Short Communication

Detection of self-incompatible oilseed rape plants (*Brassica napus* L.) based on molecular markers for identification of the class I *S* haplotype

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Abstract

The selection of desirable genotypes with recessive characteristics, such as self-incompatible plants, is often difficult or even impossible and represents a crucial barrier in accelerating the breeding process. Molecular approaches and selection based on molecular markers can allow breeders to overcome this limitation. The use of self-incompatibility is an alternative in hybrid breeding of oilseed rape. Unfortunately, stable self-incompatibility is recessive and phenotype-based selection is very difficult and time-consuming. The development of reliable molecular markers for detecting desirable plants with functional self-incompatible genes is of great importance for breeders and allows selection at early stages of plant growth. Because most of these reliable molecular markers are based on discrimination of class I *S*-locus genes that are present in self-compatible plants, there is a need to use an internal control in order to detect possible PCR inhibition that gives false results during genotyping. In this study, 269 double haploid F_2 oilseed rape plants obtained by microspore embryogenesis were used to verify the applicability of an improved PCR assay based on the detection of the class I *SLG* gene along with an internal control. Comparative analysis of the PCR genotyping results *vs. S* phenotype analysis confirmed the applicability of this molecular approach in hybrid breeding programs. This approach allows accurate detection of self-incompatible plants via a different amplification profile.

Keywords: double haploid, hybrid breeding, MAS, S locus, *SLG*.

An efficient pollination control system is necessary in hybrid breeding in order to avoid unwanted self-pollination or sib-pollination of the female parental line. In self-pollinating plants such programs are limited in their ability to overcome and prevent pollination by their own pollen. Consequently, hybrid breeding depends on a variety of approaches to protect maternal plants from self-pollination. Self-incompatibility (SI) prevents self-fertilization by rejecting pollen from plants with the same genotype (Charlesworth, 2010; Goring and Indriolo, 2010) and has been successfully used for seed production in F_1 hybrid cultivars of *Brassica napus* (Zhang *et al.*, 2008a). In *Brassica*, the specificity of pollen-stigma interactions in SI systems is controlled sporophytically by two linked genes with multiple allelic forms (*S* haplotypes) of a single Mendelian polymorphic locus, designated as the *S* locus (Bateman, 1955; Nasrallah *et al.*, 1991). The male and female determinants of SI encoded by the *S*-locus genes have been identified as a peptide ligand (*S*-locus protein 11; *SP11*, or

S-locus cysteine-rich protein; *SCR*) (Schopfer *et al.*, 1999; Suzuki *et al.*, 1999) and its cognate receptor (*S*-locus receptor kinase; *SRK*; Stein *et al.*, 1991) respectively. Recognition specificity is achieved by interaction of the female determinant *SRK* with its ligand, the male determinant *SP11* (Chapman and Goring, 2010). The interaction between *SP11* and *SRK* triggers the signaling cascade in an *S*-haplotype-specific manner and results in the rejection of self-pollen, but the signal components involved are still not well characterized (Zhang *et al.*, 2011). S-locus glycoprotein (SLG) encoded by the *SLG* gene is the second female determinant involved in SI reaction. SLGs accumulate in the mature papilla cell wall and affect self-pollen tube development (Kishi-Nishizawa *et al.*, 1990). Gain-of-function experiments have demonstrated that SLG enhances the recognition reaction of SI (Watanabe *et al.*, 2003).

The *S* haplotypes are divided into classes I and II (Nasrallah *et al.*, 1991). Class I is generally dominant over class II in pollen and confers a strong SI phenotype (Nasrallah and Nasrallah, 1993). Cultivated *B. napus* (2n = 38, AACC) is a self-compatible allotetraploid that originated through spontaneous interspecific hybridization between two self-incompatible species, *Brassica rapa*

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(2n = 20, AA) and *Brassica oleracea* (2n = 18, CC). Theoretically, *B. napus* lines are considered to be homozygous at two *S* loci, with one *S* haplotype being derived from *B. rapa* and the other from *B. oleracea*. *Brassica napus* is considered to be self-incompatible like its two ancestors and artificially produced lines, although most cultivated lines are self-compatible (Zhang *et al.*, 2011). Most *B. napus* contain a class I *S* haplotype (similar to S^{47} of *B. rapa*) in the A genome and a class II haplotype (similar to S^{15} of *B. oleracea*) in the C genome (Okamoto *et al.*, 2007; Zhang *et al.*, 2008a). Self-compatibility in *B. napus* is caused by independent mutations in dominant *S* haplotypes that result in suppression of the functional recessive *S* haplotype on different chromosomes (Okamoto *et al.*, 2007). Four and three class-II *S* haplotypes have been identified in *B. rapa* (S^{29}, S^{40}, S^{44}, S^{60}) and *B. oleracea* (S^2, S^5, S^{15}) (Sato *et al.*, 2006). Not surprisingly, in homozygous plants with two recessive *S* haplotypes, the character of the recessive *S* haplotype appears as an SI phenotype that is usually a mutant phenotype. However, genetic changes in polyploid *B. napus*, including chromosomal rearrangements (Udall *et al.*, 2005) and epigenetic phenomena (Gaeta *et al.*, 2007; Zhang *et al.*, 2011), can alter gene expression and phenotype. No original Czech hybrid cultivar based on SI has yet been released, but SI is currently intensively used in Czech breeding programs. An approach based on molecular marker technology for the genotyping of breeding populations in rapeseed hybrid breeding could provide a greater incentive for investment.

A double haploid (DH) SI line (SI20) with stable recessive *S* haplotypes derived from crosses between an SI donor line (WRG15) and self-compatible donors of 00-quality (2051; Viking) and four self-compatible (SC) cultivars (Da Vinci, Cabernet, Chagall and Wisent) were used in this study (Figure 1). Four SC cultivars were used as the male parent in crosses with the SI20 line to obtain F_1 hybrids, and the F_1 plants were used to produce 269 DH of the F_2 population via microspore embryogenesis. These populations are regularly created by breeders to improve the properties of self-incompatible plants and obtain desirable genotypes of comparable or excess properties equal to current best cultivars. The variation of newly created DH plants reflects primarily in additive gene effects and additive epistasis, enabling fixation in only one cycle of selection compared to about seven years using the conventional system to generate near homozygous lines. All of the materials used in this study were obtained from the Research Institute of Crop Production in Prague, Czech Republic.

The *S* phenotype was measured as follows: when the first flowers appeared on the major inflorescence, this inflorescence and two secondary ramifications were bagged for self-pollination. Every two days, the bags were tapped gently to ensure enough self-pollination. The bags were removed after three weeks to allow the seeds to develop in a more natural environment. After maturation of the seed siliques, the number of seeds produced was counted. The seed set obtained by selfing was checked when siliques were ripe. Plants were considered as SI when the number of seeds per silique ranged from 0 to 3. Self-seeds were obtained by bud-pollination when the SI reaction was still not fully developed.

Genomic DNA from *B. napus* cultivars and the DH line was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method (Williams *et al.*, 1992). The concentration and quality of DNA was determined with a BioMate 5 spectrophotometer. PCR amplification was done using a primer pair SLGa (Zhang *et al.*, 2008b) developed from SLG-A10 and that amplified a ~1100 bp region in SC plants but not in the SI line. The primer pair SCO3 that amplified a ~750 bp fragment (Zeng
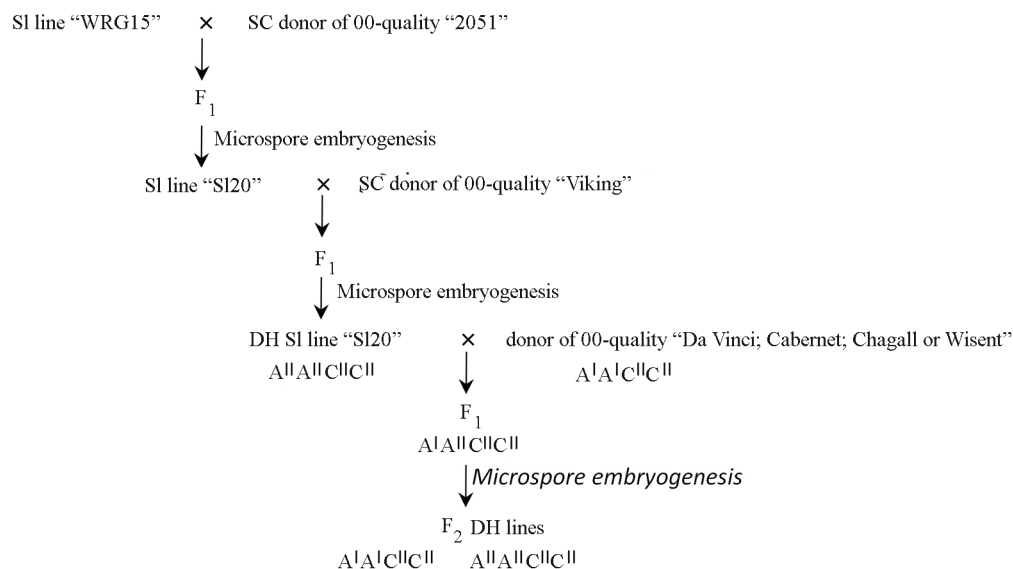

Figure 1 - Overview of the triple-cross model, including the three-component hybrid and three line hybrid in *B. napus*.
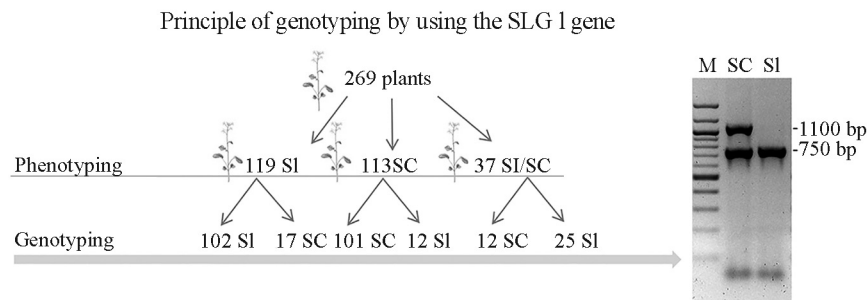
Principle of genotyping by using the SLG 1 gene

Figure 2 - Phenotypic and genotypic screening of F₂ DH plants. The plants (n = 269) initially underwent *S* phenotypic analysis followed by genotyping with molecular markers. Duplex PCR with class I SLG-specific primers (SLGa forward and reverse) resulted in the amplification of an ~1.1 kb fragment in plants considered to be self-compatible (SC); self-incompatible (SI) plants showed no such fragment. All samples (SC and SI) showed amplification of a 750-bp internal control fragment. M – molecular markers.

et al., 2009) was used as a positive control for the presence of DNA in the PCR. PCR was done with 10 ng of genomic DNA as template, mixed with 0.5 μL (10 μM) of each primer, 10 μL of PPP master mix (TopBio), 1x BSA and distilled water to give a final volume of 20 μL. The PCR reaction involved pre-denaturation for 3 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 58 °C and 1 min at 72 °C and finally, 10 min at 72 °C. The PCR products were subjected to electrophoresis on 1.5% agarose gels in 1x Tris-borate-EDTA (TBE) buffer and detected by staining with ethidium bromide.

All SI20 x (SC) Da Vinci, Cabernet, Chagall and Wisent F₁ plants were completely self-compatible, indicating that the *S* haplotype of the A genome of SI20 was recessive (A^II A^II C^II C^II) to four SC cultivars deduced to be A^I A^I C^II C^II. Self-incompatibility was assessed in the F₂ DH population of 269 plants. The distribution of the S phenotype showed two distinct classes, with a score < 3 indicating SI plants (n = 119) and > 10 indicating SC plants (n = 113). Limited number of plants (n = 37) showed intermediate character with score 4-10 indicating SI/SC plants. The segregation of SC plants to SI plants in the F₂ DH population at the molecular level agreed with the expected ratio of 1:1 (130 SC *vs.* 139 SI) for a single *S* locus (Figure 2).

An approach based on molecular markers was implemented in a traditional schedule of oilseed rape hybrid breeding. A particular technique that involved two pairs of primers was used to manage the breeding population. Comparison of the genotype (presence of the desired alleles) *vs.* phenotype (seed test) showed that selection based only on phenotypic expression (number of seeds per silique) can lead to the loss of valuable SI genotypes (in our case, with improper selection 4.5% of the plants (n = 12) would be excluded, as well as a further 37 phenotypically indistinguishable flowers, if there were no subsequent molecular characterization). In 12 plants phenotypically identified as SC we assumed that there was breakdown of the SI barrier at the end of flowering, possibly as a result of survival strategies. The 17 plants that showed the SI phenotype (contrary to their SC genotype) could have been caused by high tem-

peratures in the pollination bags that, together with humidity, can lead to complete sterility (Shah *et* al., 2011) or induce the loss of siliques in the early stages of development, as described by Young *et al.* (2004). The presence of haploid plants can also cause the SI phenotype.

Recent molecular screening for the class I *S* haplotype has been based on the detection of SLG I/SRK I genes (Gao *et al.*, 2013), and gene transformation studies have confirmed that both of these genes are important for the SI recognition reaction (Watanabe *et al.*, 2012). Self-incompatibility mechanisms associated with functional molecular markers can be particularly important in hybrid breeding, as suggested by Tochigi *et al.* (2011). These authors showed that the use of a molecular marker based selection of SI plants leads to elimination of undesirable SC plants at an early ontogenetic stage and to favourable reduction of the number of plants available for subsequent experiments. In conclusion, the results of this study indicate that we have successfully developed and validated a new approach for detecting SI.

Acknowledgments

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Associate Editor: Dario Grattapaglia