Characterization of a Common S Haplotype BnS-6 in the Self-Incompatibility of Brassica napus

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Abstract: Self-incompatibility (SI) is a pollen-stigma recognition system controlled by a single and highly polymorphic genetic locus known as the S-locus. The S-locus exists in all Brassica napus (B. napus, AACC), but natural B. napus accessions are self-compatible. About 100 and 50 S haplotypes exist in Brassica rapa (AA) and Brassica oleracea (CC), respectively. However, S haplotypes have not been detected in B. napus populations. In this study, we detected the S haplotype distribution in B. napus and ascertained the function of a common S haplotype BnS-6 through genetic transformation. BnS-1/BnS-6 and BnS-7/BnS-6 were the main S haplotypes in 523 B. napus cultivars and inbred lines. The expression of SRK in different S haplotypes was normal (the expression of SCR in the A subgenome affected the SI phenotype) while the expression of BnSCR-6 in the C subgenome had no correlation with the SI phenotype in B. napus. The BnSCR-6 protein in BnSCR-6 overexpressed lines was functional, but the self-compatibility of overexpressed lines did not change. The low expression of BnSCR-6 could be a reason for the inactivation of BnS-6 in the SI response of B. napus. This study lays a foundation for research on the self-compatibility mechanism and the SI-related breeding in B. napus.

Keywords: self-incompatibility; S haplotype; Brassica napus; SCAR (sequence characterized amplified regions) marker; SCR (S-locus cysteine rich)

1. Introduction

Self-incompatibility (SI), which is a genetic mechanism that helps to avoid inbreeding depression and promotes outcrossing by rejecting self-pollination, occurs in approximately 40% of flowering plant species [1,2]. The single polymorphic genetic S-locus regulates SI responses in many plant species [3]. In Brassicaceae, the S-locus mainly includes the pollen determinant of SI, S-locus cysteine-rich protein (SCR)/S-locus protein 11 (SP11) [4,5], and the stigma determinant of SI, a papilla cell localized in membrane-anchored Ser/Thr kinase (S-locus receptor kinase gene, SRK) [6,7]. Previous studies have indicated that SCR/SP11 is the ligand for SRK [8–11]. When the pollen lands on the stigma of the same S haplotype, specificity recognition between SCR and SRK will trigger the SI response [7,12]. This S haplotype-specific receptor–ligand interaction results in the activation of a pollen-inhibitory signaling pathway upon self-pollination [13]. There are approximately 100 known S haplotypes in B. rapa and 50 S haplotypes in B. oleracea that regulate SI response, respectively [14,15]. Based on the dominance and nucleotide sequences of SCR and SRK in...
Brassica, S haplotypes can be divided into two classes: Class I and II [16,17]. Genetically, SCR genes of class II are dominated by class I, while the SRK genes of these two classes are codominant [18]. Further studies have indicated that the dominant effect of different SCR genes is regulated by SMI (SCR-methylation-inducer) and SMI2 (SCR-methylation-inducer) [19,20].

Most B. napus accessions are self-compatible, while the two progenitors, B. rapa and B. oleracea, are self-incompatible naturally [17,21]. It is generally believed that B. napus lost SI during its speciation by crossing B. rapa and B. oleracea [22]. Eight S haplotypes have been identified in B. napus, named BnS-1 to BnS-7 and BnS-1300, among which BnS-1 to BnS-5 are Class I haplotypes and BnS-6, BnS-7 and BnS-1300 are Class II haplotypes [17,23]. Recent studies have shown that the S haplotype in the C subgenome of B. napus is BnS-6, which is the homolog of BoS-15 in B. oleracea [17,24]. The main S haplotype in the A subgenome of B. napus was found to be BnS-1. Pollination testing and Northern blotting showed that the SC (self-compatibility) of B. napus ‘Westar’ with BnS-1/BnS-6 was caused by the non-expression of BnSCR-1 [17]. Further studies have shown that a 3.6 kb Helitron-like transposon insertion in the promoter region of BnSCR-1 leads to the inactivation of BnSCR-1 in ‘Westar’ [25,26]. This Helitron-like transposon was found to be widely present in B. napus with BnS-1 and was transposed to the current region during the speciation of B. napus [26]. Moreover, the loss of function of SCR in pollen and translational repression might cause the SC of B. napus with BnS-7/BnS-6 [24]. However, the function of BnS-6 in the C subgenome has not been well studied.

In B. napus, SI is one of the main approaches for heterosis utilization. The B. napus line ‘S-1300’, a synthetically developed double-low B. napus self-incompatible accession [27], contains two class II S haplotypes: BnS-1300 and BnS-6 [23,28]. The SI of ‘S-1300’ is recessive to BnS-1/BnS-6 lines but dominant to BnS-7/BnS-6 lines [23,24,29]. A perfect tribe-cross hybrid seed produce system was established by combining ‘S-1300’ with BnS-1/BnS-6 lines (SI restorer lines) and BnS-7/BnS-6 lines (SI maintainer lines) [24,30]. However, the self-incompatible accessions in floral morphologies are similar to the self-compatible accessions, meaning that it is difficult to discriminate the contaminated plants in SI breeding lines [23]. Thus, the development of S-locus-linked genome markers would allow practical detection across the whole growth period. Here, we developed stable SCAR markers based on BnS-1300, BnS-1, BnS-6, and BnS-7 and confirmed that the major S haplotypes in 523 B. napus cultivars and inbred lines were BnS-1/BnS-6 and BnS-7/BnS-6. RNA expression analysis showed that the SRK of all S haplotypes was codominant and that the expression of SCR determined the SI phenotype in B. napus. The further functional characterization of BnS-6 showed that BnSCR-6 is functional, but in B. napus and B. oleracea with BoS-15, there may be abnormalities in the SI signal pathway or recognition specificity. These results provide stable molecular markers for the SI-related breeding of B. napus and insight into the molecular basis of loss of SI in B. napus.

2. Results
2.1. BnS-1/BnS-6 and BnS-7/BnS-6 Are the Main S Haplotypes in B. napus

The sequence colinear comparison of SCR (Figure S1) and SRK of the S haplotypes of ‘S-1300’, its restorer line ‘Westar’ and its maintainer line ‘Bing409’, indicated that the S haplotypes in the A subgenome of ‘S-1300’, ‘Westar’, and ‘Bing409’ were BnS-1300, BnS-1, and BnS-7, respectively, while the S haplotypes in the C subgenome of these three lines were BnS-6. Therefore, we developed six pairs of SCAR markers in order to identify the SCR and SRK genes of BnS-1300, BnS-1, and BnS-7 in the A subgenome. The corresponding PCR products of different SCR and SRK genes were found to be 473 bp (BnSCR-1300, Gene accession number XR_004449204) and 472 bp (BnSRK-1300, Gene accession number AB097116), 412 bp (BnSCR-1, Gene accession number AB270773) and 883 bp (BnSRK-1, Gene accession number AB086976), and 353 bp (BnSCR-7, Gene accession number AB270770) and 294 bp (BnSRK-7, Gene accession number AB008191), respectively (Figure 1).
Table 1. Genotypes of these SCAR markers were crossed with 14 self-compatible inbred lines to produce 14 F1 generation hybrids. First, the marker SRK6-1 [23] was verified in these self-compatible lines. As shown in Table 1 and Figure S2, all these materials contained the SRK6-1 products, indicating that the S haplotypes in the C subgenome of all these lines were BnS-6 or BnS-6-like. Then, the markers of BnS-1300, BnS-1, and BnS-7 were tested in these 14 lines. In the A subgenome of ‘Westar’, ‘Huangshuang2’, ‘Tapidor’, and ‘89008’, the S haplotype was BnS-1, while in the A subgenome of ‘Ningyou-7’, ‘326’, ‘614’, ‘1728’, ‘C32’, ‘Bing409’, ‘242’, ‘198’, ‘1745’, and ‘230’, the S haplotype was BnS-7 (Table 1). To further confirm whether the genotype was associated with the SI phenotype, the F1 plants were self-pollinated and the SC Index (SCI) of F1 plants of ‘Westar’, ‘Huangshuang2’, ‘Tapidor’, and ‘89008’ were crossed with ‘S-1300’ ranging from 17.68 to 21.06, which were presented as the SC phenotype. The SCI of F1 plants of the other ten lines crossed with ‘S-1300’ ranged from 0.02 to 0.64, which were presented as the SI phenotype (Table 1). These results indicated that these SCAR markers were able to identify the S haplotype in B. napus.

The S-locus was found to widely exist in B. napus lines, however, the distribution of S haplotypes in B. napus natural population was still unclear. We then investigated the S-locus in 523 B. napus inbred lines and cultivars using the present SCAR markers. In 523 B. napus inbred lines, 239 lines contained BnS-1 (45.70%); 226 lines contained BnS-7 (43.21%); and only 3 lines (‘11-P63-5Y7’, ‘11-P63-8Y32’, and ‘11-P63-3Y3’) contained BnS-1300. Interestingly, 20 lines contained the BnS-1 and BnS-7 haplotypes simultaneously, suggesting that more than one S-locus could exist in the A subgenome of these B. napus lines. There were also 35 cultivars that could not be detected in any mentioned S haplotypes, indicating that there were other S haplotypes in these 35 cultivars. We also tested the S haplotype in the C subgenome and the results indicated that all 523 lines were BnS-6 (Tables 2 and S2). Obviously, these 239 BnS-1/BnS-6 cultivars and 226 BnS-7/BnS-6 cultivars could be used as restorer and maintainer lines for ‘S-1300’ in SI-related breeding, respectively.

Figure 1. PCR fragment amplified and obtained from three kinds of parent materials and F1 generation of one-by-one pairs using SCAR markers. NTC: no template control. M: DNA marker, from top to bottom, the size of band was 2000, 1000, 750, 500, 300, and 200 bp, respectively.

| Genotypes | BnS-1 | BnS-7 | BnS-1300 |
|-----------|-------|-------|-----------|
| ‘Westar’  | +     | +     | +         |
| ‘Huangshuang2’ | +   | +     | +         |
| ‘Tapidor’ | +     | +     | +         |
| ‘89008’   | +     | +     | +         |
| ‘Ningyou-7’ | +   | +     | +         |
| ‘326’     | +     | +     | +         |
| ‘614’     | +     | +     | +         |
| ‘1728’    | +     | +     | +         |
| ‘C32’     | +     | +     | +         |
| ‘Bing409’ | +     | +     | +         |
| ‘242’     | +     | +     | +         |
| ‘198’     | +     | +     | +         |
| ‘1745’    | +     | +     | +         |
| ‘230’     | +     | +     | +         |
| ‘S-1300’  | -     | +     | +         |

To verify the effect of the SI-related SCAR molecular markers, the ‘S-1300’ line was crossed as the female parent with 14 self-compatible inbred lines to produce 14 F1 generation hybrids. As shown in Table 1 and Figure S2, all these materials contained the SRK6-1 products, indicating that the S haplotypes in the C subgenome of all these lines were BnS-6 or BnS-6-like. Then, the markers of BnS-1300, BnS-1, and BnS-7 were tested in these 14 lines. In the A subgenome of ‘Westar’, ‘Huangshuang2’, ‘Tapidor’, and ‘89008’, the S haplotype was BnS-1, while in the A subgenome of ‘Ningyou-7’, ‘326’, ‘614’, ‘1728’, ‘C32’, ‘Bing409’, ‘242’, ‘198’, ‘1745’, and ‘230’, the S haplotype was BnS-7 (Table 1). To further confirm whether the genotype was associated with the SI phenotype, the F1 plants were self-pollinated and the SC Index (SCI) of F1 plants of ‘Westar’, ‘Huangshuang2’, ‘Tapidor’, and ‘89008’ were crossed with ‘S-1300’ ranging from 17.68 to 21.06, which were presented as the SC phenotype. The SCI of F1 plants of the other ten lines crossed with ‘S-1300’ ranged from 0.02 to 0.64, which were presented as the SI phenotype (Table 1). These results indicated that these SCAR markers were able to identify the S haplotype in B. napus.

The S-locus was found to widely exist in B. napus lines, however, the distribution of S haplotypes in B. napus natural population was still unclear. We then investigated the S-locus in 523 B. napus inbred lines and cultivars using the present SCAR markers. In 523 B. napus inbred lines, 239 lines contained BnS-1 (45.70%); 226 lines contained BnS-7 (43.21%); and only 3 lines (‘11-P63-5Y7’, ‘11-P63-8Y32’, and ‘11-P63-3Y3’) contained BnS-1300. Interestingly, 20 lines contained the BnS-1 and BnS-7 haplotypes simultaneously, suggesting that more than one S-locus could exist in the A subgenome of these B. napus lines. There were also 35 cultivars that could not be detected in any mentioned S haplotypes, indicating that there were other S haplotypes in these 35 cultivars. We also tested the S haplotype in the C subgenome and the results indicated that all 523 lines were BnS-6 (Tables 2 and S2). Obviously, these 239 BnS-1/BnS-6 cultivars and 226 BnS-7/BnS-6 cultivars could be used as restorer and maintainer lines for ‘S-1300’ in SI-related breeding, respectively.
Table 1. Genotypes of the 14 B. napus lines collected and the phenotypes of the corresponding F₁ hybrids.

| Material   | Origin  | SRK1300-1/SCR1300-1 | SRK1-1/SCR1-1 | SRK7-1/SCR7-1 | SRK6-1 | SI Phenotype ¹ |
|------------|---------|---------------------|---------------|---------------|--------|----------------|
| S-1300     | China   | +                   | −             | −             | +      | 0.01 SI        |
| Huashuang2 | China   | −                   | +             | −             | +      | 21.06 SC       |
| Westar     | Canada  | −                   | +             | −             | +      | 16.95 SC       |
| Tapidor    | Europe  | −                   | +             | −             | +      | 20.60 SC       |
| 89008      | China   | −                   | +             | −             | +      | 17.68 SC       |
| Ningyou-7  | China   | −                   | −             | +             | +      | 0.02 SI        |
| 326        | China   | −                   | −             | +             | +      | 0.31 SI        |
| 614        | China   | −                   | −             | +             | +      | 0.50 SI        |
| 1728       | China   | −                   | −             | +             | +      | 0.15 SI        |
| C32        | China   | −                   | −             | +             | +      | 0.23 SI        |
| Bing409    | China   | −                   | −             | +             | +      | 0.41 SI        |
| 242        | China   | −                   | −             | +             | +      | 0.64 SI        |
| 198        | China   | −                   | −             | +             | +      | 0.34 SI        |
| 1745       | China   | −                   | −             | +             | +      | 0.21 SI        |
| 230        | China   | −                   | −             | +             | +      | 0.05 SI        |

¹: SI phenotypes of F₁ generation plants from crossing ‘S-1300’ with male parents; +: successful amplification; −: no amplification.

Table 2. Genotype statistics of 523 B. napus cultivars and inbred lines.

| Genotypes of the Lines | Number of Lines | Proportion |
|------------------------|-----------------|------------|
| SRK1300-1              | SRK1-1          | SRK7-1     | SRK6-1     |          |
| +                      | −               | −          | +          | 3        | 0.57%      |
| −                      | +               | −          | +          | 239      | 45.70%     |
| −                      | −               | +          | +          | 226      | 43.21%     |
| −                      | +               | +          | +          | 20       | 3.82%      |
| −                      | −               | −          | +          | 35       | 6.69%      |

+: successful amplification; −: no amplification.

2.2. Gene Expression of SRK and SCR in Different S Haplotypes

SRK and SCR are the key components of SI response, so we designed specific qRT-PCR primers (Figure S1, Table S1) based on the SRK and SCR of different S haplotypes in order to research the SC mechanism of B. napus. As only 5 bp differences are included between the CDS of BnSCR-1300 and BnSCR-6 (Genebank accession number AB270774), it is difficult to distinguish them as one bp artificial mismatch was introduced at the 3’ end of the reverse primer apart from the internal 2 bp substitutions (Figure S1). Using these primers, in addition to the specific primers of BnSCR-1 [23] and BnSCR-7 [24], we detected the RNA expression of BnSCR-1, BnSCR-1300, BnSCR-6, and BnSCR-7 in different tissues of corresponding materials. SCR genes were specifically expressed in the anther of self-incompatible lines and the expression level increased with the development of the anther. In ‘S-1300’, the expression level of BnSCR-1300 was consistent with that of ‘BrHB’, a BrS-60 contained B. rapa SI line (Figure S3), and reached the highest relative expression level of 6.12 in the L4 anther (Figure 2a). In the transgenic SI line ‘W-3’, the expression pattern of BnSCR-1 (Figure 2c) was similar to that of BnSCR-1300 in ‘S-1300’. However, in the SC lines ‘Westar’ and ‘Bing409’, the transcript levels of BnSCR-1 and BnSCR-7 were near 0 (Figure 2b,c). The expression levels of BoSCR-15 in the L4 anther of B. oleracea was 0.407 (Figure S3). BnSCR-6 is a homolog of BoSCR-15 and exists in all B. napus lines, but the expression levels of BnSCR-6 in all B. napus samples were very low (Figure 2a–c). Additionally, the expression levels of BnSRK-1, BnSRK-1300, BnSRK-6 (Genebank accession number AB270772), and BnSRK-7 in the mature stigma of all B. napus lines mentioned above were detected. In the stigma of all B. napus, the expression levels of SRK were increased along with the development of buds, and reached the highest level in the L4
stigma, but were not associated with SI. The relative expression levels of SRK in the L4 stigma ranged from 0.211 to 0.661 (Figure 2d-f).

Figure 2. Relative expression of SCR and SRK in different tissues of B. napus. (a–c) Relative expression of SCR in different tissues of ‘S-1300’, ‘Bing409’, ‘Westar’, and ‘W-3’, respectively; (d–f) Relative expression of SRK in different tissues of ‘S-1300’, ‘Bing409’, and ‘Westar’, respectively. The relative expression was corrected using the reference gene BnActin7.

The results revealed that the expression patterns of SCR and SRK from different S haplotypes were similar in B. napus, B. rapa, and B. oleracea. They were not expressed in vegetative organs but were highly expressed in reproductive organs. With the development of flowers, the expression levels of SCR and SRK gradually increased in the anthers and stigma, respectively. The expression levels of all SRK were normal and had no corresponding relationship with the SI phenotype. However, the expression levels of SCR in SI lines (BnSCR-1300 in ‘S-1300’ and BnSCR-1 in ‘W-3’) were three orders of magnitude higher than those in SC lines (BnSCR-1 in ‘Westar’ and the expression level of BnSCR-7 in ‘Bing409’). The above results indicate that the low expression of SCR is the reason for the SC in ‘Westar’ and ‘Bing409’.

2.3. Functional Validation of BnSCR-6

BnS-6 existed in all 523 B. napus lines, and SRK was normally expressed. In order to investigate the function of BnS-6 in the SI response of B. napus, a 1975 bp fragment that included the promoter and full coding region of BnSCR-6 was obtained from ‘S-1300’ and 14 self-compatible B. napus lines in the SCAR marker development section. The sequences of BnSCR-6 in these lines and BnSCR-15 in B. oleracea [31] were conserved. Motif search using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 18 September 2015) revealed that there were no obvious differences in the predicted cis-elements in the promoters of BnSCR-6 and BnSCR-1300 (Figure S4). Then, the 1398 bp promoter of BnSCR-6 was fused to the GUS reporter construct and introduced into wild-type Arabidopsis italiana (Col-0) plants. The GUS staining results showed that the promoter was functional and specific in the middle buds and was similar to the promoter of BnSCR-1300 (Figure 3). A series of promoter deletion cassettes of BnSCR-6 and BnSCR-1300 were also analyzed, but GUS expression could still be detected in the middle buds (Figure 3). These GUS staining results confirm the functional consistency of the promoters of BnSCR-6 and BnSCR-1300.
Figure 3. Comparison of the GUS staining of BnSCR-6 and BnSCR-1300 promoters in different Arabidopsis tissues. (a–d) are the GUS staining of BnSCR-6 in different parts of plants; (e–h) are the GUS staining of BnSCR-1300 in different parts of plants; (a,e) are flower buds; (b,f) are stems and leaves; (c,g) are siliques; (d,h) are the whole plant at the seedling stage; (i) is the GUS staining of different length of BnSCR-6 promoter in flower buds; (j) is the GUS staining of different lengths of BnSCR-1300 promoter in flower buds.
As described above, BnSRK-6 was normally expressed. To rebuild the SI response of BnS-6 in B. napus, the CDS of BnSCR-6 was expressed in ‘Westar’ under the control of 1851 bp and 684 bp promoters of BrSCR-47, a Class I dominant SCR in B. rapa (Figure 4a) [26]. At least 20 independent transgenic lines were generated in the 1851 bp and 684 bp promoter constructs, respectively. Both the T₁ and T₂ progenies were self-compatible according to the pollination assay (Figure 4c–e). The SC phenotype was also observed in seven B. oleracea with BoS-15 (Table S3). The phenotypes were compatible when pollinated with the pollen of BnSCR-6 overexpressed lines on the stigma of B. napus with BnS-6 and that of B. oleracea with BoS-15 (Figure 3c–f, Table S3). These results indicated that the loss of SI of BnS-6 (BoS-15) was a common phenomenon in B. napus and B. oleracea lines, and that only normally expressed BnSCR-6 could not recover the SI response.

Figure 4. Pollination test of BnSCR-6 overexpression lines. (a) Overexpression of BnSCR-6 vector diagram. (b) BnSCR-6 expression level detection in T₀ generation. (c) Pollination pattern diagram. (d) Typical pollen tube germination in T₂ generation, left to right: Westar, ‘Westar × We-6OE, S-1300 × We-6OE, C32 × We-6OE, 1241 × We-6OE, Gan-64 × We-6OE, Gan-118 × We-6OE. Gan-64 and Gan-118 is the B. oleracea material with S-15, Bar = 500 µm. (e) The corresponding siliques in Figure d, left to right: Westar, Westar × We-6OE, S-1300 × We-6OE, C32 × We-6OE, 1241 × We-6OE. Due to the poor seed-setting ability of B. oleracea itself, the hybrid siliques develop abnormally, Bar = 2 cm. (f) The SCI statistics of pollination tests in (e).

We hypothesize that the recognition between BnSCR-6 and BnSRK-6 or the downstream signal pathway may cause an abnormal SC phenotype of BnS-6. Pollination assays were performed between the T₂ or T₃ progeny of BnSCR-6-overexpressed lines with other B. napus lines. Interestingly, when the pollen of BnSCR-6-overexpressed lines in T₃ progeny were pollinated to the stigma of B. napus with BnS-6, little-seed-setting appeared in the
pollination between nine of fifteen individuals with ‘S-1300’, and all the seed-setting was normal in the pollination between the fifteen individuals with ‘Ningyou-7’, ‘326’, ‘Bing409’, and ‘ZS11’ (Figure 5). These results confirmed that BnSRK-1300 in ‘S-1300’ could recognize BnSCR-6 in BnSCR-6-overexpressed lines, which was consistent with the results of previous studies on BrSRK-60 and BoSCR-15 in B. rapa and B. oleracea [32,33]. The above results indicated that BnSCR-6 was functional in BnSCR-6-overexpressed lines, but there could be abnormalities in terms of the SI signal pathway or the recognition specificity in B. napus and B. oleracea with BoS-15.

![Figure 5](image.png)

**Figure 5.** Pollination test of the T3 generation of BnSCR-6 overexpression materials. W-6OE-null: genetically modified negative material; Bar = 1 cm.

### 3. Discussion

SI is an important biology phenomenon. The SI response in *Brassica* is regulated by SCR/SRK recognition. However, the distribution of the S haplotype in *B. napus* population is not very clear, which has impeded the research on the SC of *B. napus*. Through the development of SI-related SCAR markers, we analyzed a *B. napus* natural population and found that BnS-1/BnS-6 and BnS-7/BnS-6 were the most common S haplotypes in the *B. napus* population. Additionally, we preliminarily analyzed the function of BnSCR-6, finding that the low expression of BnSCR-6 is one of the reasons for the inactivation of BnS-6 in the SI of *B. napus*. The above results lay a foundation for the further analysis of the SC mechanism in *B. napus*.

SI is a mechanism in plants that prevents inbreeding through the rejection of self-pollen [34]. *B. napus* is an important oil crop around the world and heterosis is the main way to increase its yield and quality [35]. Compared to the cytoplasmic male sterility system, there are some advantages to the self-incompatible system, such as shorter breeding periods required, a wider range of restorer lines, and no negative cytoplasmic effects [23,36], which is a benefit for *B. napus* breeding. However, due to the normal flower phenotype, it is hard to distinguish the self-incompatible and self-compatible lines. The design of molecular markers based on the S-locus is very important for the SI-related breeding of *B. napus*. In the past decade, several draft molecular markers have been developed [23,28,37]. There are also some issues with the existing molecular markers, such as the results not being reproducible and the need for several PCR programs to be designed for different markers, which is time- and labor-consuming. Based on the S haplotypes in the A subgenome, we designed SCAR markers specifically for the amplification of the SCR and SRK. These SCAR markers could help to identify the S haplotype of *B. napus* in a precise and efficient manner.
In 523 B. napus cultivars and inbred lines, we identified many B napus lines that could be used as restorer lines (239 individuals, BnS-1/BnS-6) or maintainer lines (226 individuals, BnS-7/BnS-6) of ‘S-1300’ in the breeding process. In the breeding process, the maintainer lines and restorer lines found in this study could improve the SI line ‘S-1300’ and obtain lots of hybrid combinations with potential agricultural traits. In addition, these SCAR markers could accelerate the breeding process and commercial SI hybrid seeds production.

There are about 100 S haplotypes in B. rapa and 50 S haplotypes in B. oleracea [14,15]. However, only eight S genotypes have been reported in B. napus [17,23]. Recent studies have shown that the major S haplotypes are BnS-1/BnS-6 and BnS-7/BnS-6 and that the S haplotype in the C subgenome of all B. napus is BnS-6. This is consistent with previous reports that there are six S haplotypes in the B. napus A subgenome, most of which are BnS-1 [17]. Three cultivars originating from ‘S-1300’ are BnS-1300/BnS-6 (Table 2 and Table S2). Twenty cultivars were found to have both BnS-1 and BnS-7 in the A subgenome (Table 2 and Table S2), suggesting more than two S haplotypes could exist in these B. napus lines. However, there are also 35 cultivars that couldn’t detect any S haplotype could be detected in the A subgenome, indicating that there were other S haplotypes. B. napus (AACC), a young allotetraploid species, was derived from the hybridization of two diploid species, B. rapa (AA) and B. oleracea (CC), about 7500 years ago [38]. However, only a few allotetraploidization events have occurred and been stably passed on to B. napus [39], which may have resulted in fewer S haplotypes existing in B. rapa.

B. napus is self-compatible, although S-locus is present. The SC of B. napus with BnS-1/BnS-6 is due to the presence of a transposon insertion in the BnSCR-1 promoter position in the A subgenome, which results in the abnormal expression of BnSCR-1 [17,25,26]. The S haplotype in the C subgenome of all-natural B. napus accessions is BnS-6, and the SCR and SRK sequences are consistent with the homologous S haplotype BoS-15 in B. oleracea. The results of the RNA expression analysis show that all SRKs were normal, but BnSCR-6 was expressed in barely any tissues of B. napus. The sequences of SCR and SRK of BnS-7 in B. napus are consistent with the homologous gene sequences in B. rapa, and BnSRK-7 is normally expressed, but all these lines are self-compatible. Although BnSCR-7 of some materials is expressed [24], the level is far lower than that of BnSCR-1300 in ‘S-1300’ and BnSCR-1 in ‘W-3’ (Figure 2). Such differences in expression levels are likely to be the cause of phenotypic differences. Therefore, the difference in SCR expression is one of the direct causes of the self-compatible phenotype of B. napus.

The further comparison of BnSCR-6 and BnSCR-1300 showed that the predicted cis-element and GUS activation of BnSCR-6 and BnSCR-1300 were similar; meanwhile, the BnSCR-6-overexpressed lines were self-compatible in multigenerational self-pollination. Hadj-Arab et al. [40] found self-compatible individuals in a multigenerational self-pollinated B. oleracea with BoS-15, and additional self-compatible individuals emerged from the self-progenies. RNA expression analysis revealed that BoSCR-15 was not expressed in these self-compatible individuals, which accounts for the SC of these individuals [40]. In addition, B. oleracea is not strictly self-incompatible [41–44]. Combination with the present result for the BnSCR-6-overexpressed lines indicated that the inactivation of BoS-15 in the C subgenome may have occurred in the evolution of the B. oleracea C genome itself, rather than in the progress of B. napus formation.

The BnSCR-6 protein in the BnSCR-6 overexpressed lines can recognize BnSRK-1300, resulting in partial incompatibility in the cross-pollination test (Figure 5). This shows that BnSCR-6 and BnSRK-1300 have similar recognition specificity and that differences in their sequences do not change their recognition specificity. This result is consistent with the pollination experiment in B. rapa and B. oleracea carried out by Sato et al. [33], which indicates that the function of BnSCR-6 in the overexpressed lines is normal and that the reason why SCR and SRK of BnS-6 cannot recognize each other in natural B. napus is not only due to the abnormal BnSCR-6 expression. There are multiple reasons for the SC of BnS-6 in B. napus.
In conclusion, we developed stable SCAR markers for S haplotype identification in B. napus and subsequently found that all the S haplotypes in the C subgenome are BnS-6, while BnS-1/BnS-6 and BnS-7/BnS-6 are the main S haplotypes in B. napus. The expression levels of SCR and SRK in different S haplotypes were deduced from qRT-PCR detection, which indicated that the expression level of SRK did not affect the SI phenotype. The expression of SCR in the A subgenome affected the SI phenotype, and the expression of BnSCR-6 in the C subgenome of all materials was low. The further promoter and functional analysis of BnSCR-6 revealed that the low expression of BnSCR-6 is one of the main reasons for the inactivation of BnS-6 in the SI of B. napus. The present work provides stable molecular markers for SI-related breeding in B. napus and lays the foundation for the research of the SC mechanism in B. napus.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

The B. napus SI line ‘S-1300’, its restorer line ‘Westar’, and its maintainer line ‘Bing409’ were crossed with each other to produce F₁ progeny. ‘S-1300’ was also used as a female parent and crossed with another 12 B. napus SC inbred lines (Table 1) to produce F₁ progeny for SI-related SCAR marker development. A rapeseed natural population, consisting of 523 inbred lines and cultivars from 10 countries (Table S2) [45], was used to survey the S haplotype distribution in B. napus. The wild-type B. napus line ‘Westar’ was used for a test of the overexpression of BnSCR-6. The transgenic self-incompatible B. napus line ‘W-3’ was used for BnSCR-1 expression detection. B. napus plants were grown in the field at Huazhong Agricultural University. Arabidopsis thaliana plants (Col-0) were grown at 22 °C with a 16/8 h light/dark cycle in a greenhouse.

4.2. SI Phenotype Assay and Pollination Assay

SI phenotype and SCI were determined following a previously described method [28]. When 3–5 flowers were present on the major inflorescence, the open flowers were removed, the major inflorescence and 2–3 secondary ramifications were covered by paper bags and kept for two weeks. After removing the bags, the seeds and flowers were counted, and the SCI was calculated as the number of seeds divided by the number of flowers. Approximately 100–150 flowers from each plant were investigated. SI phenotype of each plant was categorized as SCI < 2 (self-incompatible), and SCI ≥ 2 (self-compatible).

Pollination assay was performed following a previously described method [46]. Floral buds of the B. napus were emasculated one day before anthesis to avoid pollen contamination. Pollination was performed on the anthesis day. Some pollinated pistils were left to set seeds. The rest were cut at the peduncle 16 hours after pollination, fixed for 2 h in ethanol:acetic acid (3:1), softened in 1 mol/L NaOH at 60 °C for 1.5 h and stained with 0.01% (w/v) decolorized aniline blue for 2.5 h in 2% (w/v) K₃PO₄. Pistils were gently squashed on a microscopic glass slide by placing the cover glass over the pistils. Samples were examined using a fluorescence microscope (Ax 10, Zeiss, Jena, Germany).

4.3. Sequence Collinear Comparison and Primer Design

The sequence alignment analysis of BnSCR-1300, BnSCR-6, and BnSCR-7 was performed in Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 26 August 2015) with the default parameters and edited with genedoc (http://nrbsc.org/gfx/genedoc, accessed on 26 August 2015). The primers were designed in the region of sequence differences using the Primer premier 6 software (http://www.premierbiosoft.com/primerdesign/index.html, accessed on 26 August 2015) with adjustment manually.

4.4. DNA Extraction and Genotyping Assay

Genomic DNA from all individuals was extracted from young leaves. To increase the efficiency and reduce the genotyping costs, total genomic DNA was extracted by a simple approach [47] using the following procedures: (1) Adding a stainless-steel bead and
300 µL of DNA extraction buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) to each well and sealing the plate with a silicone cover. (2) Grinding the leaf sample with a paint shaker for 3 min and separating the supernatants by centrifugation. (3) Transferring 100 µL of supernatants to a V-bottom plate prefilled with 50 µL of isopropanol per well, which was then mixed by pipetting, and stored at −20 °C for 30 min. (4) Precipitating the DNA by centrifuging the plate and discarding the supernatant by inverting the plate. (5) Adding 100 µL 75% ethanol and repeating step (4) and tap-drying the plate with paper. (6) Air-drying the samples overnight and resuspending the samples in 50 µL dd H₂O. For the SCAR molecular marker assay, 10 µL reaction volume was used, which contained 5 µL 2× Taq Reaction Buffer (containing Mg²⁺, dNTPs, and DNA polymerase) (Vazyme, Nanjing, China), 0.5 µM of each primer, and 1 µL genomic DNA. The PCR reaction was performed in a T100 thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following program: 3 min at 94 °C, 35 cycles at 94 °C for 30 s, primer annealing temperature for 30 s, 72 °C for 45 s, followed by 72 °C for 10 min. The PCR products were analyzed by electrophoresis on 1.0% agarose gel in 0.5× TAE buffer and were visualized by staining with ethidium bromide.

4.5. RNA Extraction and Quantitative Real-Time PCR (Qrt-PCR)

Roots, leaves, stems, flower buds (L1: 0–2 mm buds), anthers (L2–L4 anther: anther in 2–4, 4–6, and 6–8 mm buds), and stigma (L2–L4 stigma: stigmas in 2–4, 4–6, and 6–8 mm buds) were collected for RNA extraction. Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The RNA samples were quantified using a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and the first-strand cDNA was synthesized by reverse transcription with a Thermo RT kit (Thermo Fisher, Waltham, MA, USA). qRT-PCR was performed in triplicate for each sample using the SGEExcel FastSYBR MasterMix (Sangon Biotech, Shanghai, China) on a CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gene-specific primers used in the amplification are listed in Table S1, and Actin (GenBank accession no.: AF111812) was used as an internal control to normalize the transcript levels for all the expression analyses. Relative expression levels of SRK and SCR in different S haplotypes were determined using the comparative 2^−∆CT method and normalized to Actin, then the relative expression level of BnSCR-6 in overexpressed lines was determined using the comparative 2^−ΔΔCT method and normalized to BnSCR-6 in ‘Westar’ [48].

4.6. Promoter Construct and GUS Assay

The promoter sequences of BnSCR-6 and BnSCR-1300 were amplified from the DNA of ‘Westar’ and ‘S-1300’ by PCR using specific Table S1. For the GUS assay, the promoter sequences of BnSCR-6 and BnSCR-1300 of different lengths were amplified from the DNA of ‘Westar’ and ‘S-1300’ and cloned into the vector pC2300-GUS [26] to yield SCR6-P1-GUS to SCR6-P4-GUS and SCR1300-P1-GUS to SCR1300-P4-GUS constructs, respectively. All promoter-GUS constructs were introduced into A. thaliana by Agrobacterium-mediated transformation. The GUS activity was visualized by staining different tissues of the T₃ generation homozygous transgenic lines overnight in X-Gluc solution [49], then the tissues were cleaned in 75% (v/v) ethanol and imaged under a stereomicroscope (Nikon, SMZ25, Tokyo, Japan).

4.7. Vector Construction of Bnscr-6 Overexpression and Plant Transformation

The 1851 bp and 684 bp BrSCR-47 promoter sequences were obtained from our previous study [26], and subcloned into pCAMBIA2300 to yield 2300::1851P and 2300::684P constructs, respectively. The CDS of BnSCR-6 was amplified from cDNA of ‘S-1300’ using gene-specific primers, confirmed by sanger sequencing, and subcloned into 2300::1851P and 2300::684P constructs to yield 2300::1851P-SCR6 and 2300::684P-SCR6 constructs, respectively. The 2300::1851P-SCR6 and 2300::684P-SCR6 constructs were introduced into Agrobacterium tumefaciens GV3101 host cells and transformed into B. napus ‘Westar’ fol-
lowing the previous method [50]. The DNA of transformed plants were analyzed by PCR, combining the primers 47pro-1 and SCR6-R to verify the presence of BnSCR-6 transgene.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/plants10102186/s1: Figure S1: Collinear comparison of SCR genomic sequences of BnS-1300, BnS-6 and BnS-7; Figure S2: PCR fragments amplified from ‘S-1300’ and 14 B. napus SC lines using SCR markers; Figure S3: Expression analysis of SCR and SRK in the flower tissues of B. rapa and B. oleracea; Figure S4: cis-elements prediction in the promoters of BnSCR-6 and BnSRK-1300; Table S1: Primers used in this study; Table S2: The result of PCR amplification with SCR marker in the B. napus cultivars and inbred lines; Table S3: Pollination test results of T2 generation transgenic plant with B. oleracea.

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