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The self-compatibility mechanism in *Brassica napus* L. is applicable to F_1 hybrid breeding

Takahiro Tochigi[a], Hitoshi Udagawa, Feng Li, Hiroyasu Kitashiba, Takeshi Nishio*

Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan
* Corresponding author
a. Present address: Misato Research Station, Sakata Seed Co. Ltd, 6412 Misato Ogura, Azumino-shi, Nagano-ken 399-8103, Japan

Abstract

***Brassica napus*, an allopolyploid species having the A genome of *Brassica rapa* and the C genome of *Brassicca oleracea*, is self-compatible, although both *B. rapa* and *B. oleracea* are self-incompatible. We have previously reported that *SP11/SCR* alleles are not expressed in anthers, while *SRK* alleles are functional in the stigma in *B. napus* cv. 'Westar', which has *BnS-1* similar to *B. rapa S-47* and *BnS-6* similar to *B. oleracea S-15*. This genotype is the most frequent *S* genotype in *B. napus*, and we hypothesized that the loss of the function of *SP11* is the primary cause of the self-compatibility of 'Westar'. To verify this hypothesis, we transformed 'Westar' plants with the *SP11* allele of *B. rapa S-47*. All the transgenic plants and their progeny were completely self-incompatible, demonstrating self-compatibility to be due to the nonfunctional *SP11* allele in the A genome, which suppresses a functional recessive *SP11* allele in the C genome. An artificially synthesized *B. napus* line having two recessive *SP11* alleles was developed by interspecific hybridization between *B. rapa* and *B. oleracea*. This line was self-incompatible, but F_1 hybrids between this line and 'Westar' were self-compatible. These results suggest that the self-compatibility mechanism of 'Westar' is applicable to F_1 seed production in *B. napus*.**

Keywords: self-incompatibility, *SP11/SCR*, dominance relationship, F_1 hybrid breeding, *B. napus*

Introduction

Self-incompatibility in the genus *Brassica* is controlled by a single *S* locus (Bateman 1955), which contains a gene determining recognition specificity of the stigma, i.e., *SRK* (S-receptor kinase) (Stein et al. 1991), as well as a gene determining the recognition specificity of pollen, i.e., *SP11/SCR* (Schopfer et al. 1999, Suzuki et al. 1999). The *SP11/SCR* (*SP11* hereafter) allele from the majority of *S* haplotypes is expressed in the tapetum and pollen grains, while *SRK* is in stigma papilla cells. *SP11* and *SRK* have more than 50 alleles in a species and recombination between *SP11* and *SRK* seldom occurs (Takuno et al. 2007). A set of an *SP11* allele and an *SRK* allele is termed *S* haplotype (Nasrallah and Nasrallah 1993). Based on nucleotide sequence homology of *SRK*, *S* haplotypes are classified into two classes, class I and class II, class II haplotypes being generally recessive to class I haplotypes in pollen (Nasrallah et al. 1991). The factor responsible for suppression of a recessive *SP11* allele has been revealed not to be a dominant *SP11* allele but to be located at different region in the *S* locus (Tarutani et al. 2010). SP11 protein has been reported to interact with SRK protein produced by the same *S* haplotype in stigma papilla cells (Kachroo et al. 2001, Takayama et al. 2001), and such interaction is considered to trigger a series of reactions leading to rejection of self-pollen grains by stigma papilla cells. Although such downstream reactions have not been elucidated, ARC1 (Gu et al. 1998), THL1, THL 2 (Cabrillac et al. 2001), and MLPK (Murase et al. 2004) have been reported to participate in these reactions.

While *Brassica rapa* and *Brassica oleracea* are self-incompatible, *Brassica napus*, which is an allopolyploid species having a genome derived from *B. rapa* and a genome from *B. oleracea*, is self-compatible. By determining nucleotide sequences of *SP11* and *SRK* alleles, Okamoto et al. (2007) have identified *S* haplotypes in *B. napus* cultivars. Most of recently developed cultivars of double-low rapeseed (canola) with low erucic acid content and low glucosinolate content have *BnS-1* and *BnS-6*, which are similar to *S-47* in *B. rapa* (*BrS-47*) and *S-15* in *B. oleracea* (*BoS-15*), respectively. *BrS-47* is a class-I *S* haplotype that is dominant over the class-II *BoS-15* in the pollen of allopolyploid plants, although these *S* haplotypes are not allelic. *SP11* of *BnS-1* (*BnSP11-1*) has a 3,606-bp insertion in the promoter region and is not expressed (Okamoto et al. 2007). Since gene expression of dominant *SP11* alleles is not required for suppressing expression of recessive *SP11* alleles (Fujimoto et al. 2006a), Okamoto et al. (2007) have inferred the loss of function of *BnSP11-1* to be the essential cause of self-compatibility of these *B. napus* cultivars. However, this inference has not yet been confirmed.

F_1 hybrid breeding of *B. napus* using a self-incompatibility system has been projected, and self-incompatible *B. napus* lines have been developed by crossing self-incompatible *B. rapa* plants with *B. napus* (Goring et al. 1992). One problem with F_1 hybrid breeding using the self-incompatibility system is that F_1 hybrid plants are also self-incompatible, and therefore efficiency of seed setting is lower than that in self-compatible plants. If expression of recessive *SP11* alleles located on a different chromosome can be suppressed by a nonfunctional dominant *SP11* allele, as suggested by Okamoto et al. (2007), an F_1 hybrid between a *B. napus* line having two recessive *SP11* alleles and a *B. napus* line having nonfunctional dominant *SP11* alleles, e.g., 'Westar', is expected to be self-compatible.

In the present study, we introduced *BrSP11-47* cDNA under a promoter of a class-I *SP11* allele into *B. napus* cv. 'Westar' having *BnS-1* and *BnS-6*, and conducted tests to determine if the transgenic plants were self-incompatible. Furthermore, to develop an F_1 hybrid breeding method in *B. napus,* we produced a self-incompatible artificially synthesized *B. napus* line and tested

to see if F_1 hybrids between the artificially synthesized *B. napus* line and 'Westar' were self-compatible.

Materials and Methods

Plant materials

Brassica napus cv. 'Westar' was used as a material for plant transformation, an *S-47* homozygote in *Brassica rapa* (*BrS-47*) was used as a donor of *BrSP11-47*, and *S-29* and *S-46* homozygotes in *B. rapa* were materials for testing cross-incompatibility. *S-44* homozygote in *B. rapa* (*BrS-44*) and *S-15* homozygote in *B. oleracea* (*BoS-15*) were used for interspecific hybridization to develop an artificially synthesized *B. napus* line.

Vector construction for plant transformation

A cDNA clone of *BrSP11-47* isolated from a homozygote of *BrS-47* (Fujimoto et al. 2006b) was ligated with a 686-bp sequence of the promoter region of *BrSP11-47* and inserted into a 5' site of the NOS (nopaline synthase gene) terminator of pBI101-H, which contains a hygromycin phosphotransferase gene (HPT) in a pBI101 vector, yielding pBI101-HBrSP11-47 (Fig. 1a). The 686-bp promoter sequence of *BrSP11-47* was inserted into a 5' site of the GUS (beta-glucuronidase gene) of pBI101-H to produce pBI101-HBrSP11pro::GUS (Fig. 1b). These vector constructs were introduced into the *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Plant transformation

Plant transformation was carried out following the method of Takasaki et al. (1997). After elongation of 4-5 cm hypocotyls from aseptically sown seeds, 5- to 10-mm sections were used as explants. Tobacco BY-2 cells (*Nicotiana tabacum* cv. Bright Yellow 2) were spread on an agar medium containing salts and vitamins of MS (Murashige and Skoog, 1962), 200 mg/ml KH_2PO_4, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3.0 % (w/v) sucrose, and 0.7 % agar. The explants were pre-cultured on a filter paper placed on the tobacco BY-2 cells at 25˚C under dark for 24 h and inoculated with a 1/10 dilution of overnight-cultured suspension of *Agrobacterium*. After co-cultivation for three days, the hypocotyls were transferred to a B5 agar medium (Gamborg et al. 1968) containing 1mg/ml 2, 4-dichlorophenoxyacetic acid, 125 mg/ml carbenicillin, 20 mg/ml hygromycin, 30 g/l sucrose, and 0.7% agar and cultured at 25˚C for seven days. They were transferred to a B5 medium containing 3 mg/l 6-benzylaminopurine, 1 mg/l trans-zeatin, 125 mg/l carbenicillin, 20 mg/l hygromycin, 10 mg/l $AgNO_3$, 10 g/l sucrose, and 0.7 % agar. Regenerated shoots were cultured on a B5 medium containing 125 mg/l carbenicillin, 20 mg/l hygromycin, 10 g/l sucrose, and 0.7 % agar for root regeneration, and the regenerated plants were grown in a closed greenhouse for isolated cultivation.

Resynthesizing *Brassica napus*

Interspecific hybrids between *B. rapa* and *B. oleracea* were obtained with ovary culture according to the method of Inomata (1977) with a slight modification. Seven days after pollination of *B. oleracea S-15* pollen onto the stigma of young buds of a *B. rapa S-44* homozygote, ovaries were excised from the immature pods. The ovaries were surface sterilized with 1% NaClO for 15 min, rinsed three times with sterilized distilled water, and cultured on an MS medium containing 50 g/l sucrose, 400 mg/l casamino acid, and 0.8% agar. The cultured ovaries were maintained under fluorescent light for 16 h (100 μ E m^{-2} s^{-1})/8 h (light/dark) at 23 ± 2˚C. After ca. 40 days, the plantlets regenerated from the siliques were transplanted to an MS medium containing 30 g/l sucrose and 300 mg/l casamino acid. After 2-3 leaves appeared, shoots of the plantlets were excised and cultured on an MS medium containing 30 g/l sucrose, 0.8% agar, and 100 mg/l colchicine for 8 days. Subsequently, the plantlets were transferred to the same MS medium without colchicines, cultured until root system development, and transplanted to pots in a glasshouse. After flowering, plant morphology, stomata size, and pollen fertility were observed and compared with those of *B. rapa*, *B. oleracea,* and *B. napus* to estimate ploidy level.

Southern blot analysis of genomic DNA

Genomic DNA was isolated from leaves or inflorescences using the CTAB method (Murray and Thompson 1980). After electrophoresis of 3 µg genomic DNA digested with *Hin*dIII or *Sac*I on 1.0% agarose gel, DNA fragments were transferred onto a nylon membrane (Nytran N; Watman, UK). Hybridization was performed in a solution of 5x SSC, 1.0% blocking reagent (Roche, Switzerland), 0.1% sodium-N-lauroyl sarcosinate, and 0.02% SDS at 65˚C. The membrane was washed twice with a solution of 0.1x SSC and 0.1% SDS at 65˚C for 20 min. Probes were labeled with digoxigenin using PCR DIG labeling Mix (Roche). Regions of the sequences used as probes are shown in Fig. 1.

RT-PCR and real time RT-PCR

Total RNA was isolated from anthers using an SV Total RNA Isolation Kit (Promega, USA). cDNA was synthesized from 2 µg RNA using a First Strand cDNA Synthesis Kit (GE Healthcare Bioscience, USA). PCR was carried out using 1 µg cDNA as a template for detection of gene expression of *SP11*. The primer pair employed was BrSP11_47ex1F (5'-ATGAAATCTGCTGTTTATAATGCTT-3') and BrSP11_47ex2R (5'-CTAACACAATTTACATACACAAGAA-3').

Real time RT-PCR was performed with 1/10 dilution of the synthesized cDNA as a template using SYBR Premix Ex Taq (TAKARA, Japan) with a primer pair of RT_47exF2 (5'-AAGTGGAAGCTAATCTGATGAATCC-3') and RT_47exR2 (5'-GGCCTCTTGTCCATACCCTTC-3'). Using the actin gene of *B. rapa* as a standard (actF, 5'-CGGTCCAGCTTCGTCATACTCAGCC -3', and actR, 5'-AAATGTGATGTGGATATCAGGAAGG -3'), the amount of cDNA was measured and the amount of *SP11* mRNA was estimated as a relative value. Experiments were repeated three times.

Pollination test and observation of pollen tubes in stigmas

Just after anthesis, excised flowers were stood on an agar plate and pollinated. Sixteen hours after pollination, styles were hydrolyzed in 1N NaOH at 50˚C for one hour and incubated in aniline blue solution (0.1% aniline blue, 0.1M K_3PO_4) at 50˚C for one hour. Pollen tubes in the stigmas and styles were observed under a UV fluorescence microscope. The number of pollen tubes penetrating

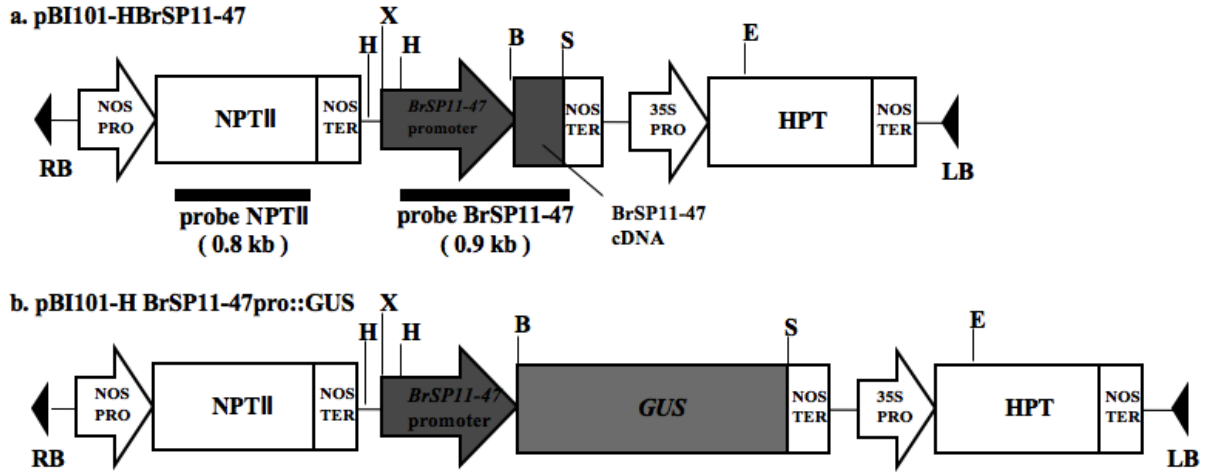

Fig. 1. T-DNA region of vector constructs for transformation of 'Westar'.
a. A construct of a *BrSP11-47* transgene. b. A construct of *BrSP11-47* promoter::*GUS*. Gray arrows and gray boxes indicate *SP11* promoter and coding region, respectively. *BrSP11-47* promoter is a 686-bp upstream region of the translation initiation site. Horizontal lines indicate regions used as probes in Southern blot analysis.
H: *Hin*dIII site, X: *Xba*I site, B : *Bam*HI site, S: *Sac*I site, E: *Eco*RI site, RB: right border, LB: left border, NOS PRO: nopaline synthase gene promoter, NPTII: neomycin phosphotransferase gene, NOS TER: nopaline synthase gene terminator, 35S PRO: cauliflower mosaic virus 35S promoter, HPT: hygromycin phosphotransferase gene.

a style were counted and scored using six indices, i.e., 1, no pollen tube; 2, 1-2 pollen tubes; 3, 3-9 pollen tubes; 4, 10-29 pollen tubes; 5, 30-99 pollen tubes; and 6, more than 100 pollen tubes penetrating a style. Indices 1 and 2 represent strong incompatibility (also shown as -), indices 3 and 4 represent partial incompatibility (±), and indices 5 and 6 represent compatibility (+).

GUS assay

Anthers of 4-mm, 5-mm, and 10-mm flower buds were taken from the plants transformed with pBI101-HBrSP11pro::GUS and incubated in GUS staining buffer (1.9 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-gluc), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.3% Triton X-100, 20% methanol, 100 mM phosphate buffer pH 7.0) at 37˚C. The flower buds were sliced by a microslicer (DOSAKA EM, Kyoto, Japan) and observed under a microscope.

Results

Copy number and expression of the transgene

Three transgenic plants, TSP11-47-1, TSP11-47-2, and TSP11-47-3, were obtained by transformation with pBI101-HBrSP11-47. Southern-blot analysis of genomic DNA detected the transgene of these transgenic plants. A 0.9-kb probe having the sequence downstream of a *Hin*dIII site in the *BrSP11-47* promoter detected two and three bands together with two bands of 'Westar' in genomic DNAs of TSP11-47-1 and TSP11-47-3 digested by *Hin*dIII (Supplementary Fig. 1). In TSP11-47-2, no additional band was observed by the analysis using *Hin*dIII, but one band was detected together with two bands of 'Westar' after digestion with *Sac*I. Therefore, TSP11-47-1, TSP11-47-2, and TSP11-47-3 were considered to have two, one, and three copies of the transgene, respectively.

Transgenic plants were obtained by introducing the *BrSP11pro::GUS* construct. Flowers, stems, and leaves of the transgenic plants were incubated with X-gluc for detection of GUS activity, and the GUS activity was detected only in anthers. In microscopic observation of anthers, the tapetums of 4-mm flower buds were stained, while tapetums of 5-mm and 10-mm flower buds, which contained pollen grains having GUS activity (Fig. 2), were degraded. Pollen grains of 5 mm and 10 mm buds also showed GUS activity (Supplementary Fig. 2).

BrSP11-47 transcripts were detected in 3- to 6-mm buds of the transgenic plants, TSP11-47-1, TSP11-47-2, and TSP11-47-3, but not in those of 'Westar' by RT-PCR. Quantitative analysis using real-time RT-PCR showed that amounts of transcripts in TSP11-47-1, TSP11-47-2, and TSP11-47-3 were 0.82, 0.35, and 2.64 times those in a *B. rapa BrS-47* homozygote (Fig. 3). The trangenic plant of a higher copy number showed a higher level of transcripts than that of a lower copy number.

TSP11-47-1, TSP11-47-2, and TSP11-47-3 showed complete self-incompatibility. All the tested flowers, more than 10 per plant, showed self-incompatibility, indicating highly stable self-incompatibility of the transgenic plants. Pollen grains of the transgenic plants were also incompatible with the stigmas of *BrS-47* homozygotes in *B. rapa* (Table 1).

Analysis of progenies of the transgenic plants

The *BrSP11-47* transgene in selfed progenies (T_1) of the transgenic plants, TSP11-47-1 and TSP11-47-2, was analyzed by PCR. Three T_1 plants having the transgene were selected from each T_1 line, and plants that had lost the transgene by segregation were named NT (non-transgenic) plants and used as controls. Southern blot analysis of three T_1 plants of TSP11-47-1 using the *BrSP11-47* probe showed the same band pattern (Supplementary Fig. 3), suggesting that two copies of the transgene detected in TSP11-47-1 are located at one locus or near loci. In three T_1 plants of TSP11-47-2, it was difficult to detect the transgene by Southern blot analysis using the *BrSP11-47* probe, but a band of

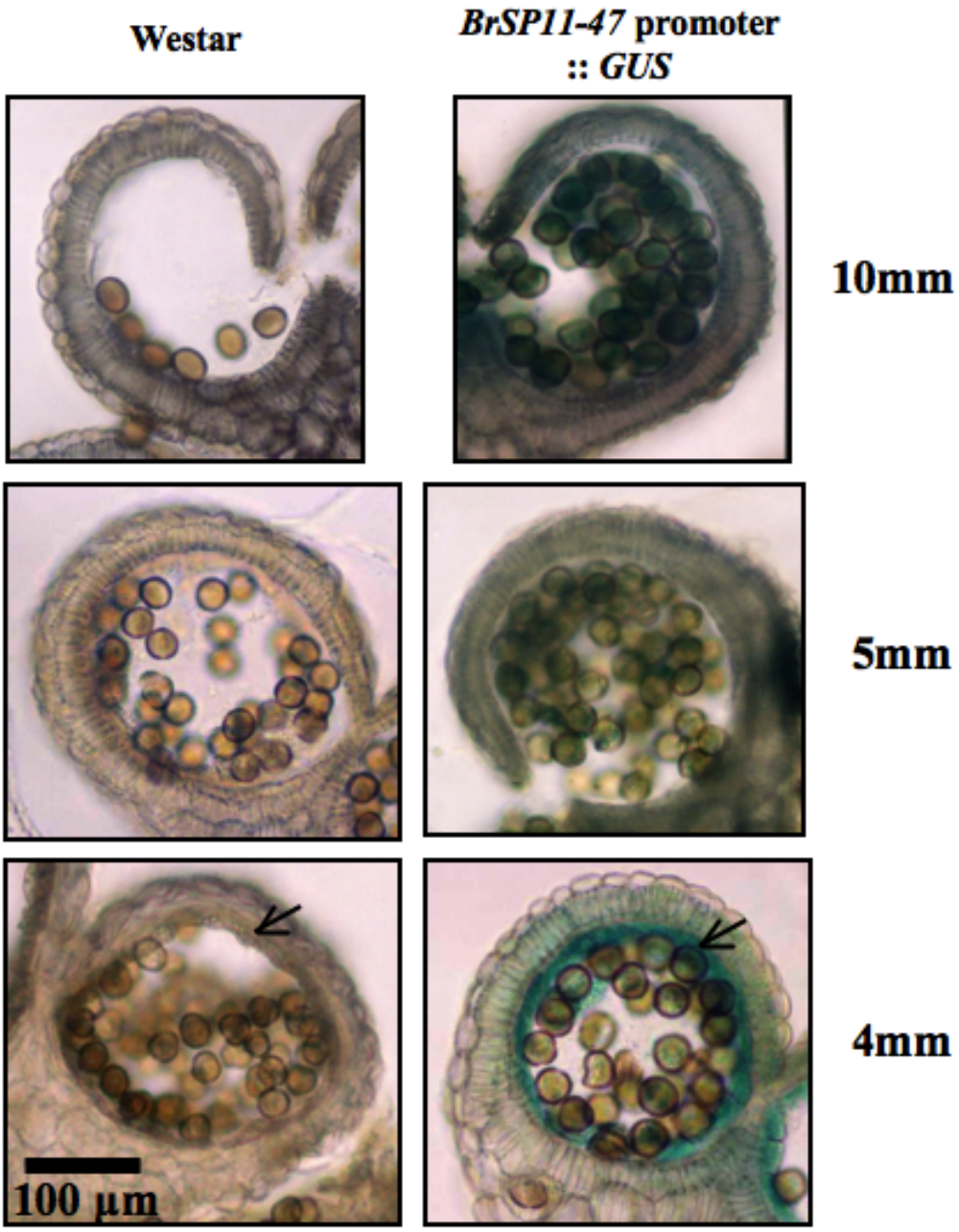

Fig. 2. GUS activity in anthers of a transgenic plant with the *BrSP11-47* promoter::*GUS* transgene Anthers of 4, 5, and 10-mm-long buds were stained by X-gluc. Arrows indicate tapetum.

NPTII was clearly detected (Supplementary Fig. 4).

Amounts of transcripts of *BrSP11-47* in 4-mm buds, before tapetum degradation, were estimated by real-time RT-PCR (Supplementary Fig. 5). The amounts of transcripts in the three TSP11-47-1 T_1 plants relative to that in the *B. rapa BrS-47* homozygote were 1.20, 0.82, and 2.14. Those of an NT plant and a wild-type 'Westar' plant were 0.04 and 0.03, respectively. The T_1 plants of TSP11-47-2 showed lower transcript levels, 0.31, 0.61, and 0.31, than those of TSP11-47-1. All the T_1 plants of TSP11-47-1 and TSP11-47-2 exhibited strong self-incompatibility (Table 2). NT plants from TSP11-47-1 and TSP11-47-2 were self-compatible like wild-type 'Westar' plants.

Self-compatibility of F_1 hybrids between self-incompatible artificially synthesized *B. napus* and 'Westar'

Eight plants were obtained by ovary culture after interspecific pollination of a *B. rapa S-44* homozygote with pollen grains of a *B. oleracea S-15* homozygote. After treatment of apical meristems of the eight plants with colchicine, selfed seeds were obtained from two plants. No pollen tubes penetrated the stigmas after self-pollination of any selfed progeny, indicating that all the selfed progeny of an artificially synthesized *B. napus* line having homozygous *BrS-44* and homozygous *BoS-15* were completely self-incompatible (Table 3).

One of the artificially synthesized *B. napus* plants was crossed with 'Westar' pollen to obtain F_1 hybrid plants. Most stigmas from three F_1 hybrid plants showed compatibility with self-pollen grains like 'Westar', indicating the F_1 plants to be self-compatible (Table 3). RT-PCR detected no transcript of either *BrSP11-44* or *BoSP11-15* in the anthers of these F_1 plants (Fig. 4).

Discussion

Transgenic plants of 'Westar' having *BrSP11-47* were

Table 1 Pollination tests of transgenic plants

Stigmas	Pollen: Westar	TSP11-47-1	TSP11-47-2	TSP11-47-3	*BrS-47* homozygote
Westar	+[a]	-	-	-	-
TSP11-47-1	+	-			-
TSP11-47-2	+		-		-
TSP11-47-3	+			-	-
BrS-47 homozygote	+	-	-	-	-
BrS-46 homozygote	+	+	+		+
BrS-29 homozygote	+			+	+

[a] +: compatible, ±: partially incompatible, -: incompatible.

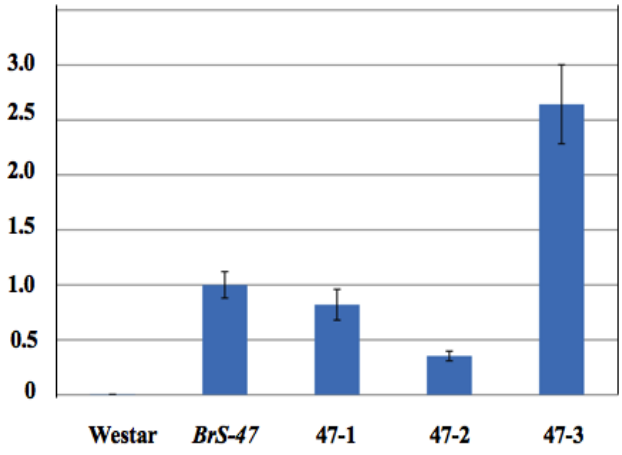

Fig. 3. Amounts of transcripts of *BrSP11-47* analyzed by real-time RT PCR in transgenic plants.
Relative values are shown with a standard of *BrS-47* (=1). Measurements were repeated three times, and bars indicate SE.

Table 2 Self-incompatibility of T_1 plants

Lines	Index[a] 1	2	3	4	5	6	Average
Westar	3[b]	0	0	0	4	10	4.88
NT-1	3	0	4	0	0	8	4.20
TSP11-47-1-1	15	2	0	0	0	0	1.12
TSP11-47-1-2	15	2	0	0	0	0	1.12
TSP11-47-1-3	17	1	0	0	0	0	1.06
NT-2	2	0	3	2	3	6	4.38
TSP11-47-2-1	13	2	0	0	0	0	1.13
TSP11-47-2-2	15	2	0	0	0	0	1.12
TSP11-47-2-3	13	3	0	0	0	0	1.19

[a] see Materials and Methods.

[b] The number in the table refers to the number of self-pollinated flowers.

self-incompatible and their pollen grains were incompatible with the stigmas of *B. rapa BrS-47* homozygotes. In T_1 plants, the presence and absence of the *BrSP11-47* transgene corresponded to self-incompatibility and self-compatibility of their phenotypes, respectively. These results clearly demonstrate the cause of self-compatibility of the commonest genotype in *B. napus* to be the inability of gene expression of *BnSP11-1*.

For breakdown of self-incompatibility in allotetraploid species, both homoeologous genes derived from different parental species must lose their function. Such mutation is rare. Since the dominance relationship functions even between homoeologous *S* haplotypes (Okamoto et al. 2007) and a nonfunctional *SP11* allele can suppress the expression of a recessive *SP11* allele (Fujimoto et al. 2006a), Okamoto et al. (2007) suggested that mutation(s) in a dominant *S* haplotype was an important factor for the development of *B. napus*. The present study, which demonstrated the self-compatibility of 'Westar' is caused by the loss-of-function of a dominant *SP11* allele, supports their hypothesis.

Based on our finding on the self-compatibility mechanism of 'Westar', we propose an F_1 hybrid breeding method of oil-seed rape (Fig. 5). First, a *B. napus* line having a functional class-II *S* haplotype from the A genome, which is recessive to *BnS-1* in pollen, is developed as a parental inbred line. Such a functional class-II *S* haplotype can be incorporated by conventional cross-breeding using an artificial amphidiploid line having functional class-II *S* haplotypes from the A genome and the C genome as a parent. Alternatively, a functional class-II *S* haplotype of *B. rapa* can be introduced by interspecific crossing between *B. napus* and *B. rapa*. Self-incompatible *B. napus* lines with class-II *S* haplotypes have been developed by interspecific crossing and suggested to be useful in F_1 hybrid breeding (Zhang et al. 2008). Second, F_1 seeds are produced only from a self-incompatible *B. napus* line using a self-compatible *BnS-1/BnS-6* line, e.g., 'Westar', as a pollen parent. F_1 plants are expected to be self-compatible.

The proposed F_1 hybrid breeding method is considered to be advantageous in seed production efficiency over the conventional F_1 hybrid breeding method using cytoplasmic male sterility. Male-sterile lines are not preferred by insect pollinators because of the absence of pollen grains and, in some lines, the abnormality of

Table 3 Self-incompatibility and self-compatibility of airificially synthesized *B. napus* an its progeny

	Index[a]						
Lines	1	2	3	4	5	6	Averag
Westar	0[b]	0	0	3	2	7	5.33
Synthesized *B. napus*	12	0	0	0	0	0	1.00
F_1-1	0	0	0	0	9	3	5.25
F_1-2	5	1	2	0	3	1	2.83
F_1-3	3	0	0	0	8	4	4.47

[a] see Materials and Methods.

[b] The number in the table refers to the number of self-pollinated flowers.

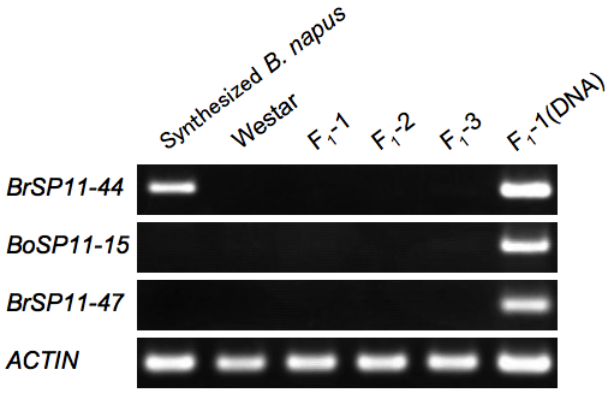

Fig. 4. *SP11* transcripts in a synthesized *B. napus* plant and F_1 hybrids detected by RT-PCR with 30 cycles.

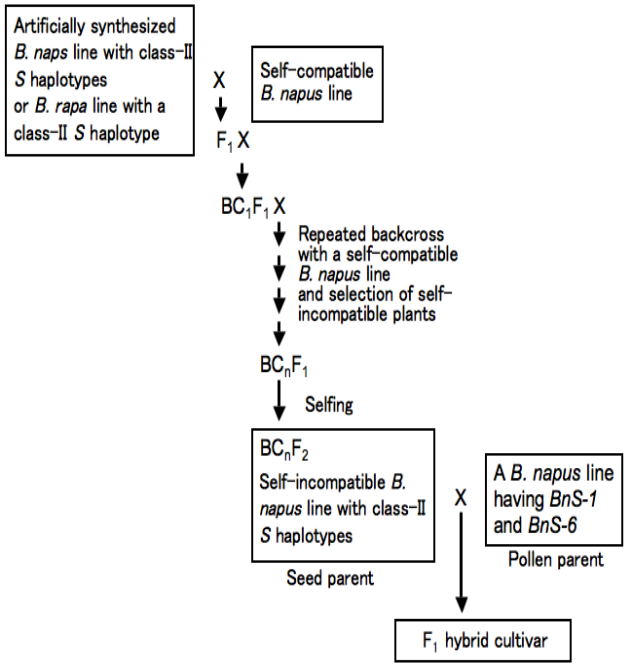

Fig. 5. Schematic representation of F_1 hybrid breeding using self-incompatibility system in oil seed rape.

nectarines or flower morphology. Inferiority of F_1 hybrid breeding using self-incompatibility to that using male-sterility is instability of self-incompatibility resulting in contamination of selfed seeds into F_1 seeds. Especially in the F_1 hybrid breeding method of *B. napus* using class-II *S* haplotypes generally resulting in weak self-incompatibility phenotype, high frequency of selfed seed contamination may become a problem. Since the strength of self-incompatibility depends on the *S* haplotype (Lowson and Williams 1976; Nasrallah et al. 1991), selection of *S* haplotype is important. In the present study, *BrS-44* was used as a functional class-II *S* haplotype from the A genome. *BrS-44* and *BoS-2* are considered to determine the same recognition specificity, and both *BrS-44* and *BoS-2* provide strong self-incompatibility. Although *BoS-15* homozygotes show weak self-incompatibility, *BrS-60*, which determines the same recognition specificity as *BoS-15*, provides strong self-incompatibility (Sato et al. 2006). *BrS-44* and *BrS-60* are considered to be useful in this F_1 hybrid breeding method. On the other hand, *BrS-40* and *BrS-29* are recessive to *BrS-60*, which is similar to *BnS-6*. Introduction of these *S* haplotypes may result in weak self-incompatibility due to mutual weakening.

There are several *S* genotypes in *B. napus* (Okamoto et al. 2007, Zhang et al. 2008). Cultivar 'Bronowski' has *BnS-2*, which is similar to *BrS-21*, a class-I *S* haplotype in *B. rapa*, and *BnS-6*. The line N344 has *BnS-3*, which is similar to *BrS-8*, a class-I *S* haplotype in *B. rapa*, and *BnS-6*. In these genotypes, frameshift mutations have been identified in *SRK* alleles (Okamoto et al. 2007). These *S* genotypes are also considered to be usable as parental lines for seed production of self-compatible F_1 hybrids. However, not all the self-compatibility phenotypes of these *S* genotypes can be explained by mutations of dominant *S* haplotypes (Okamoto et al. 2007). One *S* genotype has only class-II *S* haplotypes, i.e., *BnS-6* and *BnS-7*. *BnS-7* is similar to *BrS-29*, the most recessive *S* haplotype in *B. rapa*. *BnS-6* similar to *BoS-15* and *BrS-60* is considered to be dominant over *BnS-7*, but mutation in *BnS-6* has not been identified (Okamoto unpublished data). Zhang et al. (2008) have found *B. napus* cultivars having another class-II *S* haplotype and *BnS-6*, whose self-compatibility mechanism has not been elucidated, either. Nasrallah et al. (2007) have reported that the stigmas of interspecific hybrids between self-compatible *Arabidopsis thaliana* and self-incompatible *Arabidopsis lyrata* were compatible with pollen grains of both the parental plants and that the transcripts of an *SRK* allele derived from *A. lyrata* were considerably reduced in the interspecific hybrids, suggesting epigenetic control of *SRK* expression in the interspecific hybrids. It is of interest whether self-compatibility of the *BnS-6*/*BnS-7* line is caused by epigenetic control of gene expression. In any case, this genotype is considered to be unusable as a pollen parent of the proposed F_1 hybrid seed production method.

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Supplementary Data

Supplementary Fig. 1. Genomic Southern-blot analysis of transgenic plants using a probe of *BrSP11-47*.

Genomic DNAs of transgenic plants (1: TSP11-47-1, 2: TSP11-47-2, 3: TSP11-47-3) were digested with *Hin*dIII (left) or *Sac*I (right). Bands different from those of 'Westar' are of transgenes.

Supplementary Fig. 2. GUS activity in pollen grains of a

transgenic plant with the BrSP11-47 promoter::GUS transgene.
Pollen grains of 4, 5, and 10-mm-long buds were stained by X-gluc.

Supplementary Fig. 3. Genomic Southern blot analysis of T1 plants of TSP11-47-1using a probe of BrSP11-47.

Supplementary Fig. 4. Genomic Southern blot analysis of T1 plants of TSP11-47-2using a probe of BrSP11-47.

Supplementary Fig. 5. Amounts of transcripts of *BrSP11-47* analyzed by real-time RT PCR in T_1 plants.
Relative values are shown with a standard of *BrS-47* (=1). Measurements were repeated three times, and bars indicate SE.

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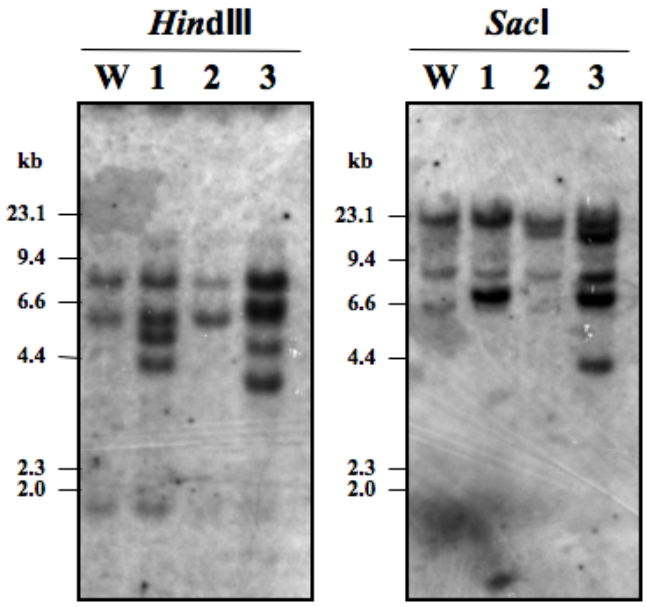
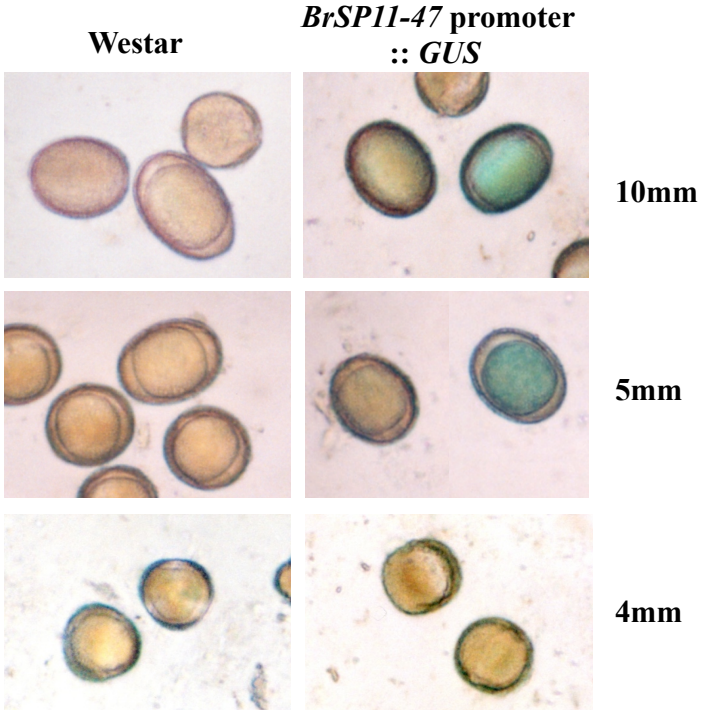


Supplementary Fig. 1. Genomic Southern-blot analysis of transgenic plants using a probe of *BrSP11-47*. Genomic DNAs of transgenic plants (1: TSP11-47-1, 2: TSP11-47-2, 3: TSP11-47-3) were digested with *Hin*dIII (left) or *Sac*I (right). Bands different from those of 'Westar' are of transgenes.



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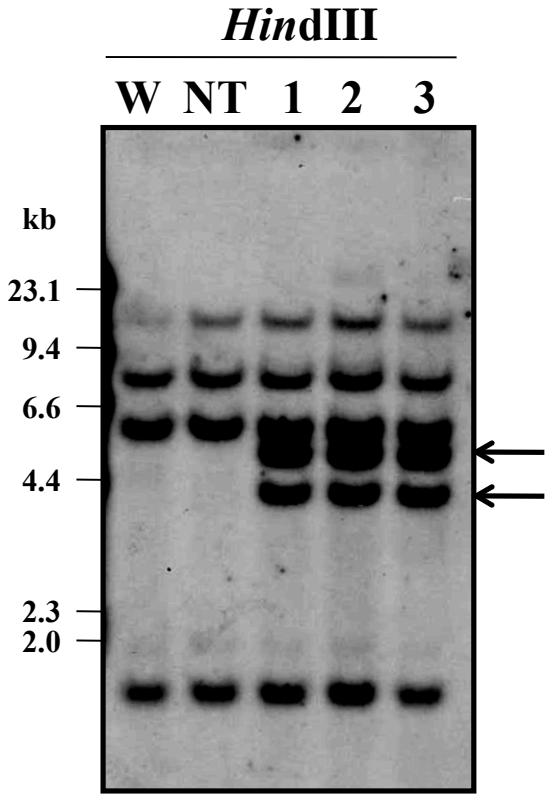
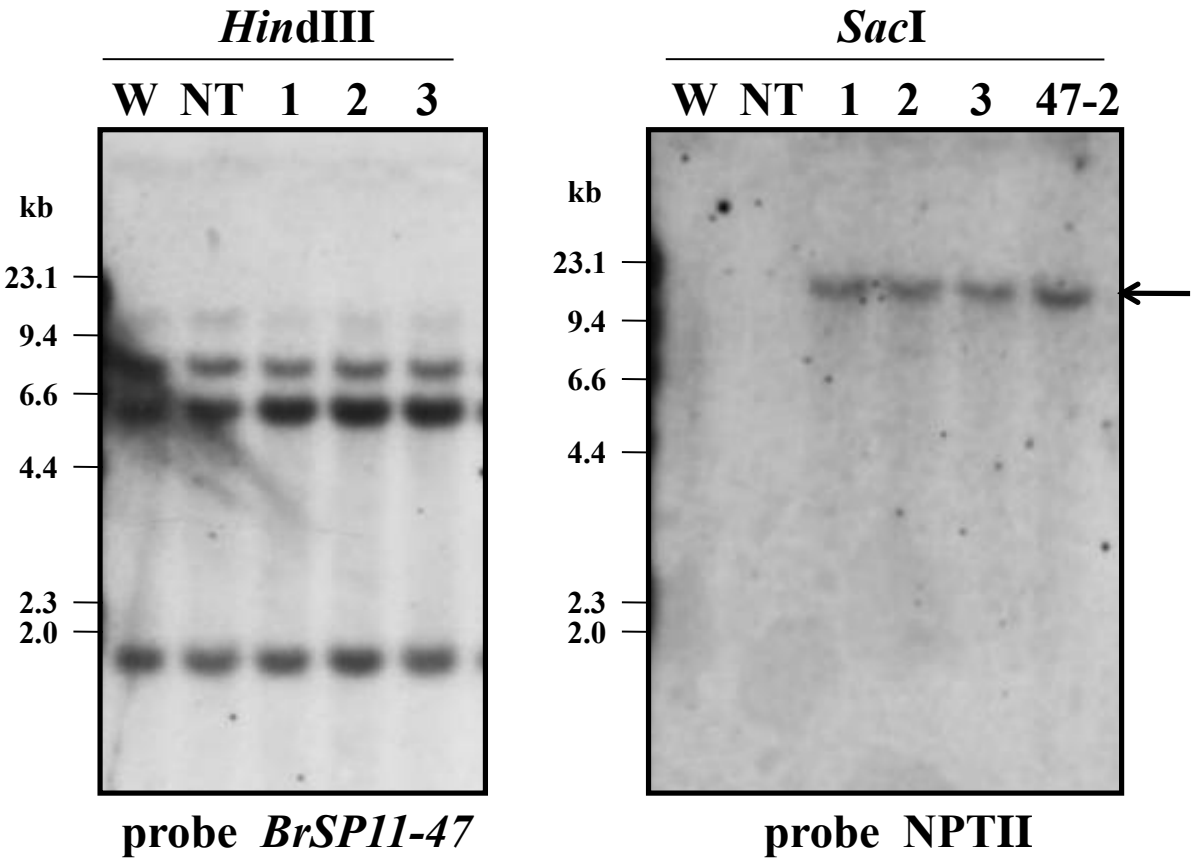
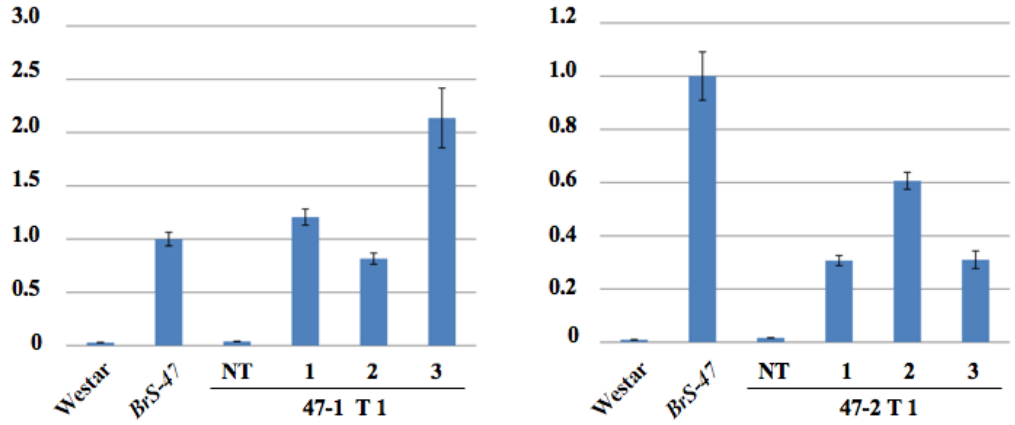


Supplementary Figure 3. Genomic Southern blot analysis of T1 plants of TSP11-47-1 using a probe of *BrSP11-47*.



Supplementary Figure 4. Genomic Southern blot analysis of T1 plants of TSP11-47-2 using a probe of *BrSP11-47*.



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