Genetic Diversity of Genes Controlling Unilateral Incompatibility in Japanese Cultivars of Chinese Cabbage

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Abstract: In recent years, unilateral incompatibility (UI), which is an incompatibility system for recognizing and rejecting foreign pollen that operates in one direction, has been shown to be closely related to self-incompatibility (SI) in *Brassica rapa*. The stigma- and pollen-side recognition factors (SUI1 and PUI1, respectively) of this UI are similar to those of SI (stigma-side SRK and pollen-side SP11), indicating that SUI1 and PUI1 interact with each other and cause pollen-pistil incompatibility only when a specific genotype is pollinated. To clarify the genetic diversity of SUI1 and PUI1 in Japanese *B. rapa*, here we investigated the UI phenotype and the SUI1/PUI1 sequences in Japanese commercial varieties of Chinese cabbage. The present study showed that multiple copies of nonfunctional PUI1 were located within and in the vicinity of the UI locus region, and that the functional SUI1 was highly conserved in Chinese cabbage. In addition, we found a novel nonfunctional SUI1 allele with a dominant negative effect on the functional SUI1 allele in the heterozygote.

Keywords: allelic diversity; *Brassica rapa*; Chinese cabbage; dominant negative effect; gene duplication; pollen-stigma interaction; self-incompatibility; unilateral incompatibility

1. Introduction

Most Japanese cultivars of Chinese cabbage (*Brassica rapa* L.) are F1 hybrids. Traditionally, their seeds have been produced using the *Brassica* self-incompatibility (SI) system. The SI system in *Brassica* is sporophytically controlled by a single S-locus with highly variable, multiple alleles [1]. The S-locus region contains two genes, SRK and SP11/SCR, which correspond to female and male S determinants, respectively [2]. SRK encodes a transmembrane receptor kinase, which is expressed specifically in stigma, and SP11/SCR encodes a small cysteine-rich ligand for SRK, which is localized on the pollen coat [3–5]. The S-haplotype-specific interaction of SP11 and the extracellular domain of SRK induces the SI reaction, in which the self-pollen fails to germinate or penetrate into the stigma [6]. The number of S-haplotypes has been estimated to be more than 100 in *B. rapa* [7–9]. Advanced understanding of the S-haplotype diversity, including dominance relationships between the haplotypes [10], is important for the efficient production of high-quality F1-hybrid seed in *Brassica* crops.

In addition to SI, we reported an interesting incompatibility relationship between Turkish and Japanese populations of *B. rapa* [11,12]. Pollen of the Turkish line was rejected on the stigma of the Japanese line, although crossing in the reverse direction showed compatibility. This cross-incompatibility operating in one direction, unilateral incompatibility...
(UI) occurred within species, in contrast to the UI that is known to occur in interspecies crossing [13,14]. Our molecular genetic studies of intraspecies UI in *B. rapa* revealed that it was controlled by the stigma-expressed gene, *stigmatic unilateral incompatibility 1*, SUI1, encoding an SRK-like receptor kinase and the pollen-expressed gene, *pollen unilateral incompatibility 1*, PUI1, encoding an SP11-like small cysteine-rich ligand. SUI1 and PUI1 are tightly linked and are considered to originate from a duplication event of the SRK-SP11 region in *Brassica* [12]. The S locus is located on chromosome A07, while the UI locus (containing SUI1 and PUI1) is on chromosome A04 of *B. rapa* [12]. From our further analysis of genetic diversity and distribution of the PUI1 and SUI1 genes in *B. rapa*, a functional PUI1-1 allele was found only in the Turkish lines and not in the Japanese lines, while the three functional SUI1 alleles (SUI1-1,-2, and -3) were found in Japanese wild populations and some cultivated varieties. Thus, loss of function of SUI1 in Turkish lines and PUI1 in Japanese lines might have resulted in the unidirectional pollen-stigma incompatibility in *B. rapa* [12].

The physiological pollen-rejection phenotype of the intraspecies UI is similar to that of SI and is consistent with the involvement of M-locus protein kinase (MLPK) in UI, which may function in SRK-mediated SI signal transduction [15,16]. It is noteworthy that the incompatibility response of UI is almost as strong as in the rigid SI phenotype in *B. rapa*. Thus, UI may have an effect on the SI-dependent breeding process in *B. rapa*. In this study, we extensively analyzed SUI1 and PUI1 alleles in Japanese cultivated lines of Chinese cabbage (*Brassica rapa* var. *pekinesis*). The results presented here give new insight into the historical relationship between UI and the breeding system of Chinese cabbage in Japan.

2. Results

2.1. Cultivars of Chinese Cabbage Produced by Japanese Seed Companies

The UI phenotype observed on the stigma (stigma-side UI phenotype) was originally identified in the Japanese commercial hybrid variety ‘Osome’ of Japanese mustard spinach, Komatsuna (*B. rapa* var. *perviridis*), from the Takii seed company [11]. To understand the role of SUI1 in Japanese *B. rapa* cultivars, here we examined 52 commercial cultivars of Chinese cabbage (*B. rapa* var. *pekinesis*) from 16 Japanese seed companies (listed in Table 1) to determine their SUI1 and PUI1 alleles in addition to their stigma-side UI phenotype. All the cultivars used in this study, except ‘Kashinhakusai’ (#8), are F1 hybrids. Because functional SUI1 alleles behave as dominant over nonfunctional alleles [11], they can be analyzed to predict the UI phenotype on the stigma side of hybrid varieties.

Table 1. UI phenotype and genotype of Japanese cultivars of Chinese cabbage.

| Sample Number | Seed Company                        | Cultivar          | Stigma-Side UI Phenotype | Genotype     |
|---------------|-------------------------------------|-------------------|--------------------------|--------------|
| #1            | Tokita Seed Co., Ltd.               | Mainoumi          | UI                       | SUI1-2/SUI1-11 | pui1-3/pui1-4/pui1-6 |
| #2            | Takii & Co., Ltd.                   | Puchihiri         | UI                       | SUI1-2       | pui1-3/pui1-4 |
| #3            | Takii & Co., Ltd.                   | Kigokoro 75       | UI                       | SUI1-2       | pui1-3/pui1-4 |
| #4            | Sakata Seed Corp.                   | Kimikomachi       | UI                       | SUI1-2       | pui1-3/pui1-4 |
| #5            | Takii & Co., Ltd.                   | Chihiri 70        | UC                       | sui1-10      | pui1-3/pui1-4 |
| #6            | Takii & Co., Ltd.                   | Banki             | UI                       | SUI1-2       | nd |
| #7            | Watanabe Seed Co., Ltd.             | Matsushima        | UI                       | nd           | pui1-3/pui1-4 |
| #8            | Noguchi Seed Co.                    | Kashinhakusai     | UI                       | SUI1-1       | pui1-3/pui1-4 |
| #9            | Watanabe Seed Co., Ltd.             | Menkoi            | UI                       | SUI1-2/SUI1-11 | pui1-3/pui1-4/pui1-6 |
| #10           | Ishii Seed Growers Co., Ltd.        | Kinami 90         | UI                       | nd           | nd |
| #11           | Kaneko Seed Co., Ltd.               | Kougetsu 77       | UI                       | SUI1-2       | pui1-3/pui1-4 |
| #14           | Kaneko Seed Co., Ltd.               | Eiki              | UC                       | nd           | pui1-3/pui1-4/pui1-6 |
| #16           | Kaneko Seed Co., Ltd.               | Moeki             | UI                       | nd           | pui1-3/pui1-4 |
| #17           | Kaneko Seed Co., Ltd.               | Kasumihakusai     | UC                       | SUI1-2/SUI1-12 | pui1-3/pui1-4/pui1-6 |
| #18           | Kaneko Seed Co., Ltd.               | Shouki            | UI                       | nd           | pui1-3/pui1-4 |
| #19           | Watanabe Seed Co., Ltd.             | Strong CR         | UI                       | SUI1-2       | pui1-3/pui1-4 |
| #20           | Watanabe Seed Co., Ltd.             | Aiki              | UI                       | nd           | pui1-3/pui1-4/pui1-6 |
### Table 1. Cont.

| Sample Number | Seed Company                  | Cultivar         | Stigma-Side UI Phenotype | Genotype            |
|---------------|-------------------------------|------------------|--------------------------|---------------------|
| #23           | Nozaki Saishujo Ltd.           | Maiko            | nd                       | nd                  |
| #24           | Nozaki Saishujo Ltd.           | Chi China        | UI                       | nd                  |
| #25           | Nozaki Saishujo Ltd.           | Eisyun           | nd                       | nd                  |
| #27           | Nozaki Saishujo Ltd.           | Retasai          | UI                       | nd                  |
| #33           | Marutane Seed Co., Ltd.        | Chikara          | UI                       | SU1-2               |
| #35           | Yamato Noen Co., Ltd.          | Kiyorokobi       | UI                       | SU1-2               |
| #41           | Watanabe Seed Co., Ltd.        | K'ai 65          | UI                       | nd                  |
| #45           | Kaneko Seed Co., Ltd.          | Gokui            | UC                       | nd                  |
| #47           | Kaneko Seed Co., Ltd.          | Taibyou nozomi 60| UI                       | nd                  |
| #49           | Mikado Kyowa Seed Co., Ltd.    | CR Ouen          | UI                       | SU1-2               |
| #50           | Mikado Kyowa Co., Ltd.         | Hakuei hakusai  | UC                       | SU1-2/SU1-10        |
| #51           | Mikado Kyowa Co., Ltd.         | Senki            | nd                       | nd                  |
| #53           | Sakata Seed Corp.              | Saiki            | nd                       | nd                  |
| #55           | Sakata Seed Corp.              | Yumebuki         | UI                       | nd                  |
| #57           | Takayama Seed Co., Ltd.        | Gokigen          | UI                       | nd                  |
| #58           | Ishii Seed Growers Co., Ltd.   | CR Seiga 65      | UI                       | SU1-2               |
| #62           | Takii & Co., Ltd.              | Oshou            | UI                       | nd                  |
| #63           | Takii & Co., Ltd.              | Musou            | UI                       | SU1-2               |
| #64           | Takii & Co., Ltd.              | Senshou          | UI                       | nd                  |
| #65           | Takii & Co., Ltd.              | Kinshou          | UI                       | nd                  |
| #74           | Tohoku Seed Co., Ltd.          | Daifuku          | UI                       | SU1-2               |
| #75           | Tohoku Seed Co., Ltd.          | Daifuku75        | UI                       | nd                  |
| #77           | Tohoku Seed Co., Ltd.          | Shinseiki        | UI                       | nd                  |
| #80           | Nanto Seed Co., Ltd.           | CR Kinshachi 75  | UI                       | SU1-2               |
| #83           | Nanto Seed Co., Ltd.           | Taibyou apolo 60| UC                       | SU1-2/SU1-10        |
| #84           | Nippon Norin Seed Co.          | Kikumusume 65    | UI                       | SU1-2               |
| #85           | Nippon Norin Seed Co.          | Kien75           | UI                       | nd                  |
| #88           | Nippon Norin Seed Co.          | Super CR Shinrisou| UC                     | SU1-2               |
| #91           | Takayama Seed Co., Ltd.        | Kinkaku 65       | UI                       | nd                  |
| #96           | Tokita Seed Co., Ltd.          | Haruhi           | UI                       | nd                  |
| #97           | Nanto Seed Co., Ltd.           | Taiki 60         | UI                       | nd                  |
| #101          | Musashino Seed Co., Ltd.       | Nanzan           | UI                       | nd                  |
| #102          | Watanabe Seed Co., Ltd.        | Seiitoku         | nd                       | nd                  |
| #103          | Watanabe Seed Co., Ltd.        | Shunjuu          | UI                       | nd                  |
| #104          | Watanabe Seed Co., Ltd.        | Kaname           | UI                       | nd                  |

nd, not determined.

#### 2.2. Stigma-Side UI Phenotype Determined by Pollination Test

To verify the stigma-side UI phenotype of the Japanese cultivars of Chinese cabbage, stigmas of 47 cultivars were crossed with the pollen from the Turkish line (S<sup>24</sup>t, S<sup>40</sup>t, or S<sup>21</sup>t) possessing PUI1-1/PUI1-1 with crossing combinations of different S-haplotypes for discriminating the UI phenotype from the SI phenotype (S<sup>21</sup>t was produced for this study) [16]. Among the 47 cultivars, 85% (40 cultivars) had the incompatibility (UI) phenotype to the Turkish pollen (Table 1). Only seven cultivars, ‘Chihiri 70’ (#5), ‘Eiki’ (#14), ‘Kasumihakusai’ (#17), ‘Gokui’ (#45), ‘Hakuei hakusai’ (#50), ‘Taibyou apolo 60’ (#83), and ‘Super CR Shinrisou’ (#88), had the compatibility (UC) phenotype to the Turkish pollen (Table 1). Thus, the majority of the Chinese cabbage cultivars we tested have the ability to reject the PUI1-1/PUI1-1 pollen, indicating that they possess functional SU11 allele(s).

#### 2.3. The SU11 Allele and Its Distribution

We isolated the full-length SU11 gene by polymerase chain reaction (PCR) amplification from the genomic DNA of each cultivar and determined its allele(s) by sequencing, as listed in Table 1. From 22 cultivars in which SU11 was sequenced, six alleles, including functional alleles (SU11-1 and -2), were identified. One cultivar, ‘Kasumihakusai’ (#8), with...
stigmatic UI phenotype, had the SUI1-1 allele, which was originally isolated from Komatsuna variety ‘Osome’ [11,12]. This may be because, among the cultivars used in the present study, only ‘Kashinhakusai’ (#8) is not an F1 hybrid, as described above. The 16 cultivars with stigmatic UI phenotype possessed the SUI1-2 allele (Table 1), which has been found in wild B. rapa populations [11,12]. Three alleles encoding putative intact SUI1 proteins, SUI1-10 (accession, LC641787), SUI1-11 (accession, LC641786), and SUI1-12 (accession, LC641785), and one allele encoding truncated protein, sui1-t10 (accession, LC641784) were newly identified alleles in this study (Figure 1). Phylogenetic analysis with amino acid sequences revealed that SUI1-11 and SUI1-12 belonged to the same clade, and this was different from the clade with the functional SUI1s (SUI1-1, -2, and -3) and SUI1-10 (Figure 2), suggesting that SUI1-11 and SUI1-12 are nonfunctional alleles. Four out of seven stigmatic UC cultivars possessed SUI1-10, -12, or sui1-t10.

Figure 1. Schematic representation of the SUI1 genomic sequences in this study. The shaded boxes represent the protein coding regions. Positions of amino acid substitutions compared to SUI1-2 are shown by bars and listed below. The extracellular domain (consisting of most of the 1st exon) is indicated. The position of the 10-bp deletion of sui1-t10 is shown in the sixth exon.

Figure 2. A maximum likelihood phylogenetic tree of SUI1s and SRKs in B. rapa. Branch support values from 100 bootstraps are indicated. Functional SUI1s that genetically interact with PUI1-1 are indicated by asterisks (*).
In the case of the SUI1-10 allele, found in cultivars ‘Hakuei hakusai’ (#50) and ‘Taibyou apolo 60′ (#83), a single base substitution at codon 413 (changing the residue from cysteine to tyrosine) was present at the C-terminus of the extracellular domain (Figure 1). Both cultivars possessing the SUI1-10 allele showed stigmatic UC phenotype, despite being heterozygous for the functional SUI1-2 allele (Table 1), indicating that there was a dominant negative effect of SUI1-10 toward SUI1-2 (as described below in detail).

On the other hand, the SUI1-11 allele had 17 amino acid changes in the extracellular domain, and cultivars ‘Mainoumi’ (#1) and ‘Menkoi’ (#9) with SUI1-2/SUI1-11 heterozygote showed the stigmatic UI phenotype (Figure 1, Table 1). Even if SUI1-11 is nonfunctional, as expected, the stigmatic UI phenotype is consistent with the dominance of SUI1-2 over SUI1-11.

The SUI1-12 allele had six amino acid changes in the extracellular domain. The cultivar ‘Kasumihakusai’ (#17) had SUI1-2 and SUI1-12 alleles as a heterozygote, and it showed the stigmatic UC phenotype (Figure 1, Table 1). It is also possible that SUI1-12 might show a dominant negative effect to SUI1-2 in ‘Kasumihakusai’ (#17), as in the case of SUI1-10 in ‘Hakuei hakusai’ (#50) and ‘Taibyou apolo 60′ (#83).

‘Chihiri 70′ (#5) possessed the truncated sui1-t10 allele (Figure 1). All the 15 SUI1 clones of ‘Chihiri 70′ (#5) isolated from two independent PCR amplifications were sui1-t10, suggesting that ‘Chihiri 70′ (#5) is homozygous for sui1-t10, which is consistent with its stigmatic UC phenotype. The sequence of the extracellular domain of sui1-t10 was perfectly matched with SUI1-1 and SUI1-2 functional alleles, but there was a 10-bp deletion in the sixth exon, as in sui1-t4, sui1-t5, and sui1-t6, which results in a frameshift and creates a premature termination codon [12].

### 2.4. The PUI1 Allele and Its Distribution

To examine the PUI1 alleles of 48 cultivars of Chinese cabbage, we cloned the PCR fragments of the full-length PUI1 and determined their sequences (Table 1, see Materials and Methods section). In these Japanese cultivars, we found three nonfunctional alleles (pui1-3, -4, and -6), which have been reported previously [12]. Out of the 48 cultivars, 34 possessed both pui1-3 and pui1-4, and 14 possessed all three alleles (Table 1). The existence of three alleles in an individual plant indicates the possibility of duplication of PUI1. To verify this duplication, we first propagated the self-pollinated progeny of ‘Super CR Shinrisou’ (#88; pui1-3/pui1-4) and determined the PUI1 genotype of the 22 segregants using a direct sequencing method. It was found that all the segregants exhibited both pui1-3 and pui1-4, suggesting that the pui1-3 and pui1-4 genes were linked and homozygous in this progeny (Table 2). Next, we propagated the self-pollinated progeny of ‘Gokui’ (#45; pui1-3/pui1-4/pui1-6) and determined the PUI1 genotype of the 32 segregants using a PCR-restriction fragment length polymorphism (RFLP) method. It was found that all individuals possessed pui1-3, pui1-4, and pui1-6, suggesting that the three PUI1 genes (pui1-3/pui1-4/pui1-6) were linked and homozygous in this progeny (Table 2). Furthermore, a similar PCR-RFLP experiment was performed using ‘Nanzan’ (#101; pui1-3/pui1-4/pui1-6) selfed progeny (Table 2, Table S1). Interestingly, the self-pollinated population (78 plants) of ‘Nanzan’ segregated to pui1-3/pui1-4 and pui1-3/pui1-4/pui1-6 plants. Their segregation ratio was 17:61 (1:3; chi-square test, $\chi^2 = 0.43, p > 0.05$) fit for a simple Mendelian inheritance. The result indicates that ‘Nanzan’ (#101) is a heterozygote of duplicated (pui1-3/pui1-4) and triplicated (pui1-3/pui1-4/pui1-6) PUI1 genes in the B. rapa genome. Thus, duplication and/or triplication of nonfunctional PUI1 genes had occurred at the UI locus region in Japanese B. rapa cultivars.
Table 2. Segregation analysis of PUI1 allele in the selfed progeny of #45, #88, and #101.

| Sample Number | Cultivar   | n  | PUI1 Genotype Detected |
|---------------|------------|----|------------------------|
|               |            |    | pui1-3/pui1-4          |
| #45           | Gokui      | 32 | 0                      | 32                      |
| #45           | Super CR   | 22 | 22                     | *                      |
| #101          | Nanzan     | 78 | 17                     | 61                      |

* ‘Super CR Shinrisou’ (#88) does not have pui1-6 allele.

2.5. Genetic Segregation Analysis of the Dominant Negative Effect of SUI1-10

As described above, ‘Hakuei hakusai’ (#50) and ‘Taibyou apolo 60’ (#83), possessing the SUI1-2/SUI1-10 genotype, exhibited stigma-side UC phenotype (i.e., accepting the Turkish PUI1-1/PUI1-1 pollen), even though they have a functional SUI1-2 allele. To confirm this dominant negative effect of SUI1-10, we performed a genetic analysis of ‘Taibyou apolo 60’ (#83).

We produced self-pollinated progeny of ‘Taibyou apolo 60’ (#83-S1 progeny) and determined their stigma-side UI phenotype and SUI1 genotype (Table 3). Stigma-side UI phenotypes of this progeny were determined by test cross-pollination using homozygous plants (S24t) as the pollen donor. The SUI1-2 and SUI1-10 alleles were discriminated by direct-sequencing detection of a single nucleotide polymorphism at codon 413 and were segregated in the #83-S1 progeny; three of eleven plants showed stigma-side UI, and the others were stigma-side UC. Stigma of the UI homozygous plants were incompatible to the S24t pollen (UI), and five SUI1-2/SUI1-10 heterozygous, and three SUI1-10/SUI1-10 homozygous individuals showed compatible pollen tube penetration with the S24t pollen (UC), indicating that SUI1-10 is nonfunctional and has a dominant negative effect to the functional SUI1-2.

Table 3. Segregation analysis of SUI1 allele in the selfed progeny of #83.

| Population | SUI1 Genotype       | n  | Stigma-Side UI Phenotype |
|------------|---------------------|----|--------------------------|
|            |                     |    | UI                       |
| #83-S1     | SUI1-2/SUI1-2       | 3  | 3                        | 0                        |
|            | SUI1-2/SUI1-10      | 5  | 0                        | 5                        |
|            | SUI1-10/SUI1-10     | 3  | 0                        | 3                        |
| #83-S2     | SUI1-2/SUI1-2       | 23 | 23                       | 0                        |
|            | SUI1-2/SUI1-10      | 41 | 0                        | 41                       |
|            | SUI1-10/SUI1-10     | 16 | 0                        | 16                       |

For further confirmation of this effect, the #83-S2 population with a higher number of plants was produced by self-bud pollination of the #83-S1 SUI1-2/ SUI1-10 heterozygous plants. In the #83-S2 population, SUI1 genotypes segregated as expected; for genotypes SUI1-2/SUI1-10: SUI1-10/SUI1-10 the observed ratio was 23:41:16 (1:2:1; chi-square test, χ² = 1.27, p > 0.05, df = 2, Table 3, Table S2). The stigma-side UC phenotype and SUI1-10 genotype of the #83-S2 population showed perfect linkage in the 80 plants (Table 3). Thus, it was concluded that the nonfunctional SUI1-10 does show a dominant negative effect on the functional SUI1-2.

To verify if this effect is observed with the other functional allele, we produced SUI1-3/SUI1-10 heterozygous plants by a cross between SUI1-3/SUI1-3 [11,12] and SUI1-10/SUI1-10 plants selected from the #83-S2 population. Stigmas of SUI1-3/SUI1-10 heterozygous plants were compatible (UC) with PUI1-1/PUI1-1 pollen from the S24t and also S40t lines, indicating that SUI1-10 also shows a dominant negative effect on the functional SUI1-3.
3. Discussion

Highly controlled pollen-stigma incompatibility is important for F₁ hybrid seed production of Brassica cultivars. The molecular mechanism of SI in Brassica has been studied for many years and is used in F₁ breeding. The recently discovered UI system, regulated by SUI1 and PUI1, can potentially provide another mechanism to control pollination in B. rapa. Therefore, determination of the UI genotype is considered as important as the SI genotype in the breeding of this major Japanese vegetable, Chinese cabbage. In this study, we determined the SUI1 and PUI1 allelic diversity of 22 and 48 cultivars, respectively, of Chinese cabbage in Japan. In addition, we confirmed the stigma-side UI phenotype of 47 cultivars. This revealed that most of the cultivars showed a stigma-side UI phenotype with a functional SUI1 allele (SUI1-2), whereas no functional PUI1 allele (PUI1-1) was found. We also searched the re-sequence data of B. rapa lines that are stored at Chungnam National University and found a functional SUI1-2 allele in a South Korean population (data not shown). The fact that functional SUI1 alleles are present in Japanese and South Korean cultivars should be taken into consideration in breeding programs for B. rapa. UI may be beneficial as the additional incompatibility, which could be used in breeding programs by the introduction of PUI1-1 to the pollen donor.

To the best of our knowledge, there is no report that traits important for Chinese cabbage are mapped to flanking regions of the UI locus in chromosome A04. Thus, for an unknown reason, the functional SUI1-2 has been selected, and its sequence has been conserved during the breeding of Chinese cabbage cultivars in Japan. It would be interesting to investigate whether SUI1 itself strengthens SI and thus increases the efficiency of F₁ seed production.

In our previous study, we isolated nine intact alleles of SUI1 and showed that SUI1-1, SUI1-2, and SUI1-3 are incompatible with PUI1-1/PUI1-1 pollen [12]. SUI1-1 was originally isolated from a Japanese commercial hybrid variety of Komatsuna (B. rapa var. perviridis) and SUI1-2 and SUI1-3 were found in Japanese wild populations of B. rapa [12]. In the current study, we isolated three novel intact SUI1 alleles; one (SUI1-10) belongs to the functional clade (with SUI1-1, SUI1-2, and SUI1-3) and the other two alleles (SUI1-11 and SUI1-12) belong to the nonfunctional clade (Figure 2). The fact that SUI1-10/SUI1-10 homozygote is stigmatic UC indicates that SUI1-10 is a nonfunctional allele (Table 3). The Cys-413 residue of SUI1-2 is the last of the 12 highly conserved cysteine residues in the SUI1 extracellular domain and is located within the PAN_APPLE domain, which is the C terminal region of the extracellular receptor region. It has been clarified that homodimerization of SRK in Brassicaceae is essential for ligand interaction [17]. The PAN_APPLE domain of SRK has been shown to be important for ligand-independent dimer formation of SRKs and is responsible for correct intracellular trafficking [18–21]. It has been reported that the last Cys residue of SRK is predicted to form an intramolecular disulfide bond [20,21]. Thus, although the SUI1-10 sequence is similar to the functional SUI1-2, the C413Y mutation of SUI1-10 might cause structural disruption of SUI1 and breakdown of incompatibility through unusual dimer formation.

A feature of the sporophytic regulation of SI is the dominance relationship between S-haplotypes [10,22,23]. The molecular mechanism of the pollen-side dominance relationship has been well studied and revealed that mono-allelic gene expression of the dominant SPI1 haplotype is controlled by small RNA-based epigenetic regulation [24–26]. On the stigma side, there is a complex allelic interaction that is as yet unexplained [10]. It was presumed that the SRK protein itself determines the dominance relationship rather than differences in SRK gene expression [23], and Naithani et al. [18] noted that the stigma-side dominance relationship may result from an increased tendency for heterodimer formation in some SRK pairs [18]. On the other hand, the existence of dominant negative alleles of receptor kinases that function as receptor complexes in many situations during plant development is widely known [27–29]. In most of these, the formation of a receptor complex with abnormal receptor proteins or receptor-related proteins encoded by dominant negative alleles causes disruption of signaling pathways. Thus, one possible explanation...
for the dominant negative effect of SUI1-10 may be an increase of SUI1-2/SUI1-10 heterodimer on the stigma surface and competitive inhibition of the interaction with the PUII ligand. We also found a dominant negative effect of SUI1-10 to SUI1-3, which has four aa substitutions (R322H, I326L, R363H, and V364D) compared to the extracellular domain of SUI1-2, suggesting that these four residues are not important for the effect.

In this study, it was found that the PUII gene of Japanese cultivars of Chinese cabbage showed very low diversity. Among six PUII alleles, of which only PUII-1 from a Turkish strain can induce UI [12], only two patterns of genotype (pui1-3/pui1-4 or pui1-3/pui1-4/pui1-6) were observed, and no cultivars with a functional PUII-1 allele could be found. Interestingly, the pui1-3/pui1-4 genotype might consist of two linked pui1-3 and pui1-4 genes (Figure S1). Similarly, the pui1-3/pui1-4/pui1-6 genotype might consist of three linked pui1-3, pui1-4, and pui1-6 genes (Figure S1). Such duplication and triplication of nonfunctional PUII have complicated the UI locus region. Although such PUII duplication or triplication cannot be found in the reference genome information of B. rapa inbred line Chiifu (B. rapa reference genome version 3.0, https://brassicadb.cn, accessed on 1 April 2021), de novo genomic sequence assembly of these Chinese cabbage cultivars using next-generation sequencing technology, including long-read sequencing, would provide new insights into the genomic structure of the UI locus [30]. In fact, we can find the two duplicated PUII genes on the UI locus of the genome sequence of B. rapa Z1 (version 1.0, https://brassicadb.cn, accessed on 19 October 2021, Figure S2) [31].

Further analysis of the genetic diversity of the UI locus in B. rapa other than Chinese cabbage (subsp. pekinensis), such as turnips (subsp. rapa), leafy Brassica crops (subsp. chinensis, periridis), and field mustard (subsp. oleifera) will not only contribute to the discovery of novel alleles but also provide new insights into the genomic structure of the pollen-side factor and the dominant recessive interaction of the stigma-side factor. It will also be interesting to determine whether the UI locus has a multi-allelic structure like the S locus.

4. Materials and Methods

4.1. Plant Material

The plant material consisted of 52 commercial cultivars of Chinese cabbage, B. rapa ssp. pekinensis (Table 1). All except one, ‘Kashinhakusai,’ were F1 hybrid cultivars. To produce self-pollinated progeny, bud pollination was performed. Petals and stamens were removed from a young flower bud (2–4 d before flowering), and the immature pistil was pollinated. The pollinated pistil was then covered with a paper bag until the seed was harvested. Plant materials were vernalized at 4 °C for 4 weeks in a refrigerator and then grown in a greenhouse.

4.2. Test Pollination

Flower buds were cut at the peduncle and pollinated. After pollination, they were stood on 1% solid agar for about 24 h under room conditions. Then, pistils of the pollinated flowers were softened in 1N NaOH for 1 h at 60 °C and stained with basic aniline blue (0.1 M K3PO4, 0.1% aniline blue). Samples were mounted in 50% glycerol on slides and observed by UV fluorescence microscopy (Figure S3) [32]. At least three flowers were used from each cross combination, and observations were generally replicated at least three times on different dates for each cross combination. For the determination of the stigma-side UI phenotype, PUII-1/PUII-1 homozygous plants (S24t, S40t, and S21t) were used as the pollen donor in test pollinations (S21t was produced for this study) [16].

4.3. Cloning, Sequencing, and Genotyping of SUII and PUII Alleles

Total DNA was extracted from young leaf tissue of B. rapa by the procedure of Murray and Thompson (1980) or using a DNeasy plant mini kit (Qiagen) [33]. For molecular cloning of full-length SUII and PUII genes, genomic PCR was performed using KOD-Plus-Neo DNA polymerase (TOYOBO) according to the manufacturer’s instructions. PCR primers
SUI1cDNA_F3 and SUI1_gR2 for SUI1 and PCP-like1-F1 and PCP-like1-R1 for PUI1 were used (Table S3). All amplified fragments were detected as a single band in the gel electrophoresis. PCR products were modified by adding 3'-A overhangs using A-attachment mix (TOYOBO) and cloned into a vector, pTAC-2, using DynaExpress TA PCR Cloning kit (Biodynamics). The nucleotide sequence was determined with a 3500 or 310 Genetic Analyzer using Big Dye Terminator version 3.1 or 1.1 Cycle Sequencing Kit (Applied Biosystems); in the case of SUI1, the SUI1-specific sequencing primers, SUIcDNA_F3, SUI_gR2, SUIinter_cF1, SUIinter_cF2, SUIinter_cF3, SUIinter_GF1, SUIinter_cF4, and SUIinter_cF5 (Figure 1 and Table S3), were used. GENETYX version 13 software package (GENETYX Corp.) was used for the sequence comparison and alignment. For the segregation analysis, we determined the genotype of SUI1 and PUI1 alleles by direct sequencing of PCR products. SUII-1 and SUII-10 alleles were amplified using primers SU1_2-10typeSDF and SU1_2-10typeSDR (Table S3). Each PUI1 allele was amplified using the primer pair for the PUI1 second exon region, PUI1-3.4.6-F, and PUI1-3.4.6-R (Table S3, Figure S4). For discrimination of PUI1 alleles by PCR-RFLP, amplified DNA fragments were cut by restriction enzyme (BamHI, SalI, or BsrI), followed by checking on an electrophoresed agarose gel (Figure S4). For the direct sequencing marker, amplified fragments were purified from the electrophoresed agarose gel and sequenced as described above.

4.4. Phylogenetic Analysis

Phylogenetic analysis was performed on the Phylogeny.fr platform (http://www.phylogeny.fr/, accessed on 21 October 2021) [34]. Full-length amino acid sequences were aligned with MUSCLE (version 3.7) configured for the highest accuracy. Accession numbers of SRKs and SUI1s are listed in Table S4. After alignment, ambiguous regions were removed with Gblocks (version 0.91b). The phylogenetic tree was reconstructed using the PhyML program (version 3.0 aLRT). The default substitution model was selected assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The reliability of internal branches was assessed using the bootstrapping method (100 bootstrap replicates). The tree was represented with TreeDyn (version 198.3).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10112467/s1, Figure S1: Schematic model of duplicated and triplicated PUI1 allele; Figure S2: Genomic organization of the SUI1 and PUI1 region of B. rapa Z1 (yellow sarson) identified from published genome sequence available at https://brassicadb.cn accessed on 29 September 2021 [31]; Figure S3: Representative results of test-pollination under UV fluorescence microscopy; Figure S4: Nucleotide sequence alignment of PUI1 alleles. Table S1: PUI1 genotype in the selfed progeny of #101, ‘Nanzan’; Table S2: SUI1 genotype and stigma-side UI phenotype of selfed progeny of #83; Table S3: Primers used in this study; Table S4: Accession number of SUI1 and SRK sequences used in phylogenetic analysis.

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