally self-incompatible.

Perennial ryegrass as a model grass

Perennial ryegrass (*Lolium perenne* L.) is a temperate (cool-season) perennial grass. It is sown as a perennial forage and turf grass noted for fast establishment, exceptional quality, and high cool-season productivity. Adapted to mild and wet climates, it is native to Europe, temperate Asia, and North Africa and it has been introduced to other temperate regions including North and South America, Europe, New Zealand, and Australia. Perennial ryegrass is bred as both diploid and tetraploid (Hannaway et al., 1997).

The molecular genetic characterization of the two locus gametophytic SI system is more advanced in perennial ryegrass than in other species, while molecular tools are also becoming increasingly available for this crop (Klass et al., 2011; Pfeiffer et al., 2013; Byrne et al., 2015). Perennial ryegrass is naturally a diploid (2n = 2x = 14) with seven linkage groups (Studer et al., 2010). The *S* and *Z* loci have been mapped to linkage groups 1 and 2, respectively (Thorogood et al., 2002; Shinozuka et al., 2010). Recently, a *domain of unknown function 247 (DUF247)* gene was found to be involved in the determination of the male component of the *S* locus (Manzanares et al., 2015). Also, an *SF* gene has been mapped to linkage group 5, while some distorted segregation related to SF was found on linkage group 3 (Thorogood et al., 2005; Arias-Aguirre et al., 2013; Manzanares, 2013). In addition, a large number of expressed sequence tags (EST)-derived simple sequence repeat (SSR) markers and single nucleotide polymorphism (SNP) markers are available and detailed genetic maps have been developed (Studer et al., 2008; Shinozuka et al., 2010; Studer et al., 2012; Pfeiffer et al., 2013). Perennial ryegrass is, therefore, a good grass model species to study such important biological mechanisms to control pollination in hybrid breeding

schemes (Arias-Aguirre et al., 2011). It has the additional advantage of having several related species of agronomic importance, and any advances can be readily transfered or adapted to other grasses.

Objectives

The aim of this work is to contribute and to increase the knowledge of SF in outcrossing grasses for the purpose of inbred line development and making hybrid breeding possible as a way to improve genetic gains. Specific objectives are: 1) review the current knowledge on SF in allogamous grasses, 2) fine map a *SF* gene in a perennial ryegrass population, 3) understand the mode of action of a *SF* gene in autotetraploids of perennial ryegrass, and 4) analyze the prospects for transferring *SF* to related species.

Organization of the thesis

The thesis contains one published review article (Chapter 2), two manuscripts in preparation for submission for publication (Chapters 3 and 4), and a conceptual manuscript (Chapter 5). Chapter 6 contains the general conclusions and summarizes the main results of this thesis along with its implications for breeding allogamous grasses. The General Introduction Chapter presents the main uses of grasses together with a brief characterization of the family, the grasses SI system and the role of perennial ryegrass as a model grass. Literature for each individual chapter and procedures are introduced and discussed within the respective chapters.

Author contributions

Chapter 2

JD contributed with the outline, writing and editing of the manuscript.

BS conceived, revised and edited the manuscript.

TL conceived, revised and edited the manuscript.

Chapter 3

JD contributed to the experiment design, conducted the phenotypic assays, genotyped the population, conducted data analysis, 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 helped with laboratory related activities.

TL conceived the experiment, helped with the experimental design, assisted with the interpretation of results and critically reviewed the manuscript.

Chapter 4

JD contributed to the experiment design, conducted the phenotypic assays, genotyped the population, conducted data analysis, 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 helped with laboratory related activities.

TL contributed with the experimental design, assisted with the interpretation of results and critically reviewed the manuscript.

Chapter 5

JD contributed with the outline of the manuscript and was the primary writer.

TL conceived the manuscript, critically revised and edited the manuscript

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CHAPTER 2

OVERCOMING SELF-INCOMPATIBILITY IN GRASSES: A PATHWAY TO HYBRID BREEDING

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Abstract

Allogamous grasses exhibit an effective two-locus gametophytic self-incompatibility (SI) system, limiting the range of breeding techniques applicable for cultivar development. Current breeding methods based on populations are characterized by comparably low genetic gains for important traits such as biomass yield. In order to implement more efficient breeding schemes, the overall understanding of the SI system is crucial as are the mechanisms involved in the breakdown of SI. Self-fertile variants in outcrossing grasses have been studied and the current level of knowledge includes approximate gene locations, linked molecular markers and first hypotheses on their mode of action. Environmental conditions increasing seed set upon self-pollination have also been described. Even though some strategies were proposed to take advantage of self-fertility, there have so far not been changes in the methods applied in cultivar development for allogamous grasses. In this review, we describe the current knowledge about self-fertility in allogamous grasses and outline strategies to incorporate this trait for implementation in synthetic and hybrid breeding schemes.

Introduction

Perennial grasses are widely used as forage crops across the globe representing an important component of animal production systems. Moreover, as turf they are essential for sport fields and key components for landscape architecture and home yards. More recently, perennial grasses appeared as an attractive option in the bioenergy market due to their intrinsic properties for biofuels and their ecological advantages over annual crops. As perennials, each evaluation trial takes two or more years since persistence is usually a trait of interest and forage yield varies with the age of the pasture, leading to breeding cycles that easily exceed five years (Wilkins and Humphreys 2003). It can take as long as 10 to 15 years to develop and release a cultivar (Lee et al. 2012).

Among perennial grasses, genetic self-incompatibility (SI), which promotes cross-pollination and prevents self-pollination, is widespread. SI in grasses is controlled gametophytically by at least two multiallelic and independent loci, *S* and *Z*. This SI system is assumed to be conserved in grass species, including annual grasses like Italian ryegrass (*Lolium multiflorum* Lam.) and rye (*Secale cereale* L.) (reviewed by Baumann et al. 2000; Klaas et al. 2011; Yang et al. 2008). A general tendency in allogamous crops has been to move towards hybrid breeding, which offers opportunities for greater uniformity, higher selection intensities, absolute parental control and maximum exploitation of heterosis. However, cultivars from species exhibiting SI can only be developed as improved populations or synthetic varieties since inbred lines cannot be obtained by continued self-pollination. Breeding methods applied in grasses varies from restricted recurrent phenotypic selection to

half-sib selection and between-within family selection among others (Posselt 2010; Vogel and Pedersen 1993; Wilkins and Humphreys 2003). Reported genetic gains obtained in perennial grasses are low, e.g., 0.43% for dry matter yield per year in perennial ryegrass (*Lolium perenne* L.) in Ireland for the past 40 years (McDonagh et al. 2014), and between 0.25 to 1.18% in temperate forage grasses in New Zealand, though higher rates were reported for seasonal yield (Woodfield 1999). Similarly, genetic gains for annual dry matter yield in Germany were 0.45% for perennial ryegrass and 0.27% for Italian ryegrass for the last 30 years. Those values were the lowest among twelve different crops evaluated (Laidig et al. 2014). Slow progress in breeding perennial grasses is due to the difficulty of altering the harvest index, which had a great impact on the improvement of most grain crops, lower financial investment compared to cereal crops, among other reasons (Laidig et al. 2014; McDonagh et al. 2014; Wilkins and Humphreys 2003; Woodfield 1999).

The efficiency of perennial grass breeding can be improved by different means. One of them is manipulation of the reproductive system to overcome the SI barrier, which would allow efficient inbreeding as an important component of hybrid breeding schemes. This offers the advantages of uniformity and perpetuation of genotypes, diminution of the genetic load by removal of deleterious alleles in heterozygous breeding germplasm and finally, hybrid breeding. Even though SI of perennial grasses is very efficient, its breakdown has been reported. The objectives of this paper are (1) to review mechanisms in perennial grasses leading to temporary or permanent self-fertility (SF), and (2) discuss practical applications of available SF for breeding allogamous grasses.

Origin of self-fertility (SF)

Within the grass family (Poaceae), both autogamous and allogamous species are found, even within species of the same genus. SI is common and ensures outcrossing, but genetic SF and pseudocompatibility have been reported within SI grass species (Table 1).

Pseudocompatibility

SI of grass species is considered to be very effective. However, small amounts of selfed seed may be obtained for some individuals, especially when forced to self-pollinate, a feature that was reported as early as 1931 (Beddows 1931; Jenkin 1931). Lundqvist (1960), working with outbred populations of rye, found up to 5 % of selfed seed set in almost all plants investigated. Similarly, averages of 0.25 to 1.63 seeds per inflorescence were obtained in perennial ryegrass when forced to self-pollinate under different environmental conditions (Foster and Wright 1970). Such selfing rates in perennial ryegrass were also obtained by other authors (Beddows et al. 1962; Cornish et al. 1980; Fearon et al. 1983; Madsen et al. 1993). These low rates of selfed seed set were considered to be due to environmental effects and referred to as pseudo-self-compatibility (Cornish et al. 1980; Fearon et al. 1983). It was shown that non-genetic effects, such as temperature, humidity, different environments, and artificial pollination techniques, were responsible for a considerable proportion of the variation in selfed seed set (Foster and Wright 1970). Particularly temperature had a strong effect (Wilkins and Thorogood 1992). Plants from one particular population went from 2.3% seed set under unheated glasshouse conditions to 30.7% when exposed to 34º C at anthesis. The same trend was found by Jones and Jenabzadeh (1981) with a six-fold increase in selfed

seed set in a hot and dry environment. Whether high temperatures affect stigma receptivity or the ability of pollen to penetrate stigma is not clear. In perennial ryegrass, increase in SF occurred only when pollen grains were exposed to heat, while stigmas exposed to heat or pollination conditions under high temperatures only had a minor effect (Wilkins and Thorogood 1992). However, in a different experiment (Elgersma et al. 1989), female parents exposed before and during anthesis to higher temperatures increased pollen performance. Even though the optimal range of temperature varies between species or genotypes, in general, increasing the temperature increases success of self-pollination.

Significant differences among genotypes were reported for pseudocompatibility (Beddows et al. 1962; Elgersma et al. 1989; Foster and Wright 1970; Gertz and Wricke 1991; Wilkins and Thorogood 1992). In rye, a positive and significant correlation in the level of selfed seed set of the plants and their offspring was found (Lundqvist 1958, 1960). In perennial ryegrass, the capacity for SF was heritable and increased over inbred generations in selected families (Jones and Jenabzadeh 1981). Following selection for pseudocompatible seed set in opposite directions, seed set was significantly higher in the population selected for high pseudocompatibility response compared to the original population, while the population selected in the opposite direction set significantly less seed upon self-pollination under high temperatures than the original (Gertz and Wricke 1991).

The genetic nature of pseudocompatibility has been suggested to be polygenic and to involve modifier genes other than *S* and *Z*. In meadow fescue (*Festuca pratensis* Huds.), the female influence on the trait was important and independent from the SI genotype, leading to the conclusion that it is controlled by other genes acting in the pistil (Lundqvist 1961a).

Additional clues come from a significant genotype by heat treatment interaction in perennial ryegrass and the weak correlation between selfed seed set with and without heat treatment, which suggests different genes acting under different temperature conditions (Wilkins and Thorogood 1992). The significant genotypic differences in a perennial ryegrass population on selfed seed set with a lack of significant effect of *S* and *Z* genotypes on the trait also supports the conclusion that pseudocompatibility is determined outside *S* and *Z* (Fearon et al. 1983). The full range of segregation in the offspring of plants with high and low selfed seed set found by Lundqvist (1958) suggest polygenic inheritance. Polygenic modifiers contributing to pseudocompatibility were also reported for dicots, which have a different SI system (reviewed by Good-Avila et al. 2008). However, there is also strong evidence for allelic variation for response to pseudocompatibility at the *S* and *Z* loci. In meadow fescue, Lundqvist (1964) obtained distorted segregation of *S* and *Z* alleles and *S*-*Z* combinations in offspring obtained by pseudocompatibility. The excess of allele S_1 over allele S_2, and the excess of Z_4 over Z_3 led to the conclusion that S_1 and Z_4 alleles favored pseudocompatibility. In rye, Gertz and Wricke (1991) used isozyme markers linked to *S* and *Z*, and analyzed the segregation of alleles at both loci in the offspring of plants self-pollinated at high temperature. Gamete selection favored specific alleles at both loci while unlinked isozyme loci were not affected. The isozyme marker linked to the *S* locus was correlated with pseudocompatible seed set with specific alleles producing more seed. In summary, while *S* and *Z* impact pseudocompatibility, other loci outside the initial SI recognition components are involved too.

In addition to environmental and genetic factors, chemical substances applied to the stigmas were able to inhibit the SI response. Calcium channel blockers lantharum chloride (La^{3+}) and verapamil allowed incompatible pollen grains to grow their tubes down the style in both rye (Wehling et al. 1994) and perennial ryegrass (Klaas et al. 2011). Additionally, the protein kinase inhibitor Lavendustin A also allowed self-pollen tubes to reach the ovary (Wehling et al. 1994). In dicot SI species, application of different hormones, transcription inhibitors, protein inhibitors and protein synthesis inhibitors showed different degrees of SI inhibition with the potential of producing selfed seed from self-incompatible species (de Nettancourt 2001).

Pseudocompatibility can be seen as a failure of the SI system and also suggests that the SI is an intricate mechanism involving the recognition genes S and Z as well as a number of unknown genes with different functions in a pathway that leads to pollen tube arrest. All these genes must require a certain range of environmental conditions to be adequately expressed with some alleles being more sensitive than others. Moving out of this range of conditions could reduce their expression affecting the effectiveness of the whole system. Thus, knowing this range of conditions for particular genotypes can be exploited to increase the selfed seed set of self-incompatible genotypes for the purpose of inbred line development. Additionally, identifying environmentally sensitive S and Z alleles can make inbred line development by pseudocomaptibility even more effective.

Genetic self-fertility

Fully self-fertile plants have been reported within allogamous grass species (Table 1). The genetic nature of SF was studied by segregation analysis in different mating designs involving self-fertile and self-incompatible parents as well as their offspring. In rye, a 1:1 segregation of self-fertile and self-incompatible plants in the offspring of SI x SF/SI crosses were found and indicated single gene effects (Voylokov et al. 1993), in agreement with earlier studies by Lundqvist (1958) and Wricke (1969). Determining the percentage of compatible pollen by *in-vitro* pollination tests (Kho and Baer 1968; Lalouette 1967; Lundqvist 1961b) allowed a more accurate segregation analysis and the discrimination of homozygous from heterozygous self-fertile plants. In perennial ryegrass, all offspring from a cross between a self-fertile and a self-incompatible inbred showed a 50 % pollen compatibility reaction, the F_2 segregated into 50% and 100% pollen compatibility in a 1:1 ratio. The fully compatible F_2 bred true in the F_3 while segregation occurred in the 50 % compatibility class (Thorogood and Hayward 1991). This segregation pattern is consistent with other studies (Arias-Aguirre et al. 2013; Manzanares 2013; Thorogood et al. 2005) and is in agreement with a single gene action with gamethophytic control, where only the pollen carrying a SF allele can accomplish pollination (Figure 1).

A different research question aims to determine how SF is controlled in autogamous species. Interspecific crosses between autogamous *Lolium temulentum* L. and perennial ryegrass followed by backcrosses to ryegrass as recurrent parent was performed and segregation of SF was assessed. A 1:1 segregation in BC_1 and BC_2 into self-fertile and self-incompatible plants as well as the self-fertile's pollen being 50% compatible agrees with a 1-

locus model for the control of SF acting gametophytically in autogamous *L. temulentum* (Thorogood and Hayward 1992; Yamada 2001).

Even though the majority of research suggests there is a single SF gene, additional loci may be involved in conferring SF. In rye, two independently segregating loci caused SF (Lundqvist 1958). Moreover, deviation from the expected segregation in some crosses suggested an additional third locus (Lundqvist 1968; Wricke 1969). Confirmation of a third locus came from a test for allelism using a set of self-fertile inbred lines. By crossing interline F_1 plants to self-incompatible plants, the segregation of SF:SI was analyzed in all possible combinations of interline crosses. In total, three independent loci for SF best explained allelism test results (Voylokov et al. 1993). Analysis of the segregation in the F_2 of crosses between a set of trisomic and self-fertile mutants, Melz et al. (1987) also identified three SF genes and later, four genes were reported (Melz et al. 1990). Similar results were obtained in *Phalaris coerulescens* Desf. where the use of isozyme markers revealed that SF genes were linked to three independent loci (Hayman and Richter 1992). In summary, the evidence across studies suggests that at least three genes can act independently. SF, as the ability to produce selfed-seed, is dominant and epistatic over SI and its expression is at the gametophytic level (Figure 1).

<u>*S* and *Z* sources of SF</u>

Since the SI system requires the coordinated action of *S* and *Z*, a logical hypothesis is that mutants at each of these two loci would provoke a lack of coordination leading to a functional breakdown of the SI reaction or a lack of recognition of self-pollen (Figure 2 A-B).

Pioneering studies confirmed that mutations at *S* and *Z* can be determinants of SF (Lundqvist 1958, 1968).

In *P. coerulescens*, self-fertile mutants showed distorted segregation at the marker for phosphoglucoisomerase (*PGI-2*), which is linked to the *S* locus, while test crosses and allelism tests showed that other self-fertile plants carried a mutation at the *Z* locus (Hayman and Richter 1992). A SF gene from *L. temulentum* introgressed to perennial ryegrass showed joint segregation with the *GOT/3* isozyme locus, which was initially thought to be linked to the *Z* locus (Thorogood and Hayward 1992). A SF QTL on linkage group 1 of perennial ryegrass mapped closely to a marker that was previously found to be linked to the *S* locus and thus very likely corresponded to the *S* locus itself (Thorogood et al. 2005). In rye, a SF gene co-segregated with the *Prx7* and *Pgi2* genes on chromosome 1R, both linked to *S*, while distorted segregation was also observed for the isozymes *B-Glu*, *Est4*, and *Est11* on chromosome 2R, which are linked to *Z* (Fuong et al. 1993). This is in agreement with Melz et al. (1987, 1990), who also found SF genes on chromosomes 1R and 2R. SF mutations at *S* and *Z* were later mapped more precisely on chromosomes 1R and 2R, respectively (Egorova et al. 2000; Voylokov et al. 1997).

S and *Z* self-fertile alleles can be a valuable source of SF to be employed in inbreeding schemes. Large screenings in many populations may be required to find such an allele in a particular species. Alternatively, introgression by interspecific crosses from closely related species could be a valuable option.

Origin of *S* and *Z* SF alleles

Mutations at the *S* and *Z* locus mostly affected pollen rather than stigma specificity, since self-fertile mutants are often compatible to their self-incompatible parents and sibs as pollen donors but not when used as females (Hayman and Richter 1992; Lundqvist 1958, 1968). The low rate at which SF genotypes arise support the idea that deleterious mutations are responsible for such genotypes. A mutation at a functional SI allele would lead to a lack of function and thus, not being recognized in the stigma as self-pollen. This is supported by reports in the gametophytic SI (GSI) systems of the dicots where deletions, base pair mutations or sequence duplications at the S locus have been described to result in SI function loss or inactivation in the pollen grain (de Nettancourt 2001).

Specificity is known to be controlled separately in pollen and stigma. Thus, each SI locus might carry two linked genes (Lundqvist 1958, 1968; and reviewed by Klaas et al. 2011; Takayama and Isogay 2005; Yang et al. 2008). Recombination between the two genes was suggested to be the origin of SF since it would disrupt the SI system, and was supported by the observed reduction in new self-fertile plants with the decrease in heterozygosity (Lundqvist 1958, 1960). This hypothesis is challenged by the fact that the *S* locus in many grasses is located in a centromeric region (Bian et al. 2004; Egorova et al. 2000; Kakeda et al. 2008; Korzun et al. 2001; Manzanares et al. 2015) with suppressed recombination (Kakeda et al. 2008), comparable to the Solanaceae S-RNase SI system (Takayama and Isogay 2005; Wang et al. 2003, 2004). In addition, presence of repetitive elements similar to retrotransposons near the *S* locus in perennial ryegrass would also reduce recombination rates (Manzanares et al. 2015). Suppressed recombination between the two genes would be a requirement to

maintain the functionality of the SI mechanism. However, evidence of recombination within the *S* locus has been reported in the Solanaceae S-RNase system (Takebayashi et al. 2003; Wang et al. 2001, 2003). Moreover, in grasses, the *Z* locus was localized in the distal portion of the long arm of chromosome 2 (Bian et al. 2004; Korzun et al. 2001), in a region showing low prevalence of repetitive sequences where recombination is not suppressed (Bian et al. 2004; Shinozuka et al. 2010). Similarly to the *Z* locus in grasses, the *S* locus in the Rosaceae family is located in a region were recombination is not suppressed though the male and female determinants are tightly linked, being less than 550 bp apart (Sapir et al. 2007). Nevertheless, evidence for recombination within the *S* locus was also found in Rosaceae (Donia et al. 2015; Ortega et al. 2006; Vieira et al. 2003). Very low recombination rates were suggested in the *S* locus region of the Brassicaceae (Charlesworth et al. 2006) though recombination was detected within the *S* locus complex of genes but not between the male and female determinants (*SP11* and *SRK*, respectively) (Takuno et al. 2007). Interestingly, the self-fertile nature of *Arabidopsis thaliana* was attributed to inter-haplotype recombination in the *S* locus (Sherman et al. 2007). Overall, recombination between the pistil and the pollen determinants at both *S* and *Z* loci may explain some of the spontaneous emergence of SF in grasses.

It has also been proposed that point mutations as well as intragenic recombination are sources of new allele formation, leading to SF even in the presence of functional alleles. In *Solanum chacoense* Bitt. (Solanaceae), new specificities can arise from few or even single base-pair substitutions at the hypervariable regions of the *S-RNase* gene (Matton et al. 1997, 1999; Saba-El-Leil et al. 1994) and thus, mutations may result in new functional alleles.

Likewise, point mutations at the hypervariable regions of the *F-box* gene in *Prunus* species, contribute to allele diversity (Donia et al. 2015). Such a new specificity in the *S-RNase* gene would lead to non-recognition by the stigma of any self-pollen. A new specificity in *F-Box* genes would be unmatched in the self-stigma and thus being unrecognized in the same way as foreign pollen. Such mutations would go unrecognized until a new complementary mutation occurs at the opposite gene (Gervais et al. 2011; Uyenoyama et al. 2001). Similarly, the process of new allele formation within *S* and *Z* may be responsible for the loss of SI activity until a new mutation at the cognate gene arises.

Other genes: non *S* or *Z* sources of SF

Genes independent of *S* and *Z* causing SF were found in rye, *P. coerulescens* and perennial ryegrass. While in rye *S* and *Z* are located on rye chromosomes 1R and 2R, respectively, genes affecting SF were located also on chromosomes 3R, 4R, 5R and 6R (Melz et al. 1987, 1990). A SF locus identified as *S5* by Voylokov et al. (1993) was found to be linked to the *Est5-7* isozymes that are located on chromosome 5R. The population segregating for this gene showed significant deviation from Mendelian ratios for these isozymes, with an excess of the allele from the self-fertile line (Fuong et al. 1993). Further mapping efforts allowed to localize the *S5* gene in the centromeric region of chromosome 5R (Egorova et al. 2000; Voylokov et al. 1997). Their gene products, when functional, could be part of a signal transduction cascade within the pollen grain, triggered by *S* and *Z* and causing the pollen tube arrest (Wehling et al. 1995).

A third gene that segregates independently of *S* and *Z* was identified in *P. coerulescens*. SF co-segregated with the leaf peroxidase isozyme (PER). This SF gene was named *T* and has no allelic variability (Hayman and Richther 1992). Similarly, in perennial ryegrass, a F_2 population derived from a cross between two inbred lines was used to map a SF gene. A region of 19.9 cM in LG5 showed the greatest distortion with an excess of one of the homozygotes classes and harboring a QTL for SF, which would be analogous to the *S5* locus of rye (Thorogood et al. 2005). The region was then narrowed to 3.9 cM using an F_3 population derived from a single heterozygous F_2 plant (Manzanares 2013). Interestingly, a SF locus was also mapped on LG5 within a 14 cM region in a completely different population and with the SF source coming from a different origin (Arias-Aguirre et al. 2013).

Whether the non-*S* and -Z locus conferring SF in rye, perennial ryegrass, and *P. coerulescens* is due to the same gene remains to be answered but it is worth noting that this region is conserved among different grasses (Alm et al. 2003; Jones et al. 2002b; Sim et al. 2005). Additionally, in all cases pollen specificity was affected and the SF locus was able to provoke self-pollen compatibility and hence SF even in the presence of functional *S* and *Z* alleles. Its gene action is epistatic over *S* and *Z*, acting at the gametophytic level (Figure 2 C). The fact that a mutation at the *T* locus results in SF, leads to two hypotheses for gene action: 1.) the mutation at *T* causes a gain of function whose product is epistatic over *S* and *Z*, either by blocking their expression or suppressing the function of their gene products in the pollen grain, probably acting as an alternative substrate competing for the recognition sites; 2.) the mutation is a loss of function which implies that the *T* locus is a functional gene expressed in the pollen grain whose product is required in the incompatibility reaction and when knocked

down, impairing self-recognition or pollen tube arrest. Because of gametophytic gene action, dominance considerations are not relevant. Gain of function mutations are less common and would imply that the allele arose once in evolution which disagrees with the fact that it has been found in three different self-incompatible species from different tribes. In comparison, a loss of function mutation is more likely since insertions or deletions at different sites within a gene could create a gene product that is no longer functional. The lack of allelic variability at the *T* locus and the fact that normally just *S* and *Z* explain the compatibility-incompatibility reaction suggests that the *T* locus is fixed among SI grasses.

Besides the locus on LG5, a region on LG3 of perennial ryegrass has shown a high degree of distorted segregation in different mapping populations (Anhalt et al. 2008; Jensen et al. 2005; Jones et al. 2002a, b). This distortion could potentially be due to the presence of another SF locus, since some markers on LG3 were found to interact with the *S* and *Z* SI loci with alleles co-segregating or even contributing to a SF reaction (Thorogood and Hayward 1992; Thorogood et al. 2002). However, this region could not be associated with the observed SF segregation and thus, has so far been disregarded as a potential SF locus (Arias-Aguirre et al. 2013; Manzanares 2013; Thorogood et al. 2002).

In the dicot S-RNase SI system, additional genes unlinked to the *S* locus conferring SF were reported and suggest roles for the grass *T* locus. A modifier gene unlinked to the *S* locus causes breakdown of the SI system in sweet cherry (*Prunus avium* L.) (Cachi and Wünsch 2011; Wünsch and Hormaza 2004; Wünsch et al. 2010) and in apricot (*Prunus armeniaca* L.) (Vilanova et al. 2006). Similarly to the grass *T* locus, in the *Prunus* non-S SF locus, only the pollen grain carrying the mutation is able to effect self-pollination, even in the presence of

intact and functional *S* alleles expressing normal levels of S-RNase and F-box pollen-expressed gene (SFB) products. Such a pollen modifier has been proposed to have a non-specific role in pollen rejection such as preventing S-RNases ubiquitination or being required in the S-RNase uptake into the pollen tube (Vilanova et al. 2006). Alternatively, in grasses the SF gene product could potentially be required to complete the interaction between *S* and *Z* products acting either upstream or downstream in the incompatibility pathway or by forming a complex molecule with the *S* and *Z* products.

A gene unlinked to *S* but essential for SI was suggested in *Fragaria* spp. (Rosaceae) but its expression was in the pistil rather than the pollen grain (Boskovic et al. 2010). In the Brassicacea SSI system, major genes not linked to the S locus have roles either in the recognition phase or downstream in the rejection process, acting in the pistil of recessive self-fertile mutants. Such a SF pistil mutant has not been reported in grasses yet, probably because it would behave as a recessive trait and is thus less likely to be noticed by traditional SF screenings. However, SF pistil mutants in grasses should also occur since other unspecific pistil factors are likely involved in the rejection process, as described for the Brassicacea SSI as well as the Solanaceae GSI system, where at least three additional non-RNase pistil proteins are known to be required in the pollen rejection process (Goldraij et al. 2006).

Breeding strategies involving SF in outcrossing grasses

SF introgression and inbreeding

For the development of inbred lines to be used as parents for synthetics or hybrids, a SF gene needs first to be introgressed into breeding populations. Due to their outcrossing nature, plants within these populations are heterozygous and heterogeneous, and thus require a modified back-cross procedure to keep the genetic variability of the original population (Figure 3). A SF donor homozygous for the mutation is crossed to different plants of the population of interest. Several plants are used in each step to sample the genetic variability of the population and seed is harvested from the SI recurrent parent to avoid selfings. The F_1 is bulked and back-crossed to another set of plants from the recurrent population. Molecular markers are used in the BC_1 generation to select SF plants for the next round of back-crossing. The process is repeated up to BC_5 – BC_7 to reduce the donor's genome contribution in the new introgressed population, followed by self-pollination to create an F_2 generation and start the inbreeding process.

After SI is overcome, development of vigorous and fertile inbreds is a concern for early breeding cycles using SF. Experimental results in perennial ryegrass showed an overall reduction of 40% in dry weight in first generation inbreds compared to outbred progeny even though the decrease was genotype dependent and some of them did not show a significant reduction even after two generations of self-pollination (Bean and Yok-Hwa 1972). Reduced germination and seedling survival was reported in perennial ryegrass after three generations of self-pollination (Jones and Jenabzadeh 1981). In orchardgrass (*Dactylis glomerata* L.), there

was a progressive decrease in vigour and seed production as well as an increase in leaf diseases and winter injuries up to the second selfing generation (Kalton et al. 1952). Another report showed no detectable inbreeding depression for dry matter yield and forage quality after one selfing generation (Van Santen and Casler 1987). Genotypes tolerant to inbreeding depression have been reported for tall fescue after four generations of selfing (Buckner 1960; Buckner and Fergus 1960; De Santis 2007), and perennial ryegrass after five selfing generations (Jones and Jenabzadeh 1981). Variation for the extent of inbreeding depression among inbred progenies suggests that cycles of inbreeding and recombination of the most vigorous plants within families might be an effective way to obtain superior inbreds. Doubled haploids (DH) by means of anther culture (Andersen, 2003) or inducers (Kindiger 2012; Kindiger and Singh 2011) can also play an important role in grasses as a fast way to achieve homozygosity, and with a clear potential for purifying populations from deleterious alleles since only DHs with low genetic load would survive. Inbreeding offers two major advantages: i) to increase in genetic variability among individuals in a population which increases expected selection gains, and ii) to effectively eliminate recessive deleterious alleles causing genetic load. Moreover, inbred lines enable hybrid breeding schemes comparable to rye and other hybrid crops.

There is promising evidence of heterosis that justifies hybrid breeding approaches. In many grass species, mid-parent heterosis for yield under sward conditions between 5 and 20% has been detected (Posselt 2003). A recent study showed up to 21% for high parent heterosis in perennial ryegrass (O'Connor et al, 2015), while 38% was reported in switchgrass (*Panicum virgatum* L.) (Vogel and Mitchell 2008). Significant high parent heterosis for quality

traits in orchardgrass were also observed (Robins et al 2015). Such heterosis levels however were obtained mainly from population hybrids, non-inbred and partial inbred parents and largest amounts of heterosis are expected in crosses between two inbred parents.

Self-fertility for synthetic variety development

The introduction of inbreeding as part of breeding programs has been proposed for different grass species, including rye (Voylokov 2007; Wricke 1976), tall fescue (Buckner 1960; De Santis 2007), *Bromus inermis* and timothy (*Phleum pratense* L.) (Drolsom and Nielsen 1969). Basically, after inbreeding, parents are selected and intercrossed to produce a synthetic 1 generation (Syn1), where vigor is restored. Since such inbred parents are self-fertile, a varying degree of self-pollination is expected when polycrossing selected inbred parents, leading to a certain degree of inbreeding depression in the resulting Syn1. For that reason, Posselt (2010) suggested that the use of self-fertile materials should be avoided when breeding synthetic varieties. However, this issue can be overcome in two different ways: i) self-incompatible plants can be selected within the Syn1 generation to generate the Syn2 (Figure 4A), and ii) use self-incompatible inbred parents developed using pseudocompatibility (Figure 4B). In agreement with this, Voylokov (2007) proposed a scheme where an *S* locus SF mutation is introgressed into elite lines. The resulting progeny would be self-fertile and heterozygous for the SF gene. Progeny would eventually be advanced or backcrossed to the recipient parent to reduce the donor genome contribution. The use of isozyme *Prx7* marker, which is linked to the *S* locus, was proposed to identify the heterozygous plants. Once the desired level of homozygosity is achieved, heterozygous plants for SF are identified from

selected families and intermated. In this way, self-incompatible progeny can be recovered, since self-incompatible genotypes arise only when heterozygotes for the SF mutation from different families are intermated. The resulting progeny would represent the Syn2 with restored SI (Figure 4A). The same scheme is possible using SF mutants at the locus *Z* or *T*, and using molecular markers linked to them to identify heterozygotes.

Alternatively, self-incompatible inbreds can be produced using pseudocompatibility. Breeders could use heated greenhouses (30º - 34º C) and develop inbred lines by single seed descent, a method that is less space demanding, or choose warmer locations and develop inbreds by the pedigree method. Selection to increase pseudocomaptibility at high temperatures as described by Gertz and Wricke (1991) would be advantageous. Chemical inducers applied at anthesis could also be an option though further research is needed to identify suitable chemical substances. Once self-incompatible inbreds are developed, the selected parentals are polycrossed to develop a Syn1 with very few or without selfings (Figure 4B).

It is worth noting that for synthetic breeding, parents should be selected for superior combining ability and thus only a small number of plants are required to produce test crossed seed for evaluation and after that, few plants would be sufficient for polycrossing the selected lines. Thus, the amount of parent seed required for synthetic breeding is fairly low, which makes both methods viable.

An additional advantage of utilizing inbred parents for synthetic breeding is that inbred seed can be stored, eliminating the need of maintaining the parent clones vegetatively over long periods (Wilkins and Thorogood 1992). Also, the use of inbred lines instead of single

plants in test-crosses to assess combining ability provides more seed that can be used in larger plots or larger trials (Gertz and Wricke 1991).

Pseudocompatibility for hybrid breeding

A method to produce F_1 hybrids in grasses based on SI was proposed by England (1974) and later tested by Posselt (1993). It involves the creation of inbred families ("lines") with a high degree of within-line incompatibility previous to the final interline cross to produce the hybrid. Plants within a line share the same alleles at S and Z though some plants are homozygous at one of the loci while others are heterozygous at both limiting the crossability among plants from the same line. The method takes advantage of low levels of selfed seed that self-incompatible plants are able to produce by pseudocompatibility in order to create such lines. The expected percentage of hybridization under this scheme is 83% and was speculated that non-hybrid plants would not compete under sward conditions. The same strategy can be applied using markers to restrict SI allele diversity within families without the need of a self-fertilization step (Pembleton et al. 2015). Moderate levels of heterosis were obtained in some crosses under this scheme (Posselt 1993; Eickmeyer 1994) but in the contrary, the realized hybridization percentage greatly deviated from the expected and was highly variable ranging from 25 to 80% (Eickmeyer 1994).

Gertz and Wricke (1991) proposed to use pseudocompatibility to produce parent lines for hybrid seed production. This involves creation of self-incompatible inbred lines, as described for synthetics, by repeated self-pollination under high temperature conditions. Seed from selected inbred lines developed in this way could then be mixed in equal

proportions and planted in the field under normal environmental conditions to produce 100% hybrid seed. A problem with this strategy is the multiplication of the self-incompatible inbreds at large scale. For that purpose, hot and dry locations would have to be selected for seed multiplication of the inbreds, while cooler locations should be used for the hybrid seed production to avoid selfings. Yet, the viability of this strategy still needs to be tested, which has the advantage of avoiding genetic manipulation but has the challenge to be dependent on environmental conditions. Application of SI inhibitors is a tempting strategy that would also avoid environmental dependency. However, proper techniques and products still need to be developed.

Hybrid breeding with self-fertile inbreds

Once the SI barrier is overcome by the introduction of SF genes, inbred lines can be developed by repeated self-pollination. Once inbred lines from different heterotic groups show superior testcross performance hybrid seed can be produced. However, hybrid breeding requires effective control of self-pollination. Two biological mechanisms can potentially be exploited for hybridization: SI and cytoplasmic male sterility (CMS) (Arias-Aguirre et al. 2012; Posselt 2010).

Ideally, after inbred line development, SI would be reestablished and crossing two self-incompatible inbred lines would ensure 100% hybridization. However, when selfing a heterozygous self-fertile plant, only pollen grains carrying the mutation are able to fertilize. Thus, no self-incompatible individual is recovered in the progeny, unless two heterozygous self-fertile plants with a different *S-Z* constitution are crossed. A breeding scheme involving

the development of isolines differing only at the alleles present in one of the SI loci and crossing them at the final step could be used to develop a self-incompatible inbred line. However, such a cross produces both self-incompatible and self-fertile progeny, requiring the identification of the self-incompatible plants. Further seed multiplication of the self-incompatible inbred line is prevented by SI, and plants would have to be clonally propagated to a commercial scale to produce hybrid seed.

Alternatively, CMS is currently the preferred mechanism of hybridization control in many species. Hybrid breeding in rye is an example of a grass species possessing the 2 locus SI that moved from population/synthetic breeding to a CMS-based hybrid system and where SF was a prerequisite for the development of inbred lines (Geiger and Miedaner 1999). The system requires a male sterile line (A line), which is used as the female parent for the hybrid seed production, and a maintainer line (B line), which is genetically identical to the A line except that it is male fertile and used to produce seed of the A line (Figure 5). An A line is obtained by crossing the inbred of interest (line B) to a CMS donor followed by repeated backcrossing to the line B. A restorer line (R line) is required in grain crops so that the F_1 can produce grain. However, in forage crops, the R line would not be required. A few sources of CMS have been identified in perennial grasses, which were obtained either by interspecific crosses, protoplast fusion or mutagenesis (Islam et al. 2014; Sykes et al. 2016). Another source of male sterility was obtained in tall fescue by using Chimeric REpressor gene-Silencing Technology (CRES-T) to silence genes that specify the formation of stamens (Sato et al. 2012), though understanding of the inheritance of this trait is still incomplete. Instability of male sterility has been a limitation for practical use of CMS in forage breeding programs (Posselt

2010). However, an *in silico* pipeline to identify genomic regions containing potential restorer fertility genes (R*f*) has recently been developed, facilitating marker design and screenings to prevent fertility restoration (Sykes et al. 2016). Interestingly, one of the main challenges for CMS in grasses has been their outcrossing nature that favors the accumulation of restorer genes in heterozygous background (Islam et al. 2014). Therefore, with the current knowledge on the genetics and mode of action of SF, stable CMS lines could potentially be developed, and both traits could be combined in a single breeding program to develop single cross hybrids in grasses.

Conclusions

The genetic progress achieved to increase dry matter yield in allogamous grasses is rather low when compared to other crops. The SI system, although it contributes to high levels of heterozygosity within populations, also limits the breeding methods that can be applied in cultivar development. The overall understanding of the SI system is crucial as are the mechanisms involved in the breakdown of SI. Pseudocompatibility offers a temporary option to overcome SI without affecting its functionality under normal environmental conditions. However, large-scale inbred line production exploiting this mechanism still needs to be tested. A functional breakdown of the SI system is possible, which leads to self-fertile individuals and inbred production at a much larger scale. SF alleles at *S*, *Z*, and *T* loci have been identified which arose from mutations or recombination within them, provoking a lack of function. The number of self-incompatible species, where self-fertile mutants have been reported is rather low. However, such mutants are likely present in any grass species with a

functional SI system. In addition, interspecific crosses between related species would allow the introgression of SF into a self-incompatible relative. Although the genes involved have not been cloned, nor gene products or biochemical pathways elucidated in grasses, the current knowledge of their mode of action plus the availability of molecular markers linked to these loci, allow their application in novel breeding approaches that take advantage of inbreeding to develop new types of cultivars.

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Table 1. Grass species characterized for SI and SF (Connor, 1979; Li et al., 1997; Baumann et al., 2000).

Subfamily	Tribe	Species	SI	GSI	S-Z	SF	PsC	
Pooideae	Poeae	*Briza media*	Yes	Yes	Yes			
		Briza elatior	Yes	Yes				Murray, 1974
		Briza australis	Yes	Yes				Murray, 1974
		Briza minor	No					
		Bromus inermis	Yes					McKone, 1985
		Bromus tectorum	No					McKone, 1985
		Cynosurus cristatus	Yes	Yes				
		Dactylis aschersoniana	Yes	Yes	Yes			
		Festuca pratensis	Yes	Yes	Yes		Yes	Lundqvist, 1964
		Festuca rubra	Yes	Yes				
		Lolium perenne	Yes	Yes	Yes	Yes	Yes	
		Lolium multiflorum	Yes	Yes	Yes			
		Lolium temulentum	No					
	Aveneae	*Anthoxanthum odoratum*	Yes					
		Arrhenatherum elatius	Yes	Yes				
		Avena barbata	No					
		Alopecurus myosuroides	Yes	Yes				
		Alopecurus pratensis	Yes	Yes				
		Deschampsia flexuosa	Yes	Yes				
		Holcus lanatus	Yes	Yes				
		Phalaris arundinacea	Yes	Yes				
		Phalaris coerulescens	Yes	Yes	Yes	Yes		
	Triticeae	*Secale cereale*	Yes	Yes	Yes	Yes	Yes	
		Hordeum bulbosum	Yes	Yes	Yes			
		Hordeum vulgare	No					
Chloridoideae	Chlorideae	*Chloris gayana*	Yes					
		Chloris striate	No					
Ehrhartoideae	Oryzeae	*Oryza barthii*	Yes					
		Oryza sativa	No					
Panicoideae	Andropogoneae	*Sorghastrum nutans*	Yes					
		Zea mays	No					
	Paniceae	*Panicum virgatum*	Yes	Yes	Yes*		Yes	Martinez-Reyna and Vogel, 2002; Liu et al., 2014

SI: Self-incompatibility; GSI: gametophytic self-incompatibility; S-Z: S-Z incompatibility system; SF: self-fertility; PsC: pseudocompatibility. * S-Z incompatibility system not determined but suggested.

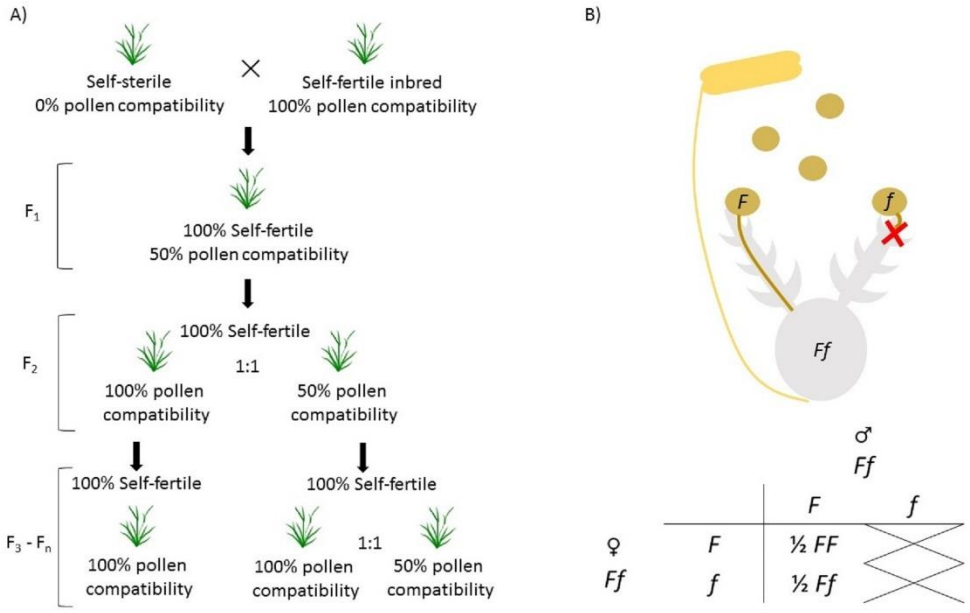

Figure 1. Single gene model for self-fertility and pollen compatibility. A) Observed segregation of SF and pollen compatibility. Note that SF is dominant and gets fixed after the first selfing generation. B) Scheme of the single SF gene acting gametophytically in the pollen grain and the genotypic segregation.

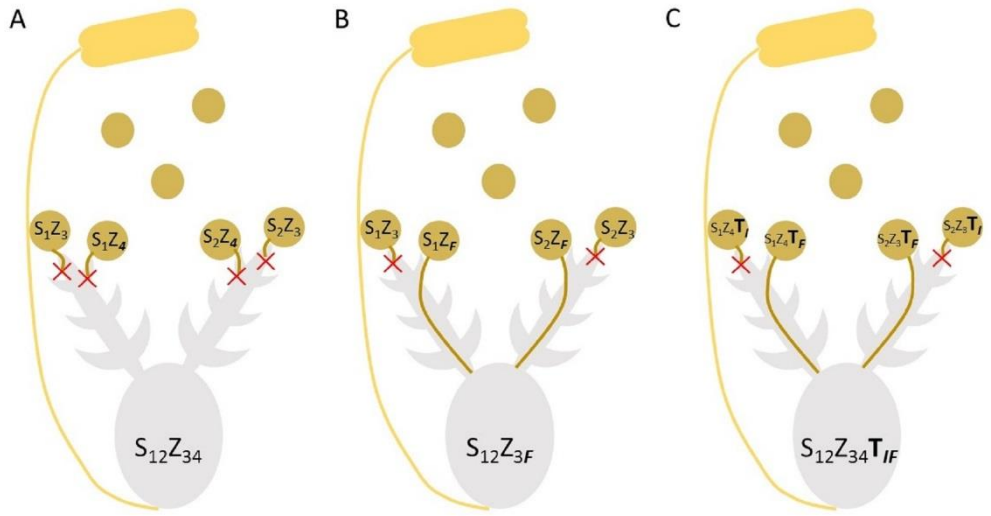

Figure 2. Self-fertility mutations affecting pollen specificity: A) SI: the allele at *S* and the allele at *Z* of all self pollen grains are matched in the stigma and consequently inhibited; B) mutation at a SI locus (*Z*), pollen grains carrying the mutation are not recognized in the stigma and the pollen tubes are able to grow through the pistil and reach the ovary; and C) mutation at the *T* locus, if a pollen grain carries the wild type *T* allele (T_I), SI is determined by *S* and *Z*, if the pollen grain carries the mutant *T* allele (T_F), the pollen is compatible independently of the alleles at *S* and *Z*.

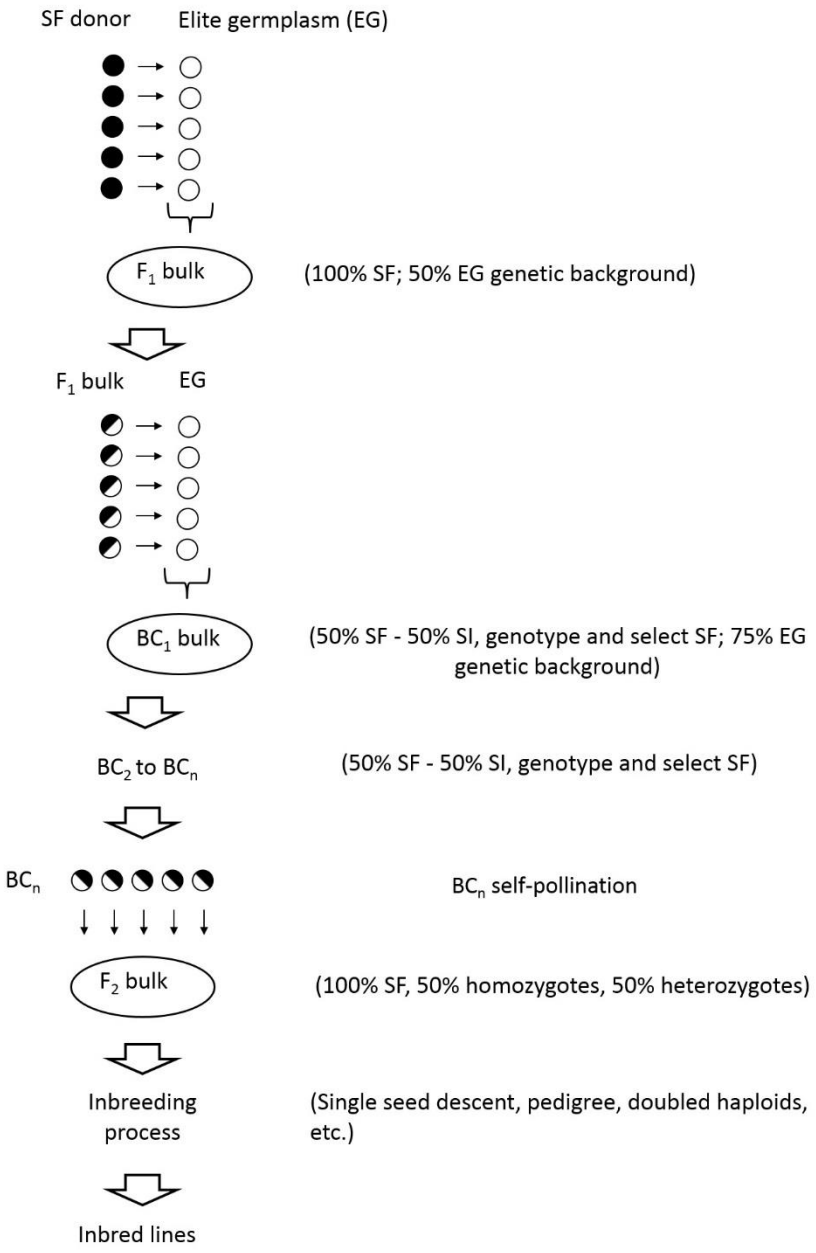

Figure 3. Scheme of a modified back-crossing procedure to introgress a SF gene into a breeding population. ● = homozygous for SF, ◐ = heterozygous for the SF gene, and ○ = SI plant.

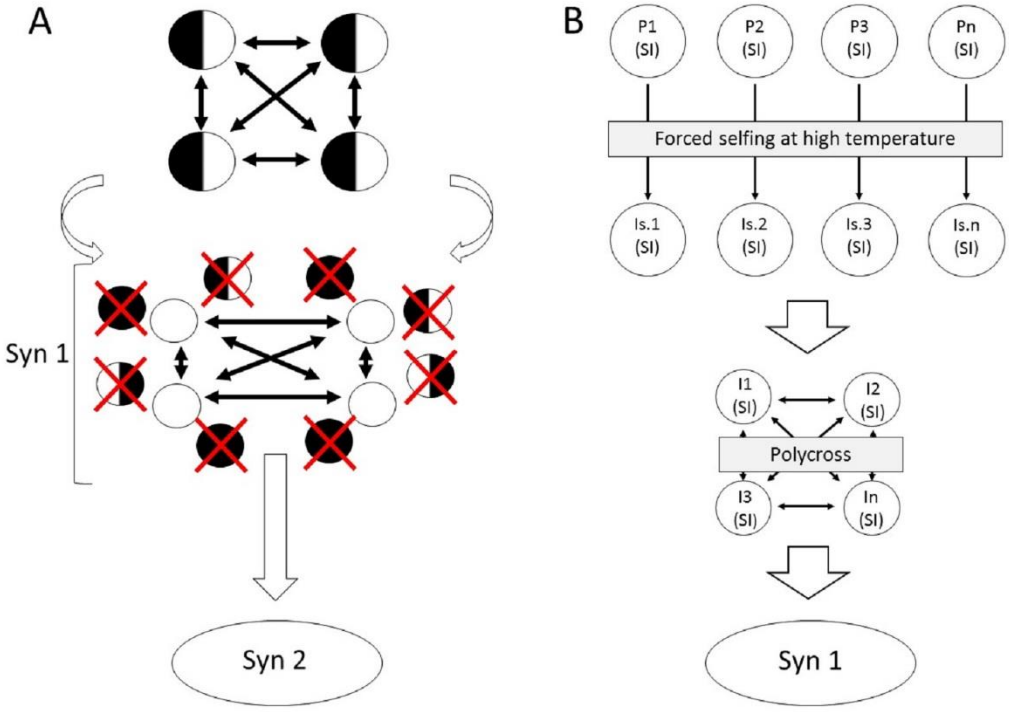

Figure 4. Synthetic breeding using inbreds developed with SF or pseudocompatibility. A) Inbred lines heterozygous for the SF gene, developed by repeated self pollination and selected for high general combining ability, are used as parents and polycrossed to create the Syn 1 generation. Self-incompatible plants within Syn 1 are selected with molecular markers and polycrossed to develop Syn 2 while reestablishing SI (Black circles: homozygous for the SF gene; black/white circles: heterozygous for the SF gene; white circles: self-incompatible plants). B) Self-incompatible inbreds (Is.1, ls.2, etc.) are developed by pseudocompatibility from different origins (P1, P2, etc). Selected inbreds (I1, I2, etc.) are polycrossed under natural conditions to develop the Syn 1 generation.

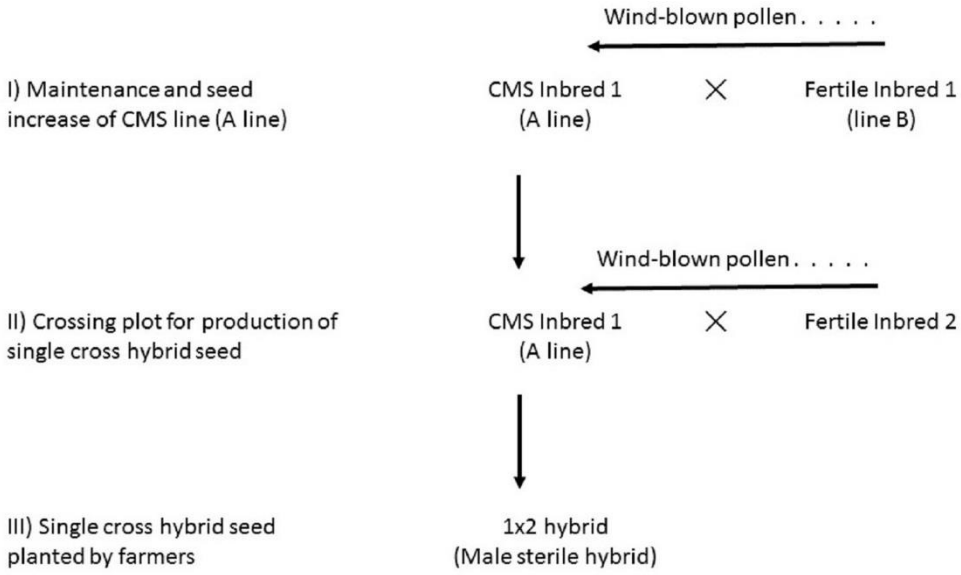

Figure 5. Method for producing hybrid seed using CMS (adapted from Stephens and Holland, 1954). The A line is maintained and multiplied by crossing to the B line which is the pollen donor. The A line is then crossed to a selected fertile inbred (pollen donor) to produce the single cross F_1 hybrid.

CHAPTER 3

FINE MAPPING A SELF-FERTILITY LOCUS IN PERENNIAL RYEGRASS

A paper in preparation to be submitted for publication to Theoretical and Applied Genetics

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Abstract

In grasses self-incompatibility (SI) is characterized by a gametophytic two locus (*S* and *Z*) mechanism acting together in the recognition and inhibition of self-pollen. Mutations affecting the expression of SI have been reported in a few grass species. In perennial ryegrass (*Lolium perenne* L.) a mutation independent from *S* and *Z*, and mapping on linkage group 5 (LG 5) was responsible for producing self-fertile plants. The main objectives of this work were to fine map the location of the self-fertility (*SF*) gene in a perennial ryegrass population and to determine whether there is any effect of other genomic regions on pollen compatibility. A sample of the population was used to identify flanking markers of the *SF* locus region and assess possible interactions with other regions, using segregation and linkage analysis. Recombinants were identified in a large population and the *SF* region was densely covered with additional markers. The phenotypic segregation of SF showed a bimodal distribution with one mean at 49% pollen compatibility and the other at 92%. Marker-trait association analysis showed that only markers on LG 5 were significantly associated with the trait. A single gene model explained 94% of the observed variability and

no effects of other regions were detected. Linkage analysis mapped the *SF* locus to a 1.6 cM region on LG 5. The flanking marker sequences were aligned to a 1.35 Mbp region in the rice reference genome, and to an 807 kbp region in *Brachypodium distachium*. Here, we provide markers tightly linked to SF that can be used for introgression of this trait into advanced breeding germplasm. Moreover, our results represent a further step towards the identification of the *SF* gene in LG 5.

Introduction

The Gramineae family displays a wide range of reproductive mechanisms among its more than 10,000 species, some promoting self-pollination, and others cross-pollination due to mechanisms such as dioecy and self-incompatibility (Connor, 1979). Different to other known self-incompatibility (SI) systems, SI in grasses is characterized by a gametophytic two locus (*S* and *Z*) mechanism acting together in the recognition and inhibition of self-pollen. A pollen grain is incompatible if its alleles at both *S* and *Z* are present in the female genotype (Lundqvist, 1954). This is a strong mechanism and thus seed from self-pollination is difficult to obtain. This particular SI system is restricted to the tribes Poaceae, Aveneae, and Triticeae, which encompass a great number of forage and cereal grasses. It was also shown that this SI system is conserved across different genera within these tribes (Li et al., 1997; Baumann et al., 2000).

Where SI is present, cultivar development is restricted to synthetic or population varieties, maintaining high levels of heterozygosity but also heterogeneity and often segregation for deleterious alleles. However, mutations affecting the expression of SI have

been reported in a few grass species (Do Canto et al., 2016), but with the exception of rye (*Secale cereale* L.), it has not been exploited for breeding purposes. This self-fertility enables inbred line development, which is the first step in hybrid breeding and has the potential to purge deleterious alleles.

In perennial ryegrass (*Lolium perenne* L.) mutations independent from *S* and *Z*, and mapping in linkage group 5 (LG 5) were responsible for producing self-fertile plants in three different populations (Thorogood et al., 2005; Arias-Aguirre et al., 2013; Manzanares, 2013) implying that SI is more complex than initially thought. If a mutant at this locus impairs SI, the functional gene is likely part of either the recognition or the pollen tube arrest response. Its mode of action is simple: only pollen grains carrying the mutant allele develop pollen tubes being able to penetrate the stigma and to reach the ovary. Offspring by self-pollination of a heterozygous genotype for the mutation segregate into homozygous and heterozygous at a 1:1 ratio and no self-incompatible individuals are recovered (Do Canto et al., 2016).

Whether inbred lines or selfed progeny are required, a self-fertility (SF) mutation has to be introgressed into breeding populations first. Even though such a mutant is likely to be present in any self-incompatible grass species, so far it was only reported in three species: rye (Lundqvist, 1958), perennial ryegrass (Thorogood and Hayward, 1991), and *Phalaris coerulescens* Desf. (Hayman and Richter, 1992). Alternatively, SF can be introgressed from self-fertile relatives, when interspecific hybridization is possible as was the case of the SF introgression from *Lolium temulentum* L. into perennial and Italian ryegrass (*Lolium multiflorum* L.) (Thorogood and Hayward 1992; Yamada, 2001). Whichever path is chosen,

markers closely linked to the gene are needed for an effective introgression and subsequent elimination of the donor genome.

The *SF* gene was mapped to a 14 cM region in LG5 by Arias-Aguirre et al. (2013) in a F_2 population derived from a cross between a self-fertile and a self-incompatible genotype. Markers linked to the *S* locus in LG 1 and *Z* in LG 2 segregated independently from SF but deviated significantly from Mendelian ratios. The same was true for a region in LG 3. The main objective of this work was to narrow down the *SF* locus region in LG 5 to a few cM in the same mapping population used by Arias et al. (2013), and connect this region to physical maps of sequenced grass genomes. In addition, we aimed to determine, whether there is any effect of the *S*, *Z,* and LG 3 genotypes on pollen compatibility.

Materials and Methods

To fine map the *SF* gene on LG 5, a sample of the population was used to identify flanking markers and determine possible interactions with other regions known to control SI, using segregation and linkage analysis. All plants of the population were then genotyped with these flanking markers to identify recombinants. Only recombinants were phenotyped and genotyped with additional markers to finally map the trait to a narrow genetic region.

Plant materials

A F_2 mapping population was developed by selfing a single F_1 plant from a cross between a self-fertile inbred and a self-incompatible plant from the VrnA mapping population (Jensen et al., 2005). This population is segregating for a *SF* gene in LG 5 as

described by Arias-Aguirre et al. (2013). A total of 1248 plants were employed in the experiment from which a sub-set of 94 plants was initially used to identify flanking markers in LG 5 and to study possible interactions with other genome regions.

Phenotyping

The percentage of self-compatible pollen of every plant was determined by *in-vitro* pollination tests (Lundqvist, 1961) using fluorescence microscopy (Kho and Baer 1968). For each plant, five mature and unpollinated pistils were dissected in the morning before pollen was shed and transferred to 47 mm petri dishes with growing medium consisting of 2% agarose, 10% sucrose, and 100 ppm of boric acid. Anthers from the same plants were collected late in the morning and shaken over the pistils so that fresh pollen was evenly spread over the stigmas. A two hour period was given to allow compatible pollen tubes to grow and incompatible pollen to get inhibited. After that, the pistils were briefly submerged in a staining solution containing 0.2% aniline blue and 2% K_3PO_4 by applying a few drops. The stigmas were thereafter separated from the ovaries using a razor blade, placed over a microscope slide with another drop of the staining solution, and finally covered with a cover slip. 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. Compatible pollen grains are usually translucent, their pollen tubes have a bright color and can be observed to grow towards the style. Inhibited pollen grains are usually bright and a short and thickened pollen tube can be observed. Compatible and incompatible pollen grains were counted, and in every stigma, between 10 and 60 pollen grains were

observed. The percentage of compatibility was calculated for every stigma and used to calculate the mean for each plant. Replication at different days was done for a few plants at the beginning of the experiment and showed high consistency among them.

DNA extraction and genotyping

DNA was extracted from young leaves following a CTAB (cetyl trimethylammonium bromide) based protocol (Doyle and Doyle, 1987). Leaf samples were frozen in liquid nitrogen and ground to powder before being suspended in CTAB buffer and incubated at 65° C for 1 hour. Chloroform and RNase were used for purification from proteins, lipids, and RNA. Nucleic acids were precipitated with isopropanol and pelleted by centrifugation. Two washing steps with 75% ethanol were performed before re-suspending the DNA pellet in sterile distilled water.

Genotyping was done using High Resolution Melting analysis (HRM). The PCR reaction mix contained 0.2 mM of dNTPs, 25 mM of magnesium chloride, 20 to 30 µM of each, forward and reverse primers depending on the primer set, 1x LCGreen dye (BioFire Diagnostics, Inc., Salt Lake City, UT, USA), and 20 ng of DNA. To prevent evaporation during light scanning exposure, 20 µl of mineral oil was added. PCR amplification was conducted in a BIO RAD T100TM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and based on the protocol of Studer et al. (2009). 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 LightScannerTM 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.

Fine mapping - Phase I

The initial set of plants was genotyped with six markers surrounding the region of interest on LG 5 plus markers linked to the SI loci *S* and *Z*, and five markers in LG 3. Markers on LG 5 included the four markers that were previously found to be significantly associated with SF and the two flanking markers of the region comprising a 52 cM segment (Arias-Aguirre et al., 2013). Markers on LG 3 were within a region that showed segregation distortion previously (Arias-Aguirre et al., 2013).

Phenotypic segregation was analyzed to determine whether it could be explained by a single qualitative gene. Plants with more than 80% pollen compatibility were considered fully compatible and assigned to the 100% compatibility class. Plants with 30% to 65% pollen compatibility were considered partially compatible and assigned to the 50% compatibility class. No plants were found outside these ranges. The segregation into the two classes in a 1:1 ratio was then tested with a chi-square test of goodness of fit. Single marker association with the compatibility classes was analyzed by chi-square tests of independence.

An analysis of variance (ANOVA) was performed to test the effect of the genotype at the *SF* locus. In addition, we tested whether the observed variation around the mean of both partial and full compatibility groups was due to quantitative effects of S or Z alleles as well as alleles of loci in LG 3, or due to error. The data were separately analyzed for the two groups using the GLM procedure in SAS® software version 9.4 (SAS Institute Inc., Cary, NC)

with the marker genotypes as the explanatory variables and pollen compatibility as the response variable.

Genotypic data were used to create a linkage map using the OneMap software (Margarido et al., 2007). Recombination fractions between all pairs of markers were estimated with a two-point test and linkage groups were determined using a maximum recombination fraction of 0.5 and a LOD score of 3.0. Marker orders within LGs were obtained by estimating the multipoint likelihood of all possible orders using the *compare* function which identifies the most likely order. The Kosambi mapping function was selected for estimation of mapping distances. In a different run, phenotype classes for SF were included and treated as markers to find a tentative position of the *SF* locus and identify its flanking markers.

Fine mapping - Phase II

The 1154 remaining plants of the population were genotyped with the flanking markers identified in *Phase I*. All plants showing a recombination between the flanking markers were selected. They were vernalized in growth chambers for 8 weeks at 5° C and short photoperiod conditions (8 hours day/16 hours night). Plants were then transferred to the greenhouse and kept at 23° C and long photoperiod conditions (16 hours day/8 hours night) for flowering. The recombinant plants were phenotyped by *in vitro* pollination tests as described above.

The phenotypes obtained were classified into two phenotype classes as described in *Phase I* and the segregation compared to a 1:1 ratio using a chi-square goodness of fit test.

The *Lolium* genome zipper (Pfeifer et al., 2013) was used to identify and obtain the primary sequence of additional markers using the flanking markers as reference. Microsatellites were identified within the sequences using WebSat software (Martins et al., 2009). For SNPs, alternative sequences were obtained from the genome zipper when available or from published sources (Studer et al., 2012). Primers were designed with Primer-BLAST (Ye et al., 2012) using 60° C as the primers melting temperature (Tm). Primer sets with the smallest product size and lowest self- and self 3'-complemantarity (self-binding affinity) were selected making them more suitable for HRM. An additional set of markers designed on perennial ryegrass cDNA reads aligned with the conserved region in rice (Manzanares, 2013; Manzanares unpublished) were also screened. Markers were tested for polymorphism in a small sample of the population. Fifteen new polymorphic markers were used to genotype the recombinants.

A marker order was first obtained using the software OneMap with the genotypic information from all recombinants. Initially, a map was constructed using a two-point algorithm. The map was then optimized by selecting a sample of equally spaced markers to build a frame based on the multipoint likelihood of all possible orders between them and choosing the most likely one. The remaining markers were added one at a time based on LOD scores at different positions. The whole process is automated in the function *order.seq* and *try.seq*. The procedure was repeated using four different ordination algorithms to obtain the set of markers for the initial frame: Rapid Chain Delineation (Doerge, 1996), Seriation (Buetow and Chakravarti, 1987), Recombination Counting and Ordering (Van Os et al., 2005), and Unidirectional Growth (Tan and Fu, 2006). The ordering coincidence between

the different methods was used as an indication of the right order and any conflicting markers, those assigned to different positions depending on the method, were eliminated. A similar process was used to obtain a second marker order which included the SF phenotype as marker. For the latter, only information from those recombinants that could be phenotyped were used. Recombination frequencies between adjacent markers were obtained via the expectation maximization (EM) algorithm with the *rf.2pts* function in Onemap based on the total population size. Recombination frequencies between markers and the *SF* locus were obtained by estimating the number of recombinant gametes in the 210 recombinants based on the recombination frequency in the 113 phenotyped plants and then dividing by the total number of gametes in the 1248 plants as follows:

$$rf_{1248} = (rf_{113} \times \text{total gametes}_{210}) / \text{total gametes}_{1248}$$

where rf_{1248} is the estimated recombination frequency in the whole population and rf_{113} is the recombination frequency in the 113 phenotyped plants.

The recombination frequencies were then used to calculate the map distances based on Kosambi mapping function:

$$d = \left(\frac{1}{4}\right) \ln\left(\frac{1+2r}{1-2r}\right)$$

where *d* is the mapping distance and *r* is the recombination frequency (Kosambi, 1943).

Results

Fine mapping - Phase I

Genotypic segregation

The molecular markers were assigned to two different linkage groups except for the *S* locus (05_02911) and *Z* locus (LpGK1) markers that segregated independently from each other and from the other two linkage groups as was expected and were manually assigned to linkage groups 1 and 2, respectively (Figure 1).

Two markers in LG 5 (G01_045 and G03_096) had one of the homozygous classes under-represented, while for the other four markers the allele from the self-incompatible parent was totally missing. For these four markers, segregation between the other homozygous and the heterozygous genotype did not differ from a 1:1 ratio ($P = 0.05$). For the *S* locus one of the homozygous and the heterozygous class segregated at a 1:1 ratio, while the other homozygous class was missing. For the *Z* locus, all three genotype classes were present, but one of the homozygous classes was under-represented and segregation deviated significantly from a Mendelian ratio ($P \leq 0.01$). In LG 3, markers G05_090, G07_071, and G04_098, did not significantly differ from a 1:2:1 ratio or 3:1 (G07_058) ratio ($P \leq 0.05$). Marker G02_079 in LG 3 was underrepresented for one of the homozygous classes, while the other two classes segregated at a 1:1 ratio ($P \leq 0.05$).

Phenotypic segregation

The phenotypic segregation of *SF* showed a bimodal distribution with no overlap between both groups. The first group consisted of 55 plants ranging from 31% to 63% compatibility with a mean compatibility of 49%. The second group included 39 plants with 78% to 100% compatibility with a mean of 92% (Figure 2). The bimodal distribution indicates a qualitative mode of gene action.

Under the hypothesis of a single gametophytic gene causing *SF*, only the heterozygote SF_{IF} and the homozygote SF_{FF} (SF_I is the incompatible allele and SF_F is the self-fertile) are expected in the F_2 generation at a 1:1 ratio, resulting in the expected 50% and 100% pollen compatibility classes. The chi-square test of goodness of fit showed no significant differences between observed and expected segregation ratios (Table 1).

The ANOVA analysis showed that the effect of a single *SF* gene with the homozygous for the self-fertile allele producing fully compatible phenotypes (100% compatibility) and the heterozygous producing partial compatible phenotypes (50% compatibility) was highly significant ($P \leq 0.0001$). The model explained most of the observed phenotypic variation ($R^2 = 0.937$).

Marker-trait associations

The chi-square test of independence for marker-trait associations showed highly significant p-values for all markers in LG 5 indicating a strong co-segregation of markers in this region with the tentative *SF* locus. All markers in LG 3 as well as the *S* and *Z* locus

segregated independently from the compatibility groups indicating no major effect on the phenotype ($P \leq 0.05$) (Table 2).

Within the 50% compatibility group, the phenotypic means of the different genotypes at *S*, *Z,* and markers at LG 3 were very similar. The analysis of variance showed that there was no significant effect of the genotype at these loci on the level of pollen compatibility ($P = 0.4369$). The same was true for the 100% compatibility group ($P=0.3419$).

Assuming a single gene with qualitative action, SF was included as a marker and a linkage analysis was performed to find a tentative position. The *SF* locus was mapped in LG 5, 4.3 cM distal from marker G05_065 and 15.9 cM proximal to marker G03_096 (Figure 3).

Fine mapping - Phase II

The remaining 1154 plants of the population were genotyped with the flanking markers identified in *Phase I* (G05_065 and G03_096). A total of 210 recombinant individuals were found. From these, 85 plants did not flower and 12 failed to shed pollen and could not be phenotyped. The remaining 113 plants were phenotyped, 59 of them were assigned to the 50 % compatibility group and 54 to the 100 % pollen compatibility group (Table 3). The observed segregation was not significantly different from the expected 1:1 segregation ratio ($P \leq 0.05$).

Two linkage maps were created, the first one with the 210 recombinants and the second using only the 113 plants that were genotyped and phenotyped. Marker orders were compared and found to be identical. A third map was created with the 133 phenotyped plants but this time with the *SF* phenotype as a marker. *SF* was located

between markers G05_065 and G06_096 without changing the order of markers. Recombination fractions between markers were obtained based on 1248 plants (Table 4). Genetic distances where converted to mapping distances using Kosambi mapping function. The 18 markers, including the *SF* locus covered a region of 19 cM. A densely covered region of 4.9 cM between markers 12_19535 and rg1_012d_d09 surrounds the *SF* locus, with 13 markers at an average distance of 0.38 cM. The *SF* locus maps within a 1.6 cM region, being 0.61 cM away from marker G05_065 to one side and 1.0 cM from G06_096 to the other side.

A BLAST search on the plants databases of the PGSB Plant Genome and Systems Biology (http://pgsb.helmholtz-muenchen.de/plant/search.jsp) using the *Oryza sativa* RGAPv7 CDS.fa dataset, indicated that marker G05_065 sequence has a 389 bp alignment with 88% identity with rice locus *LOC_Os12g37415.1*, while marker G06_096 has a 354 bp alignment with 84% identity with locus *LOC_Os12g35550.2* both located on chromosome 12, 1.35 Mbp apart from each other. The same search was repeated using the *Brachypodium distachium* MIPS_V1.2_CDS.fa dataset. Marker G05_065 has 398 bp alignment to locus *Bradi4g04720.1* located on *Brachypodium* chromosome 4 with 93% identity, and marker G06_096 has a 377 bp match with *Bradi4g05590.1* at 87% identity also on chromosome 4, the distance between them being 807 kbp.

The *B. distachium* physical interval between both loci was used to search for annotated genes in Gramene database release 52 (Gupta et al., 2016) (http://www.gramene.org). A total of 89 protein coding genes are located within this region. Among them, a gene coding a Leucine-rich repeat domain (*LRR*) protein with

medium to high expression in anthers (533 FPKM) and low or no expression on other parts of the plant was found. Another gene, coding a universal stress protein A (*Usp A*), is expressed only in anthers. A different gene, with a medium-high expression in the pistil (474 FPKM) but low or no expression in other organs, codes a protein with a Thaumatin family domain. Another one codes a basic-leucine zipper domain (*bZIP*) transcription factor with medium-high levels of expression in anthers, pistils and other reproductive organs. Other genes with no organ-specific expression are: an integral component of membrane with a domain of unknown function (*DUF4149)*, a universal stress protein (*USP*), and a Thioredoxin domain protein.

Discussion

Our results revealed that *SF* in the fine mapping population is caused by a single locus. Only markers on LG 5 were significantly associated with the trait indicating no major effects of genes outside this region.

According to their phenotypes, the plants were separated in two clearly differentiated groups and the ratio of plants in each of these groups was not different from a 1:1 ratio. A single gene model accounted for almost all the variability. The single gene mode of action is in agreement with previous studies in perennial ryegrass (Thorogood and Hayward, 1991; Thorogood et al., 2005; Arias-Aguirre et al., 2013; Manzanares, 2013), *Phalaris coerulescens* Desf. (Hayman and Richter, 1992), and rye (Voylokov et al., 1993).

Thorogood et al. (2005) mapped a *SF* mutation in LG 5 (*T* locus) and found an interaction with a *SF* mutation in the *S* locus, the latter coming from a different parental

source. However the *T* locus was not tested independently from the *S* locus mutation and thus, interactions with functional *S* alleles are not known. In our population, the 1:1 segregation of *S* locus genotypes with one of the homozygous classes completely missing was intriguing. However the segregation was completely independent from the SF segregation and thus, other factors are responsible for it such as gamete selection against this particular allele in the female side.

A quantitative interaction with alleles at other loci was never tested before, since all previous works used a discrete classification of phenotypes into compatibility classes, which eliminates any possible variation on pollen compatibility due to quantitative effects. The genotypes at *S* and *Z* loci did not affect the mean pollen compatibility at any of the two phenotype groups, and the same was true for markers in LG 3. Thus, for the alleles present in our population, interactions can be disregarded. Our results are consistent with the hypothesis of a single gene acting gametophytically and epistatic over *S* and *Z*, where a pollen grain carrying the *SF* mutation is able to overcome the SI barrier no matter what *S-Z* allele combination is present (Arias-Aguirre et al., 2013; Do Canto et al., 2016).

We were able to fine map the position of the *SF* locus to a 1.6 cM region between markers G05_065 and G06_096. These two markers are within the interval found to be significantly associated with SF by Arias-Aguirre et al. (2013). Our two-step approach and phenotyping only the recombinants allowed us to use a large population size, which in turn resulted in a higher precision and the possibility to map markers that are less than 0.5 cM apart.

It was not possible to compare our mapping results with those of Thorogood et al. (2005) because there are no markers in common between the two experiments. However there are some interesting similarities to the map obtained by Manzanares (2013) using a different population. Three markers are common in these two experiments: G05_065, G06_096, and 12_19937. Markers G05_065 and G06_096 mapped side by side within the *T* locus region (analogous to our *SF* locus) along with three other markers, while marker 12_19937 is one of the flanking markers of the *T* locus region. In our population, marker 12_19937 also mapped very close to marker G06_096, and just 1.57 cM away from the *SF* locus. Three of the markers employed by Manzanares (2013) were not polymorphic or unspecific in our population: G01_095, PTA.2547.C1, and 12_21248 (data not shown), while the opposite was true for markers rg1_012d_d09, 12_22211, and 12_23502. The difference in polymorphism for these six markers is consistent with the different genetic backgrounds of the two populations. The three markers linked to the *SF* gene in both populations indicate that *T* and *SF* are the same locus, and that it can be functional in different genetic backgrounds.

It remains to be answered whether there is only a single conserved *SF* locus on LG 5 in perennial ryegrass and other grasses or if different *SF* loci are present in different species and populations. An indirect comparison can be performed by aligning the sequences of the markers linked to the *T* locus of rye (Egorova et al., 2000), *P. coerulescens* (Hayman and Richter, 1992) and perennial ryegrass (Thorogood et al., 2005; Manzanares, 2013) to the sequenced genomes of rice and *B. distachium.* It can reveal if they map in the same or

different physical region, indicating the likelihood of being a single conserved locus or different loci.

Alignment of the *SF* locus region to the physical maps of rice and *B. distachium* provided an estimation of the physical distance. These regions are still large and have a large number of genes within them making it difficult to identify candidate genes. However, in *B. distachium* a few of them are mainly expressed in the anthers or in the stigmas. The *LLR*-domain protein coding gene, even though it has no biologycal process or cellular component predicted, *LRR*-domains seem to facilitate the formation of protein-protein interactions and are involved in a range of biological processes. This includes signal transduction, cell adhesion, disease resistance, apoptosis, and the immune response (Rothberg et al 1990), all of which can potentially be involved in the inhibition of self-pollen tube growth in the stigma. The *UspA* genes are predicted to be related to the MADS-box proteins and bind to DNA. Transcriptional induction of the *UspA* gene of *Escherichia coli* occurs when conditions cause growth arrest, while *in vitro*, UspA undergoes autophosphorylation (Freestone et al., 1997). Phosphorylation is involved in the signaling cascade of the SI reaction in the Papaver SI system (Takayama and Isogai, 2005), and notably, an increase in phosphorylation activity in pollen was found in incompatible pollinations in rye (Wehling et al., 1994). The *Thaumatin* family domain gene expressed only in the pistil, also known as pathogenesis-related group 5, is involved in acquiring resistance and stress response, and accumulate in plants in response to infections (Ruiz-Medrano et al., 1992). A similar response may be triggered in the stigma to block the pollen tube growth in incompatible pollinations. The *bZIP* domain proteins are sequence specific DNA-binding

proteins and are involved in different biological processes (Hurst, 1994). The *bZIP* gene product has probably a more general role during flowering and embryo development, since it was expressed in different organs.

Next steps to target the gene include identification of molecular markers between the flanking markers identified in our population. The sequences of rice and *B. distachium* syntenic to the ryegrass *SF* locus are good candidates for microsatellite and SNP identification. In our population 23 plants have a recombination between the flanking markers. This group of plants can be used to identify recombinations and further reduce the target region. Sequencing and cloning approaches can be applied next. Specific polymorphisms within this region could also be assessed by Genotyping-by-Sequencing (GBS) technology (Elshire et al., 2011; Poland and Rife, 2012) in order to find the specific region responsible for the trait variation.

With flanking markers at a short distance, introgression of the *SF* gene into breeding populations of perennial ryegrass and related species can be made in a more efficient way using marker assisted selection and backcrossing. The flanking markers identified in our experiment are 1.6 cM distant from each other making double recombination events between them and misclassification of genotypes very infrequent. It also allows selection of genomes that have recombination events close to the target gene and hence reduce linkage drag (Frisch et al., 1991a, b; Ribaut and Hoisington, 1998).

Conclusions

Our results are a step forward towards the identification of the *SF* gene in LG5. Interaction with other loci were discarded suggesting a major role of this gene in the completion of the SI response when the non-mutant allele is present. Future work may include sequencing technologies to find the specific polymorphism causing the breakdown of the SI mechanism as well as gene expression and function analysis that will ultimately contribute to figure the genetic and biochemical pathways behind SI and SF in grasses.

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Table 1. Phenotypic segregation in the initial set of 94 plants and chi-square test of goodness of fit under a 1:1 expected segregation ratio.

Plants with 50% pollen compatibility	Plants with 100% pollen compatibility	χ^2 for a 1:1 segregation ratio	DF	P-value	Significance
55	39	2.723	1	0.099	ns

Table 2. Marker-trait association analysis by chi-square test of independence in phase I of the experiment.

Molecular marker	LG	DF	X^2	P-value	Marker-trait segregation
05_02863[a] (*S* locus)	1	1	0.001	0.97354	independent
LpGK1[b] (*Z* locus)	2	2	0.077	0.99621	independent
G04_098[c]	3	2	0.546	0.76117	independent
G03_089[c]	3	1	0.367	0.54442	independent
G02_079[c]	3	2	0.346	0.84105	independent
G05_90[c]	3	2	0.195	0.90728	independent
G07_071[c]	3	2	2.427	0.29711	independent
G01_045[c]	5	2	29.32	< 0.00001	not independent
G05_071[c]	5	1	37.19	< 0.00001	not independent
G03_052[c]	5	1	48.24	< 0.00001	not independent
G06_096[c]	5	1	63.93	< 0.00001	not independent
G05_065[c]	5	1	63.93	< 0.00001	not independent
G03_096[c]	5	2	24.61	< 0.00001	not independent

[a] Manzanares et al (2016); [b] Studer et al. unpublished; [c] Studer et al (2010).

Table 3. Phenotypic segregation within the recombinants and chi-square test of goodness of fit under a 1:1 expected segregation ratio.

Plants with 50% pollen compatibility	Plants with 100% pollen compatibility	Total	χ^2 for a 1:1 segregation ratio	DF	P-value	Significance
59	54	113	0.221	1	0.638	ns

Table 4. Marker order, recombination fractions, and distances. The marker order was based on 210 recombinant plants. Recombination frequencies and distances calculations were based on 1248 plants.

Marker order	Recombination fraction	Genetic distances in cM	Two-point distances to the *SF* locus[a]	Map distances[f]
G03_096[b]	-	-	8.41	0
12_19535[d]	0.0790	7.90	1.57	8.0
12_22211[c]	0.0089	0.89	0.76	8.9
12_23502[c]	0.0032	0.32	0.76	9.2
12_23149[d]	0.0008	0.08	0.68	9.3
12_22975[d]	0.0016	0.16	0.61	9.4
G05_065	0.0016	0.16	0.61	9.6
SF locus	0.0061[a]	0.61	0.00	10.2
G06_096[b]	0.0100[a]	1.00	1.00	11.2
PTA.1433.c1[e]	0.0064	0.64	1.57	11.8
12_19937[c]	0.0012	0.12	1.57	11.9
12_18921[d]	0.0012	0.12	1.73	12.1
rg3_007a_d05[e]	0.0052	0.52	2.50	12.6
PTA.133.c1[e]	0.0024	0.24	2.32	12.8
rg1_012d_d09[e]	0.0008	0.08	2.32	12.9
PTA.280.c1[e]	0.0506	5.06	7.30	18.0
PTA.218.c1[e]	0.0089	0.89	7.92	18.9
Ve_005b_h05[e]	0.0008	0.08	7.92	19.0

[a] Estimated from 113 recombinants; [b] Flanking markers from *Phase I*; [c] Manzanares, 2013; [d] Manzanares unpublished; [e] Studer et al (2012). [f] Map distances based on Kosambi mapping function.

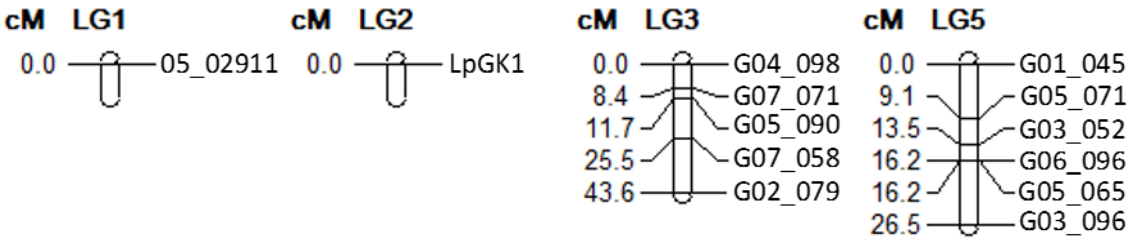

Figure 1. Map location of markers used in *Phase I*.

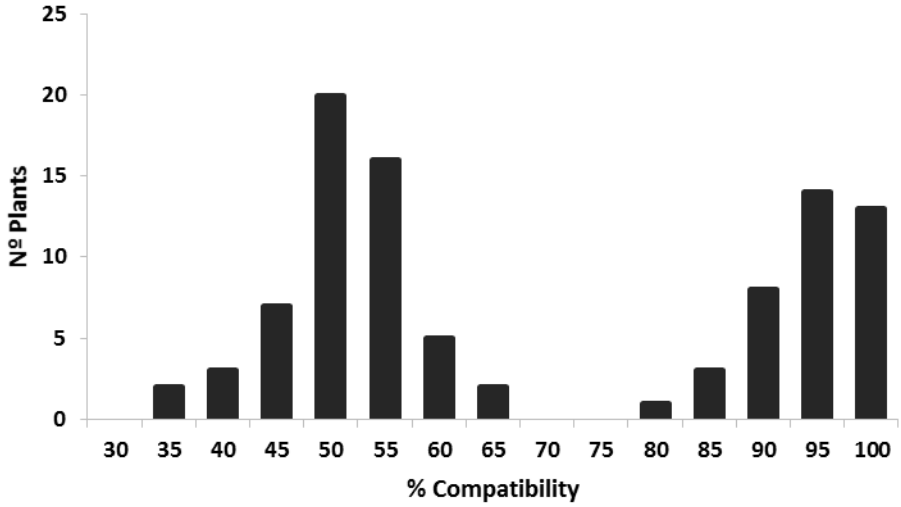

Figure 2. Phenotypic segregation in the initial set of 94 plants of the mapping population.

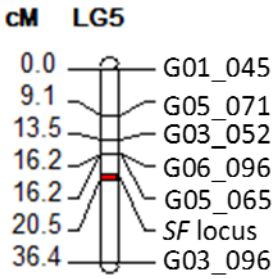

Figure 3. Linkage map of LG 5 including *SF* as a marker showing the position of the *SF* locus and the flanking markers.

CHAPTER 4

MODE OF GENE ACTION OF A SELF-FERTILITY GENE IN AUTOTETRAPLOID PERENNIAL RYEGRASS

A paper in preparation to be submitted for publication to Heredity.

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Abstract

Many of the agronomically important perennial grass species are polyploids. It has been shown that self-incompatibility (SI) in grasses is not impaired after chromosome doubling and a strong SI mechanism is maintained. However, it is not known whether self-fertility (*SF*) mutations would remain functional at the tetraploid level. The aim of this work was to determine a) whether *SF* remains functional in a tetraploid perennial ryegrass (*Lolium perenne* L.) population; b) if the *SF* mutation expresses dominance in heterozygous pollen grains; and c) how this gene segregates at the tetraploid level. An F_2 population was developed by self-pollination of a tetraploid F_1 plant. The F_2 individuals had mean levels of pollen compatibility between 38% and 84% showing that *SF* remained functional in this particular population. The *SF* locus genotype was the main determinant of pollen compatibility explaining 54% of the variation. The observed segregation was significantly different from the segregation models under both SF_F dominant and recessive ($P_{(\chi 2)} \leq 0.001$), and tended to be intermediate between them, indicating incomplete dominance.

The frequency of the different genotypes also suggested that SF_{FF} pollen grains have a competitive advantage over the SF_{IF} ones. The type of tetrasomic inheritance for this locus could not be discerned from our data. The implications of our results for breeding polyploid grasses are discussed.

Introduction

Polyploidy in the grass family is prevalent and variation in ploidy level often occurs at both the intra-genera and intra-species levels (Keeker, 1998). It encompasses many of the agronomically important perennial grass species including tall fescue (*Festuca arindinacea*, 2n = 6x = 42), bromegrass (*Bromus inermis*, 2n = 8x = 56), orchard grass (*Dactylis glomerata*, 2n = 4x = 28) and switchgrass (*Panicum virgatum*, 2n = 4X – 8X = 36 - 72) among others.

Polyploidy is one of the main mechanisms of speciation in plants, which can provide advantages over the diploid parents allowing a more successful adaptation to new or changing environments (Comai, 2005; Van de Peer et al., 2009; Sattler et al., 2016). Increased heterozygosity results in an increase in vigor when compared to diploid parents (Gallais, 2003; Comai, 2005; Sattler et al, 2016). This is also enhanced in allopolyploids by allele complementarity between homeologues (Paterson, 2005). Gene redundancy results in masking deleterious alleles by one or multiple copies of wild type alleles. This also happens in diploid gametes of autotetraploid species, where otherwise deleterious alleles are exposed. Transgressive segregation is often observed and may be caused by increase in heterozygosity or allele dosage provoking an increase in gene expression, which may

explain the appearance of extreme phenotypes (Paterson, 2005; Van de Peer et al., 2009). Not surprisingly, chromosome doubling by artificial means is one of the tools employed by breeders to create improved varieties, which often show an increase in organ size known as the “gigas” effect caused by an increase in cell size which in turn is provoked by a larger gene copy number (Satter et al, 2016). Good examples are tetraploid perennial ryegrass (*Lolium perenne,* 2n = 4x = 28), tetraploid Italian ryegrass (*Lolium multiflorum,* 2n = 4x = 28), and tetraploid meadow fescue (*Festuca pratensis*), where cultivars of artificially produced tetraploids are commercialized.

The breeding methods employed for cultivar development in outcrossing polyploids do not differ from diploids: synthetic varieties are the preferred type of cultivar (Posselt, 2010). However, gene segregation differs from diploids. In strict allopolyploids, preferential pairing between homologous chromosomes resembles diploid behavior. In diploids there is an increase in homozygosity with every selfing generation. In allopolyploids the same is true within homologous chromosomes but different alleles may be fixed in homeologous chromosomes. Thus, homeologous “heterozygosity” becomes fixed (Levy and Feldman, 2002; Comai, 2005; Satter et al., 2016).

In contrast, in autotetraploids quadrivalents are formed (multisomic inheritance) and chromosomes segregate randomly (Muller, 1914). Moreover, 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). Sister chromatids may end up in the same gamete, thus the homologous chromosomes in a gamete can be identical. Once crossing-over occurred, sister chromatids become attached to different centromeres and during the

first division, any of the homologous chromosomes can migrate to the same pole. Thus two sister chromatids, now attached to different centromeres, may migrate to the same pole at first division (first reduction). At second division, depending on the orientation of the chromosomes, the two sister chromatids may separate to the same gamete cell (second reduction). This is known as double-reduction and the two chromosomes in a gamete are the same, increasing the expected fraction of homozygous gametes (Haldane, 1930; Mather, 1935, 1936). However, even in strict autotetraploids not all chromosomes join in quadrivalents, some will pair forming bivalents. This reduces the number of chiasmata compared to quadrivalents and no double reduction occurs. The meiotic configuration in bivalents and quadrivalents can vary even within the same species. Ahloowalia (1969) found 2II (bivalents) + 6IV (quadrivalents), 4II + 5IV, 6II + 4IV and 8II + 3IV chromosome associations in tetraploid perennial ryegrass. Altogether, gamete frequencies in autopolyploids will vary depending on which type of configuration the chromosomes form in meiosis I, and the distance of the locus to the centromere.

An interesting feature of grasses is that self-incompatibility (SI) is not impaired after chromosome doubling and many of the polyploid grasses have a strong SI. 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 the diploid level, some mutations can mask the SI mechanism and plants can become fully self-fertile. One of these mutations is a self-fertility (*SF*) locus on linkage group 5 (LG5) in perennial ryegrass. Half of the pollen grains from a heterozygous mutant are

compatible in self-pollinations and half remain incompatible, while pollen from homozygous plants for the mutation are 100% compatible. The offspring from self-pollination of a heterozygous plant segregates in a 1:1 ratio into partial and full pollen-compatible plants (Thorogood et al., 2005; Arias-Aguirre et al., 2013). However, it is not known whether SF would remain functional at the tetraploid level and no attempts have been made to understand the mode of action of the *SF* gene in tetraploids. For instance, the effect of such a mutation could be suppressed or compensated by a larger gene copy of incompatibility alleles at other loci, or eventually, allelic relationships at the *SF* locus in the diploid pollen could modify the expectation of pollen compatibility percentages.

The overall objective of this study was to improve the understanding of *SF* in perennial ryegrass at the tetraploid level. Specific objectives were to determine a) whether SF remains functional in a tetraploid perennial ryegrass population; b) if the *SF* mutation expresses dominance in heterozygous pollen grains; and c) how this gene segregates at the tetraploid level.

Materials and methods

The genotypic segregation in a tetraploid F_2 population was used to infer the mode of action of the *SF* gene at the tetraploid level. Genotypic segregation is affected by allelic dominance relationships in diploid pollen as well as gamete frequencies at this particular locus under tetrasomic inheritance.

Plant materials and chromosome doubling

A diploid self-fertile plant heterozygous for the *SF* locus on LG5 was selected. This is an F_1 plant from a cross between a self-fertile plant (Arias-Aguirre et al., 2013) and a self-incompatible plant from the VrnA mapping population (Jensen et al., 2005). This is the same F_1 plant that produced the *SF* mapping population described in the previous chapter as well as the mapping population of Arias-Aguirre et al (2013). This plant 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*. Alleles for *S* and *Z* were arbitrarily designated as S_1, S_2, Z_3, and Z_4.

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 genome 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. 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. DNA content of the isolated nuclei was measured by flow cytometry at the Flow Cytometry Facility at Iowa State University using a BD FACSAria^{TM} 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, panicles 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 based DNA extraction protocol was followed to obtain DNA from the tetraploid F_2 population, the tetraploid F_1 plant and from diploid F2 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 markers linked to the *SF* locus as well as markers linked to the SI loci *S* and *Z*, on linkage groups 1 and 2 respectively (Table 1), using High Resolution Melting analysis (HRM). PCR reaction mix containing 0.2 mM of dNTPs, 25 mM of magnesium chloride, 20 to 30 µM of each forward and reverse primers depending on the primer set, 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 60ºC to 98ºC.

Diploid plants with known marker genotypes were used as controls. For a particular locus, homozygous diploids for either allele were used as controls for the nulliplex and quadruplex genotypes since they produce similar curves in HRM (Table 2). A heterozygous diploid genotype was used as control for the duplex. For the simplex and the triplex, the controls were created by mixing DNA of a heterozygous and a homozygote for either allele in a 1:1 ratio. Due to segregation distortion in the diploid population, no homozygous self-incompatible plant was found for the *SF* locus. Thus, there was no control for the nulliplex (SF_{IIII}), and a control for the simplex (SF_{IIIF}) could not be created. The same situation occurred for the *S* locus where one of the homozygous genotypes is missing in the diploid F_2

population, and thus, there were no controls for the nulliplex (S_{2222}) and the simplex (S_{1222}) genotypes.

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 tetraploid F_1 plant, except for the vernalization period, which was extended to eight weeks to improve induction. Before anthesis, panicles were covered with paper bags to avoid pollen contamination and produce self-pollinated seed.

Plants that flowered were phenotyped using an *in vitro* pollination test (Lundqvist, 1961) and fluorescence microscopy (Kho and Baer, 1968). From each individual, five unpollinated pistils were dissected from spikelets at anthesis and planted in 47 mm petri dishes containing growing media composed of 2% agar, 10% sucrose, and 100 ppm Boric acid. Stigmas were pollinated with fresh pollen from the same plant and left for two hours at 25°C to allow compatible pollen tubes to grow and incompatible pollen tubes to get inhibited. A few drops of a staining solution consisting of 0.2% aniline blue in 2% K_3PO_4 were applied over the pistils. The ovaries were then separated from the pistils and the stigmas placed on microscope slides with another drop of the staining solution. 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, take a bright color and a short and thickened pollen tube can be observed. Compatible pollen grains are translucent and pollen tubes can 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 3):

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.

c) Incomplete dominance: partial dominance results in either late inhibition or slow pollen tube growth. 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 for different types of segregation under tetrasomic inheritance were estimated (Table 5), which account for differences in chromosome pairing at meiosis I, crossing-over and the expected amount of double reduction. The different segregation types considered were:

I) Random chromosome segregation (RC) (Muller, 1914), pairing takes place between any two of the four homologous chromosomes but no crossing over is considered. For a duplex genotype (AAaa), the possible gametes are: 1 AA : 4 Aa : 1 aa.

II) Maximum equational segregation (ME) (Mather, 1936), 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. This is known as double-reduction and is symbolized by α, and when maximum equational segregation occurs α = 1/6. Thus, the expected gamete frequencies are calculated as follows:

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

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

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

This model accounts for the maximum amount of double reduction assuming random separation of chromosomes.

III) Prediction from meiotic configurations (MC) (Jackson and Jackson, 1996), the maximum chiasmata depends on the meiotic configuration of chromosomes at meiosis. Gamete genotypes frequencies are calculated based on the proportion of bivalents (II) and chain (cIV) and ring quadrivalents (oIV) formed and the maximum chiasmata on each of these configurations. Based on the observations of Ahloowalia (1967), the 6 II + 2 cIV + 2 oIV configuration was selected for the calculations. Gamete frequencies for each configuration were obtained from Jackson and Jackson (1996) and prorated by the relative contribution of each chromosome configuration (Table 4). Expected frequencies from this method are intermediate between Random Chromosome and Maximum Equational segregations.

Based on the gamete frequencies expected under the different segregation types and pollen compatibility under the different dominance models, the expected genotype frequencies in the resulting tetraploid F_2 population were derived (Table 6).

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.

Analysis of variance was performed using the GLM procedure implemented in SAS® software version 9.4 (SAS Institute Inc., Cary, NC.) to test the effect of the *SF* genotype as well as the interactions with *S* and *Z* on the percentage of pollen compatibility. Mean comparisons were done using least square means with Tukey-Kramer adjustment for multiple comparisons (SAS Institute Inc., 2013).

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 (Figure 1). Individual tillers of those plants were labeled and reanalyzed to prevent having mixoploids, which is a common phenomenon in chromosome doubled individuals. A single tetraploid tiller, now the F_1 tetraploid plant (FTF_1), was selected for producing a 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 polymorphic markers linked to the *SF* locus and the SI loci *S* and *Z*. For the *S* locus, only three genotype classes appeared. The

diploid controls allowed us to identify them as quadruplex (S_{1111}), triplex (S_{1112}) and duplex (S_{1122}) with 18, 12, and 47 individuals, respectively (Figure 2A). Both the nulliplex (S_{2222}) and simplex (S_{1222}) were absent. The *Z* locus in turn had the 5 expected genotype classes (Figure 2B). The controls allowed identification of each of the genotype classes: 6 Z_{3333}, 14 Z_{3334}, 28 Z_{3344}, 16 $Z_{3444,}$ and 13 Z_{4444}. At the *SF* locus four genotype classes appeared (Figure 2C). 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 visual comparison between the observed *SF* genotype segregation and the expected under the different models is shown in Figure 3. The SF_{IIIF} genotype is not expected if the SF_F allele is recessive. The most abundant genotype expected in the recessive models is SF_{IFFF}, while SF_{IIFF} is most frequent in the dominant ones. The most important difference between the models is due to allele dominance relationships rather than tetrasomic segregation types. The differences between the types of tetrasomic segregation within dominance models were minor for the population size employed. The most abundant observed genotype is SF_{IIFF}. The observed number of SF_{FFFF} is similar to the expected under SF_F recessive. The number of SF_{IFFF} is closer to the SF_F dominant models and the number of SF_{IIIF} genotypes is lower than the expected under SF_F dominant.

The lowest chi-square value obtained was 18.6 for SF_F allele being dominant under MC prediction and the highest was 102.6 for the dominant model and RC segregation. With a 95% probability the chi-square threshold values were 7.81 for the dominant models and 5.99 for the recessives. Chi-square values were lower for the recessive model under RC and ME segregations. The observed segregation was found to be significantly different from all the segregation models under both SF_F dominant and recessive (Table 7).

From the 77 plants only 32 were phenotyped since the rest did not flower. The most striking observation was that the SF_{FFFF} genotype is not 100% compatible (Table 8). The SF_{IFFF} in turn is very close to the 50% compatibility which would be expected if SF_F were recessive under RC segregation. The SF_{IIFF} compatibility is intermediate between the recessive and dominant SF_F models (22-56% expected in ME; 17-67% in RC). The compatibility of SF_{IIIF} is very high but is probably biased by the low number of observations.

Overall there is a medium positive correlation between the number of SF_F alleles and the percentage of compatible pollen grains.

When considering only the *SF* allele dosage, the analysis of variance showed that there is a significant effect of the number of SF_F alleles on the pollen compatibility ($Pr > F \leq 0.0001$), with the number of SF_F alleles explaining 53% of the variation ($R^2 = 0.53$). There is still a strong effect even after accounting for the *S* and *Z* genotypes which in turn are not significant after accounting for the *SF* genotype ($P \leq 0.05$). When interactions are included, SF_F allele number is still highly significant ($P = 0.0015$), and the genotype at the *Z* locus becomes significant ($P \leq 0.0001$). The *S* locus genotypes and their interaction with the *SF* genotypes are not significant ($P = 0.097$). However, the interaction between *SF* and *Z*

genotypes, the *S* and *Z* genotype interaction and the triple interaction are highly significant ($P \leq 0.0001$, $P = 0.0032$ and $P \leq 0.0001$ respectively). The model improves compared with the one without interactions (R^2=0.32) but is still lower than taking SF_F alleles alone.

Discussion

In gametophytic SI systems, the increase in ploidy level has been associated with the breakdown of the SI system. 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 the grass two-locus SI system, however, chromosome duplication does not impair the mechanism (Baumann et al., 2000) and such allele competition has not been reported. The mode of action is similar to the one at the diploid level and only one of the two alleles at both *S* and *Z* need to be matched in the stigma to inhibit the pollen tube growth (Fearon et al., 1984; Arias-Aguirre et al., 2014). While the stability of the SI system across ploidy levels has been reported, mutations conferring *SF* were only studied at the diploid level. As such, it was not known, whether gene duplication of a self-fertile genotype would result in a self-fertile tetraploid, or if allele dosage at the SI loci would be able to compensate the effect of the *SF* locus and restore SI. Our results show that SF stays functional in autotetraploid perennial ryegrass. It was possible not only to obtain selfed progeny but also the SF allele was transmitted to the offspring, which in turn showed variable levels of pollen compatibility.

In diploid pollen, dominance between alleles is possible and occurs at the *S* locus in different tetraploid dicots. For example, in *Oenothera organensis*, a dicot with S-RNase SI system, S_{46} pollen grains produce seed in an S_{2344} female, but not in an S_{2366} (Lewis, 1947; de Netancourt, 2001). In grasses it was unknown whether *SF* or SI would prevail with a heterozygous pollen grain. 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} genotype favors the SF_F dominant alternative. The SF_{IIFF} genotype is the most abundant also supporting the dominant mode of action. However, the observed number of SF_{FFFF} genotypes would only be possible if SF_{IF} pollen grains were incompatible but the frequency of the other genotypes differ greatly from the expected. 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 SF_F recessive indicating some degree of incomplete dominance. 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 noticed before 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 exhibit 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 phenotypes of the different *SF* genotypes showed that there are complex interactions between the *SF* locus, and the genetic background. 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. Still, all genotypes showed some degree of pollen compatibility, the minimum observed value was 18%. There is a medium but positive correlation between the number of SF_F alleles and the percentage of pollen compatibility. While in the diploids the *SF* locus has a clear qualitative mode of action, in the tetraploids it only explained 53% of the observed variability. Despite of this the *SF* genotype is still the main determinant of pollen compatibility. In sour cheery (*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.

The type of tetrasomic inheritance could not be deduced. For a particular dominance model, the difference between the expected proportions in genotypic classes of the different segregation types is low (i.e., from Table 6 the expected proportions of SF_{FFFF} under SF_F dominant are 3.3 for RC, 4.7 for MC, and 6.3 for ME) and a very large population would be needed. While a minimum population size of 32 individuals is required to recover

a single SF_{IIIF} individual with a 99% probability (Sedcole, 1977) and thus, in order to differentiate between dominance and recessive gene action, a population size of 810 plants would be necessary to discriminate between all tetrasomic segregation types (Bailey, 1961). However, since SF_F showed incomplete dominance it would be very difficult to discern between tetrasomic segregation types even in large populations because the expected ranges of the genotypic classes under incomplete dominance overlap between segregation types (see Table 6 and Figure 3). This absence of a clear cut segregation between phenotypes is one of the main obstacles in analyzing segregation in polyploids (Little, 1945).

A more descriptive statistic in autopolyploids is the index α that characterizes to which extent double reduction is affecting the data (Mather, 1936). 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, consequently both effects are confounded. The reciprocal cross eliminates the competition effect and assuming the rate of double reduction is the same in both gametophytes this would be the real double reduction value. The difference between the indexes of both crosses can be attributed to the competitive advantage of the SF_{FF} pollen grain.

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 in tetraploids the mutation load is higher but always decreases with increasing selfing rates, proving 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 deceases 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).

Conclusions

This is the first study that analyses the segregation and mode of action of a *SF* mutation in the *S-Z* loci SI system in autotetraploid grasses. An F_2 population was developed by self-pollination and the F_2 individuals had variable levels of pollen compatibility showing that SF remained functional in this particular perennial ryegrass tetraploid population. There was an important genetic background effect but the *SF* gene is still the main

determinant of self fertility. The alleles SF_I and SF_F in the pollen grain exhibited incomplete dominance, SF_{IF} was not fully inhibited but SF_{FF} has a competitive advantage over it. Our results are important for breeding polyploid grasses. The trait has the potential to be introgressed into related polyploid species with the purpose of purging deleterious alleles or inbred line development within breeding populations.

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Table 1. Molecular markers employed in the experiment.

Primer	Linked to	distance	Reference
12_23502	*SF* locus, LG5	0.8 cM	unpublished
05_02911	*S* locus, LG1	< 0.5 cM	Manzanares et al., 2015
LpGK2	*Z* locus, LG2	0 cM*	unpublished

* cosegregating with the *Z* locus in a mapping population of more than 10,000 individuals

Table 2. Controls used to determine the tetraploid genotypes by HRM analysis.

SF locus		*S* locus		*Z* locus	
diploid control	tetraploid genotype	diploid control	tetraploid genotype	diploid control	tetraploid genotype
SF_{FF}	SF_{FFFF}	S_{11}	S_{1111}	Z_{33}	Z_{3333}
SF_{II}	SF_{IIII}*	S_{22}	S_{2222}*	Z_{44}	Z_{4444}
SF_{IF}	SF_{IIFF}	S_{12}	S_{1122}	Z_{34}	Z_{3344}
SF_{II} + SF_{IF} mix	SF_{IIIF}*	S_{11} + S_{12} mix	S_{1112}	Z_{33} + Z_{34} mix	Z_{3334}
SF_{IF} + SF_{FF} mix	SF_{IFFF}	S_{22} + S_{12} mix	S_{1222}*	Z_{44} + Z_{34} mix	Z_{3444}

* No controls available

Table 3. Pollen from the tetraploid F_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 4. Gamete frequencies calculations based on meiotic configurations.

	II	cIV	oIV
Configurations per meiocyte	6	2	2
Chromosome frequency per meiocyte[a]	0.43	0.29	0.29

Gamete genotype frequencies (Jackson & Jackson, 1996)[b]

	II	cIV	oIV
AA	0.167	0.208	0.222
Aa	0.667	0.583	0.556
aa	0.167	0.208	0.222

Calculated gamete frequencies based on relative contributions of each configuration

	II	cIV	oIV	Total
AA	0.072	0.060	0.064	0.196
Aa	0.287	0.169	0.161	0.617
aa	0.072	0.060	0.064	0.196

[a] configurations per meiocyte X number of chromosomes in that configuration / total number of chromosomes (28). [b] Assumes a minimum of 1 chiasmata, and a maximum of 2 per II, 3 per cIV and 4 per oIV.

Table 5. Expected gametic frequencies for a given locus according to different segregation models.

Segregation type	*AA*	*Aa*	*aa*
RC	0.167	0.667	0.167
ME	0.222	0.556	0.222
MC	0.196	0.617	0.196

RC: random chromosome segregation; ME: maximum equational segregation; MC: meiotic configuration prediction.

Table 6. Expected *SF* locus genotypic proportions in the F_2 generation according to different models.

	Segregation type	SF_{FFFF}	SF_{IFFF}	SF_{IIFF}	SF_{IIIF}
SF_F recessive	RC	16.7	66.7	16.7	0
	ME	22.2	55.6	22.2	0
	MC	19.4	61.2	19.4	0
SF_F dominant	RC	3.3	26.7	56.7	13.3
	ME	6.3	31.7	46.0	15.9
	MC	4.7	29.4	51.1	14.7
Incomplete	RC	> 3.3; < 16.7	> 26.7; < 66.7	> 16.7; < 56.7	< 13.3
dominance	ME	> 6.3; < 22.2	> 31.7; < 55.6	> 22.2; < 46.0	< 15.9
	MC	> 4.7; < 19.4	> 29.4; < 61.2	> 19.4; < 51.1	< 14.7

RC: random chromosome segregation; ME: maximum equational segregation; MC: meiotic configuration prediction.

Table 7. Chi-square test of goodness of fit for different segregation models and allele relationships at the *SF* locus.

Segregation type	SF_F allele	χ^2	Degrees of Freedom	P-value
RC	recessive	42.5	2	< 0.00001
	dominant	102.6	3	< 0.00001
ME	recessive	19.4	2	0.00006
	dominant	42.7	3	< 0.00001
MC	recessive	68.2	2	< 0.00001
	dominant	18.6	3	0.00034

Table 8. Pollen compatibility for different *SF* genotypes and the correlation coefficient on the number of SF_F alleles and pollen compatibility. The number of plants of each genotype is given in brackets.

	SF_{FFFF}	SF_{IFFF}	SF_{IIFF}	SF_{IIIF}	Correlation
% compatibility	84 (5)	46 (12)	38 (13)	70 (2)	0.46

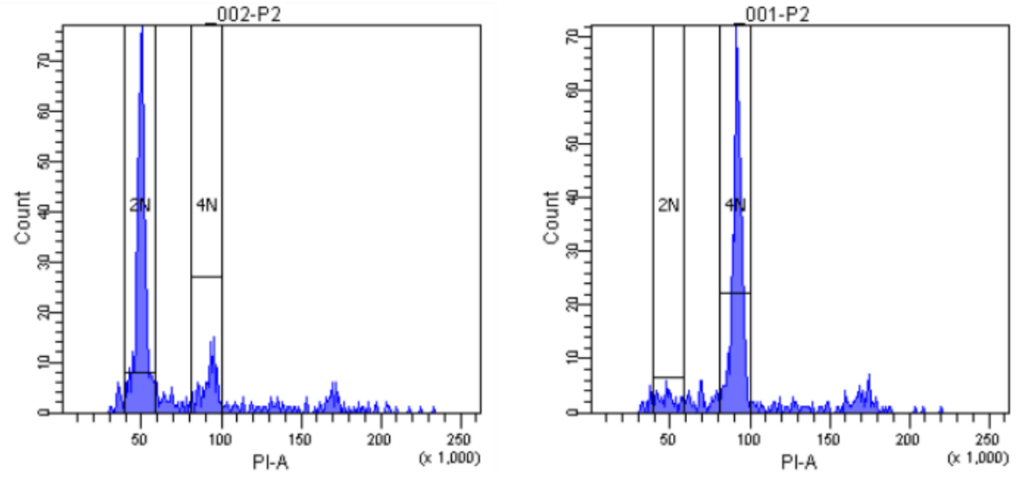

Figure 1. Flow cytometry histograms of distribution of cells with different propidium iodide fluorescence (PI-A) indicating differences in nuclei size. Left: a diploid control showing G0/G1 cells with a PI-A mean of 49.5; right: one of the tetraploid samples with a PI-A mean of 92.

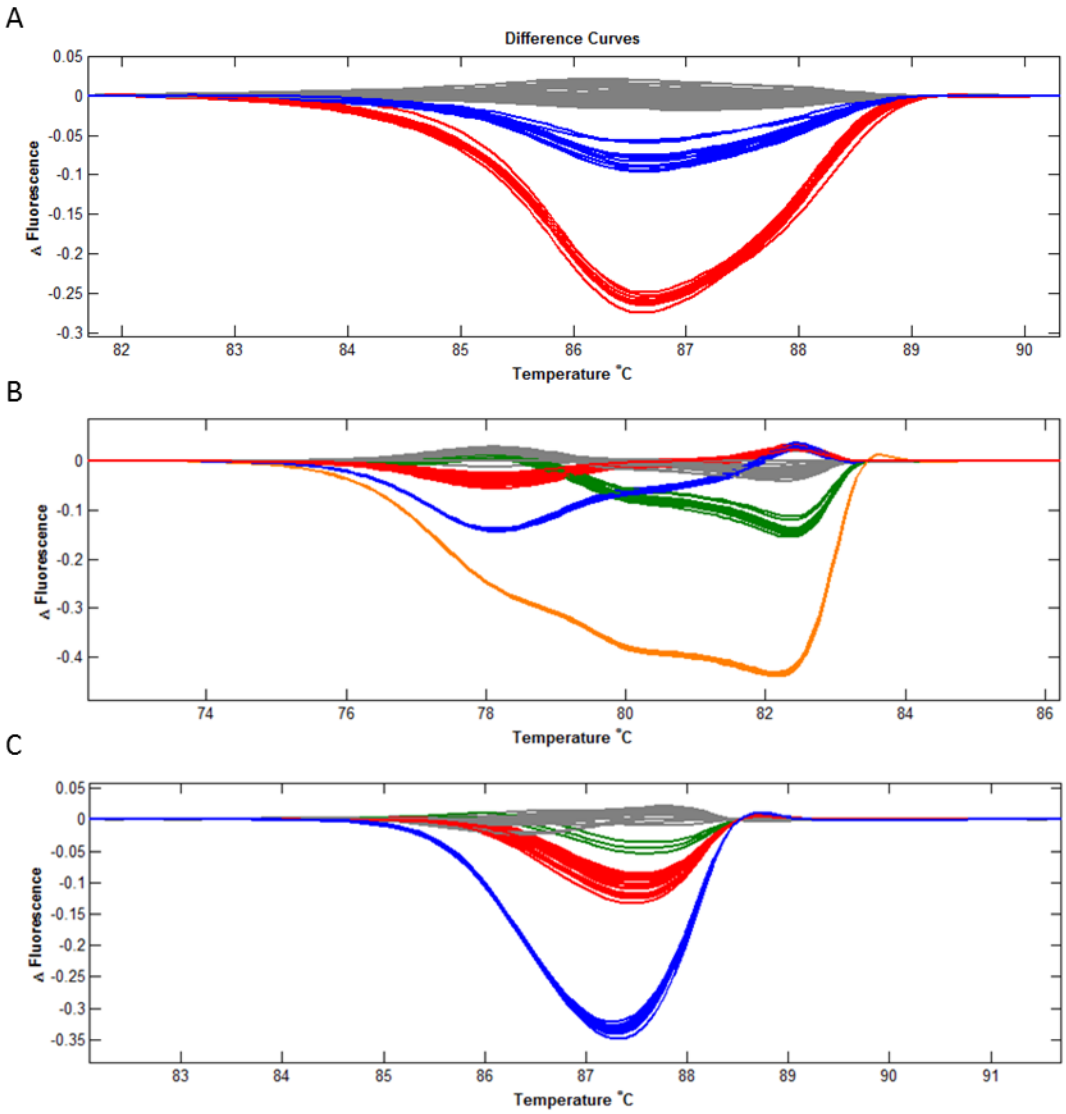

Figure 2. Difference fluorescence curves for *S, Z,* and *SF* loci markers. A) *S* locus, red curves: S_{1111} genotypes; blue curves: S_{1112} genotypes; gray curves: S_{1122} genotypes. B) *Z* locus, orange curves: Z_{3333}; green curves: Z_{3334} genotypes; gray curves: Z_{3344}; red curves: Z_{3444}; blue curves: Z_{4444}. C) *SF* locus, blue lines: SF_{FFFF} genotypes; red lines: SF_{IFFF} genotypes; gray curves: SF_{IIFF} genotypes; green curves: SF_{IIIF} genotypes.

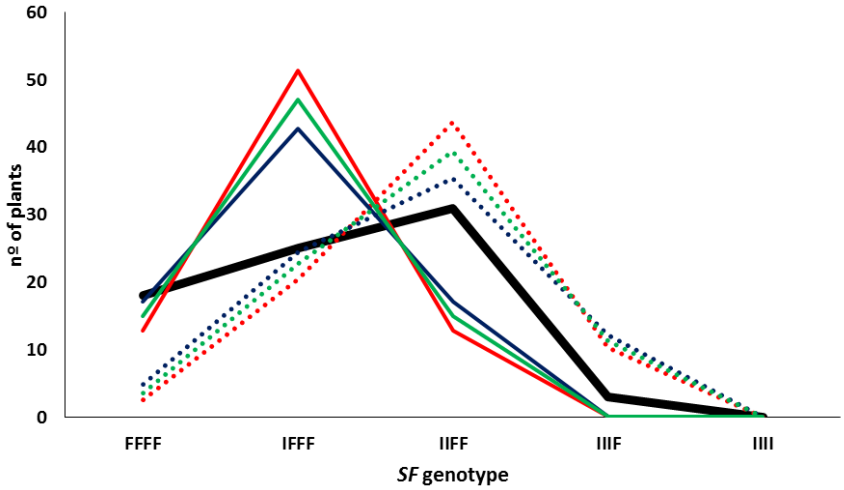

Figure 3. Number of observed and expected *SF* genotypes under different models. Black solid line: observed; solid colored lines: SF_F allele recessive; doted lines: SF_F allele dominant; red lines: RC segregation; blue lines: ME segregation; green lines: MC prediction.

CHAPTER 5

PROSPECTS FOR MIGRATING A SELF-FERTILITY LOCUS FROM PERENNIAL RYEGRASS TO RELATED SPECIES

Abstract

The prospects of migrating the *SF* locus from perennial ryegrass to other related self-incompatible and agronomically important species are discussed. Italian ryegrass (*Lolium multiflorum* Lam.), tall fescue (*Festuca arundinacea* Schreb.), and meadow fescue (*Festuca pratensis* Huds.) breeding programs would be benefitted with the incorporation of this trait. Hybridization and gene transfer to Italian ryegrass does not impose any constraints, hybrids are fertile and the Italian ryegrass genome can be recovered by backcrossing. For meadow fescue creating triploid hybrids and backcrossing to diploid meadow fescue is the more promising approach for incorporating small chromosome segments of perennial ryegrass and recover the meadow fescue genome. The best approach for introgression into tall fescue is by creating pentaploid hybrids and backcrossing to tall fescue, but it is unknown how the SF locus would behave in the alloploid background of tall fescue. Meiotic behavior and fertility of the hybrids and backcrosses are discussed.

Introduction

The festucoids are a large group of temperate grasses within the grass tribe Poeae that have morphological and molecular similarities. It comprises the *Lolium* and the large *Festuca* genera among others (Gaut et al., 2000; Catalan et al., 2004). Among them, a clade

known as broad-leaved contains four of the most important temperate forage grasses of the world: perennial ryegrass (*Lolium perenne* L.), Italian ryegrass (*Lolium multiflorum* Lam.), tall fescue (*Festuca arundinacea* Schreb.), and meadow fescue (*Festuca pratensis* Huds.).

Perennial ryegrass is cultivated as a forage, both for grazing and silage. Due to its exceptionally high quality and palatability it is highly valued in dairy and sheep production systems. It has fast establishment and good productivity. It is better adapted to mild and wet climates tolerating heavy and waterlogged soils, but is less persistent than other grasses especially under high temperatures or drought conditions. It is also used as turf and for conservation purposes (Hannaway et al., 1997; Humphreys et al., 2010). Italian ryegrass is highly palatable and has high digestibility and nutritional value, making it an attractive forage crop in dairy and other livestock production systems either for direct grazing, hay or silage. It typically has a faster establishment than perennial ryegrass. It is also used as a winter cover to prevent erosion and nitrate leaching, as a winter forage overseeded in perennial warm-season grasses swards, and in turf mixes for rapid ground coverage (Evers et al., 1997; Humphreys et al., 2010). Meadow fescue is also a high quality forage grass with high yield potential and tolerant to biotic and abiotic stresses. It is better adapted to colder regions than the ryegrasses. Due to its good persistence and tolerance to frequent grazing or cutting, it is also part of turf mixes (Rognli et al., 2010). Tall fescue is adapted to a wider range of soils and climates. Although less palatable than the ryegrasses, its superior growth provides forage over extended periods over the year and it is highly tolerant to grazing. Its uses also extend to turf and conservation purposes (Rognli et al., 2010).

Of these, only Italian ryegrass has an annual life cycle, while the other three are perennials. The four of them are naturally outcrossing, and with the exception of tall fescue, they have been characterized for self-incompatibility (SI) exhibiting the two locus SI typical for grasses. As a result, cultivars are created as synthetic or population varieties. It is in the breeder's interest to have the flexibility to produce self-pollinated seeds in order to develop inbred lines and multiply them at a large scale. Transferring a self-fertility (*SF*) locus from perennial ryegrass to other important grass species through interspecific hybridization is one way to achieve this objective.

In this article, the prospects of migrating the *SF* locus from perennial ryegrass to other related agronomically important species is discussed based on the available information on hybridization among them.

Hybridization and gene transfer within the *Lolium-Festuca* complex

The festucoid species have a basic chromosome number n = 7. They are all closely related to the point that interspecific hybridization is possible, and genetic diversity among them can be exploited for the genetic improvement of its agronomically most important species. For this purpose, interspecific and intergeneric hybridization has been studied with two objectives: I) create stable interspecific hybrids that combine the best attributes of both species, and II) transfer small chromosome segments that improves a specific trait on the recipient species, like biotic and abiotic stress tolerance. Table 1 summarizes the most relevant species that have been successfully hybridized.

The likelihood of transferring specific target genes from one species to another depends on the genomic similarities between the 2 species (King et al., 1999). Gene transfer in introgression breeding programs depend on high levels of homoeologous chromosome pairing and recombination at meiosis (Canter et al., 1999; King et al., 1999). However, Canter et al. (1999) pointed that unstable amphiploids represent also a potentially useful source of recombined genes in backcrossing programs. Restricting the amount of interspecific recombination by maximizing the amount of preferential pairing provides a controlled introgression of small portions of the donor genome and facilitates the recipient genome recovering in successive backcrosses (Lewis, 1980; Thomas et al., 1988; Canter et al., 1999; Zwierzykowski et al., 1999a).

The use of Genomic *in situ* Hybridization (GISH) for the observation of chromosomes at meiosis in hybrids and its derivatives has played a key role in determining whether homeologous recombination exists as well as the physical position of the chromosome segments introgressed (Thomas et al., 1994). A main concern in *Lolium* and *Festuca* hybridization has been the reduction of abnormalities at meiosis, and its relation to fertility. In the majority of the research however, the direction of the introgression is from *Festuca* to *Lolium* and less information is available for introgressions in the opposite direction. The best inferred approaches for migrating the *SF* locus to Italian ryegrass, meadow fescue and tall fescue are detailed below.

Introgression of *SF* from *Lolium perenne* into *Lolium multiflorum*

Both are diploid species with a basic chromosome number n=7 (2n=14) and hybrids between them show regular formation of seven bivalents. Direct crossing with no embryo rescue is often sufficient to obtain hybrids. These two species are easily crossable and are inter-fertile (Jauhar, 1975). For instance, in a polycross involving *L. perenne* and *L. multiflorum* plants, 47.5% hybrid progeny was obtained in *L. perenne* plants and 26.7% hybrids in *L. multiflorum* plants (Arcioni and Mariotti, 1983). The diploid hybrids had almost as good chromosome pairing and chiasmata as the parental species. Amphidiploids, chromosome doubling of the diploid hybrids, have high frequency of quadrivalents and high chiasmata frequency (Jauhar, 1975).

By crossing tetraploid *L. multiflorum* (2n = 4x = 28) x diploid *L. perenne* (2n = 2x = 14), triploid hybrids are obtained in an attempt to create preferential pairing. The expected result is that there is less interspecific recombination and smaller portions of the donor genome are introgressed, facilitating the recovery of the recurrent parent genome. In addition, the proportion of progeny expected without any portion of the donor genome is greater in a backcross with a triploid hybrid than with a diploid hybrid (Thomas et al., 1988). This method requires embryo rescue to be more effective, at least on the F_1. The triploids are sufficiently male fertile to be backcrossed reciprocally to the diploid *L. multiflorum*, although the triploid hybrid as female gives a higher frequency of aneuploids.

A high frequency of trivalents with little evidence of preferential pairing was observed. Genetic markers segregated randomly with the exception of a particular cross. Cytologically the hybrids behave as autotriploids but segregation ratios of some loci indicate

some degree of preferential pairing. A non-random position of the *L. perenne* chromosome in a trivalent formation can reduce chiasmata between both genomes and also favor the elimination of *L. perenne* genome. Overall it was proved that using triploid hybrids a more controlled introgression of genes from one species to the other is possible (Thomas et al., 1988).

Direct crossing between tetraploids of both species (*L. perenne* 2n = 4x = 28 x *L. multiflorum* 2n = 4x = 28) with no embryo rescue is also very efficient and results in allotetraploid hybrid progeny. In direct crosses without emasculation, 82% hybrid progeny were obtained in *L. perenne* mother plants and 93% in the reciprocal cross. Tetraploid hybrids are also highly fertile (Ahloowalia, 1977). Chiasmata associations in tetraploid hybrids also occurs. High multivalent frequency and homeologous chromosome pairing was reported by Ahlowwalia (1977), with 3-4 quadrivalents per cell, which represents 43-57% of the chromosomes. However, in another report, hybrids gave significantly higher proportion of bivalents compared with the tetraploid parents, and lower levels of multivalents (mean 8.98 bivalents and 2.11 quadrivalents) and lower chiasma frequency, which suggested a high degree of preferential pairing in the hybrids (Lewis, 1980). Although bivalent formation must result in disomic inheritance to support preferential pairing, segregation analysis actually agreed with tetrasomic inheritance (Lewis, 1981). It was also shown that this variation is genotype dependent (Lewis, 1980, 1981). This genotypic variation provides a means of reducing homeologous pairing. A preponderance of bivalents was observed when crossing specific genotypes of both species suggesting the possibility of using genotypes that reduces or suppresses homeologous paring in interspecific crosses between *L. perenne*

and *L. multiflorum.* The frequency of multivalents per pollen mother cell (PMC) in such a cross ranged from 2.15 to 0.6, proving that this could be an effective way of reducing homeologous recombination (Aung and Evans, 1985).

Markers in common within the two species have been developed and used for different purposes (Xiong et al., 2007; Brown et al., 2010; King et al., 2013). This helps in the identification of introgressed segments as well as the elimination of the donor genome with backcrosses. When using triploids and tetraploids hybrids, backcrossing to a diploid recipient should be an effective way to recover diploids with the introgressed gene. This was true for intergeneric crosses (Humphreys and Thorogood, 1993; Zwierzykowski et al., 1999a; Kosmala et al., 2006) and no limitations to this approach are anticipated.

The type of hybrid of choice when transferring *SF* from *L. perenne* to *L. multiflorum* would largely depend on the breeder's choice and germplasm available. The more straightforward approach is to cross diploid plants to produce the first hybrid generation (Figure 1). Diploid hybrids may require more efforts to recover the *L. multiflorum* genome and an adequate marker-assisted backcrossing program should be used depending on the resources and time frame available. This should be similar to any intraspecific backcross program. Triploid and tetraploid hybrids provide a more controlled introgression of chromosomes segments from *L. perenne* but require the additional step of creating tetraploid plants to be employed. Although tetraploid cultivars and tetraploid breeding populations are available for most breeding programs. The choice can be facilitated if genotypes, that promote low homeologous recombination, were previously identified.

Introgression from *Lolium perenne* and *Lolium multiflorum* into *Festuca pratensis*

F. pratensis is also a diploid with the same basic chromosome number as the *Lolium* spp. (2n = 14). Crosses between them show regular chromosome pairing in bivalents, though in some crosses some univalent, trivalents, and quiadrivalents can be observed. There is a slight variation in chiasmata frequency. Hybrids are often pollen sterile with non-dehiscent anthers, but some female fertility is observed. Direct crosses can be obtained but when *F. pratensis* is the female, better success is achieved by embryo rescue (Jauhar, 1975; Gymer and Whittington, 1975). Success in backcrosses of the hybrid to *F.pratensis* differs depending on the fertility of the hybrid used but fertility is recovered in subsequent backcrossing generations (Jenkin 1955).

Auto-allotriploids (2n = 3x = 21) are also obtained by crossing an induced autotetraploid of one species to a diploid of the other. Embryo rescue improves success and crossability is higher when the diploid is used as female in *L. multiflorum* x *F. pratensis* crosses (Naganowska et al., 2001). At meiosis they have 2 genomes of one species and one from the other, most of them have 21 chromosomes in both *L. perenne* and *L. multiflorum* x *F. pratensis* crosses (Jauhar, 1975; Naganowska et al., 2001). Large numbers of trivalents are observed and the majority of this hybrids behave as autotriploids (Jauhar, 1975), but relatively high numbers of bivalents were also found in *L. multiflorum* x *F. pratensis* crosses (Naganowska et al., 2001). Those with 2 *Lolium* genomes have a higher trivalent frequency and with little evidence of preferential pairing of chromosomes of the duplicated genome (Jauhar, 1975) and they are all male and female fertile in both *L. perenne* and *L. multiflorum* x *F. praensis* crosses (Jauhar, 1975; Naganowska et al., 2001). Those with two *Festuca*

genomes have a relatively lower trivalent frequency with some evidence of preferential pairing between the *Festuca* chromosomes, and a significantly higher frequency of chiasmata (Jauhar, 1975). In contrast, lower chiasmata frequency in those with two *Festuca* genomes was observed by Naganowska et al. (2001), and also those with the single genome cytoplasm had lower chiasmata in *L. multiflorum* x *F. pratensis* crosses. Pollen fertility was reduced or pollen was completely sterile in these crosses (Jauhar, 1975; Naganowska et al., 2001). Even with a high frequency of trivalents, preferential pairing between the *L. perenne* chromosomes in the *LpLpFp* hybrids was observed by King et al. (1999). The position of the *F. pratensis* chromosome at meiosis is more frequent at the end of the meiotic configuration, having fewer opportunities for chiasmata. Hybrids with two *F. pratensis* genome sets were not analyzed by King et al. (1999), but a similar pattern can be anticipated. The frequency can be lower than expected under random pairing but there is a general agreement that recombination between homeologous occurs.

Extensive genome recombination was observed also in the first backcross (BC_1) generation with frequent homeologous pairing (King et al., 1998; Zwierzykowski et al., 1999a). An average of 4.6 chiasmata binding homeologous arms per meiocyte was estimated and the translocation breakpoints were distributed along the entire length of the chromosome arms (Zwierzykowski et al., 1999a). At the triploid level gametic and zygotic selection limited the rate of introgression of the donor chromatin into the recipient. Male gametes with the haploid number of chromosomes (n=7) contained fewer complete and translocated chromosomes of the donor (Zwierzykowski et al., 1999a) which agrees with Thomas et al. (1988) in that with triploids the recovery of the recurrent parent genome is

facilitated. The direction of the backcrosses have an impact on the outcome. The cytoplasm of F_1 hybrids affected the chromosome number and composition of male gametes recovered when backcrossed to *L. multiflorum*. There seems to be a faster recovery of the diploid chromosome number, a lower chromosome number from the donor, and lower number of translocations when the cytoplasm of the F_1 triploid hybrid is from the intended recipient species.

In the BC_2 generation, after selecting diploid BC_1 plants with *F. pratensis* introgressions, the transmission rate of *F. pratensis* alleles through pollen was significantly lower than of *L. mutliflorum* alleles, but this difference was locus-specific. No difference was found in the transmission of female gametes. When intercrossing BC_2 plants the deficiency of *F. pratensis* alleles was less marked (Humphreys and Thorogood, 1993). Once a chromosome segment has been introgressed into a diploid background recombination occurs freely in the *F. pratensis*/*L. perenne* bivalents with recombination points along all the chromosome arm (Armstead et al., 2001; king et al., 2002).

Introgressions from *F. pratensis* into *L. multiflorum* have been successfully done. For freezing-tolerance, a partially fertile triploid obtained from a cross between a tetraploid *L. multiflorum* and a diploid *F. pratensis* was backcrossed twice to a diploid *L. multiflorum*. From the selected BC_2 plants, 80% had recovered the diploid chromosome number. Diploid plants with single *F. pratensis* chromosome segments and improved freezing tolerance were identified. Seed set and seedling survival was greater when *L. multiflorum* was used as female, and the diploid chromosome number in BC_2 predominated also when *L. multiflorum* was used as female. The number of introgressions was higher when the BC_1 plant was used

as female. The use of F_1 and BC_1 plants as male parent accelerated the loss of the donor parent genome (Kosmala et al., 2006). For introgressing a stay-green gene in the same direction, emasculated tetraploid *L. multiflorum* were crossed to a diploid *F. pratensis* as pollen donor (Moore et al., 2005). The triploid hybrid was used as pollen donor in crosses to diploid *L. multiflorum.* Plants with 14 chromosomes in the BC_1 generation and within these, plants with a single introgression were identified. Selected BC_1 plants were crossed to diploid *L. multiflorum* to generate the BC_2 generation, where the *F. pratensis* introgression segregated in a 1:1 ratio. Similarly, a crown rust resistance gene was introgressed into *L. multiflorum* (Roderick et al., 2003). Again the tetraploid *L. multiflorum* was used as female to produce the triploid hybrid. For the first backcross, hybrids were used both as male and female since some of them were male sterile, but in the following generation BC_1 plants were used as pollen donors.

Gene introgression through triploid hybrids combines the advantages of a more controlled chromosome segment translocations from the donor, the relatively easy recovery of diploid chromosome number and the elimination of non-target donor segments in few backcrosses. Transfer of the *SF* locus from *L. perenne* to *F. pratensis* with this approach should be more efficient than using diploid interspecific hybrids.

Another alternative is to create allotetraploid hybrids by crossing two autotetraploid plants (*L. perenne* 2n=4x=28 x *F. pratensis* 2n=4x=28). Synthetic amphiploid *festulolium* cultivars have been developed, favored by the meiotic stability that this hybrids show (Canter et al., 1999). Meiosis in early generations is stable with meiotic configurations predominantly bivalents (11.5 bivalents/cell, Zwierzykowsky et al., 2008) and pairing is

predominantly intragenomic (Canter et al., 1999; Zwierzykowsky et al., 2008). Intergenomic pairing happens at a low frequency showing that there are some opportunities for intergenomic recombination but at a low level (Zwierzykowsky et al., 2008). However, there is extensive recombination between the genomes in subsequent generations of open pollination of the hybrids. Recombinant chromosomes and recombination breakpoints increased from generation to generation, as well as substitution of large chromosome sections of *F. pratensis* by *L. perenne* (Canter et al., 1999; Zwierzykowsky et al., 2006). A mean of 17.9 *Lolium* and 9.7 *Festuca* chromosomes were observed, with a mean chromatin length per genotype of 62.1% *Lolium* and 37.9% *Festuca* (Canter et al., 1999). However, this can be the result of selection favoring *Lolium* in the absence of complete preferential pairing. Fertility also increased reaching 65% pollen stainability and 33% seed set (Zwierzykowsky et al., 2006).

Chromosome segments conferring winter hardiness were introgessed from *F. pratensis* to *L. perenne* using an amphiploid *L. perenne* x *F. pratensis* material as a starting point and by backcrossing, diploid introgression lines were obtained Gronnerod (2004) (cited by Kosmala 2006).

Similarly, allotetraploid hybrids between tetraploid *L. multiflorum* (2n=4x=28) x tetraploid *F. pratensis* (2n=4x=28) can be obtained. Advanced generations also show increased recombination between homeologues chromosomes but no chromosome substitution was observed (Thomas et al., 1994) and the proportion of the total genome length occupied by *L. multiflorum* was between 49% and 67% (Zwierzykowski et al. 1998b). Translocation breakpoints ranged from 22 to 38 per cell and the majority of chromosomes

were hybrid chromosomes composed of segments of chromatin from the different species (Zwierzykowski et al. 1998b).

Crossing tetraploids is a way of obtaining partial male and female fertile hybrids but crossability is low and requires embryo rescue. Fertility is recovered after cycles of recombination, and fertile diploids can also be efficiently recovered from allotetraploids using androgenesis (Lesniewska et al., 2001; Guo et al., 2005).

Considering the fertility levels of the triploids and the possibility of recovering diploid plants with the introgressed segments by backcrossing, this may be the best approach to introgress the *SF* locus from *L. perenne* to *F. pratensis* (Figure 2). Overall, the decision of which of the parents are used as male and female can have a profound effect on the rate of transmission of the donor allele. In all cases introgressions have been made from *F. pratensis* to *Lolium* spp. and less is known about introgressions in the opposite direction. However, other than a lower pollen fertility of the *LmFpFp* triploid hybrids (Jauhar, 1975; Naganowska et al., 2001) similar patterns should be observed. Difficulties can be expected to be genotype dependent and should be reduced by subsequent backcrosses with *F. pratensis* as the female parent.

Introgression from *Lolium perenne* and *Lolium multiflorum* into *Festuca arundinacea*

F. arundinacea is an allohexaploid composed by two progenitor genomes: *F. pratensis* (2n=2x=14) and *Festuca arundinacea* var. *glaucescens* (2n = 4x = 28) which after hybridization and subsequent chromosome doubling led to the evolution of *F. arundinacea* (Humphreys et al., 1995). Tall fescue is described as moderate self-incompatible (Gibson

and Newman, 2001) and its SI mechanism has not been characterized. *F. pratensis* has the 2 locus SI system of the grasses but there is no information on *Festuca arundinacea* var. *glaucescens*. Consequently, it is unknown how the SI loci interact with the different genomes to provide the levels of incompatibility in *F. arundinacea*, and how the *SF* locus would act in this background. Introgressing *SF* alleles into *F. arundinacea* can provide some answers as well as facilitate the production of selfed-seed at a larger scale.

Interspecific crosses between *L. perenne* (2n = 2x = 14) x *F. arundinacea* (2n = 6x = 42) were done with emasculation of the seed parent. From these crosses, tetraploid hybrids (2n = 4x = 28) are obtained and often embryos have to be cultured for better success (Buckner et al., 1961; Evans and Aung, 1986; Pasakinskiene, 2000). When *L. perenne* was used as female, more hybrid progeny was recovered but they were all male sterile. The reciprocal yielded less progeny but they all had normal floral structures (Evans and Aung, 1986). In these tetraploid hybrids, homeologous pairing was dependent on the genotype of the *L. perenne* parent but mostly bivalents were formed (Evans and Aung, 1986). The F_1 can be backcrossed as females to both parental species. Female fertility of the hybrid is rather low (0-22 seeds per plant) but the majority produced some seed (Buckner et al., 1961). Chromosome doubling of *L. perenne* x *F. arundinacea* hybrids (2n = 4x = 28) in an attempt to create balanced octoploids and to restore fertility, has been done. Some male fertility has been obtained, though about 50% remained pollen sterile (Buckner et al., 1961; Pasakinskiene, 2000).

Few studies involving *L. perenne* x *F. arundinacea* hybrids were reported. Fertility recovery by either backcrossing and colchicine treatment was better for *L. multiflorum*

suggesting that *L. multiflorum* is more compatible to tall fescue than *L. perenne* (Buckner et al., 1961). However no explicit limitation on *L. perenne* x *F. arundinacea* hybridization has been mentioned. Introgression of the *SF* locus to *F. arundinacea* through repeated backcrossings is the most straightforward approach. The information generated in crosses with *L. multiflorum* can be used as a guide in hybridizations with *L. perenne* considering the relatedness of both *Lolium* species. An alternative is to use *L. multiflorum* as a bridge for the introgression of the *SF* locus from *L. perenne* to *F. arundinacea*.

Hybrids of *L. multiflorum* (2n = 2x = 14) x *F. arundinacea* (2n = 6x = 42) can be produced by bagging panicles of both parents together with or without emasculation of the seed parent. The F_1 is also a tetraploid (2n = 4x = 28), pollen sterile and can be backcrossed as females to both parental species. Embryo rescue of the F_1 is often required. Female fertility of the hybrid is also low but is genotype dependent (Buckner et al., 1961; Zwierzykowski, 1980a; Pasakinskiene et al., 1997).

Irregularities at meiosis occur in these hybrids. The predominant chromosome configuration reported by Springer and Buckner (1982) was bivalent, grouping between 82% and 84% of the chromosomes. Zwierzykowski (1980b) observed about half of the chromosomes grouped in bivalents but all configurations from univalent to pentavalents were observed. At anaphase I and II lagging chromosomes and chromatin bridges were also observed (Zwierzykowski, 1980b; Springer and Buckner, 1982). From the genome composition of *F. arundinacea*, *L. multiflorum* chromosomes have higher affinity for pairing with *F. pratensis* than *F. arundinacea* var. *glaucescens* chromosomes. However, homologous paring happened between all the genomes present in the hybrids. More recombination

points were actually observed in *L. multiflorum- F. arundinacea* var. *glaucescens* pairs. This suggests that chromosome segments can be introgressed in all genome combinations (Kopecky et al., 2009).

Backcrossing the F_1 hybrid to tall fescue produced low number of seeds with low vigor resulting in a low number of plants. Plants of the BC_1 progeny are pentaploids (2n = 5x = 35), which are also male sterile (Zwierzykowski, 1980a).

Chromosome doubling of the F_1 hybrid resulted in octoploids (2n = 8x = 56) with some male fertility in most genotypes, and increased female fertility as well (Buckner et al., 1961; Zwierzykowski, 1980a, b; Pasakinskiene et al., 1999). Zwierzykowski (1980a) found viable pollen based on staining between 19% and 75%, with seed set with open pollination ranging from 3% to 21%, although meiosis irregularities were still observed (Zwierzykowski, 1980b).

Backcrossing the octoploid hybrids to the parental species improved the crossing results compared to tetrataploid hybrids. Plants resulting from the crossing to *L. multiflorum* had 29 to 35 chromosomes while those from the cross to *F. arundinacea* had 42 to 49. Following backcrosses to tall fescue, the cultivar Kenhy was released, which had the hexaploid chromosome number of tall fescue but with some *Lolium* traits introgressed (Buckner et al., 1977). Other cultivars, obtained following backcrosses to both *L. multiflorum* and *F. arundinacea*, were described by Kopecky et al., (2009).

Pentaploid hybrids can be obtained by crossing tetraploid *L. multiflorum* (2n = 4x = 28) x *F. arundinacea* (2n =6x = 42). Pentaploid hybrids are sufficiently fertile to be used in reciprocal crosses to *L. multiflorum*. Using the hybrid as the pollen donor, the diploid

constitution can be recovered in 2 backcrosses: 76%-80% BC2 plants were found to be diploids vs 35% when the hybrid was used as female (Humphreys, 1989; Humphreys and Thomas, 1993). Most of the chromosome associations were between *L. multiflorum* chromosomes but some homeologous association occurs and recombinants involving all genomes were observed, enabling the transfer of genes from tall fescue (Humphreys, 1989; Humphreys et al., 1998). Recombinations were also detected in backcrosses, and the three *F. arundinacea* homeologous recombined with *L. multiflorum* chromosomes (Humphreys and Ghesquiere, 1994). It was also proven that a complete genome of *L. multiflorum* is required for gamete viability in the hybrids (Humphreys et al., 1998). Intercrossing the hybrids before backcrossing to *L. multiflorum* increases the changes of interspecific recombination (Humphreys, 1989).

Using this approach, following backcrosses to diploid *L. multiflorum*, drought and cold tolerance has been introgressed into *L. multiflorum* from *F. arundinacea* (Humphreys, 1989; Humphreys and Thomas, 1993; Humphreys et al., 1997; Kosmala et al., 2007), as well as crown rust resistance (Oertel and Matzk, 1999). This path seems to be more effective than using diploid *L. multiflorum* for the interspecific cross. Even though backcrosses to *F. arundinacea* were not made, it can be inferred that it should present less difficulties considering the increased fertility and stability of these pentaploids.

Androgenesis from pentaploids has proven very effective in recovering fertile euploid plants with either 14, 21, or 28 chromosomes with some *Lolium-Festuca* recombinant chromosomes (Humphreys et al., 1997; Humphreys et al., 1998; Zwierzykowski

et al. 1998a, 1999b; Zare et al., 1999; Zare et al., 2002). Gene combinations resulting from rare meiotic events can be recovered with this method.

Tetraploid *L. perenne* with four copies of the *SF* gene could be crossed to *F. arundinacea*. Using a tetraploid as donor instead of a diploid, fertility and stability of the hybrid should be improved considering the results obtained in *L. multiflorum* x *F. arundinacea* crosses. Successive backcrosses to *F. arundinacea* can potentially result in the recovery of *F. arundinacea* plants with the *SF* introgression from *L. perenne* (Figure 3). Alternatively, *L. multiflorum* or *F. pratensis* can be used as a bridge if *SF* introgression lines of these species have previously been obtained.

Conclusions

According to the experimental results in interspecific hybridization within *Lolium-Festuca* species, the prospects for migrating the *L. perenne SF* locus to *L. multiflorum*, *F. pratensis* and *F. arundinacea* are promising. Transfer to *L. multiflorum* would require the least effort and this locus could be readily incorporated into *L. multiflorum* backgrounds following any of the crosses described. Introgression into *F. pratensis* background imposes some fertility constrains though not severe. Since several traits have been introgressed from *F. pratensis* into *L. multiflorum*, the opposite should not offer any serious constraints. The use of triploid hybrids followed by few backcrosses to the recipient species have shown to be an effective path. Diploid recovery and elimination of non-intended donor genome segments is achieved very efficiently. Incorporation of the *SF* locus into *F. arundinacea* by direct hybridization with a *L. perenne* donor seems possible but also with some fertility

limitations. However, there is abundant information on *L. multiflorum x F. arundinacea* hybridization and gene introgressions that may be applicable when using *L. perenne*. Pentaploid hybrids are more fertile and would facilitate the recovery of stable *F. arundinacea* genotypes with the introgressed gene. An alternative not described here is using *F. pratensis* as a bridge. Reports on *F. pratensis* x *F. arundinacea* hybridization are scarce but less limitations are anticipated considering that *F. pratensis* is one of the progenitor species of *F. arundinacea.* The main concern however is the lack of information on the SI system in both *F. arundinacea* and in its other progenitor *F. arundinacea* var. *glaucescens*. This prevents any anticipation on the mode of action of the *SF* locus in such a background.

Transfer self-fertility to other agronomically important grasses were not addressed. Orchardgrass and its related subspecies (*Dactylis* spp.) would be an interesting target considering its phylogenic proximity to the festucoids. The genus *Phalaris* has a few agronomically important species and a *SF* locus has been identified in one of them. Self-fertility can be equally transferred within *Phalaris* species through interspecific hybridization. To more distant grass species, conserved synteny within the Poaceae family could help identify candidate regions by comparative approaches. Such regions could then be targeted with site-directed mutagenesis to generate putative self-fertile mutants.

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Table 1. Species reported to hybridize with *L. perenne*, *L. multiflorum*, *F. pratensis* and *F. arundinacea.* All of them have a basic chromosome number n = 7.

Species (ploidy)	Hybridizes with	Reference*
Lolium perenne (2x)	*Lolium temulentum* (2x)	1, 2
	Lolium multiflorum (2x and 4x)	5, 13-14
Lolium perenne (4x)	*Lolium multiflorum* (4x)	12, 15-18
	Festuca arundinacea var. *glauscencens* (4x)	12
Lolium perenne (2x and 4x)	*Festuca arundinacea* (6x)	6, 12, 31
	Festuca gigantea (6x)	12
	Festuca pratensis (2x and 4x)	3-5, 12, 18-19, 21, 27-28
Lolium multiflorum (2x)	*Lolium temulentum* (2x)	2
	Lolium perenne (2x)	12
	Festuca mairei (4x)	12
Lolium multiflorum (4x)	*Lolium perenne* (2x and 4x)	12, 14-18
Lolium multiflorum (2x and 4x)	*Festuca pratensis* (2x and 4x)	5, 12, 20, 22-26, 29-30
	Festuca arundinacea (6x)	6, 9, 32-41
	Festuca arundinacea var. *glauscencens* (4x)	11-12
	Festuca gigantea (6x)	8
	Festuca arundinacea var. *atlantigena* (8x)	12
	Festuca arundinacea var. *letourneuxiana* (10x)	12
Festuca arundinacea (6x)	*Festuca rubra* (6x)	12
	Festuca pratensis (2x and 4x)	12
	Lolium multiflorum (2x and 4x)	6, 9, 12, 32-41
	Lolium perenne (2x and 4x)	6, 12, 31
	Lolium rigidum (2x)	12
	Lolium loliaceum (2x)	12
	Lolium persicum (2x)	12
	Lolium temulentum (2x)	12
	Festuca gigantea (6x)	12
	Dactylis glomerata (4x)	10
Festuca pratensis (2x)	*Festuca mairei* (4x)	12
	Lolium temulentum (2x)	7, 12
Festuca pratensis (2x and 4x)	*Festuca arundinacea* (6x)	12
	Festuca arundinacea var. glauscencens (4x)	12
	Lolium persicum (2x)	12
	Lolium multiflorum (2x and 4x)	5, 12, 20, 22-26, 29-30
	Lolium perenne (2x and 4x)	3-5, 12, 18-19, 21, 27-28

* [1] Yamada (2001); [2] Thorogood and Hayward (1992); [3] King et al. (1999); [4] Jenkin (1955); [5] Jauhar (1975); [6] Buckner et al. (1961); [7] Thomas et al. (1999); [8] Morgan et al. (1988); [9] Humphreys (1989); [10] Matzk (1981); [11] Humphreys et al. (2005); [12] Zwierzykowsky (1996); [13] Arcioni and Mariotti (1983); [14] Thomas et al. (1988); [15] Ahloowalia (1977); [16] Lewis (1980); [17] Lewis (1981); [18] Aung and Evans (1985); [19] Gymer and Whittington (1975); [20] Naganowska et al. (2001); [21] King et al. (1998); [22] Zwierzykowski et al. (1999a); [23] Humphreys and Thorogood (1993); [24] Kosmala et al. (2006); [25] Moore et al. (2005); [26] Roderick et al. (2003); [27] Canter et al. (1999); [28] Zwierzykowsky et al. (2006); [29] Thomas et al. (1994); [30] Zwierzykowski et al. (1998b); [31] Evans and Aung (1986); [32] Zwierzykowski, 1980a; [33] Pasakinskiene et al. (1997); [34] Springer and Buckner (1982); [35] Kopecky et al. (2009); [36] Buckner et al. (1977); [37] Humphreys and Thomas, 1993; [38] Humphreys et al. (1998); [39] Humphreys and Ghesquiere (1994); [40] Kosmala et al. (2007); [41] Oertel and Matzk (1999).

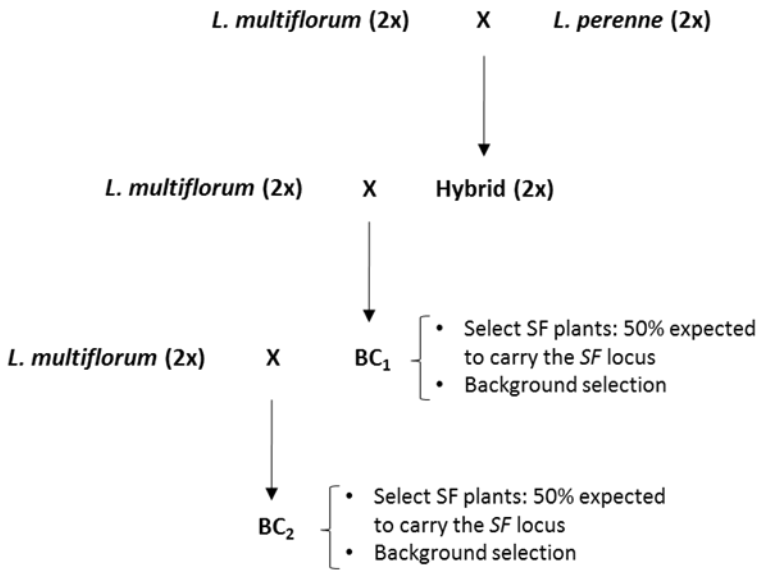

Figure 1. Simplified scheme for the introgression of the *SF* locus from *L. perenne* to *L. multiflorum*.

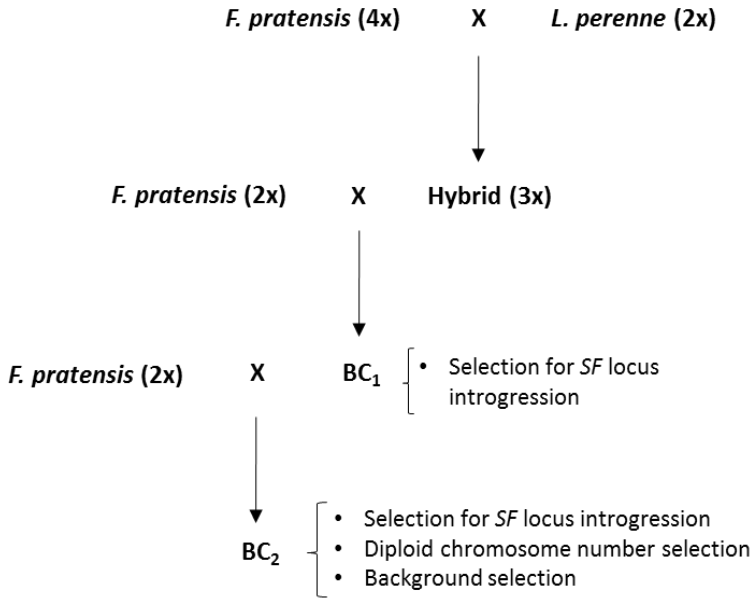

Figure 2. Simplified scheme for the introgression of the *SF* locus from perennial ryegrass into meadow fescue following the triploid hybrid approach.

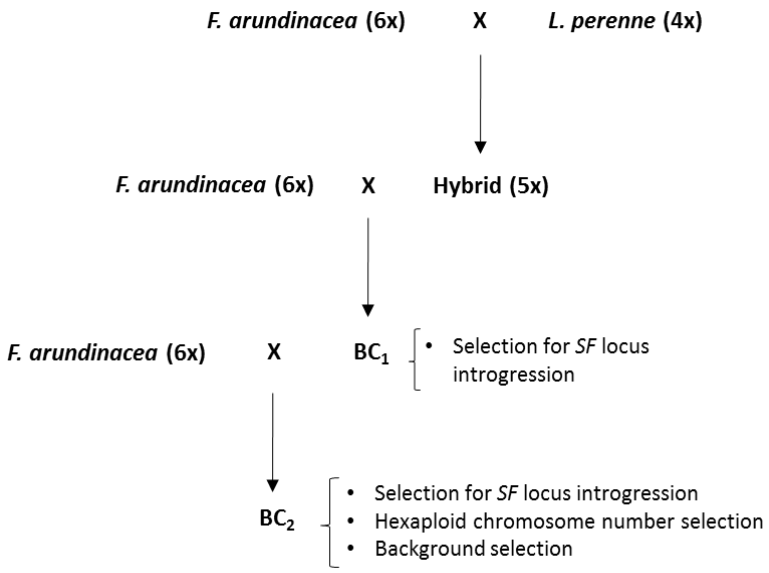

Figure 3. Simplified scheme for introgression of the SF locus form perennial ryegrass into tall fescue following the pentaploid hybrid approach.

CHAPTER 6

GENERAL CONCLUSIONS

The goal of this project was to contribute to improve the understanding on self-fertility (SF) in outcrossing grasses for the purpose of inbred line development and move a step forward towards hybrid breeding as a way to improve genetic gains. This was achieved throughout the development of the different chapters of this thesis. Reported SF mutations and their mode of action in different grass species were reviewed along with their relationship with the self-incompatibility (SI) loci, and alternatives for the incorporation of this trait in breeding programs were proposed. The focus was then put in one of this mutations in perennial ryegrass. A *SF* locus in perennial ryegrass, which is epistatic over *S* and *Z* locus, was fine mapped to an interval of 1.6 cM in LG5 using segregation and linkage analysis. The segregation and mode of action of this gene was also studied at the tetraploid level, considering the prevalence of polyploids among cultivated grasses. Finally, the prospects of transferring this gene to other related species were discussed, broadening the range of grass species that can benefit from it.

Two main sources of SF can be exploited in grasses, either genetic or environmentally induced SF, the later known as pseudocompatibility. Pseudocompatibility can be utilized when a genetic source of SF is not available, when only small amounts of selfed seeds are required, and when controlled environments are available, such as greenhouses or locations with specific temperatures ranges. It has the advantage of being temporary, and SI is recovered which can then be used as an effective pollination control

mechanism. Genetic SF is a stable mechanism and it is advantageous when full selfed-seed set is sought, as in large scale inbred line multiplication, and it is independent of the environmental conditions, not requiring special facilities or particular environments. Genetic SF has been reported in only three species: rye, *Phalaris coerulescens* and perennial ryegrass. The sources of SF found are either mutations at the *S* or *Z* locus, or mutations at a third locus, causing the SI system to be inoperative. Such mutations incorporated into breeding populations enable the development of inbred lines which can then be used as parents in narrow-based synthetic or hybrid varieties. For the later a combination of SF and male sterility is required in order to make controlled crosses and avoid selfings in hybrid seed production fields.

Fine mapping the *SF* mutation in LG5 of perennial ryegrass is an advance towards the identification of this gene. This was achieved by a gradual approximation using first a sample of the population to identify flanking markers. Identification of recombinants within the flanking markers was done in the entire population of 1248 plants. Only these recombinants were then phenotyped and used for linkage mapping. The *SF* locus was found to be located between two markers in a 1.6 segment. The flanking markers were aligned to an 807 kbp region in *Brachipodium distachium* which contains 87 annotated genes, making the search for candidate gene still vague. Further steps are still required to get to a distance short enough to allow sequencing and cloning approaches. For breeding purposes however the 1.6 cM region is close enough to facilitate marker assisted backcrossing for the incorporation of this gene into breeding populations, and reduce linkage drag by eliminating donor genome portions located close to the gene.

The grass SI system is still functional at the tetraploid level in contrast to the SI present in other group of species. However there were no reports about SF in tetraploids. It was found that SF is still functional and that incomplete dominance occurs in the diploid pollen grain between the incompatible and compatible alleles of the *SF* locus. Heterozygote pollen grains are not fully inhibited but those homozygous for the mutation have a competitive advantage resulting in an excess of quadruplex and a reduction in the simplex genotypes after selfing a duplex plant. The *SF* locus is still the main determinant of the trait in the tetraploids but there is an extensive interaction with the genetic background. This resulted in a more continuous distribution of pollen compatibility rather than the expected discrete variation. Self-fertility in autotetraploids can be used for purging deleterious mutations and reducing the mutation load, while in allopolyploids inbred line development for both synthetic and hybrid breeding could be done.

Perennial ryegrass can hybridize with other species within the *Lolium-Festuca* complex suggesting the possibility of transferring the *SF* locus to other related species. Italian ryegrass, meadow fescue and tall fescue are recipient candidates due to both their agronomic importance and their relatedness to perennial ryegrass. Italian and perennial ryegrasses are easily crossable and their hybrids are fertile. A backcrossing program similar to any intraspecific one should be sufficient for gene introgression and donor genome elimination. Meadow fescue also hybridizes with perennial ryegrass but hybrids are pollen sterile. Based on hybridization studies, the best approach is to create triploid hybrids by crossing a tetraploid induced meadow fescue to a diploid perennial ryegrass. Triploids have a better fertility and by successive backcrosses to a diploid recipient, diploid progeny with

introgressed chromosome segments are obtained. For tall fescue the best approach is to use a tetraploid perennial ryegrass donor as parent. The resulting hybrid is a pentaploid with good fertility levels. Subsequent backkcrossings to tall fescue should restore the hexaploid chromosome number of tall fescue with ryegrass chromosome segments introgressed. The main concern with tall fescue is the lack of information on its SI system and the uncertainty about the mode of action of the *SF* locus in an alloploid background.

The results and observations presented here contribute to a better understanding of the trait at both diploid and tetraploid levels and are promising as self-fertility may readily be incorporated into breeding programs.

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