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SELF-FERTILITY: KEY FOR HYBRID BREEDING OF ALLOGAMOUS GRASSES

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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*) and rye (*Secale cereale*) (reviewed by Baumann et al., 2000; Yang et al., 2008; Klaas et al., 2011). 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 (Vogel and Pedersen, 1993; Wilkins and Humphreys, 2003; Posselt, 2010). Reported genetic gains obtained in perennial grasses are low, e.g., 0.43% for dry matter yield per year in perennial ryegrass 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 diffculty of altering the harvest index in grasses, which had a great impact on the improvement of most grain crops, lower financial investment compared to cereal crops, among other reasons (McDonagh et al., 2014; Woodfield, 1999; Wilkins and Humphreys, 2003; Laidig et al., 2014).

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 the most common genetic mechanism to ensure 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-pollination. 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-pollination under different environmental conditions (Foster and Wright, 1970). Such selfing rates in perennial ryegrass were also obtained by other authors (Madsen et al., 1993; Fearon et al., 1983; Cornish et al., 1980; Beddows et al., 1962). These low rates of selfed seed set were considered to be due to environmental effects and referred to as pseudo-self-compatibility (Fearon et al., 1983; Cornish et al., 1980). 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, increasing the temperature increases success of self-pollination.

Significant differences among genotypes were reported for pseudocompatibility (Wilkins and Thorogood, 1992; Gertz and Wricke, 1991; Elgersma et al., 1989; Foster and Wright, 1970; Beddows et al., 1962). Thus, selection for pseudocompatibility is possible. In rye, a positive and significant correlation in the level of selfed seed set of the plants and their offspring was found (Lundqvist, 1960; Lundqvist, 1958). In perennial ryegrass, the capacity for SF was heritable and increased over inbred generations in selected families (Jones and Jenabzadeh, 1981). By selecting for pseudocompatibility 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 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, 1961). 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 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).

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, 1961) 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* (Yamada, 2001; Thorogood and Hayward, 1992).

Even though the majority of research suggests 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 *P. coerulescens* 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).

S and Z sources of SF

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 *L. perenne* 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. (1990, 1987), 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).

Origin of S and Z SF alleles

To date, there is no concluding evidence on the identity of the *S* and *Z* genes, but expression studies in grasses showed common features to other SI systems (Klass et al., 2011). Transcripts were identified displaying homologies to protein kinases and receptor kinases, and notably, a receptor kinase is the female determinant in the Brassicaceae sporophytic SI (SSI) system (Van Daele et al., 2008; Yang et al., 2009; Byrne et al., 2015), and two of the receptor kinases co-located with the *Z* locus (Byrne et al., 2015). Actins, P-type ATPases and GTP-binding proteins, which may be involved in a calcium signaling cascade similar to the Papaveraceae GSI system, were also found [specify “found”: mapping, expression analysis – which approach ?] (Van Daele et al., 2008). *F-box* genes expressed in anthers and linked to the *S* locus were reported [specify “reported”], which are known to be the pollen determinant in the Solanaceae, Plantaginaceae and Rosaceae S-RNase GSI systems (Kakeda, 2009), ans well as ubiquitins, which promote pollen tube degradation in both the Brassicaceae and Solanaceae SI systems (Van Daele et al., 2008; Yang et al., 2009; Shinozuka et al., 2010). Other transcripts [how identified ?] showed no sequence homologies to SI genes of other families. Expansins (Yang et al., 2009; Byrne et al., 2015), myb-like proteins, pathogenesis-related protein (Yang et al., 2009), as well as obtusifoliol 14 α-demethylase, a sterol 14 α-demethylase, a member of the cytochrome monooxygenase superfamily and a endoribonuclease dicer homolog 3b-like (DCL3b) were also present (Byrne et al., 2015) and may have a role in the degradation of the incompatible pollen tube or facilitating compatible pollen tube penetration. Interestingly, two α-expansins and a DCL3b co-located with the *S* locus in perennial ryegrass (Byrne et al., 2015). Recently, a pollen determinant candidate has been identified (Manzanares et al., 2015). The gene, encoding a protein containing a DUF247 domain, was up-regulated in pollen, cosegregated with S and had a sufficiently high allelic sequence diversity at both nucleotide and protein level to discriminate different *S* alleles. In related self-compatible species, the corresponding ortholog showed loss of function mutations. Intriguingly, a *DUF247* gene was also suggested as one of the *Z* candidate genes (Shinozuka et al., 2010). Thus, the SI system in grasses is unique in terms of the number of loci [this relates to my comments above: candidate genes based on differential expression do not necessarily play a role. They are candidates, until a role can

be demonstrated. Usually for only few genes such hard evidence can be established. Should thus be carefully interpreted.] involved but also may combine features of the different SI systems with other novel mechanisms. *S* and *Z* in grasses are probably complexes of linked genes including specificity genes, regulatory genes, and genes involved in the downstream process leading to pollen tube growth or arrest, similarly to the *S* locus in dicots (Takuno et al., 2007; Wang et al., 2003). Failure in the expression of any of those genes can potentially cause the breakdown of the SI system.

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, 1968; Lundqvist, 1958). 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 pollen [do you mean SI, not pollen ?] function loss or inactivation (de Nettancourt, 2001).

Specificity is known to be controlled separately in pollen and stigma. Thus, each SI locus might carry two linked genes (Lundqvist, 1968; Lundqvist, 1958 Yang et al., 2008; Takayama and Isogay, 2005, reviewed by Klaas et al., 2011;). 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, 1960; Lundqvist, 1958). This hypothesis is challenged by the fact that the *S* locus in many grasses is located in a centromeric region (Egorova et al., 2000; Korzun et al., 2001; Bian et al., 2004; Kakeda et al., 2008; 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 (Wang et al., 2001; Wang et al., 2003; Takebayashi el al., 2003). Moreover, in grasses, the *Z* locus was localized in the distal portion of the long arm of chromosome 2 (Korzun et al., 2001; Bian et al., 2004), 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 (Vieira et al., 2003; Ortega et al, 2006; Donia et al., 2015). 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* (Solanaceae), new specificities can arise from few or even single base-pair substitutions at the hypervariable regions of the *S-RNase* gene (Saba-El-Leil et al., 1994; Matton et al., 1997; Matton et al., 1999) 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 (Uyenoyama et al., 2001; Gervais et al., 2011). 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

While *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 (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 Ritcher, 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).

The two-locus SI system is assumed to be conserved among grasses after an important number of species have shown the same incompatibility characteristics (reviewed by Baumann et al., 2000, Li et al., 1997). Additionally, there is a high degree of synteny among triticeae (wheat, barley, and rye), poeae (*Lolium* spp., *Festuca* spp.), aveneae (*P. coerulescens*, *Holcus lanatus* and oat) and rice around the *S* and *Z* regions (Leach and Hayman, 1987; Thorogood et al., 2002; Jones et al., 2002b; Alm et al., 2003; Bian et al., 2004; Sim et al., 2005, Manzanares et al., 2015), which helped to make progress in understanding the genetics of SI by comparative genomics. It is, therefore, a reasonable hypothesis, that the non-*S* and -*Z* locus conferring SF in rye, perennial ryegrass, and *P. coerulescens* is due to the same gene, considering that the region around it is conserved among different grasses (Jones et al., 2002b; Alm et al., 2003; Sim et al.,

2005). 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 mode of action is epistatic gene action 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. [Mention, that dominance considerations are not relevant because of gametophytic gene action] Gain of function mutations are less common and would imply that the allele arose once in the 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 et al., 2002; Thorogood and Hayward, 1992). 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.) (Wünsch and Hormaza, 2004; Wünsch et al., 2010; Cachi and Wünsch, 2011) and in apricot (*Prunus armeniaca*) (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. This gene was located in the lower part of LG3 of sweet cherry (Cachi and Wünsch, 2011) and surprisingly, also in LG3 of two unrelated apricot cultivars (Zuriaga et al., 2012; Zuriaga et al., 2013). 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) when half of a population carrying functional S-RNase genes were found to be self-fertile, indicating that they carry a non-functional allele at an additional locus. Since heterozygotes for that gene favored incompatibility, it was concluded that 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, inbreeding depression 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 a second selfing generation [do you mean: 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 and Singh, 2011; Kindiger, 2012) can also play an important role in grasses as a fast way to achieve homozygosity, and with a clear potential for purfying 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 maize and other hybrid crops.

Self-fertility for synthetic variety development

The introduction of inbreeding as part of breeding programs has been proposed for different grass species, including rye (Wricke, 1976; Voylokov, 2007), 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 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).

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.

Self-fertility for hybrid breeding

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) (see Posselt, 2010, and Arias-Aguirre et al., 2012).

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 pollinate. 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.

[In the section below, you could briefly mention rye as example, where SF was prerequisite to move from population/synthetic breeding to a cms – based system. As you point out – rye does need restorers, which is a difference which you can nicely highlight whem mentioning rye as example]

Alternatively, CMS is currently the preferred mechanism of hybridization control in many species. 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 as for the F_1 to be able to produce grains which otherwise would be male sterile. 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 [Please check, if this is usually included in TAG reviews – it may not. Could be left out]

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				
		Deschampia 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.

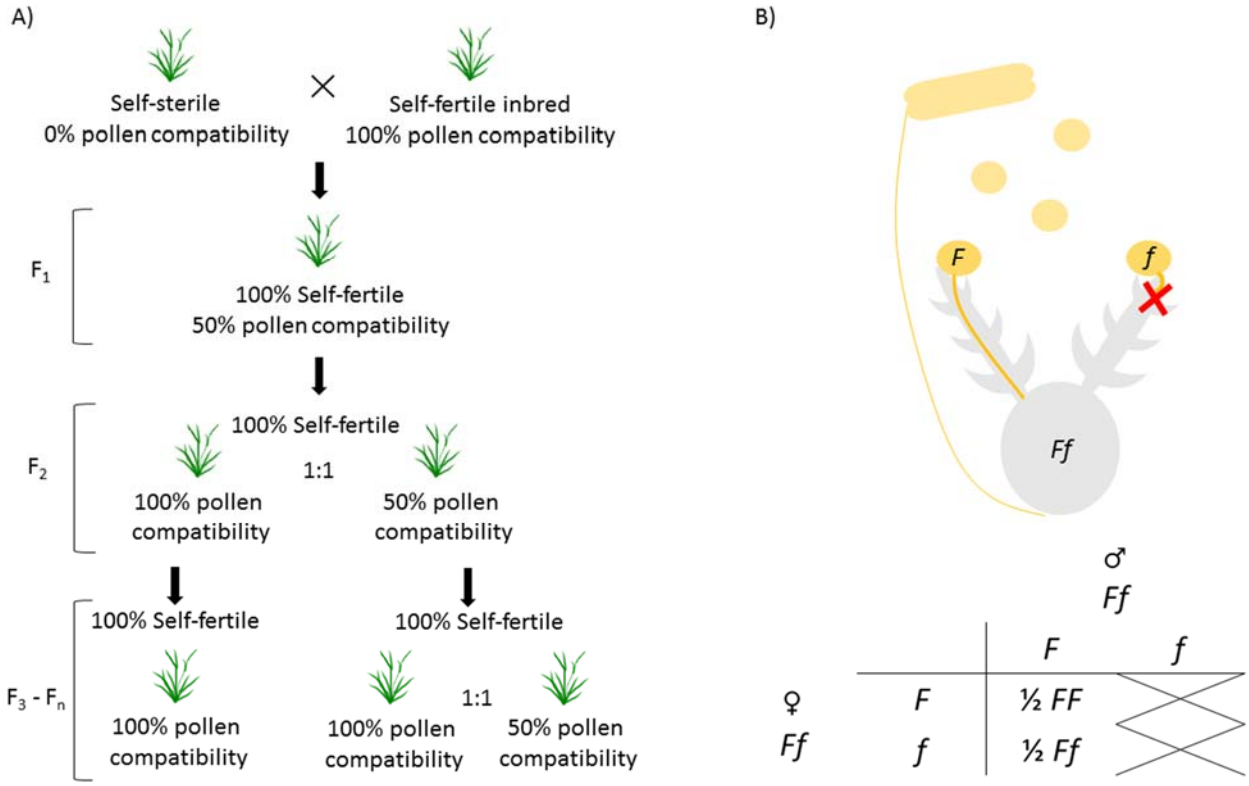

Figure 1. Single gene model for self-fertility and pollen compatibility. A) Observed segregation of SF and pollen compatibility. Note that SF is dominant and gets fixed after the first selfing generation. B) Scheme of the single SF gene acting gametophytically in the pollen grain and the genotypic segregation.

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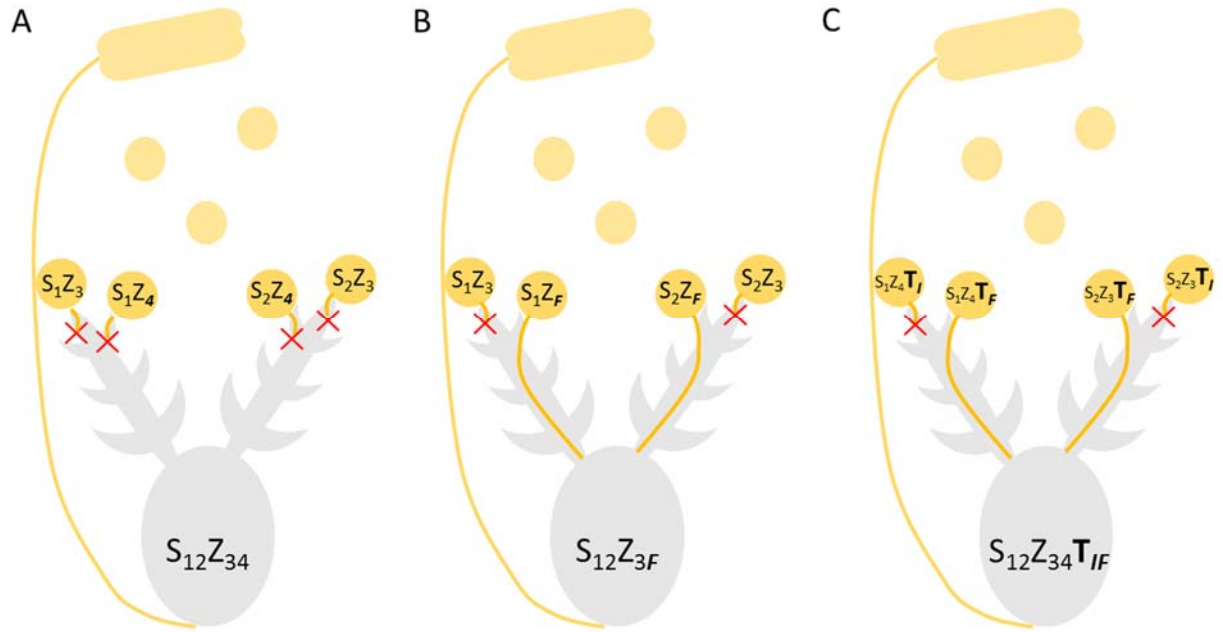

Figure 2. Self-fertility mutations affecting pollen specificity: A) SI: the allele at *S* and the allele at *Z* of all self pollen grains are matched in the stigma and consequently inhibited; B) mutation at a SI locus (*Z*), pollen grains carrying the mutation are not recognized in the stigma and the 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*.

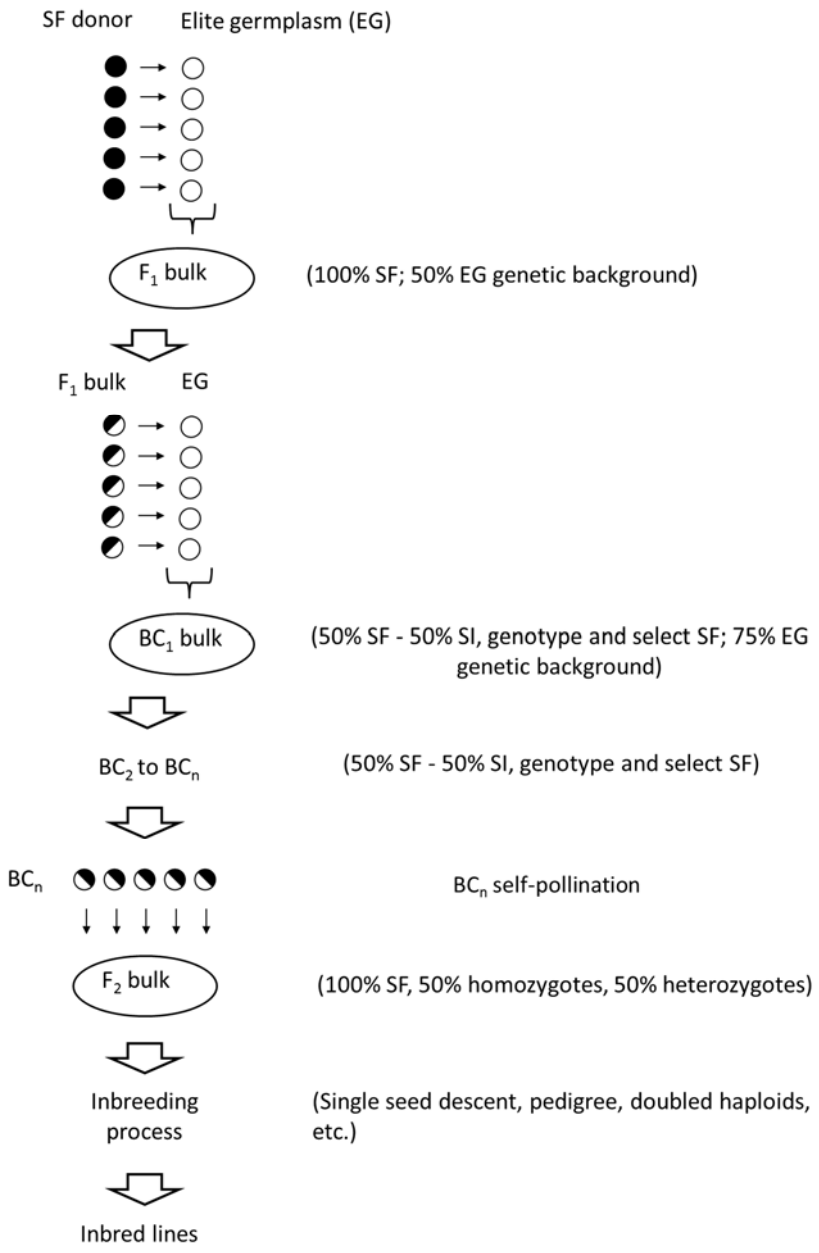

Figure 3. Scheme of a modified back-crossing procedure to introgress a SF gene into a breeding population. ● = homozygous for SF, ◐ = heterozygous for the SF gene, and ○ = SI plant.

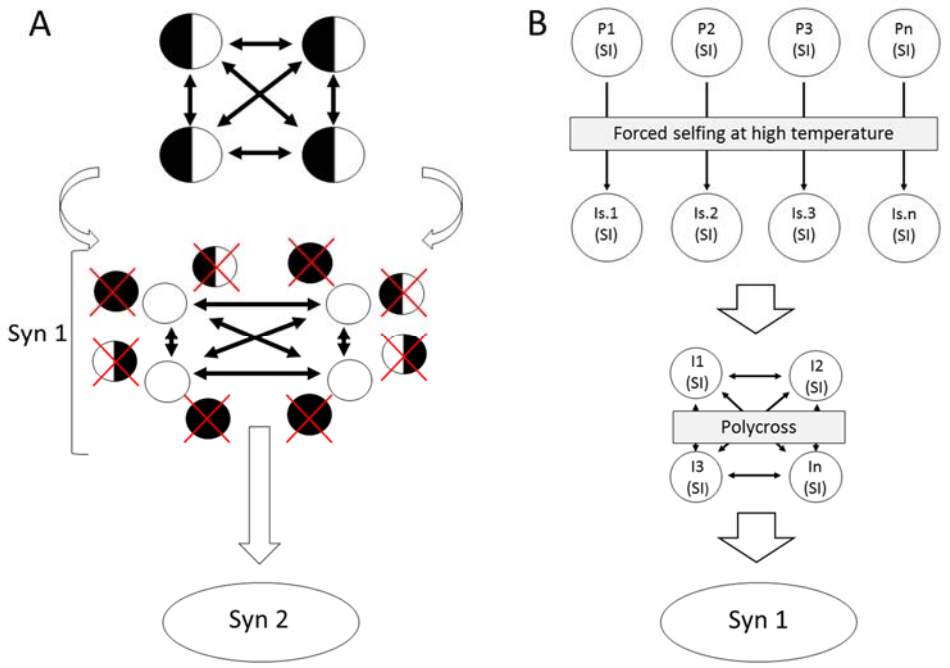

Figure 4. Synthetic breeding using inbreds developed with SF or pseudocompatibility. A) Inbred lines heterozygous for the SF gene, developed by repeated self pollination and selected for high general combining ability, are used as parents and polycrossed to create the Syn 1 generation. Self-incompatible 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.

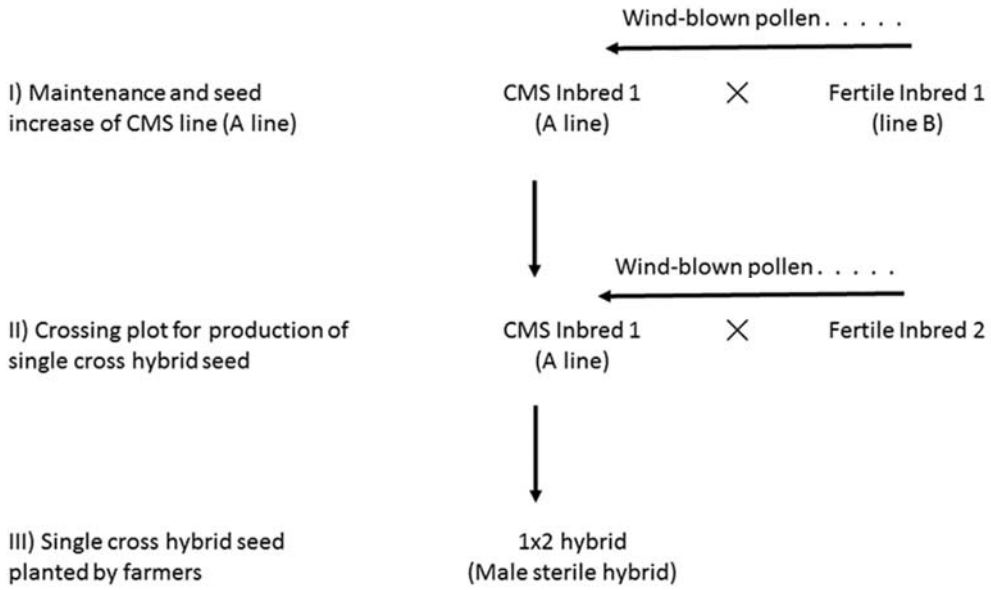

Figure 5. Method for producing hybrid seed using CMS (adapted from Stephens and Holland, 1954). The A line is maintained and multiplied by crossing to the B line which is the pollen donor. The A line is then crossed to a selected fertile inbred (pollen donor) to produce the single cross F_1 hybrid.