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Different expression of *S*-locus cysteine-rich protein *(SCR)* alleles in self-incompatible and self-compatible *Brassica napus* breeding lines and cultivars: Can be *SCR/SP11* used as a selectable marker in breeding?

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There are several approaches available for hybrid breeding in oil seed rape, *Brassica napus*, as cytoplasmic male sterility (CMS), genic male sterility (GMS), self-incompatibility (SI), and chemical hybridizing agens (CHA). In comparison with others, SI is regarded as one of the most valuable strategies in hybrid breeding. Unlike self-incompatible (SI) *B. rapa* and *B. oleracea*, two ancestor species, *B. napus* is naturally self-compatible (SC). However, occasionally SI also occurs in rapeseed cultivars. SI in *Brassicaceae* plants is sporophytically controlled by a single multi-allelic locus (S-locus), which contains at least three highly polymorphic genes expressed in the stigma (S locus glycoprotein, *SLG and* S receptor kinase, *SRK*) and in the pollen (*SCRlSP11*). In segregating population derived from crosses between DH SI lines and 00-quality donors we found two recessive alleles of a *SCR class II* gene. We developed new primers for detection of unique cv. Tandem derived allele and this allele was successfully amplified in SI donor plants and SI plants after first cycle of crossing. Analyses of other accessions (SI donor different from cv. Tandem) and varieties did not show so clear pattern of segregation and different expression of both alleles does not correspond to phenotypic manifestation of self-incompatibility and we can assume that it is caused by the presence of repressor gene that does not lie on the *S*-locus.

Key words: Self-incompatibility, *Brassica napus*, S receptor kinase (*SRK)*, S locus glycoprotein **(***SLG)*, S locus cysteine-rich protein (*SCR)*.

INTRODUCTION

Brassica napus is one of the most important source of edible oil (Tan et al., 2011). At present, hybrid cultivars have higher productivity than conventional ones and their seed quality (contents of erucic acid and glucosinolates) has also been greatly improved (Li et al., 2011; El-Beltagi and Mohamed, 2010; Ahmad et al., 2013). Therefore, the breeders are interested in development of commercial F_1 hybrids of *Brassica* species. There are several ways available for hybrid breeding in *Brassica* species, including cytoplasmic male sterility (CMS), genic male sterility (GMS), self-incompatibility (SI), and chemical hybridizing agents (CHA) (Li et al., 2011). In comparison with other ways for hybrid breeding, SI is regarded as one of the most valuable strategies for the following reasons: (1) it does not have any of the negative effects that exist in male sterility system (Dong et al., 2013); (2) it

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can be easy overcome; and (3) it can be selected easily in a breeding programme. Self-incompatibility is a widespread mechanism used by flowering plants to prevent inbreeding depression and helps to create and maintain genetic diversity within a species (Goring and Indriolo, 2010). Oil seed rape (*B. napus* L.) is an optionally cross-pollinating species, but self-pollination prevails. In an effort to increase the production, breeding is focused on the F1 hybrid breeding programmes and self-incompatibility here could find its application.

After genetic background investigation of SI in *Brassica* species was found out that SI is under control of *S*-locus (Bateman, 1955). Three genes of the *Brassica S*-locus (*SP11/SCR*, *SRK,* and *SLG*) are inherited together as a unit, and each comes in many versions, or *S*-alleles; different *S*-allele combination of these genes is referred to as *S* haplotypes (Goring and Indriolo, 2010). For SI to be maintained, alleles from each *S*-haplotype must remain as a tightly linked genetic union. Single alleles of *S*-locus were divided according their phenotype effects into two classes, Class I and II supposed to be dominant and recessive, respectively (Nasrallah et al., 1991). Recently many review articles focused on molecular mechanism of sporophytic SI in *Brassica* species were published (Takayama and Isogai, 2003; Isogai and Hinata, 2002; Kachroo et al., 2002; Takayama and Sakagami, 2002; Watanabe et al., 2003, Ivanov and Gaude, 2009, Haasen and Goring, 2010, Chapman and Goring, 2010, Charlesworth, 2010). Physical maps have been constructed of the *S*-locus region of several *S*-haplotypes from *B. oleracea* (Boyes et al., 1997), *B. rapa* (Boyes et al., 1997; Suzuki et al., 1999), and *B. napus* (Cui et al., 1999, Casselman et al., 2000). Using different techniques (direct sequencing, cDNA selection techniques, and RNA differential display), a number of genes have been identified in the *S*-locus region (Cui et al., 1999; Suzuki et al., 1999; Casselman et al., 2000).

Even though the female determinant was known, the pollen determinant was always elusive. Candidate for the pollen determinant was predicted to have several characteristics. As well as S receptor kinase (*SRK)* and S locus glycoprotein (*SLG)* gene it should be located at the *S*-locus. The pollen grain would have showed doubled haploid character in *S*-haplotype of male determinant as it is required in sporophytic form of SI. To fulfil this condition pollen determinant should be expressed before meiosis in pollen mother cells or expressed later in the tapetum cells (Watanabe et al., 2003). Characterization of the coating has revealed the presence of several families of gametophytically expressed small cysteine-rich proteins pollen coat proteins (PCPs). PCP-A class proteins have specific affinities for stigmatic and S- and S-related proteins (Doughty et al., 2000).

Two independent studies have revealed a gene for pollen determinant at the same time. *S*-locus cysteine-rich protein *(SCR)* (Schopfer et al., 1999), syn. *SP11* (Suzuki et al., 1999) is expressed in anthers only. Anther tapetum cells produce SCR/SP11 protein that bind with a high affinity to its receptor, the pistil-specific SRK protein (Goring and Indriolo, 2010). Putative pollen determinant fulfilled all requirements such as highly polymorphic character, anther-specific expression, physical linkage with SLG and SRK. *SCR/SP11* is invariably located between *SLG* and *SRK* in upstream orientation to *SLG*, closer to *SRK* than to *SLG* (Takayama et al., 2000). *SCR/SP11* encodes a small (<8 kDa) hydrophobic and positively charged peptide that exists as a monomer (Takayama et al., 2001). Based on immunostaining is suggested that S_8 SCR/SP11 was secreted from the tapetal cell into anther locule as a cluster and translocated to the pollen surface at the early developmental stage of the anther. During the pollination process, SCR/SP11 was translocated from the pollen surface to the papilla cell, and then penetrated the cuticle layer of the papilla cell to diffuse across the pectine cellulose layer (Iwano et al., 2003). Eight conserved cysteine residues, a glycine residue, and an aromatic amino acid residue are characteristic for amino acid arrangement of *SCR/SP11* gene product. *SCR/SP11* gene consist of two ORF, the first ORF for putative signal protein is highly conservative among *Brassica* species, while the second is highly polymorphic. L1 loop, designated as the hypervariable (HV) region is the most variable region of the SCR, suggesting to be responsible for imparting the allele-specific interaction with receptors. It folds into an α/β sandwich that resembles those of plant defensins (Mishima et al., 2003). As well as *S*-haplotypes the *SCR/SP11* gene is divided into two classes. *SCR/SP11* alleles are highly divergent (Watanabe et al., 2000). In four *B. rapa* class-II *S*-haplotypes, linear dominance relationship were observed. Using RNA gel blot analysis, linear dominance relationship regulation by expression of *SCR*/*SP11* was found (Kakizaki et al., 2003). The mRNA of class II SCR/SP11 was detected predominantly in the anther tapetum in homozygotes and was not detected in the heterozygotes of class I and class II *S*-haplotypes, suggesting that the dominant/recessive relationships of pollen are regulated at the mRNA level of SCR/SP11 (Shiba et al., 2002). The 522 bp 5' upstream region support the correct function of SCR gene (Shiba et al., 2001). It was demonstrated that SCR/SP11 alleles determined *S*-haplotype (Shiba et al., 2001). Duplicated SCR found in *B. oleracea* S^{15} haplotype produced two different sizes of transcripts (Shiba et al., 2004). The linear dominance relationship of SI phenotype on pollen side in class II SP11 is regulated by the expression of *SP11* (Kakizaki et al., 2003). In A self-compatible *B. rapa* of class I *S*-haplotype insertion of retrotransposon-like sequence in first introne of *SRK* and 89-bp deletion in the SP11 promoter was revealed (Fujimoto et al., 2006). Transcription of functional *SP11-60* allele of *B. rapa* was suppressed in heterozygotes with S-f2 allele originating from class I self-compatible *B. rapa* (Fujimoto et al., 2006).

The present research work was therefore designed to: (1) study the genetic variability of the *SCR/SP11* gene in SI donors, self-compatible cultivars and segregating population derived from crosses between these DH SI lines and 00-quality donors respectively; (2) explore a possible way to identify specific marker associated with SI trait (recessive alleles of a *SCR* class II gene); and (3) verify this molecular marker in segregating population.

MATERIALS AND METHODS

Plant material

A segregating doubled haploid (DH) populations of 118 oilseed rape plants was derived from four crosses between self-compatible (SC) cultivar 'Lisek' and self-incompatible (SI) line 'AIK 6', SC cultivar 'Rasmus' and SI line 'AIK 6', SC cultivar 'Rasmus' and SI line 'AIK 3', and finally SC line 'OP BN-03' and SI line 'AIK 3'. 'AIK 3' and 'AIK 6' SI lines were derived from SI line 'Tandem' with recessive type of self-incompatibility. DH lines were produced in the Crop Research Institute Prague – Ruzyne, CZ.

Analysed DH lines:
AIK-6 x Rasmus DH lines No. 1-33
AIK 6 x OP-BN-03 DH lines No. 34-66
AIK-6 x Lisek DH lines No. 67-92
AIK-3 x Rasmus DH lines No. 93-118

Analysed SI donors:
'Start (86/1)', 'WRG 15', 'Tandem 6/85', 'Tandem 1/85', 'AIK 3', 'AIK 6'.

Analysed cultivars:
Rasmus, Lisek, Jesper, Regent, Lirajet, Solida (genotypes used in specified breeding programme).

Isolation of genomic DNA and PCR-RFLP of SCR gene

Genomic DNA of *B. napus* cultivars and DH lines was extracted from young leaves of 2-week-old seedlings by the DNeasy Plant Mini kit (QIAGEN). *SCR* gene was amplified with Class II *SCR*-specific oligonucleotide primers for genomic DNA (Shiba et al., 2002). Allele specific amplifications were performed with sets of primers (allele 1: 5'-TTTGATTTTGACATATGTTC-3' and 5'-CCCCTCAACTTCATAGTGTT-3'; allele 2: 5'-TTGGACTTTGACATATGTTC-3' and 5'-CTCTGAAGTGGGTTTTACAG-3') designed according to highly variable segments between two analysed *SCR* alleles. Plant genomic DNA approximately 50 ng was mixed with 10 pmoles primers, 10x buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 1% Triton X-10), 100 µM dNTP, 1U *Taq* polymerase (TaKaRa) in a final volume of 25 µl. The PCR conditions were 45 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. Polymerase Chain Reaction (PCR) fragments were analysed using agarose and polyacrylamide gel electrophoresis and stained with ethidium bromide. PCR products were then cleaved by specific restriction endonucleases (*Mnl*I, *Hinf* I, *Hha*I). The resulting fragments were then divided into 10% PAGE or 1.5% agarose.

mRNA isolation and *cDNA* synthesis

Anthers of the DH SI lines and rapeseed cultivars were collected from buds at 2 to 3 days before opening. Total RNA was isolated using RNasy Plant Mini Kit (Qiagen). Isolation included DNA degradation step using DNase. The mRNA was isolated from total RNA using Oligotex mRNA Kit (Qiagen). Approximately 20 µg of RNA was subjected first-strand cDNA synthesis using Omniscript (Qiagen) with an oligo$(dT)_{18}$ primer. Second strand was amplified with a set of SCR II specific primers (5'-GCGAAAATCTTATATACTCATAAG-3' and 5'-TTCGTTGATCAATTATGATT-3' Shiba et al., 2002). Reverse transcription-polymerase chain reaction (RT-PCR) was performed under these conditions: 42 cycles of 30 s at 93°C, 30 s at 45°C, 1 min at 72°C and one cycle of 72°C for 5 min.

DNA cloning and sequencing

For determination of nucleotide sequences, PCR fragments were extracted from gel with QIAquick Gel extraction kit QIAGEN and ligated with TOPO TA Cloning kit (invitrogen). The insert of the expected size was analysed using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (*Mnl* I, *Hha* I) and individual clones were sequenced. Sequencing reaction was prepared with cycle sequencing ready reaction kit (Applied Biosystem). Sequence analysis was performed on the ABI PRISM 3130 sequencer.

Seed test

On one branch with buds from each plant was isolated and number of seed were counted in open-pollinating and isolated flowers/siliquae. Phenotypic expression of SI was evaluated by the number of seeds in the silique.

RESULTS

With Class II specific *SCR/SP11* primers a 450 bp band from genomic DNA was amplified. Subsequently this product was cloned and sequenced. Resulting sequence had all characteristic of SCRs such as eight conserved cysteines, one glycine, and one tyrosine in assumed positions. After analysis of genomic DNA only one single allele was found in analysed plants from segregating population. To detect other expected allele, cDNA synthesis of Class II *SCR* from anthers was performed and 350 bp long fragment was obtained and subsequently again cloned and sequenced. In order to find allelic polymorphism, screening of clones was performed by PCR-RFLP method. Two different PCR-RFLP pattern were revealed corresponding to two Class II *SCR* alleles (Figure 1). The two Class II *SCR/SP11* gene alleles were sequenced and marked as allele 1 and 2. The sequence similarity between these two alleles was 85% (Figure 2). Comparison with database NBCI (http://www.ncbi.nlm.nih.gov) showed that allele 1 was identical with S^{15} allele from *B. oleracea* and allele 2 was unique and typical for cv. Tandem as SI donor. Using PCR-RFLP of single cloned cDNAs we have found out that both alleles were present in SC plants as well as SI plants (Figure 1). It seemed that allele 2 expression is often much higher in both phenotypes. Occurrence of single nucleotide mutations in both alleles was quite

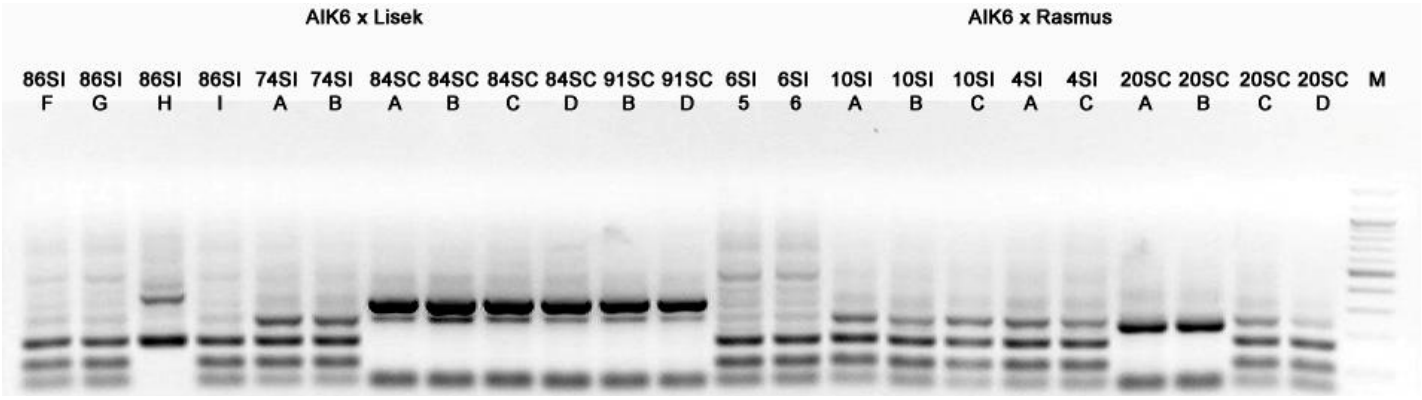

Figure 1. PCR-RFLP of *SCR* class II cDNA clones. Cloned cDNAs were amplified by PCR and then restricted with *Mnl* II. Allele 1 had three restriction sites (sample 84SC A to 91SC D, 20SC A to 20SC B). Allele 2 had two restriction sites (sample 86SI F to 74SI B, 6SI 5 to 4SI C, 20SC C to 20SC D). Clone 86SI H had 1 bp mutation in one recognition site. Putative phenotype was marked with letter SI or SC.

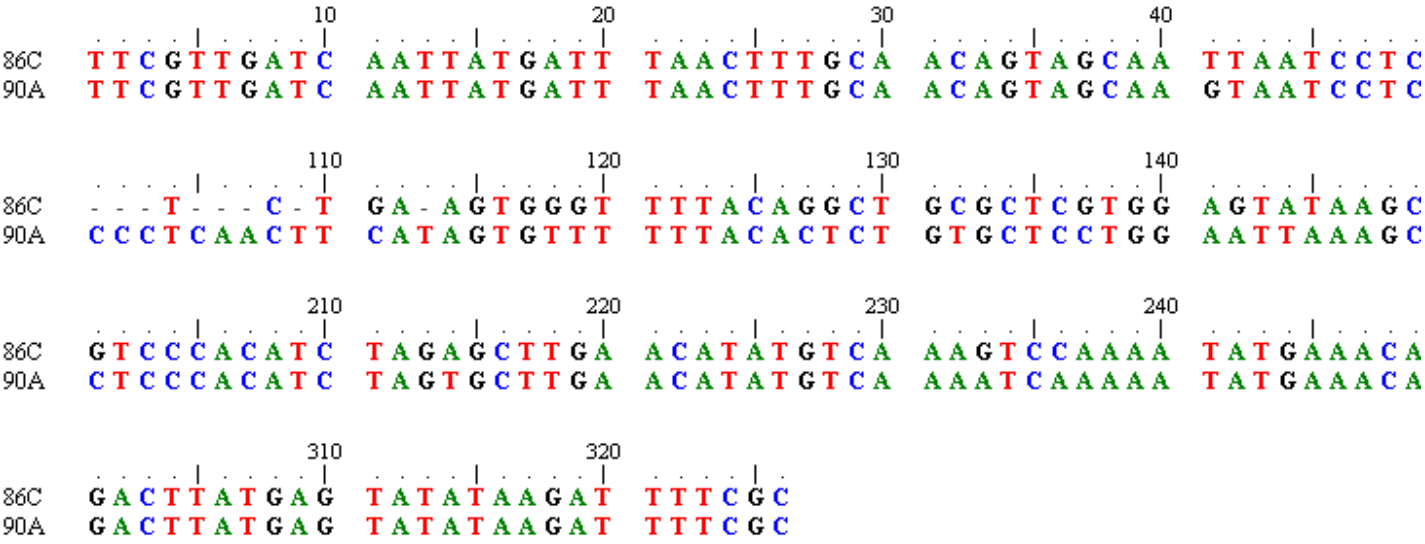

Figure 2. Comparison of cDNA sequences of two revealed *SCR* class II alleles in *Brassica napus*. The sequence similarity between these two alleles was 85%.

frequent. Furthermore, we found that S^{15} allele was duplicated. Similarly we observed that two forms of transcripts exist. We performed screening of segregating doublehaploid population with allele specific primers. A single approximately 280 bp band was present in SI DH lines only (Figure 3). Original donor of SI in this set of DH lines was Tandem 6/85 and screening of other accessions and varieties did not show so clear segregation. Amplification of allele 1 carried out at temperature of annealing 55°C but did not carry out at temperature of annealing 60°C. Also presence of allele 1 and 2 in wide group of cultivars and in several SI lines was tested. Amplification of allele 1 in cultivars resulted in about 700 bp long fragment in cultivar 'Jesper' and about 550 bp long fragment in cultivar 'Regent' despite these specific primers are designed to amplify approximately 300 bp long fragment. Allele 2 specific primers amplified 280 bp long fragment in cultivar 'Lirajet' and cultivar 'Solida'. In SI lines two fragments about 480 and 520 bp long of allele 1 in SI line 'WRG 15' and 'AIK 6' and one single fragment about 500 bp long in SI line 'AIK 3' were amplified. Allele 2 was detected in SI lines 'Start (86/1)' (1), 'Tandem 6/85' (3), 'AIK 3' (5) and 'AIK 6' (6).

DISCUSSION

SCR/SP11 is sole pollen determinant of self-incompatibility (Schopfer et al., 1999; Suzuki et al., 1999). In *B. oleracea* S^{15} haplotype Class II duplicated *SP11*

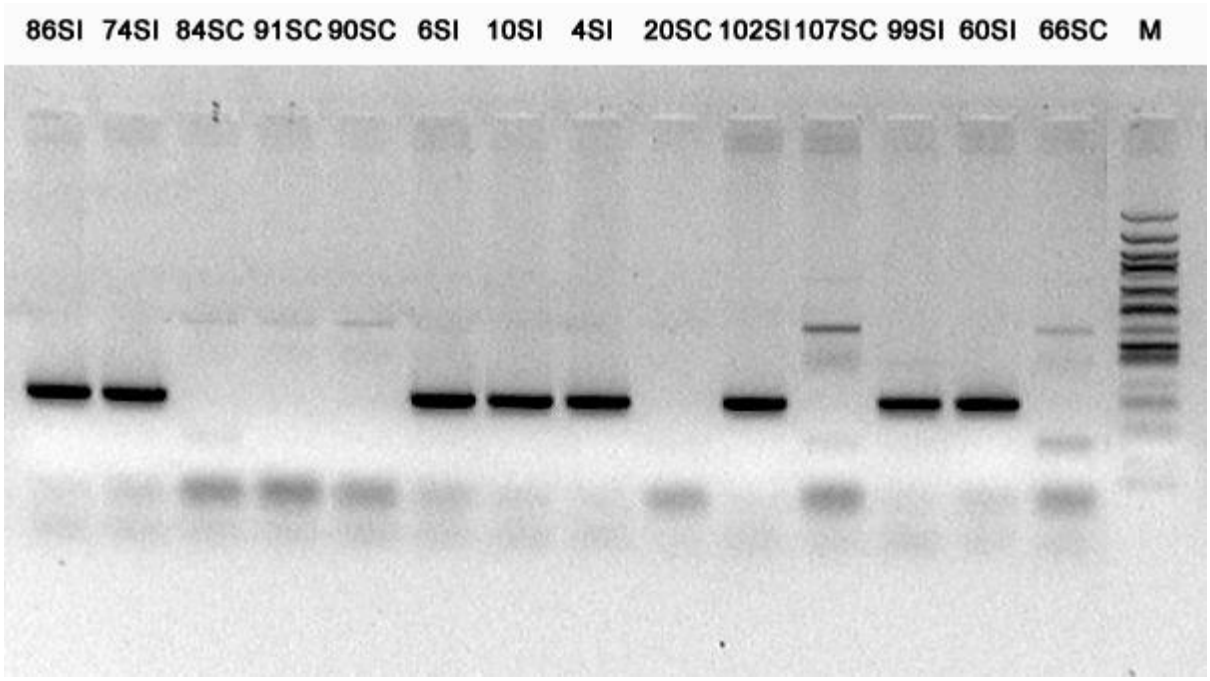

Figure 3. Screening of segregating doubled haploid population with allele 2 specific primers. A single approximately 280 bp fragment was amplified in putative SI DH lines.

gene was found - *SP11 – SP11a*, *SP11b* a *SP11b´*, and these duplicated genes were connected by integeneric spacer. The difference between *SP11a* a *SP11b* were only in one deletion and were oriented in the same direction. As result of this duplication also transcrips of different length were found (Shiba et al., 2004). In *B. napus* we also found duplicated *SCR II* gene but without presence of different transcripts. Cabrillac et al. (1999) found duplicated *SLG* genes in *B. oleracea*, line S^{15}. Allele 1 is probably derived from *B. oleracea*, because it is also duplicated and has 100% similarity to S^{15} alele from *B. oleracea*. This could suggest that this allele originates from *B. oleracea*. The duplication could lead to the producing of incorrect lengths of transcripts responsible for dysfunction. The second allele was specifically occurred in SI lines. Two types of transcripts, AL+ and AL- were observed in *B. oleracea* and it is supposed that AL- type of transcript is the later form (Shiba et al., 2002). This finding is in consistent with our results. Both types of transcripts were expressed in SI and SC DH plants. Considering that the allele specific primers were designed on the basis of sequences obtained from specific plant material and the *SCR* polymorphic nature, it is evident that they cannot easily amplify single alleles in different cultivars. Moreover, there is a big sequence similarity among genes at the *S*-locus and for instance SCR binding domain area of *SRK* gene can be amplified as well. Previously we do not succeed in amplification of allele 2 with Class II specific *SCR* primers from genomic DNA. The difference between allele 2 amplification from cDNA and genomic DNA may be caused by different numbers of copies of which the expression might be affected in a certain way. The level of expression of *S*-alleles from *B. oleracea* in interspecific crosses was dependent on the different backgrounds of *B. napus*, that is, the presence of specific *S*-alleles (Ripley and Beversdorf, 2003). Probably there is another gene present and this gene affects the expression of SCR II alleles and response to SI. We can assume that this unknown gene have multiple alleles with different relations of dominance and recessivity, because of character of suppression of particular *S*-alleles and different effects on SI reaction. Ekuere et al. (2004) believes that self-incompatibility is controlled by two loci, the *S*-locus and supressor *sp* locus, which also has multiple alleles. Latent *S*-alleles were discovered in DH population derived from F_1 hybrids after crossing of resynthetised rape and different varieties. Ekuere et al. (2004) supposed that these latent alleles are hidden due to presence of common supressor system and this suppressor system is not associated with *S*-locus. SCR/SP11 protein is quite small and for interaction with eSRK just a few specific amino acids it is necessary, but the SI reaction is much more complicated and any specific protein is needed (Chookajorn, 2004). Hypothetical suppressor should be specifically expressed in tapetal cells and is located outside of *S*-locus. We found that SC phenotype correlates well with the presence of class I *SLG* gene. Correlation with *SCR* gene was unambiguous only in specific crosses when AIK3 and AIK 6 DH lines were used as SI donors and only after first cycle of hybridization with 00 quality donors.

After second cycle of hybridization correlation between SCR marker and SI phenotype was disrupted and specific correlation was only between SLG marker and SI phenotype (data not shown). Assuming that there is a suppressor gene at another locus, the question arises, how it can correlate with *SLG I* gene. *SLG* gene is not strictly necessary, but it supported full performance of self-incompatibility (Takasaki et al., 2000). Self-incompatibility is associated with normal expression of *SRK*, but with low expression *SLG* in naturally occurring variants of *B. rapa* (Nasrallah et al., 1992). Also in many SC *Brassica* lines A10 allele was found (*SLG I* gene), which was at the same locus as *SRK* gene with one nucleotide deletion (Goring et al., 1993). We can assume that SLG protein (I) could have a greater affinity for suppressor protein than to SCR. After interaction SLG protein with suppressor binding to eSRK is possible and this reactions lead to blocking of the SI reaction.

Conclusion

The results of this study showed that rapeseeds SI donors and SC cultivars differed in their SCR/SP11 transcripts. Two different transcripts of *SCR/SP11* gene observed in cv. Tandem revealed unique recessive allele 2 and new primers amplifying 280 bp band only in SI plant derived from cv. Tandem (as SI donor) were designed. It is important to note that that the detected allele 2 (and its related sequence) was unique and typical for cv. Tandem and that therefore the allele specific primers designed for specific plant material here cannot easily amplify single alleles in different SI material derived from different SI donors. Thus, our results could be used also as a guide describing how to develop new specific primers for particular population.

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