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Research Paper

Allele specific DNA marker for fusarium resistance gene *FocBo1* in *Brassica oleracea*

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The fusarium yellows resistance (YR) gene *FocBo1* was previously identified and the DNA markers were developed to assist the breeding of YR cultivars in *Brassica oleracea*. However, the further analysis revealed discrepancies between the phenotypes and the genotypes predicted by those DNA markers in cabbage commercial cultivars. Since this discrepancy seemed to be due to unknown susceptible alleles of *focbo1*, we sequenced the gene in 19 accessions to determine the sequence variations between alleles and found that there were two resistant *FocBo1* alleles and six susceptible alleles in the investigated population. The newly designed PCR markers detected three mutations in the susceptible alleles that generate premature termination codons. These were shown to accurately distinguish resistant and susceptible alleles in more than 200 accessions of *B. oleracea* inbred lines and cultivars. The study revealed that the locus is represented by 37.2% resistant and 62.8% susceptible alleles within seventy-eight commercial cultivars. Structural analysis of the gene revealed that a part of the allelic variation comes from intragenic recombination between alleles. Our results enable a more precise prediction of the phenotype by marker assisted selection, promoting the production of YR cultivars in *B. oleracea*.

Key Words: allelic variation, *Brassica oleracea*, disease resistance, *Fusarium*, intergenic recombination, marker assisted selection, yellows.

Introduction

B. oleracea contains many agriculturally important crops such as cabbage, broccoli and cauliflower, and they are extensively cultivated worldwide. Fusarium yellows is caused by *Fusarium oxysporum* f. sp. *conglutinans*, a soil-borne disease that causes leaf yellowing and eventually dying. The pathogen forms durable thick-walled spores, so it is difficult to control by agricultural chemicals and measures, whereas resistant varieties can effectively control against this disease. Two types of yellows resistance (YR) are reported; type A resistance is inherited as a single dominant gene and effective even under high temperature, while type B resistance is inherited in a quantitative manner and abolished at ground temperatures above 24°C (Blank 1937).

QTL analysis has shown that the resistance of type A is controlled by a single dominant gene, designated as *FocBo1* (Pu *et al.* 2012). The *FocBo1* gene was molecularly isolated by map-based cloning, and the DNA marker, MTK-C, distinguishing resistant and susceptible alleles was developed (Shimizu *et al.* 2015). This marker was designed to amplify the region covering exon 9 to the 3′ untranslated region of *FocBo1*, where there are the two indels distinguishing the resistant allele in the plant accession AnjuP01 and the susceptible allele in the accession GCP04. The MTK-C marker, a dominant marker for the resistant allele, amplifies a 759 bp long PCR fragment from the resistant parent AnjuP01, but not from susceptible GCP04. The genotyping results of fifty F_1 cabbage cultivars obtained using MTK-C marker completely correlated with their phenotype except for cv. Rakuen, which was predicted as resistance by the appearance of the MTK-C band but susceptible to yellows.

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The sequence analysis revealed that *focbo1* of cv. Rakuen contains an additional nucleotide in exon 4, resulting in a loss of function of *FocBo1* (Shimizu *et al.* 2015). Shimizu *et al.* (2015) also designed co-dominant marker, MTK-1, to the position at ca. 30 kbp away from *FocBo1* and found a perfect matching between phenotype and genotype detected by the marker in F_2 populations derived from the self-pollination of four commercial F_1 cultivars. Generally, when the linkage between the marker and the target gene is not very strong, the marker meets with limited success for accurate identification because of the crossing-over that occurs between the marker and the target gene locus during the breeding programs (Collard and Mackill 2008). In addition, no comprehensive information on the DNA sequence variation between alleles makes it difficult to design DNA markers able to accurately distinguish between alleles.

DNA marker assisted selection (MAS) has several advantages compared to conventional breeding selection. MAS is not affected by the cultivation environment and the interaction of other genes. Additionally, MAS is applicable at any growth stage, since it is not affected by developmental stages of plants. Therefore, various DNA markers have been developed to select important agronomic traits such as disease resistances (Fukuoka *et al.* 2015, Matsumoto *et al.* 2012), product quality (Karim *et al.* 2016), seed contamination (Kawamura *et al.* 2017) and flowering time (Shea *et al.* 2018). Generally, accurate selection of the target trait in MAS is sometimes difficult due to insufficient information on DNA polymorphisms of the target gene or crossing-over between the target gene and its linked DNA marker, as above-mentioned.

In this study, we determined DNA sequences of several alleles of *FocBo1* locus in commercial cultivars and inbred lines and developed allele-specific DNA marker sets for MAS of fusarium resistance in *B. oleracea*. The data unveiled a wide variety of DNA sequences among susceptible alleles in contrast with only one polymorphic site among resistant alleles, suggesting high evolutionary rate in the susceptible alleles by the loss of selective pressure. Phenotype prediction done by the newly designed DNA markers was shown to be valid since perfect correlation between the detected DNA polymorphisms and the phenotypes were observed using 134 inbred lines and 78 cultivars of *B. oleracea*.

Materials and Methods

Plant materials

For the screening of *FocBo1* genes, 134 inbred lines of cabbage developed in Ishii Seed Growers CO., Ltd. and 78 varieties of commercial varieties of cabbage, broccoli, cauliflower were used (**Supplemental Table 1**). Three DH lines, AnjuP01, GCP04, and A12, derived from *B. oleracea* var. *capitata*, var. *italica*, and var. *alboglabra*, respectively, were used.

For DNA sequence analysis of *FocBo1* and *focbo1* genes, the following commercial cultivars were used; Satsuki Joou (Nippon Norin Seed Co.) and Wakamine (Takii & Co., Ltd.) in cabbage, Endeavor (Takii & Co., Ltd.) and Shaster (Takii & Co., Ltd.) in broccoli, Yukidarum (Kaneko Seeds Co., Ltd.) in cauliflower, and the cabbage inbred lines P3 and 6T-1 provided by Ishii Seed Growers Co., Ltd. and the cabbage inbred lines of NR1, NR2, NR9, and NR 12–17 by Nippon Norin Seed Co.

Genomic DNA extraction

Plant DNA was extracted by CTAB (Cetyl Trimethyl Ammonium Bromide) method with some modification (Murray and Thompson 1980). Leaf tissue of about 1 cm in diameter was collected, placed in a 1.5 ml Eppendorf tube, and frozen with liquid nitrogen. Immediately, the leaf material was ground to a fine powder with a mortar and pestle, to which 600 µl of 1.5 × CTAB Buffer was added, stirred with a vortex, and then kept at 65°C for 15 minutes. 600 µl of chloroform/isoamyl alcohol (24:1) was added, and the mixture was stirred with a vortex and centrifuged at 14000 rpm for 10 minutes. Thereafter, 450 µl of the supernatant was taken into a new 1.5 ml Eppendorf tube, and then 400 µl of cooled isopropyl alcohol was added, mixed, and centrifuged at 14000 rpm for 25 minutes. After centrifugation, the pellet was rinsed with 70% ethanol and air-dried, and then 100 µl of TE-Buffer was added to dissolve the pellet. For DNA sequencing, plant DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Screening of B. oleracea commercial cultivars

The Cong: 1-1 strain of *F. oxysporum* f. sp. *conglutinans* was provided by Dr. Kadota (National Agriculture Research Center for Tohoku Region, Japan), and was used to prepare inocula. The inoculation method was followed by the method reported by Shimizu *et al.* (2015). Twelve-day-old seedlings were transplanted to the inoculated soil medium. The test was carried out in the growth room where the room temperature was kept at 28°C with the photoperiod of 16 h at light intensity of 200 µmol m^{-2} s^{-1}. The disease index (DI = 0 to 3) of inoculated plants was determined 28 days after transplantation (Shimizu *et al.* 2015) as follows; DI = 0 with no symptoms, DI = 1 with some yellowing/atrophy of leaves, DI = 2 with fallen leaves and extremely yellowing/atrophy of leaves, DI = 3 with plant death.

Screening of B. oleracea inbred lines

The inoculation test of the inbred lines was carried out in the artificially inoculated greenhouse nursery bed of Ishii Seed Growers Co., Ltd. Seeds of inbred lines were sown directly in early August, and then their mortality was observed periodically until mid-September. The phenotype evaluation was carried out in the different three years. Plant death was identified as susceptible and surviving plants as resistant.

To investigate what kind of fusarium strains grow in the soil of this field, an inoculated plant was collected from this field. After sterilizing the leaf petiole of the infected plant, it was cultured in the acidified PDA medium. After overnight

culture at 25°C, the hyphae grown from the tissue was collected. Then, the pathogenicity and conidia morphology (shape, number and size of cells) of the isolate were examined, and consequently, the characteristics were the same as Cong: 1-1 strain, excepting that the isolate grew faster than Cong: 1-1 strain. Additionally, the intergenic spacer (IGS) of rDNA of our newly collected fungus isolate was checked using the FIGS11/FIGS12 primer set (Enya *et al.* 2008), and found that the sequence was identical to that of Cong: 1-1 strain.

Polymorphism analysis and sequencing

For PCR, each reaction mix contained 2.5 µl of EmeraldAmp PCR Master Mix (Takara Bio, Shiga, Japan), 0.5 µl of the template DNA and 5 pmol each of the forward and reverse primers, and adjusted to a total of 5 µl by adding water. The reaction mixture was incubated in a thermal cycler at 94°C for 3 min for denaturation, followed by 35 cycles of 94°C for 30 seconds (s), 55°C to 60°C for 30 s, and 72°C for 1 min for denaturation- annealing-primer extension, and finally 72°C for 3 min. The annealing temperature was set to the property of the primers. PCR products were electrophoresed on 1% agarose gel or 8–13% acrylamide gel (Kikuchi *et al.* 2004). The acrylamide gel was stained with a Gelstar solution (0.1 µl/10 ml; Takara Bio Inc., Japan). The allele specific primers and the primer sequences are listed in **Supplemental Table 2**.

The whole DNA sequences of *FocBo1* and *focbo1* genes were acquired by two methods. Whole gene sequences encompassing from the 5′UTR to 3′UTR were amplified by primeSTAR Max DNA Polymerase and cloned into pUC vector with In-fusion technology (Takara Bio Inc., Japan). Some of *focbo1* genes were separately amplified using eight primer sets. For sequence analysis of the *focbo1-3* allele, the primer BrTN 32-5R-2 was additionally used. After fractionating the PCR products by 1% agarose gel electrophoresis, the portion of the target band was cut out and purified using FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan). Direct nucleotide sequence analysis was conducted by Sanger's chain termination method (FASMAC Co. Ltd., Kanagawa, Japan). The obtained sequences were analyzed using sequence analysis software, GENETYX v.12 (Genetyx Corporation, Tokyo, Japan) and Sequencher v 2.0 (Hitachi Software, Tokyo, Japan), and then were aligned along with the *FocBo1* sequence and the *FocBr1* sequence in *B. rapa* (Shimizu *et al.* 2014, 2015) using MEGA 7 (Kumar *et al.* 2016). The phylogenetic relationship was inferred by using the Maximum Likelihood method (Hasegawa *et al.* 1985) added to MEGA 7.

Results

Polymorphism analysis of FocBo1

In this study, we determined the genotypes of commercial cultivars and inbred lines of *B. oleracea* using the existing marker, MTK-C, and found that some cases (9.6%) were inconsistent to that of the inoculation test (data not shown). It is considered that those cultivars/lines have the resistance specific sequence at the site of MTK-C marker while mutation hides in other regions of the genes, so we determined their genomic sequences along with the *FocBo1* genes of some resistant cultivars. As a result, nineteen sequences of the genes in commercial cultivars and inbred lines were determined (**Table 1**). Previously, we reported the identification and characterization of *FocBo1*, a functional resistant allele in a cabbage DH line, AnjuP01 (*B. oleracea* var. *capitata*) (Shimizu *et al.* 2015). In this study, we newly identified a broccoli cultivar, Shaster, as resistant to yellows in the inoculation test. Shaster possesses the allele identical to that of AnjuP01 except for the region encompassing from the end of exon 3 to the middle of in the intron 3, which is identical to the susceptible alleles (**Fig. 1**, **Supplemental Figs. 1**, **2**). This intragenic conversion occurred at the intron 3 between the AnjuP01 allele and one of the susceptible allele suggests that it doesn't affect the amino acid sequence and the function of the gene. Therefore, those resistant alleles were designated as *FocBo1a* and *FocBo1b* in AnjuP01 and Shaster, respectively.

We previously identified the three susceptible alleles i.e., *focbo1-a12*, *focbo1-gc*, and *focbo1-rakuen* in *B. oleracea* var. *alboglabra* (A12), var. *italica* (GCP04), and var. *capitata* (cv. Rakuen), respectively (Shimizu *et al.* 2015). Among the three alleles, both *focbo1-gc* and *focbo1-a12* have the 1 bp insertion in exon 4 (#31 in **Supplemental Fig. 2**) and the large-scale deletion after intron 9 (#179 in **Supplemental Fig. 2**), suggesting close relationship between them regarding the structure, thus here we renamed them as *focbo1-1a* and *focbo1-1b*, respectively (**Fig. 1**). In this study, we found a new susceptible allele in NR1 whose sequence is most closely related, but not identical to that of *focbo1-1b* (**Fig. 2**); the sequence polymorphism exists

Table 1. List of the cultivars and lines determined their genomic sequences of the target gene

Accession	Allele	Gene Sequence	Morphotypes	Origin
AnjuP01	*FocBo1a*	AB981181.1†	Cabbage	DH line
Wakamine	*FocBo1a*	Same as AnjuP01	Cabbage	Cultivar
Yukidaruma	*FocBo1a*	Same as AnjuP01	Cauliflower	Cultivar
NR12	*FocBo1a*	Same as AnjuP01	Cabbage	Inbred line
NR13	*FocBo1a*	Same as AnjuP01	Cabbage	Inbred line
NR14	*FocBo1a*	Same as AnjuP01	Cabbage	Inbred line
NR15	*FocBo1a*	Same as AnjuP01	Cabbage	Inbred line
NR16	*FocBo1a*	Same as AnjuP01	Cabbage	Inbred line
NR17	*FocBo1a*	Same as AnjuP01	Cabbage	Inbred line
Shaster	*FocBo1b*	LC424798	Broccoli	Cultivar
GCP04	*focbo1-1a*	LC424799	Broccoli	DH line
A12	*focbo1-1b*	LC424800	Chinese Kale	DH line
NR1	*focbo1-1c*	LC424801	Cabbage	Inbred line
Rakuen	*focbo1-1d*	LC424802	Cabbage	Cultivar
Satsuki Joou	*focbo1-2*	LC424803	Cabbage	Cultivar
P3	*focbo1-2*	Same as Satsuki	Cabbage	Inbred line
NR2	*focbo1-2*	Same as Satsuki	Cabbage	Inbred line
NR9	*focbo1-2*	Same as Satsuki	Cabbage	Inbred line
6T-1	*focbo1-3*	LC424804	Cabbage	Inbred line

†: cited from the paper of Shimizu *et al.* (2015).

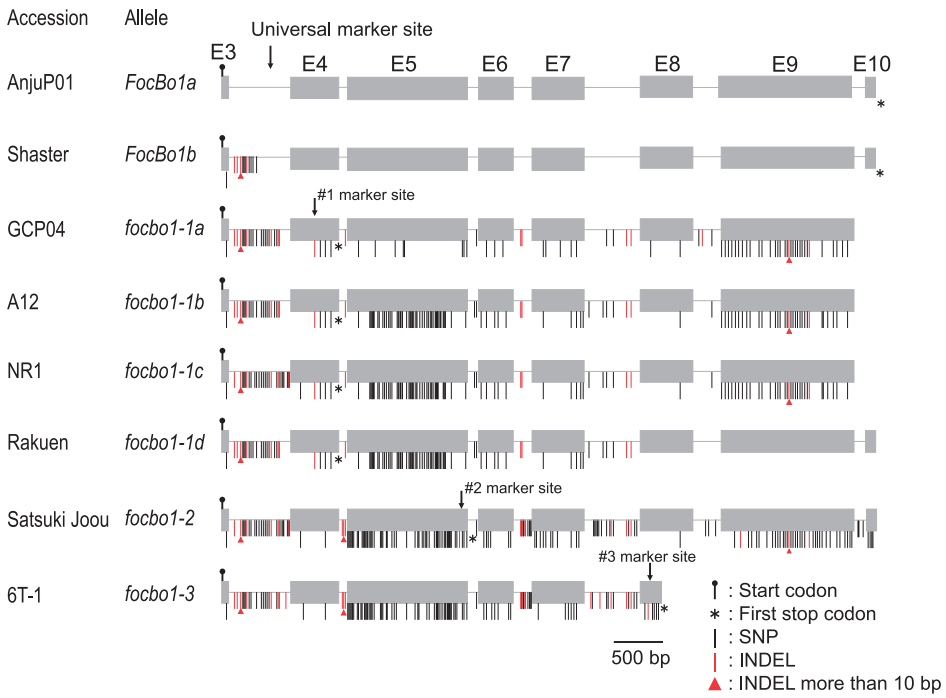

Fig. 1. Schematic comparison of *FocBo1* and the mutant genes. Boxes and horizontal lines indicate exons and introns, respectively.

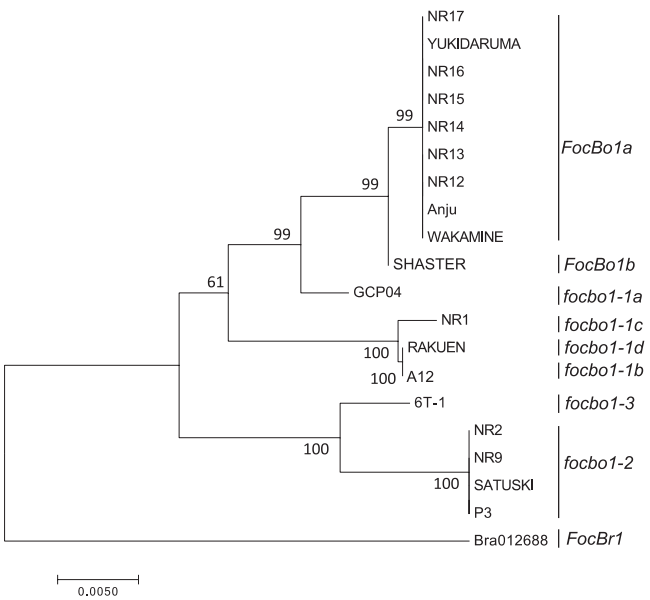

Fig. 2. Phylogenetic tree using the genomic sequences of the *FocBo1* and *focbo1* genes. The numbers above the nodes represent bootstrap value expressed as percentage of 1,000 bootstrap replications.

between intron 3 (**Supplemental Fig. 2**) and the overall sequence identity is 99.8%. It was designated as *focbo-1-1c*. Although *focbo1-rakuen* has the 1 bp insertion in exon 4 same as *focbo1-1a/b/c*, but its exon 8–exon 10 region is replaced with that of the resistance allele (**Fig. 1**), so renamed as *focbo1-1d*.

Another mutant allele was found in a cabbage inbred line (P3) and a commercial cabbage cultivar (Satsuki Joou), which was noted by the existence of a 10 bp deletion in exon 5 (**Fig. 1**, #91 in **Supplemental Fig. 2**). This type has been already reported in cabbage inbred lines developed in China, but has not been named (Lv *et al.* 2014). Since the phylogenic analysis distinguished this type from *focbo1-1a/b/c* (**Supplemental Fig. 3**), it was designated as *focbo1-2*. The other mutant allele found in a cabbage inbred line (6T-1), designated as *focbo1-3 allele*, has a 1 bp insertion mutation in exon 8 (#148 in **Supplemental Fig. 2**), causing the appearance of a stop codon at the 35 bp downstream of the mutation point. In addition, it has a large deletion from 159 bp downstream of this mutation point to the middle position of the downstream gene. The comparison of the gene structures (**Fig. 1**) and the phylogenic analysis revealed that *focbo1-2* and *focbo1-3* alleles are closely related. Regarding four *focbo1-1a–d* alleles having the same functional mutation, the *focbo1-1b–d* alleles have high similarity in the DNA sequences, while the *focbo1-1a* allele is closely related to *FocBo1a/b* alleles. This may be due to the high DNA sequence similarity from exon 5 to exon 7 in *focbo1-1a* and *FocBo1* alleles, probably caused by the intragenic recombination.

Development of DNA markers

According to the structural allelic variation revealed by the sequence analysis, we determined three regions to design DNA markers distinguishing the alleles. The regions were in the exon 4, 5, and 8, respectively (**Fig. 3**). The #1 marker was designed to distinguish between *FocBo1* and *focbo1-1*. This is an *Eco*RI-based CAPS marker which can detect the 1042 bp and 31 bp-long band from the resistant

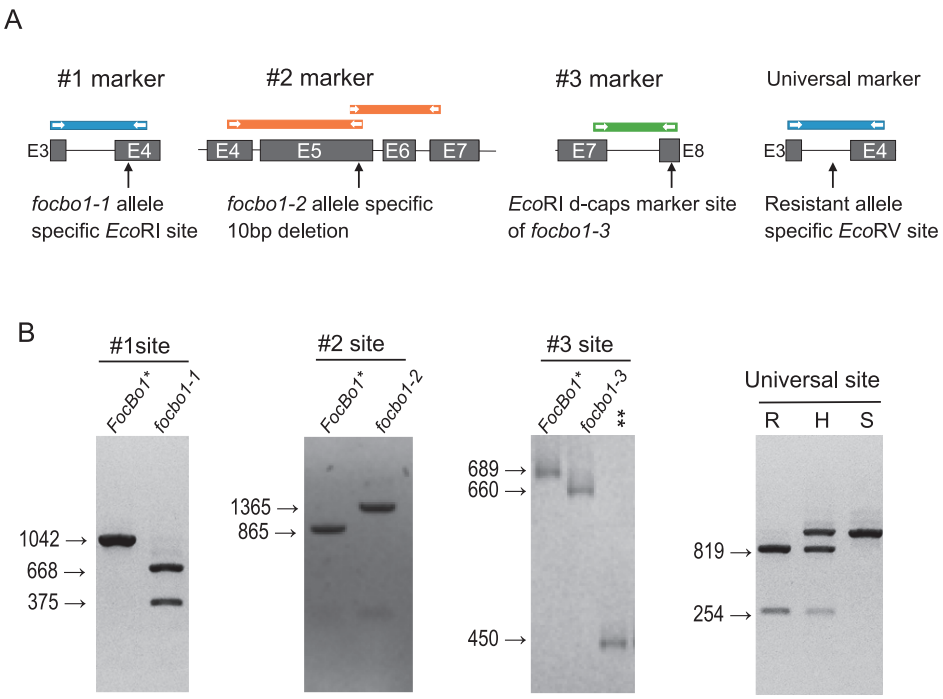

Fig. 3. Identification of the mutant alleles using allele specific and universal primers. (A) Marker positions, their target sites and amplicon sizes. (B) DNA banding pattern of #1 marker for detection of *focbo1-1*, #2 marker for *focbo1-2*, #3 marker for *focbo1-3*, and the universal. * Other susceptible alleles except for the target site also show similar size to the *FocBo1* band (See more details for band sizes in **Supplemental Table 3**). ** The #3 marker of *focbo1-3* also detected the *focbo1-2* allele specific band (450 bp) in the susceptible plant having *focbo1-2* allele (See more details in the main text). R, H and S = Resistant, Heterozygous, and susceptible alleles.

FocBo1 and the 668 bp, 375 bp, and 31 bp-long band from *focbo1-1* (**Fig. 3B**) (the 31 bp bands are not shown in **Fig. 3B**). Other susceptible alleles except for *focbo1-1* also show similar size to the *FocBo1* band (**Supplemental Table 3**).

The #2 marker was designed to detect the 10 bp indel polymorphism in exon 5 between *FocBo1* and *focbo1-2*. The #2 marker consists of two different primer sets, which were separately used for genotyping in each sample. In the PCR, SATSU-F/SATSU-R primer set amplified a fragment of 865 bp from *FocBo1*, while #2-2F1/#2-2R1 primer set amplified a fragment of 1365 bp from *focbo1-2*.

The #3 marker is an *Eco*RI-based dCAPS marker to distinguish *FocBo1*, *focbo1-2* and *focbo1-3* alleles. It produces a fragment of 689 bp from *FocBo1* allele, 450 bp from *focbo1-2*, and 660 bp from *focbo1-3* in 8% acrylamide electrophoresis. Another 240 bp-long fragment, not represented in **Fig. 3** though, is produced from *focbo1-2* by this reaction because of the presence of another *Eco*RI site in the intron 7, which is absent in other alleles identified to date.

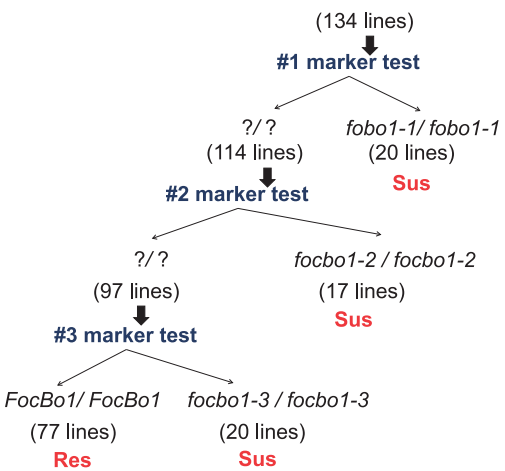

Fig. 4. Schematic diagram showing allelic genotypes determined by step-by-step analysis using the #1 to #3 markers in cabbage inbred lines. Sus and Res = susceptible and resistant phenotypes expected in each genotype, respectively.

Examination of the new DNA markers using cabbage inbred lines

Genotype test was performed in 134 cabbage inbred lines using the three DNA markers. The strategy we took to discriminate the alleles is shown in **Fig. 4**. First, we used the #1 marker to identify *focbo1-1/focbo1-1* and others. The analysis classified 20 lines as *focbo1-1/focbo1-1* and the rest 114 lines were left as unknown. Next, the 114 lines were examined by the #2 marker to distinguish *focbo1-2/focbo1-2* and others. The analysis identified 17 lines as *focbo1-2/focbo1-2* and the rest 97 lines as unknown. Then, we used the #3 marker to classify the remaining 97 lines into the two groups; *FocBo1/FocBo1* (77 lines) and *focbo1-3/focbo1-3* (20 lines). Finally, we identified 77 lines (57%) having homozygous resistant allele (*FocBo1/FocBo1*) and 57 lines (43%) having homozygous susceptible alleles. All of these

Table 2. Summary of genotypes and phenotypes determined by allelic specific PCR marker and inoculation test in *B. oleracea* cultivars, respectively

Ecotype	*FocBo1/ FocBo1*	*FocBo1/ focbo1-3*	*FocBo1/ focbo1-2*	*focbo1-2/ focbo1-2*	*FocBo1/ focbo1-1*	*focbo1-1/ focbo1-3*	*focbo1-1/ focbo1-2*	*focbo1-1/ focbo1-1*	Total
Cabbage	17	3	5	4	9	2	10	6	56
Broccoli	0	0	2	0	4	0	2	4	12
Cauliflower	0	0	0	0	1	0	0	9	10
Subtotal	17	3	7	4	14	2	12	19	78
Phenotype	R	R	R	S	R	S	S	S	

genotypes were consistent with their phenotypes in the field inoculation trials without exception (data not shown). In other words, all the resistant phenotypes of the inbred lines were accurately predicted by the three DNA markers.

Allele frequency in B. oleracea commercial varieties

Next we applied the three DNA markers to commercial cultivars of cabbage, broccoli and cauliflower. As described above, the genotypes were determined by step-by-step analysis using the #1 to #3 markers. Among the 78 cultivars analyzed, the #1 marker classified *B. oleracea* cultivars into the three groups having different PCR band pattern; unknown (31 cultivars), heterozygote of *focbo1-1* (28), and *focbo1-1/focbo1-1* (19) (**Supplemental Fig. 3**). The result indicates that the 19 cultivars could be susceptible. Next, the 28 cultivars heterozygote at #1 site were examined by the #2 marker, classifying them into *focbo1-1/focbo1-2* (12) and heterozygote of *focbo1-1* and unknown allele (16). Following the same scheme, finally we found that allele frequency in *B. oleracea* accessions was 37.2% of the resistant *FocBo1* allele, 42.3% in the *focbo1-1*, 17.3% in *focbo1-2*, and 3.2% in the *focbo1-3* allele (in total; 62.8% of the susceptible alleles). Again the result of inoculation test was perfectly matched with the prediction derived from the DNA maker genotyping (**Supplemental Table 1**). This analysis revealed that 52.6% and 47.4% of the accessions are resistant and susceptible, respectively, due to the dominant nature of *FocBo1* (**Table 2**, **Supplemental Table 1**). The *focbo1-3* allele was found only in cabbage and the *focbo1-1* allele was found in all morphotypes. Some resistant cultivars are present in broccoli, while only one resistant cultivar in cauliflower.

Test of universal primer set detecting all susceptible alleles

The three DNA markers were designed to detect frameshift mutations which abolish the gene function. Beside them, we identified an SNP in the intron 3, where an *Eco*RV site was commonly found in the resistant *Focbo1a* and *Focbo1b*, but not in all of the susceptible alleles (**Figs. 1**, **3A**). Using this *Eco*RV site, we designed a universal PCR marker which can distinguish the existing alleles into the two groups, resistant and susceptible allele, with a single PCR. This primer set produced the 265 bp and 810 bp bands in the resistant cultivars after *Eco*RV digestion while the fragment (1075 bp) is not cut in the susceptible cultivars (**Fig. 3B**). Then we tested the universal marker using the commercial cultivars to see the correlation between the resistant/susceptible phenotype and DNA marker polymorphism, and found the perfect match between them (**Supplemental Table 1**). This result indicates that the use of universal marker could be easier to distinguish resistant and susceptible alleles than the step-by-step analysis using the #1 to #3 markers. However, it should be noted that if the SNP recombines intragenic link with the other functionally important mutations such as those of #1, 2, and 3, the linkage could be lost. Nevertheless, the SNP-based CAPS marker might be useful to distinguish between resistant and susceptible alleles within a certain range of *B. oleracea* population.

Discussion

As far as we searched, we found only two alleles of *Focbo1*, and designated these as *FocBo1a* and *FocBo1b*. On the other hand, there are six susceptible alleles (*focbo1-1a–1d*, *focbo1-2*, *focbo1-3*). *FocBo1b* in Shaster possesses the region encompassing from the end of exon 3 to the middle of in the intron 3, which is identical to the susceptible alleles. This structure is most likely a recombinant derived from the functional *FocBo1* allele of AnjuP01 and the nonfunctional allele. Similarly, the susceptible allele, *focbo1-1d* in Rakuen, is derived from intragenic recombination at the exon 8–exon 10 region between the functional *FocBo1* allele of AnjuP01 and the nonfunctional allele (Shimizu *et al.* 2015, this study). Furthermore, *focbo1-1a* allele of GCP04 revealed a chimeric structure; the exon 5–exon 7 region in this gene has high similarity with the resistant allele, while the other region is closely related to the susceptible alleles of var. *alboglabra* A12. These results indicate that intragenic recombination in the *FocBo1* alleles occurred, sometimes resulting in a loss of function of the gene.

Most of the R-proteins like FocBo1 have an NBS-LRR structure where the LRR domain is thought to recognize pathogens, and the NBS domain plays a role in transmitting recognition signal to downstream proteins (Césari *et al.* 2014, Monteiro and Nishimura 2018). However, pathogens diversify Avr protein coding genes in order to avoid recognition from R-proteins and this relationship is the often described "arms race between plants and pathogens" (Kanzaki *et al.* 2012). To recognize various Avr proteins, plant R-genes evolve rapidly under positive selection. In addition, R-genes are tandemly clustered and duplicated in genomes,

and consequently, more diverse via genetic recombination between alleles or between related sequences and lateral gene conversion (Leister 2004, Monteiro and Nishimura 2018, Zhong and Cheng 2015). In this context, our study provided a good example that the intragenic crossing-over is an important source to create the variation in the R-gene like *FocBo1* alleles. Additionally, it is noted that non-functional R-genes (pseudo-gene) lacking positive selection accumulate DNA variations, which have potential to *de novo* create hyper-variation of the R-genes or diversify via intragenic recombination between this pseudo-gene and the functional R-gene.

Cabbage cultivars carrying resistance to fusarium wilt were initially developed in the United States at the beginning of the twentieth century (Anderson 1933, Walker *et al.* 1927). After that, Type A resistance is known to have been widely distributed in cabbage (Dixon 2007). In this study, we showed the real allele frequency of Type A resistance gene, *FocBo1*, in cabbage, with 17 cultivars (30.4%) being homozygous resistant, 17 cultivars (30.4%) being heterozygous, and 22cultivars (39.3%) being homozygous susceptible. This result indicated that further improvement is needed in the YR breeding of *B. oleracea* in the global warming age, because Fusarium wilt is a warm-weather disease. In addition, it is noted that the plants heterozygous at the *FocBo1* allele are usually resistant to yellows, but become more susceptible than the *FocBo1*-homozygous plants in the high temperature condition (Pu *et al.* 2012). Several YR cabbage cultivars are reported to be distributed into the commercial market, whereas it has been not reported that there are YR cultivars in broccoli and cauliflower so far. Therefore, the cultivars of broccoli and cauliflower, identified as resistant to yellows in this study, are important resources in YR breeding of these crops.

These days, the application of MAS is limited in a lot of the crops including Brassica crops, due to the insufficient information on DNA polymorphisms of the target gene. Considering this, the information and procedure presented here will effectively promote *B. oleracea* YR breeding and become a good reference to establish MAS breeding programs in other crops.

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