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The Heteromorphic Sporophytic Self-Incompatibility System of Buckwheat (*Fagopyrum esculentum* Moench)

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ABSTRACT

In flowering plants self-incompatibility (SI) systems prevent self-fertilization and preserve genetic diversity of species. The SI systems are genetically under the multiloci control, with female and male determinant involved in self/non-self recognition encoded by *S* locus. Although much have been learned for other existing SI systems (the homomorphic sporophytic and the gametophytic), learning about the heteromorphic sporophytic SI system has only just begun in *Primula* and buckwheat. Common buckwheat is a eudicot dimorphic species with either of two flower morphs per plant: pin (long pistil and short anthers) or thrum (short pistil and long anthers). Fertilization is effective only between pollen and pistil of different flower morphs. Upon self-pollination or pollination between same flower morphs, the pollen tube stops at the junction between stigma and style in thrum morph, while it stopps along 2/3 of style's length in pin morph. Breeders' reports in common buckwheat suggest existence of two *S*-determinant classes in dominant relation $S > s$, with thrum plants displaying dominant *Ss* genotype and pin plants displaying recessive *ss* genotype. In addition, thrum pollen expresses dominant *S* phenotype independently of pollen's own haploid genotype being *S* or *s*, therefore, it is concluded that buckwheat SI system is sporophytically determined. Analitical SDS-PAGE of protein extracted from pollen grains and unpollinated styles revealed differences in protein segregation pattern between morphs and further analyses are needed. Treatments of isolated pistils of both morph buckwheat flowers using different metabolic inhibitors and calcium antagonists showed an overcoming of SI reaction, similar to that obtained in the homomorphic sporophytic and the gametophytic SI systems. It follows that different SI types involve the same basic processes triggered by system specific *S*-determinants of self/non-self recognition, that are yet to be determined in the heteromorphic sporophytic SI species.

Keywords: flowering plants, heteromorphic flowers, distily, *S* locus, *S*-supergene
Abbreviations: **AC**, actinomycin D; **BAC**, bacterial arteficial chromosome; **CH**, cycloheximide; **CN**, cantharidin; **GSI**, gametophytic self-incompatibility; **OA**, ocadaic acid; **PCD**, programmed cell death; **RNA**, ribonucleic acid; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; **SI**, self-incompatibility; **SSI**, sporophytic self-incompatibility

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INTRODUCTION

Plants are immobile organisms and cannot choose their mating partners, thus the most important historic event enabling flowering plants to preserve a genetic diversity of species was evolution of self-incompatibility (SI) systems. The function of SI system is to prevent self-fertilization through organized cessation of self-pollen tube growth and to promote out-crossing. Diverse SI systems arose independently many times during the evolution of angiosperms (Bateman 1952; Charlesworth *et al*. 2005; Allen and Hiscock 2008; Igić *et al*. 2008) and are now widely distributed within plant families (more than a half of all flowering species display some type of the SI system, according to Haring *et al*. (1990) and the more recent report shows that more than 100 plant families display SI system, but the estimated SI frequency is *ca*. 40% of species, Igić *et al*. 2008).

The main classification of SI systems is based on the pollen phenotype determination: in the homomorphic gametophytic SI (GSI) the pollen phenotype is determined by its own haploid genotype and in the sporophytic SI (SSI) it is determined by diploid genotype of its mother plant. Further, the SSI exists as homomorphic and heteromorphic type (Barrett 1992) with differentiation based on how many flower morphs one species displays (homomorphic displays

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single flower morph per species, while heteromorphic displays 2 or 3 different flower morphs per species). The heteromorphic system is also characterized by different position of stigmas and anthers within the flower (Dulberger 1992). According to the last published review distyly was more widely distributed since it was displayed in at least 24 families of angiosperms such as *Polygonaceae*, *Primuliaceae*, *Gentianaceae*, *Acanthaceae*, *Plumbaginaceae*, etc., while the tristyly was identified only among three plant families: the *Lythraceae*, *Oxalidaceae* and *Pontederiaceae* (Lloyd and Webb 1992a). These numbers are not final since new data keep arriving (i.e. tristyly-distily transition reported in the *Oxalidaceae* species by Sosenski *et al*. 2009).

Genetically, SI is controlled by the *S* locus which is a highly polymorphic genome region comprising at least two closely linked genes encoding the female and the male determinant of self/non-self recognition (Hiscock and Tabah 2003; Takayama and Isogai 2005; Sherman-Broyles *et al*. 2007). These tightly linked genes, inherited as a single unit, are called the *S*-haplotype. *S*-haplotypes display high allelic polimorphism of determinants of self/non-self recognition in GSI and the homomorphic SSI, but there are still no data for the heteromorphic SSI. In the course of pollination, through interaction of these *S*-determinants it is provided that when pollen and pistil share the same allelic variant of *S*-determinant, the pollen grain is recognized as self and pollen tube growth is stopped by triggering of a complex cascade of processes.

Besides genes of *S* locus, genes residing in non-*S* linked genome regions may have important even crucial role(s) in SI response, like those of: *M* locus in SSI in *Brassica* (Murase *et al*. 2004; Kakita *et al*. 2007), modifier genes in *S*-RNase based GSI (Roalson and McCubbin 2003; Kao and Tsukamoto 2004) or *Z* locus in two locus GSI system in grass (Thorogood *et al*. 2002; Kakeda 2009). There is a report for buckwheat that genes outside *S*-complex linkage group suppress *S*-functions (Matsui *et al*. 2004). Therefore it is highly possible that genes outside *S* locus are included in determining SI response in buckwheat, which is yet to be examined in detail.

In the homomorphic SSI, mostly studied in *Brassica* plants, the main *S* locus gene products are characterized and they are the following: *S* locus receptor kinase (SRK) and *S* locus glycoprotein (SLG) as female SI components and *S* locus cystein rich protein (SCR) acting as a male component of SSI response. The SSI reaction takes place at the papilla cell surface upon binding the pollen coat protein SCR to SRK, a transmembrane serine-threonine kinase (Kachroo *et al.* 2001). Its extracellular domain shares very high homology with SLG, the third *S* locus gene product (Nasrallah *et al*. 1987; Takayama *et al*. 1987). Recently, a novel protein expressed outside the *S* locus that acts as a positive mediator of SI response was found to be MLPK (*M* locus protein kinase). The MLPK binds to SRK and promotes its autophosphorylation followed by SCR binding (Murase *et al*. 2004). This initial step triggers a signaling cascade leading to a rapid arrest of self-pollen tube growth within the stigma. In the opposite situation, when a compatible pollination takes place, it is necessary to block the SRK autophosphorylation and this role was proposed for stigma expressed thioredoxin-H proteins, recognized as a negative mediator proteins in SI (Cabrillac *et al*. 2001; Haffani *et al*. 2005).

HETEROMORPHIC SI SYSTEM OF BUCKWHEAT

Heteromorphic buckwheat

Common buckwheat (*Fagopyrum esculentum* Moench) is a distylous plant displaying heteromorphic sporophytic SI system (de Nettancourt 1977). Flower morph with long pistil and short anthers is designated as pin and morph with long anthers and short pistil as thrum. The fertilization is only possible between different flower morphs, which are usually equally distributed among the populations.

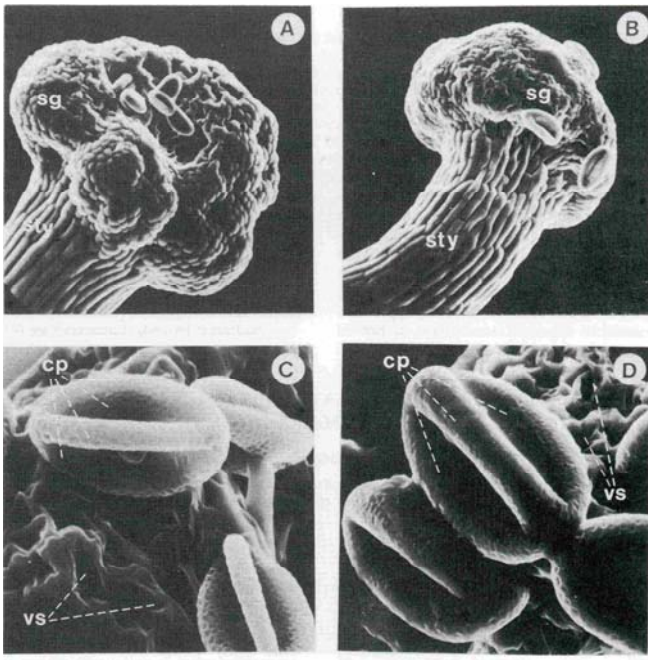

Fig. 1A-D Scanning electron micrographs (JOEL JSM T.35 scanning microscope) of a stigma receptive surface. (**A**) A stigma of pin (X200) and (**B**) a stigma of thrum (X200) morph; sg - stigma, sty - style, vs - vesicle. (**C**) A pollen grain of pin (X1000) and (**D**) a pollen grain of thrum (X1000) morph, cp - colpi. Reprinted with kind permission from **Miljuš-Đukić J, Nešković M** (1998a) Morphological study on self-incompatible buckwheat (*Fagopyrum esculentum* Moench). *Archives of Biological Sciences* **50**, 105-108, with kind permission of University of Belgrade, Belgrade, Serbia, ©1998.

It is an interesting question whether heterostyly promotes cross-pollination or not, having in mind that majority of SI plants are homomorphic. During the evolution, heterostyly appears to be one of the factors that promote outcrossing and make it more effective (Barrett 1992). Quite the opposite, Bjorkman (1995) reported that in buckwheat heterostyly does not help cross-pollination, because the majority of pollen grains that come to stigma by insects are incompatible and more pollen grains come to pin morph than thrum. However, we agree with the observation of Barrett and Shore (2008) that heterostyly in general promotes cross-fertilization by reducing the rate of pollen loss on incompatible stigmas, therefore increasing fitness of species through the male component (Lloyd and Webb 1992a, 1992b). On the other hand, SI prevents self-pollination and inbreeding depression through the female component. It is in this way that evolution of heterostyly promotes efficiency of cross-pollination while preventing self-interference (Barrett 2002).

Morphologically, buckwheat pistils consist of three closely adhering styles. In pin morph, the style is three times longer than thrum, while in thrum, the stamens are 1.5 times longer than in pin (Namai and Fujita 1995) Also, while the receptive stigma surface of the thrum morph is smaller than that of pin, pollen grains of thrum morph flower are larger than pollen grains of pin (Dulberger 1992). According to Heslop-Harrison and Shivanna (1977) the buckwheat stigma belongs to class IIA, non-papillate, dry stigmas, with no secretion. Our investigation on self-pollen tubes growth and scanning of two morphs stigmas (Miljuš-Đukić and Nešković 1998a) confirmed that there are no differences between receptive stigma surfaces of the pin and thrum morph, except those in the size of the receptive area, and no secretion was noticed on any of stigmas (**Fig. 1**).

Genetically, as learned by breeding experiments, the buckwheat SI is controlled by *S* locus displaying two classes of *S*-determinants in dominant relation $S > s$, with thrum plants displaying dominant *Ss* genotype and pin plants displaying recessive *ss* genotype (Matsui *et al.* 2004).

Moreover, the SI in buckwheat is sporophytically determined, since thrum pollen phenotype is always *S* – independently of its own haploid genotype. Previous experiments using X-ray irradiation of common buckwheat seeds (Sharma and Boyes 1961) showed that plants obtained from irradiated seeds bore homomorphic self-compatible flowers, as the result of *S*-gene breaking. Therefore, it is postulated that buckwheat *S* locus comprises at least five closely linked genes, which control style length, stylar incompatibility, pollen incompatibility, pollen grain size and anther height (commonly, this *S*-complex linkage group is named *S*-supergene) similar to that proposed for heteromorphic system in *Primula* plants (Dowrick 1956).

The site of self-pollen tube arrest after self-pollination in the sporophytic SI can be at the surface of the stigma, or within the stigma tissue, which is opposite to the gametophytic SI, where the self-pollen tubes grow along 1/3 to 2/3 of the style length before being stopped (Dulberger 1992). However, it was later discovered that in GSI displaying poppy, incompatible pollen tube growth is stopped at stigma surface as in most SSI systems. In buckwheat after self-pollination of the thrum morphs, self-pollen tube growth is stopped at the junction between the stigma and the style, while in pin it is stopped along 2/3 of the style length before being arrested (Miljuš-Đukić *et al*. 1998a). This shows how buckwheat displays both known manifestations of this process. Having these facts in mind, it is clear that there is no strict rule regarding the place of self-pollen tube growth arrest and the displayed SI type.

Homomorphic buckwheat

Ohnishi and Yoshiriko (1996) report finding a wild buckwheat species *Fagopyrum homotropicum*, in southwestern China. This species possesses self-compatible homomorphic flowers of the pin type (long homostyle). Japanese and Canadian scientists succeeded in crossing homomorphic *F. homotropicum* with common buckwheat *Fagopyrum esculentum* pin plants in order to study the inheritance of heterostyly and self-compatibility (Campbell 1995; Aii *et al*. 1998; Woo *et al*. 1999; Matsui *et al*. 2003). The flower morphology of the derived self-compatible lines was long homostyle. The results suggested that flower morphology and SI response are controlled by a single gene named *Sh*. The relationship of *Sh* to *S* and *s* is $S > Sh > s$ (Woo *et al*. 1999). Matsui *et al*. (2003, 2007) report that *Sh* gene, derived from *F. homotropicum*, arose from a recombination in the *S*-supergene in which each gene remained functional. Wang *et al*. (2005) report that there are two complementary dominant genes, *Sh* and *Sc*, that control self-compatibility and homostyly in buckwheat and that one gene or two gene segregation patterns are the result of interspecific crosses with different *F. esculentum* genotypes. Moreover, Matsui *et al*. (2007) report that there are two types of self-compatibility: one using a self-compatible gene and the other using modifier genes outside the *S* locus that control the intensity of SI response.

ELUCIDATING THE SI MECHANISM IN BUCKWHEAT

Experiments using metabolic inhibitors

Contrary to the investigated homomorphic SSI systems, the data on biochemical mechanisms of SI reaction in heteromorphic systems are still very scarce (de Nettancourt 1997), with studies which have just started in *Primula* and buckwheat species. In order to get an insight into buckwheat SI response, we used metabolic inhibitors that might modify the pistil response upon self-pollination. Data obtained from the literature on other SI systems, homomorphic SSI as well as GSI systems, showed evidence that RNA synthesis inhibitor AC (Asher and Drewlow 1970; Franklin *et al*. 1992) and protein synthesis inhibitor CH (Ferrari and Wallace 1976a, 1976b; Sarker *et al*. 1988) acted in abolishing SI

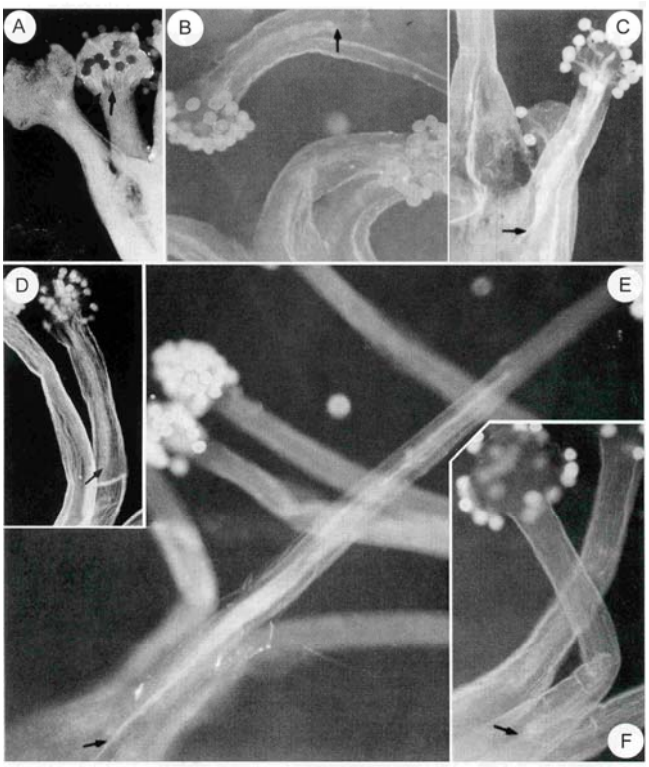

Fig. 2A-F The overcoming of the self-incompatible reaction in differently treated thrum (A, B, C) and pin (D, E, F) pistils. (**A**, **D**) controls, (**B**) 5 μM OA, (**C**) 1 mM verapamil, (**E**) 20 mM CN, (**F**) 50 μM A23187. Arrows indicate the site of self-pollen tube arrest. Reprinted with kind permission from **Miljuš-Đukić J, Ninković S, Nešković M** (2003) Effects of protein phosphatase inhibitors and calcium antagonists on self-incompatible reaction in buckwheat. *Biologia Plantarum* **46**, 475-478, with kind permission of The Institute of Experimental Botany, Prague, The Czech Republic, ©2003.

reaction. In *Brassica* (Sarker *et al*. 1988) it is stated that CH inhibited the first phase of SI reaction – the pollen germination, which is in concordance with report of Hiscock *et al.* (1995) that SI reaction induces synthesis of groups of high molecular weight pollen proteins which cannot be seen upon compatible pollinations. The results obtained on isolated buckwheat pistils treated with AC and CH (Miljuš-Đukić *et al.* 1998b) demonstrated that in common buckwheat SI reaction is completely overcome by these inhibitors of RNA and protein synthesis. These findings implicate that at the moment of anthesis when flowers became self-incompatible, proteins necessary for SI response are present at basic level and further and/or *de novo* protein synthesis is obligatory in the SI response.

Experiments using another group of inhibitors, protein phosphatase inhibitors and calcium antagonists have given us further insight into the heteromorphic SI of buckwheat. It is known that in plant cells, the same as in animal cells, the protein phosphorylation state depends on the activity of both phosphatase and kinase. Protein phosphatases PP1, PP2A and PP2C have been found in plant cells with signal transduction being one of their roles (Smith and Walker 1996). As SRK is one of the main components of SI reaction in *Brassica* homomorphic SSI, it can be expected the protein phosphatases are a part of it as well, but most likely encoded independently of *S* locus. Data obtained in *Brassica* (Rundle *et al*. 1993; Scutt *et al.* 1993) gave evidence that treatments with OA and other phosphatase inhibitors led to cessation of the SI reaction. We treated the isolated buckwheat pistils with OA and CN (**Fig. 2**) and the results showed self-pollen tubes elongation along the treated styles (Miljuš-Đukić *et al*. 2003), implying an active role for both phosphatases and kinases in the SI reaction of buckwheat.

In the *Papaver rhoeas* GSI system, besides its necessity for pollen tube growth (Trewavas and Malhó 1998), Ca^{2+}

has an important role as a second messenger in SI response (Franklin-Tong *et al*. 1997). Franklin-Tong (1999) and Franklin-Tong *et al*. (2002) report that increase in intracellular Ca^{2+} concentration in pollen tubes is necessary for stigma *S*-protein action leading to self-pollen tubes growth arrest. Extracellular calcium influx, connected with SI response and *S*-proteins action, is blocked with verapamil that overcomes SI response. Wehling *et al*. (1994) show for *Secale cereale*, another GSI displaying plant, that treatments using Ca^{2+} antagonists also block SI reaction. Our results in buckwheat calcium antagonists treatments (verapamil, La^{3+} – in a form of $LaCl_3$, and jonophore A 23187) also showed the cessation of SI response (Miljuš-Đukić *et al*. 2003). While in GSI and buckwheat heteromorphic SSI Ca^{2+} functions as a signal messenger, in homomorphic SSI Ca^{2+} fluxes do not seem to be involved in SI reaction as a signal messenger (Dearnaley *et al*. 1997).

Protein segregation patterns of pin and thrum pollen and stylar proteins

Our study on heteromorphic SSI reaction in buckwheat continues with protein analyses of styles and pollen grains of two morphs, and it is presently at the beginning. The aim is to relate the morphological differences between the morphs of flowers with those in protein profiles as well as to identify SI involved proteins. We have separated proteins extracted from unpollinated pin and thrum styles and pollen grains using SDS-PAGE (unpublished data). Differences in protein segregation pattern between the proteins extracts of unpollinated styles and that of pollen grains of two morphs have been found, but further analyses are yet to be done.

Today, a repository of data concerning this subject is not so large. A study on *Averrhoa carambola* (Wong *et al.* 1994a, 1994b) demonstrates the presence of several proteins specific for anthers and style of both short and long morph. Athanasiou and Shore (1997) report the identification of short style specific protein in distylous *Turnera*, with an unknown function. A paper on SI in *Primula* (McCubbin *et al*. 2006) reports finding a group of differentially expressed genes among the pin and thrum morphs. The identified products relate only to the flower and morph development and not to *S* locus and SI response. However, Li *et al.* (2007) report finding predicted small membrane protein of a yet unidentified function in *Primula*.

IS THE PROCESS OF PCD INCLUDED IN SI RESPONSE OF BUCKWHEAT?

Programmed cell death (PCD) is a regulated complex process of a cell death trigerred during plant development, infection or in a self-incompatibile reaction. In GSI response of *Papaver rhoeas*, a process of PCD is a part of the SI response (Thomas and Franklin Tong 2004). It is reported that the fragmentation level of DNA, as a marker of PCD process, is elevated during SI and can be reduced by caspase inhibitor treatment. The most recent data on GSI in poppy (Bosch and Franklin Tong 2008) propose a model where SI response consists of two phases, the first being connected to the *S*-proteins action leading to self-pollen tubes growth inhibition and the second including PCD. We investigated the involvement of proteases in buckwheat SI by treating isolated pistils with protease inhibitors (Miljuš-Đukić *et al.* 2007). In experiments using PMSF, Pepstatin A and Antipain protease inhibitors, SI response was overcome in self-pollinated pistils: self-pollen tubes elongated along the styles treated by protease inhibitors while compatible crosses were not affected. These findings indicate that proteases could be important players in the SI system operating in buckwheat. Still, the question "Which mechanism activates proteases?" remains, proposing different mechanisms operating in different morphs but sharing common triggers of PCD.

FUTURE WORK ON BUCKWHEAT SI

Buckwheat heteromorphic SSI system represents an interesting type of SI as physiologically it comprises the features of both the sporophytic and the gametophytic systems. McCubbin (2008) postulates the same possibility for another, previously mentioned heteromorphic species *Primula vulgaris*. We are continuing our investigation by searching for differentially expressed proteins between two morphs, especially those directly involved in SI reaction, using 2D-PAGE (two-dimensional polyacrilamid gel electrophoresis) and mass spectrometry (MALDI TOF) for protein identification.

An important part of this search is finding the female and the male component of self/non-self recognition in buckwheat SI system. Proteins found to differ between morphs are expected to be connected with flower development and morphology, but there is also a posibillity that some of them are directly involved in different phases of SI response. The male component is expected to be found among the pollen egsine proteins due to necessary recognition interaction with the transmembrane stigma proteins. Moreover, considering different manifestation of self-pollen tubes arrest in pin and thrum morphs it is possible to expect that underlying SI mechanisms are different and may be revealed in different protein segregation patterns. According to the results of the above mentioned experiments using different inhibitors it is possible that SI reaction induces protein synthesis in both stigma and pollen (of both flower morphs). To examine these results further it is necessary to separate proteins isolated from self- and cross-pollinated styles, because only upon self-pollination are those proteins expected to be revealed.

Based on the earlier mentioned results in *Papaver rhoeas* and on our previous research in buckwheat, other unsolved issues that need to be answered are the role of calcium in buckwheat SSI and possible involvement of PCD. We expect to find differences in underlying mechanisms for the two morphs. Besides proteins at the stigma surface directly involved in self-pollen discrimination and cessation of pollen tube growth in thrum morphs, there must be other proteins involved in the processes leading to interruption of self-pollen tube elongation in pin morphs. Therefore, it is most likely that different mechanisms leading to PCD activation may be at work in two morphs. There are still many questions to be answered. One answer is clear: buckwheat SI response is a complex process that involves *S*-locus encoded determinants of self/non-self discrimination, includes protein synthesis upon self-pollination and uses Ca^{2+} as a signal messenger and proteases that may take action in phase two of the reaction as in *Papaver rhoeas* (Bosch and Franklin Tong 2008).

This search, when resolved, will contribute to understanding of diversity, evolution and preservation of SI systems in angiosperms and will also provide information regarding so far most scarcely investigated heteromorphic SSI systems. Moreover, once resolved, it can be applied in control of crosses to obtain lines with desirable traits. Although the SI systems enable plants in maintaining genetic diversity and heterozygosity, they represent a drawback for breeders creating new lines with desired traits. Among the heterostylous plants, buckwheat is of the highest economical importance (Ganders 1979). It is grown mostly in the Northern hemisphere (China, Japan, Korea, Canada, Australia and northern European countries) where it is used as a cereal. Buckwheat seeds are rich in proteins (contain high concentration of all essential amino acids especially lysine, threonine, tryptophan and the sulphur-containing amino acids) and minerals (like iron, zink and selenium) which makes them desirable for human consumption. Considering the great agronomical importance of buckwheat, there is no doubt that the work on SI is worth continuing.

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