Characterization of self-incompatible Brassica napus lines lacking SP11 expression

Takumi Okamoto¹, Misaki Okamoto¹, Eri Hikichi¹, Moena Ogawa¹, Yoshinobu Takada¹, Go Suzuki², Seiji Takayama³ and Masao Watanabe¹*

¹Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-8577, Japan
²Division of Natural Science, Osaka Kyouiku University, Kashiwara, Osaka 582-8582, Japan
³Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

(Received 7 October 2019, accepted 27 January 2020; J-STAGE Advance published date: 4 June 2020)

Recognition of self-incompatibility (SI) is regulated by the SRK and SP11 genes in Brassicaceae. Brassica rapa and B. oleracea are self-incompatible, while most cultivated species of B. napus, which arose from hybridization between B. rapa and B. oleracea, are self-compatible. Various studies of the SRK and SP11 genes in self-compatible B. napus have been reported, but details of the mechanism in different B. napus lines are not fully understood. In this study, we confirmed the S haplotypes, SI phenotypes and SP11 expression in 10 representative lines of B. napus, and identified two SI lines (N110 and N343) lacking SP11 expression. In N343 (with BnS1 and BnS6 haplotypes), we confirmed that there is a 3.6-kb insertion in the promoter region of BnSP11-1, and that BnSP11-1 and BnSP11-6 are not expressed, as reported previously (expression of BnSP11-6 is suppressed by the BnS1 haplotype), although this line is self-incompatible. Similarly, in N110, with two novel S haplotypes (BnS8 and BnS9) in addition to BnS6, a 4.3-kb insertion was identified in the promoter region of BnSP11-9, and expression levels of BnSP11-6, BnSP11-8 and BnSP11-9 were all suppressed (BnSP11-6 and BnSP11-8 may be suppressed by BnS8 and BnS9, respectively), although the phenotype was self-incompatible. This observation of an SI phenotype without SP11 expression suggests the existence of unknown factor(s) that induce pollen–stigma incompatibility in B. napus.

Key words: Brassica napus, self-compatibility, self-incompatibility, S haplotype

INTRODUCTION

The genomic relationship among Brassica species is known as “U’s triangle” (U, 1935), in which B. napus (AACC) is an amphidiploid species developed from A- and C-genome donor diploid species, B. rapa (AA) and B. oleracea (CC). Brassica rapa and B. oleracea show self-incompatibility (SI), whereas B. napus shows self-compatibility (SC) (Hinata et al., 1994). B. napus is known as oilseed rape, and is one of the most important high-yield crops in the world (FAO statistics; http://www.fao.org/home/en/). Commonly, cultivars of B. napus show SC, although lines of B. napus artificially synthesized from B. rapa and B. oleracea show SI (Beschorner et al., 1995; Tochigi et al., 2011). There have been many studies of SC in B. napus; however, all components of the SC mechanism are not yet fully understood.

SI in Brassica is controlled by the S locus, with multiple haplotypes (Bateman, 1955), which contains genes for S locus receptor kinase (SRK) and S locus protein 11 (SP11) (Schopfer et al., 1999; Suzuki et al., 1999; Takasaki et al., 2000; Takayama et al., 2000). SRK and SP11 are stigma-side and pollen-side factors, respectively, and SP11 and SRK with the same S haplotype recognize each other and interact in self-pollination, triggering the SI reaction in which pollen germination is inhibited on the stigma (Takayama et al., 2001). Using this mechanism, Brassicaceae plants avoid inbreeding and maintain genetic diversity (reviewed by Watanabe et al., 2012). There are about 100 and 50 S haplotypes in B. rapa and B. oleracea, respectively (Nou et al., 1993; Ockendon, 2000; Sato et al., 2006). In addition to SRK and SP11, the gene (SLG)
encoding the S locus glycoprotein SLG, which has high sequence similarity to the extracellular domain of SRK, is also located on the S chromosome (Watanabe et al., 1994; Suzuki et al., 1999). SLG-specific primer sets have been used for the determination of S haplotypes in various Brassica species for SI research and breeding of Brassica crops (Brace et al., 1993; Nishio et al., 1994, 1996; Sakamoto et al., 1998; Park et al., 2001). The S haplotypes can be divided into two classes, class I and class II, which differ in the nucleotide sequences of SRK alleles (Nasrallah and Nasrallah, 1993). Class-I S haplotypes are dominant over class-II S haplotypes. Class-II S haplotypes are generally recessive to class-I haplotypes in pollen, but the two classes are co-dominant in the stigma (Thompson and Taylor, 1966; Ockendon, 1975; Visser et al., 1982; Hatakeyama et al., 1998b). The dominance relationship on the pollen side is controlled by regulation of SP11 expression via an epigenetic mechanism involving small RNA and DNA methylation (Shiba et al., 2002; Kakizaki et al., 2003; Tarutani et al., 2010; Yasuda et al., 2016).

The dominance relationship in pollen-side SI between class I and class II is thought to be responsible for the SC in B. napus in contrast to the SI in artificially synthesized B. napus. Okamoto et al. (2007) investigated the SC mechanism in B. napus cultivar Westar. In the S haplotypes, Westar has BnS1 in the A genome and BnS6 in the C genome, which are similar to S17 in B. rapa and S12 in B. oleracea. BnS1 is a class-I S haplotype and BnS6 is a class-II S haplotype, so BnS1 should show dominance over BnS6. Usually, if B. napus has this S haplotype combination, the phenotype is SI. However, the phenotype of Westar is SC, because there is an approximately 3.6-kb insertion in the promoter region of BnSP11-1, and the non-expressed BnSP11-1 induces suppression of the recessive BnSP11-6. Okamoto et al. (2007) analyzed the combination of class-I and class-II S haplotypes, but the offspring were not analyzed. On the other hand, Zhai et al. (2014) analyzed the combinations of class-II S haplotypes in other cultivars. Using SI and SC lines of these cultivars, they generated F1 hybrids and clarified the relationships between SI/SC phenotypes and SRK/SP11 genes. Their results showed that expression of SP11 was not linked to the SI/SC phenotypes of the hybrids or their parents, suggesting that novel factor(s) are involved in SC of B. napus. However, these novel factor(s) have not yet been characterized in the B. napus SI/SC system.

In this study, we further analyzed relationships among S haplotypes, SI phenotypes and SP11 transcripts in four SI and six SC lines of B. napus, and obtained useful information suggesting novel factor(s) in the SI/SC system of B. napus. Elucidating this mechanism is important for understanding SI and other plant reproduction systems, and developing new methods to improve agricultural efficiency.

**MATERIALS AND METHODS**

**Plant materials** The ten accessions (N110, N113, N115, N122, N343, N351, N352, N354, N476, Westar) used in this study are listed in the Tohoku University Brassica Seed Bank (http://www.agri.tohoku.ac.jp/phreede/Seed_Stock_DB/SeedStock-top.html; Watanabe et al., 1991, 1992). Eight of the ten accessions have been analyzed by Okamoto et al. (2007). BrS44 and BrS47 plants of B. rapa originated from Oguni Town, Yamagata Prefecture, Japan (Nou et al., 1991, 1993; Hatakeyama et al., 1998b). All plants were grown in a greenhouse.

**Determination of SC and SI phenotype** For the determination of pollination phenotype, pollen tube behavior on the stigma was observed by the aniline blue staining method as described in Takada et al. (2017). Non-pollinated flower buds were cut at the peduncle, and, after pollination, stood on 1% solid agar for about 24 h under room conditions. The pollinated pistils were then softened in 1 M NaOH for 1 h at 60 °C, and stained with basic aniline blue (0.1 M K3PO4, 0.1% aniline blue). Pistils were mounted in 50% glycerol (fluorescence microscopic grade) on slides, and observed by UV fluorescence microscopy (Zeiss). To determine pollination phenotype, we obtained test-cross data from at least 14 replicates on different dates. The degree of compatibility (compatibility score; CS) in each test pollination was scored on a five-point scale based on pollen tube penetration as follows: CS 5, penetration of more than 10 pollen tubes into the style; CS 4, penetration of four to ten pollen tubes into the style; CS 3, penetration of one to three pollen tubes into the style; CS 2, germination of pollen but no pollen tube penetration into papilla cells; and CS 1, no germination of pollen. Average scores less than 3 were defined as incompatible and scores 3 and above as compatible (Takada et al., 2017).

**Determination of S haplotype and sequence analysis of SP11 in B. napus** The S haplotype of each B. napus line was determined by allele-specific polymerase chain reaction (PCR) and sequence analysis of the SLG gene. All the primer sequences used in this study are listed in Supplementary Table S1. For the SI lines (N110, N115, N122, N343), at least ten different individuals were used for the determination of S haplotype. Genomic DNA of plants from the 10 accessions was isolated from young leaves using the cetyltrimethylammonium bromide method and DNeasy Plant Mini Kit (QIAGEN). For the confirmation of S haplotypes reported by Okamoto et al. (2007), allele-specific primer sets were used. PCR products were separated on a 2.0% agarose gel in 0.5 × TAE buffer, and detected by staining with ethidium bromide. For determination of the S haplotype of N110 and N343 lines, parts of the SLG genes
of class I and class II were amplified using specific primers PS5/PS15 and PS3/PS21, respectively (Nishio et al., 1996). Amplified DNA fragments were cloned using the pTAC2 vector (Biodynamics), and their sequences were determined (BigDye ver. 3.1, Life Technologies). For comparative sequence analysis of SP11s from B. napus and B. rapa, we determined the sequence of the SP11 region of BnS8 and BnS9. Furthermore, 5’-upstream regions of about 3.6 kb and 4.3 kb for BnS1 and BrS44, respectively, were amplified by KOD-Plus-Neo (TOYOBO) using primer sets InSP11-1 F2 and InSP11-1 R4, and InSP11-9 F and InSP11-9 R4, respectively, with 35 cycles of denaturation for 10 s at 98 °C and annealing/extension for 3.5 min at 68 °C. PCR products, following addition of 3’ A overhangs, were cloned into the pTAC2 vector, and sequences were determined. Identified sequences were compared and aligned using the GENETYX ver.11 software package.

RNA analysis for RT-PCR Total RNA was isolated from anthers of immature flower buds of plants from each line using the RNeasy Plant Mini Kit (QIAGEN). For synthesis of cDNA, 5 µg of total RNA was treated with DNase I (TaKaRa), and first-strand cDNAs were synthesized by the PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer’s methods. Quantitative real-time PCR (qRT-PCR) was performed on a Bio-Rad MiniOpticon system with SYBR Green (Bio-Rad) using primers for each S haplotype. Ubiquitin-conjugating enzyme 21 was used as a control (Osaka et al., 2019). Relative transcript levels were determined by the delta-delta threshold cycle relative quantification method in three replicates.

RESULTS AND DISCUSSION

S haplotypes of 10 B. napus lines In the present study, we used 10 lines of B. napus; all except N110 and N115 have been previously reported (Okamoto et al., 2007). We confirmed the S haplotype of these lines using allele-specific primer sets (Supplementary Table S1). As previously reported by Okamoto et al. (2007), seven lines (N113, N343, N351, N352, N354, N476 and Westar) possessed BnS1 (class I) and BnS6 (class II) haplotypes, and N122 had BnS6 (class II) and BnS7 (class II) haplotypes (Fig. 1A). To determine the S haplotypes of the novel N110 and N115 lines, we first used class-I and class-II SLG-specific primers, PS5/PS15 and PS3/PS21, respectively (Nishio et al., 1996). For N110, we obtained amplicons from PCR using both PS5/PS15 (class I) and PS3/PS21 (class II) primer sets, while only class-II S haplotype-specific amplification (using the PS3/PS21 primer set) was successful for N115 (Fig. 1B). Sequence analysis of these SLG fragments revealed that the N110 line has three S haplotypes: one known (BnS6) and two novel (BnS8 and BnS9) haplotypes were identified. BnSLG6 and BnSLG9 were identical to BrSLG22 (class I; accession number, AB054060) and BrSLG44 (class II; AB054059) in B. rapa (Hatakeyama et al., 1998a), respectively. On the other hand, the N115 line possesses two previously reported class-II S haplotypes, BnS6 and BnS7, as in line N122. We further confirmed these S haplotypes of N110 and N115 lines by SP11 amplification (Fig. 1B); amplicons of BnSP11-6, BnSP11-8 and BnSP11-9 were detected for N110, and BnSP11-6 and BnSP11-7 for N115.

SI phenotype of the B. napus lines SI phenotypes of the 10 B. napus lines were determined by observation of pollination penetration. From these results, N110, N115, N122 and N343 lines showed a strong SI phenotype with average CS < 2, whereas N113, N351, N352, N354, N476 and Westar showed stable SC with average CS > 3 (Fig. 2A). The four SI lines are noteworthy. Among them, N110 and N343 have both class-I and class-II S

Fig. 1. Confirmation of S haplotypes by genomic PCR analysis of B. napus lines. (A) Detection of SP11 amplicons of known S haplotypes reported by Okamoto et al. (2007). (B) Detection of SLG and SP11 amplicons of unknown S haplotypes in N110 and N115. V-ATPase was used as a positive control. M represents DNA ladder marker.
haplotypes, while N115 and N122 have only class-II S haplotypes. All six SC lines possess the BnS^S_1 and BnS^S_6 haplotypes, indicating that these SC phenotypes are caused by suppression of the class-II BnSP11-6 gene and the insertion in the BnSP11-1 promoter, as reported previously (Okamoto et al., 2007).

**Gene expression of SP11 in the B. napus lines** To investigate relationships between these SI phenotypes and SP11 expression, we performed qRT-PCR analysis of each SP11 gene in anthers of the B. napus lines. In all six SC lines (N113, N351, N352, N354, N476 and Westar), SP11 transcripts (BnSP11-1 and BnSP11-6) were absent or detected at very low levels (Fig. 3), which is consistent with the previous report (Okamoto et al., 2007). In the two SI lines, N115 and N122, BnSP11-6 transcripts were present at high levels in the anther, whereas expression of BnSP11-7 was dramatically suppressed (Fig. 3), suggesting that the recessive BnSP11-7 was suppressed by the dominant BnSP11-6, and that BnSP11-6 might trigger SI with BnSRK^S_1.

Surprisingly, although both N110 and N343 showed a strong SI phenotype, SP11 transcript levels were low (Fig. 3). In N343, the lack of SP11 transcripts may be caused by suppression due to the combination of BnS^S_1 and BnS^S_6 haplotypes, as in other SC haplotypes, although its SI phenotype cannot be explained by this mechanism. In N110 with three S haplotypes, transcripts of BnSP11-6, BnSP11-8 and BnSP11-9 were almost undetectable. Expression of the class-II BnSP11-6 gene may be suppressed by the class-I BnS^S haplotype, as seen in the combination of BnS^S_1 and BnS^S_6. In addition, in N110, BnSP11-8 (which is identical to BrSP11-22) may be epigenetically suppressed by the BnS^S haplotype (which is identical to BrS^44), because it has recently been reported that the class-I BrS^22 is recessive to the class-II BrS^44 in B. rapa, and the expression of BrSP11-22 was found to be suppressed by the BrS^44 allele in an epigenetic manner (Wang et al., 2019). However, this does not explain why transcripts of BnSP11-9 were not detected by expression analysis. Therefore, we further analyzed the genomic organization of the BnSP11-9 gene in the N110 line, as well as that of the BnSP11-1 gene in the N343 line.

**Insertions in the BnSP11-1 and BnSP11-9 promoter** To explain suppression of BnSP11-1 and BnSP11-9 transcription, we analyzed the SP11 genomic regions. In the case of BnSP11-1, Okamoto et al. (2007) indicated that there was a 3.6-kb insertion in the promoter region of BnSP11-1, and transcripts of BnSP11-1 were undetected. In our genomic PCR analysis using a specific primer set to amplify this region followed by sequencing
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of the amplified fragments, the 3.6-kb insertion in the *BnSP11-1* promoter region was identified in N113, N343, N351, N352, N354, N476 and Westar lines (Fig. 4A). A similar approach to examine the *BnSP11-9* genomic region of N110 detected a 4,295-bp insertion at 59 bp upstream of the initiation codon of *BnSP11-9* (Fig. 4B). Thus, transcriptional suppression of *BnSP11-1* and *BnSP11-9* may be caused by large insertions in their promoter regions. It is noteworthy that these insertions found in N343 and N110 are consistent with the qRT-PCR results that showed low amounts of the *BnSP11-1* and *BnSP11-9* transcripts in N343 and N110, respectively. Despite the lack of *SP11* expression, N110 and N343 showed the SI phenotype (Fig. 2A).

**Cross-pollination experiments in N110 and N343**
To confirm the presence of functional stigma and pollen in the N110 and N343 SI lines, we performed test cross experiments with Westar as a parent whose male SI recognition is disrupted (Okamoto et al., 2007). In the N343 line, reciprocal crosses showed compatibility with stigma and pollen from Westar (Fig. 2B), indicating that both male and female functions of N343 are normal (it should be noted that the *S* haplotypes of N343 and Westar are the same, although the cross Westar × N343 was compatible). In N110, the cross between Westar stigma and N110 pollen was compatible, whereas the cross with the reverse combination, N110 stigma and Westar pollen, was incompatible (Fig. 2B). Bud self-pollination of N110 showed successful pollination and seed production (data not shown). Therefore, its immature stigma was functional.

**CONCLUSION**
Results from this study of the *S* haplotypes, SI phenotypes, and the expression and genomic organization of the *SP11* gene in *B. napus* lines N110 and N343 point to an apparently contradictory conclusion, namely that in these lines SI is functional, even though *SP11* is not expressed. We speculate that this phenomenon is caused by the existence of another factor(s) related to the rejection of pollen tube penetration on the stigmatic papilla cells. If this is a novel factor, it is possibly a duplicated SI-related gene, as seen in intraspecies unilateral incompatibility (UI) in *B. rapa* (Takada et al., 2017). Interestingly, the UI phenomenon was detected in a combination between N110 (SI) as stigma parent and Westar (SC) as pollen parent. The incompatibility phenotype induced in such a specific combination can be considered sepa-
Segregation analysis of stigma- and pollen-side UI will shed light on our understanding of the molecular factors regulating UI in *B. napus*.

Another possibility is the involvement of SI signaling-related genes. In the Brassicaceae, the thioredoxin h-like proteins THL1 and THL2 are known to be negative regulators of SRK/SP11-mediated SI signal transduction (Cabrillac et al., 2001). Suppression of *THL1/2* gene expression in *B. napus* Westar, with an SC phenotype, showed inhibition of pollen germination and pollen tube penetration (Haffani et al., 2004). Therefore, in our study, it is possible that *THL1/2* gene expression is suppressed in the N110 and N343 lines for some reason. In the N110 stigma, which rejects Westar pollen (Fig. 2B), it is also possible that some compatibility factors are lacking at the mature stage.

The *SLG*-specific primer set used for the detection of...
the S haplotype has been known to have high capability, but is not perfect. In particular, it was reported that the PS5/PS15 primer set can detect more than 90% (but fewer than 100%) of class-I S haplotypes in *B. oleracea* (Sakamoto et al., 2000). Therefore, we cannot rule out the existence of an undetected S haplotype, which should be dominant/co-dominant over BnS9 (N110) or BnS9 (N343). Future genetic and molecular analyses of the N110 and N343 lines will lead to the identification of the novel incompatibility factor(s) and to a more detailed understanding of the complicated SI/SC mechanism of *B. napus*.

The authors thank Hiromi Masuko-Suzuki, Kana Ito, Moe Takeda, Mako Uchino, Maaya Hiraiwa, Chinatsu Ogasawara, Noni Sugawara, Megumi Ito, Keita Yamaki and Tai Takemoto (Tohoku University) for technical assistance. This work was supported in part by MEXT KAKENHI (Grant Numbers 16H06467 to S. T.; 16H06470, 16H06464 and 16K21727 to M. W.), JSPS KAKENHI (Grant Numbers 16H06380 to S. T.; 19K05963 to Y. T.; 16H04584, 16K15085, 17H00821, 18K00481, 19K23422 to M. W.) and JSPS Bilateral Programs (Grant Number 16032211-000461 to M. W.). This research was also supported by the Japan Advanced Plant Science Network to M. W.

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