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Genetic mapping of a fusarium wilt resistance gene in *Brassica oleracea*

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Abstract Fusarium wilt caused by *Fusarium oxysporum* f. sp. *conglutinans* is one of the most important diseases of *Brassica* crops, resulting in severe reductions in yield and quality. To characterize the inheritance pattern of fusarium resistance, a cross between a susceptible broccoli and a resistant cabbage was subjected to segregation analysis. Results indicated that resistance was controlled by a single dominant allele. This gene was named *Foc-Bo1* and mapped to linkage group seven (O7) by both the segregation test and quantitative trait locus (QTL) analysis. The QTL on O7 was detected with a logarithm of odds score (LOD) of 19.5, which was above the threshold value with genome-wide 1% significance level (2.01). A minor QTL was also detected on O4 with a LOD score of 2.06. Inoculation tests indicated that stable expression of fusarium resistance at high temperatures required *Foc-Bo1* homozygosity. The association between *Foc-Bo1* and the closest simple sequence repeat marker (KBrS003O1N10) was analyzed in three F3 populations. Based on these studies, KBrS003O1N10 represents an effective marker-assisted selection (MAS) tool for breeding fusarium wilt resistance into *Brassica oleracea* crops. To our knowledge, this is the first paper to map the fusarium-resistance gene in *Brassica* species and to validate the effectiveness of MAS in improving fusarium resistance in these important plants.

Keywords *Brassica oleracea* · Fusarium wilt · MAS (marker-assisted selection) · Type A resistance · QTL (quantitative trait locus)

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Introduction

Cole crops derived from the *Brassica oleracea* L. species include cabbage, broccoli, cauliflower, and

Brussels sprouts. These important vegetables are cultivated throughout the world, but most suffer from a damaging disease called fusarium wilt (Ramirez-Villupadua et al. 1985; Morrison et al. 1994; Farnham et al. 2001). Fusarium wilt of *Brassica* crops is caused by the soil-borne pathogen *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) (Snyder and Hansen 1940). Fusarium colonization leads to leaf yellowing, wilting, defoliation (in older plants), stunted growth, and plant death. As a result, crops suffer severe losses in both yield and quality (Walker 1930). Fusarium wilt is a warm-weather disease that is typically not a problem in cool conditions (i.e., cool seasons or environments). However, increased levels of cole crop production in temperate climates and during warm seasons have made this disease a serious problem (Bosland et al. 1988; Farnham et al. 2001). Global warming may also contribute to the rise in fusarium wilt incidence (Berrocal-Lobo and Molina 2007). Crop rotation, improved sanitation, and fungicides are ineffective measures against this disease, as these ubiquitous pathogens cannot be eradicated from the soil. Thus, the development of resistant cultivars is the most cost-effective method for controlling fusarium wilt in *Brassica* crops.

Cabbage cultivars carrying resistance to fusarium wilt were initially developed in the United States at the beginning of the twentieth century (Walker et al. 1927; Anderson 1933). Progenies obtained from crosses between resistant and susceptible cultivars were subsequently analyzed to determine the genetic basis of fusarium wilt resistance (Walker 1930; Walker and Smith 1930; Blank 1937). Two types of cabbage resistance against fusarium wilt have been reported: Type A and Type B (Blank 1937). Type A resistance is controlled by a single dominant gene and is phenotypically stable at temperatures lower than 26–28°C. In contrast, Type B is polygenic and becomes unstable as temperatures rise above 24°C (Walker and Smith 1930; Blank 1937; Walker 1953). Type A represents the more advantageous form of resistance because it is inherited in a monogenic fashion, which facilitates transfer between varieties, and it is phenotypically stable at soil temperatures as high as 28°C (Walker and Smith 1930; Blank 1937; Walker 1953). For these reasons, Type A resistance has been used effectively in cabbage and is often cited as a successful example of long-lasting, monogenic, dominant resistance (Dixon 2007). Identification of the Type A resistance gene is important to improve fusarium wilt resistance. To date, however, no studies concerning the molecular or genetic basis of fusarium wilt resistance have been conducted in *B. oleracea*.

The objectives of this study, therefore, were (1) to analyze the inheritance pattern of fusarium resistance in an F2 population of plants, both by segregation tests and quantitative trait locus (QTL) analysis, and (2) to develop a marker-assisted selection (MAS) process for fusarium wilt resistance in *B. oleracea*.

Materials and methods

Plant materials

Microspore culture was used to obtain double-haploid lines of the fusarium wilt-resistant cabbage cultivar Anju (Nippon Norin Seed Co., Japan), and the fusarium wilt-susceptible broccoli cultivar Green Comet (GC) (Takii & Co., Ltd., Japan). The GC line was crossed to the Anju line to generate the F1 population (GC was used as the female). An F1 plant was randomly selected and self-pollinated to generate 94 F2 progenies of single-plant descent. These F2 plants were used by Nagaoka et al. (2010) to construct a genetic linkage map. F3 seeds were obtained from bud self-pollination of each F2 line.

Pathogen isolation and inocula preparation

The Cong: 1-1 strain of *F. oxysporum* f. sp. *conglutinans* obtained from cabbage was provided by Dr. Kadota (National Agricultural Research Center for Tohoku Region, Japan), and was used to prepare inocula. This strain was maintained on potato dextrose agar medium (Wako Pure Chemical Industries, Ltd., Japan). Liquid inocula were obtained by inoculating potato sucrose broth medium (200 g/l potato extract and 20 g/l sucrose in distilled water) with the isolate, and shaking at 130 rpm on a rotary shaker for 1 week. After shaking, 50 ml of liquid inocula was poured into a sterilized soil mixture that contained 80 g of soil mix (Yasaibaido No. 1, Hone Agri Co., Ltd., Japan), 20 g of rice bran powder, and 30 ml of a 4% sucrose solution. The inoculated soil medium was incubated at 25°C for 2 weeks and then used for inoculation tests in both the greenhouse and growth chambers. For growth chamber tests, 2 g of perlite (a soil conditioner that improves soil aeration) was added to the inoculated

soil medium. As the inoculated soil medium contained a high dose of conidia from *F. oxysporum*, a healthy soil mix was added to the inoculated soil medium at transplantation. Ratios of either 4:1 or 9:1 of healthy soil mix to inoculated soil medium were used.

Evaluation of fusarium resistance

Artificial inoculation tests were performed in either a greenhouse or a growth chamber. In the greenhouse, inoculation tests were performed from August 16 to September 5, 2008. The mean temperature in the greenhouse during that time was 26.4 ± 2.1°C (mean ± SD), with a mean daily high temperature of 33.2 ± 3.1°C and a mean daily low temperature of 22.2 ± 1.9°C (Electronic Supplementary Material Fig. S1). The disease severity index (DI) of F2 was evaluated from the average of 12 F3 plants (F2:3). Eighty-five F2:3 populations were screened for fusarium wilt resistance. These lines were selected from the 94 F2 populations that were previously used to develop a linkage map (Nagaoka et al. 2010). Twelve seeds from each F3 family (derived from the 85 F2 plants) were sown in a plastic tray. Twelve-day-old seedlings were transplanted into plastic pots (8 cm in diameter) that contained both the healthy soil mix and the inoculated soil medium (mixed 4:1). Soil was kept moist throughout the test. The DI of each inoculated plant was determined 14 days after transplantation. A DI scale of 0–2 was used (0: no symptoms, 1: plant yellowing, 2: plant death). DIs of the 12 F3 seedlings were averaged to calculate the DI for each F2 plant.

In the growth chambers, 12-day-old seedlings were transplanted into plastic pots (8 cm in diameter). The ratio of the healthy soil mix to the inoculated soil medium was adjusted to 9:1. The DI of each inoculated plant was determined 14 days after transplantation, using the same 0–2 scale that was used for the greenhouse experiments. The growth chamber tests were carried out using three F3 lines (see "Marker validation" in "Materials and methods") and two temperature regimens: (1) 25°C for 7 days, followed by 28°C for 7 days, or (2) 28°C constant temperature for 14 days.

Detection of DNA polymorphism

Each 5-μl polymerase chain reaction (PCR) mixture contained 0.125 U Takara Ex-Taq (Takara Bio Inc., Otsu, Japan), 0.5 μl 10 × Ex-Taq buffer, 2 nmol of each dNTP, 5 pmol of each primer, and ~10 ng genomic DNA. Amplification was performed using a PCR thermal cycler (Takara Bio). The reaction mixture was incubated in the thermal cycler at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s (denaturation), 50°C for 30 s (annealing), and 72°C for 1 min (elongation). PCR products were separated by electrophoresis using polyacrylamide gel (Kikuchi et al. 2003). Gels were stained with Gelstar solution (diluted 1:10,000) (Takara Bio), and visualized under UV light. Segregation of each marker was scored with reference to parental genotypes. To detect the homologues of *RESISTANCE TO FUSARIUM OXYSPORUM 1* (*RFO1*), which confers resistance to fusarium in *Arabidopsis thaliana* (Diener and Ausubel 2005), the PCR primers (forward: 5′-GTTACAGTAACTGTTACTGC-3′ designed from exon 1; reverse: 5′-GTTAATCTGCGAGAGAACAC-3′ designed from exon 3) were developed based on the *RFO1* gene sequence (At1g79670) and used to amplify the homologous fragments in the *B. oleracea* genome. Polymorphism detection between the Anju and GC genomes was carried out using the same procedure as above-mentioned.

Segregation test and QTL analysis in F2 plants

DIs of individual F2 plants were deduced from F3 progeny tests. Since a gap appeared in the DI distribution of the F2 population, plants with a DI below 1.4 or above 1.6 were classified as disease-resistant or disease-susceptible, respectively (Fig. 1). The ratio of resistant to susceptible plants in the F2 population was then calculated and analyzed by the χ^2 test.

A linkage map was constructed from the 94 F2 population previously described by Nagaoka et al. (2010). The linkage map generated by Nagaoka et al. (2010) was modified in this study by including additional markers in the analysis. Linkage analysis was performed using the Antmap 1.2 program (Iwata and Ninomiya 2006). To identify QTL that contribute to fusarium wilt resistance, an interval mapping method developed by Hayashi and Awata (2006) for the analysis of categorical traits was used, in which the genomic regions (genetic markers) significantly influencing the probabilities of an individual being classified into some specific categories are sought. The F2 population was categorized into resistant and

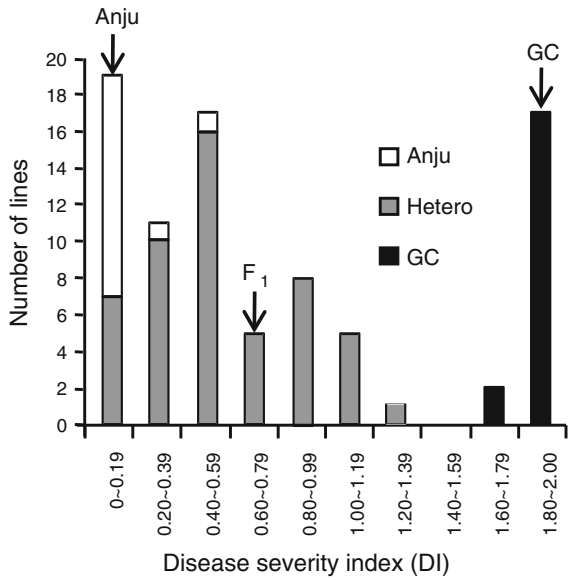

Fig. 1 Frequency distribution of the disease severity index (DI) for F2 progeny. *Arrows* indicate approximate values obtained for parental (Anju and GC) and F1 plants. The DI for each F2 plant was deduced by averaging the DIs of 12 F3 plants. F2 plants homozygous for the Anju KBrS003O10N1 locus, homozygous for the GC KBrS003O10N1 locus, or heterozygous at the KBrS003O10N1 locus are indicated by *open*, *black*, or *gray bars*, respectively

susceptible based on the DI distribution gap, which was same as the segregation test. A permutation test was carried out with 5,000 replications to obtain the significance threshold of the logarithm of odds (LOD) scores.

Marker validation

Three F2 plants (F2-37, F2-73, and F2-78), that were heterozygous for the KBrS003010N1 marker (forward: 5′-TCACTCCTCTCGCAGATTCA-3′; reverse: 5′-TGGAATCGCTTTAAGCAGATGC-3′) were selected, and F3 lines were generated by self-pollination. To detect DNA polymorphisms, healthy leaves were harvested before fusarium wilt symptoms appeared. Total genomic DNA of each plant was isolated from either fresh or freeze-dried leaves using the CTAB method (Murray and Thompson 1980). Thereafter, the same procedures which were used to analyze F2 plants were used to characterize the genotypes and DIs of F3 plants. Inoculation tests were conducted in growth chambers. The F2-37 line (F3 plants) were incubated at 25°C for 7 days following transplantation, and then the temperature was changed to 28°C. Inoculation tests for the F2-78 line were maintained at a constant 28°C. Two populations of F2-73 were incubated in each of the two temperature conditions.

Comparative analysis of QTL region

To identify syntenic regions between the *Foc-Bo1* region and *Brassica rapa*, markers designed with the bacterial artifical chromosome (BAC) sequences released from BrGP were aligned with the JWF3p map published on the BrGP web site (http://www.brassica-rapa.org/BRGP/index.jsp). The nucleotide sequences of each *B. rapa* BAC were aligned with *Arabidopsis* genome sequences by BLASTN in DDBJ (http://blast.ddbj.nig.ac.jp/). Based on a threshold value of less than e^{-10}, the regions having conserved collinearity with *A. thaliana* were regarded as homologous syntenic regions.

Results

Response to fusarium resistance

In greenhouse inoculation tests, individual plant DI scores were recorded for the parents (Anju and GC), and both F1 and F3 progenies. DI scores were determined 14 days after inoculation (Fig. 1, Electronic Supplementary Material Fig. S2). Anju parental plants are typically resistant to fusarium wilt, and in our experimental protocol these plants exhibited complete resistance (DI = 0). In contrast, plants of the GC susceptible parent succumbed to the disease (DI = 2.0). F1 plants were uniformly resistant to the disease, with a mean DI of 0.73 ± 0.46. For the F2 segregation test, the DIs of individual F2 plants were deduced from inoculation test results of F3 progeny (F2:3). Based on the gap in DI distribution, plants having a DI below 1.4 ($n = 66$) or over 1.6 ($n = 19$) were classified as disease-resistant or disease-susceptible, respectively (Fig. 1). The observed segregation ratio was tested using χ^2 analysis to determine whether the data fit the 3:1 segregation ratio that was expected for a single dominant gene (Table 1). The resulting χ^2 value for the observed segregation ratio was 0.14 ($0.7 < P < 0.8$), indicating that the F2 segregation test supported the single dominant gene model.

Table 1 χ^2 tests for goodness of fit, comparisons between observed phenotypic segregation, and the expected 3:1 ratio

Line	Number of plants		Total	χ^2	*P* value
	Resistant	Susceptible			
F2:3	66	19	85	0.32	$0.5 < P < 0.75$
F2-37 (25/28°C)	31	11	42	0.02	$0.8 < P < 0.9$
F2-73 (25/28°C)	24	8	32	0	$P = 1$
F2-73 (28°C)	34	25	59	9.49	$P < 0.01$
F2-78 (28°C)	56	29	85	3.77	$0.05 < P < 0.1$

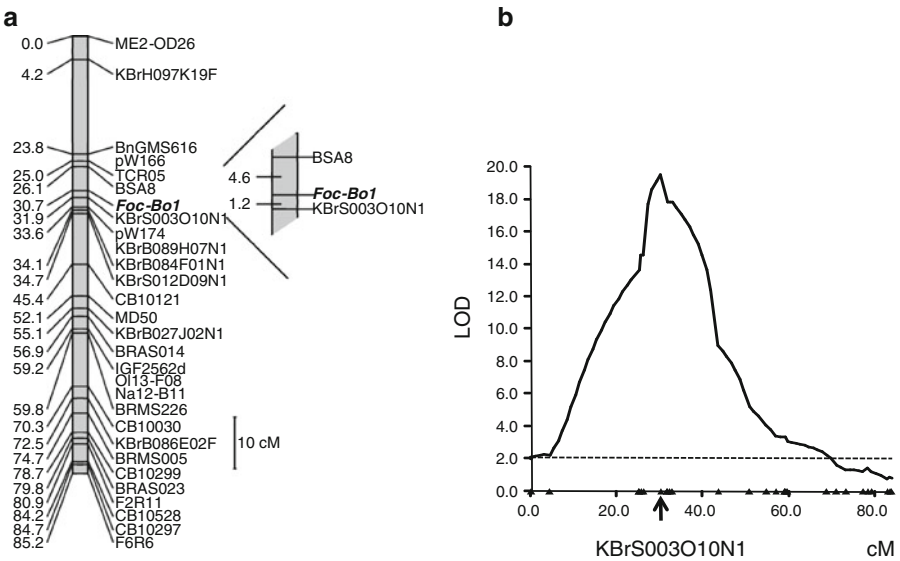

Fig. 2 Detection of the position of fusarium wilt resistance gene in *B. oleracea*. **a** Map of *B. oleracea* O7 resulting from analysis of F2 population. Marker positions are indicated in centimorgans (cM) on the *left side* of the linkage group, and locus designations are provided on the *right side*. The *Foc-Bo1* locus controls resistance to fusarium wilt. Distances between *Foc-Bo1* and the adjacent markers are indicated in the enlarged genomic region containing *Foc-Bo1*. **b** LOD plots for *QTL2* of fusarium wilt resistance by QTL analysis. LOD score and map distance are given on the *x* and *y* axes, respectively. *Triangles* on the *x* axes indicate the marker positions. Threshold of 2.01 is shown by *broken line*

Linkage analysis

The segregation data indicated that a single dominant gene controlled the disease-resistance trait. Based on this assumption, we analyzed DI data from 85 F2:3 plants and identified a single genetic locus for fusarium wilt resistance. This locus, which was named *Foc-Bo1*, was mapped to the interval between the markers BSA8 and KBrS003O10N1 on linkage group seven (O7) (Fig. 2a). The linkage map constructed by Nagaoka et al. (2010) was modified in this study by adding new markers; a total of 261 markers were distributed in nine linkage groups covering 943.7 cM, and the average interval between markers was 3.7 cM. The modified linkage map of O7 contained 28 DNA markers that included 20 simple sequence repeat (SSR), three sequence-related amplified polymorphism, two cleaved amplified polymorphic sequence, one sequence characterized amplified region and two single nucleotide polymorphism/insertion–deletion markers (Electronic Supplementary Material Table S1).

The phenotypic DI at the KBrS003O10N1 marker closely linked to the *Foc-Bo1* locus indicated that higher disease resistance was associated with the homozygous Anju genotype versus the homozygous GC genotype. Plants that were heterozygous at this locus had varying levels of resistance (Fig. 1).

Table 2 Position and effect of QTL for fusarium resistance in *B. oleracea*

QTL	Proximal marker	Linkage group	Position (cM)	LOD score[a]	Probability of fusarium resistance[b]		
					QQ	Qq	qq
QTL1	CB10159	O4	42.2	2.06 (2.01)	0.56	0.87	0.83
QTL2 (*Foc-Bo1*)	KBrS003O10N1	O7	30.1	19.5 (2.01)	1.00	0.98	0.00

[a] The figure in parenthesis indicates a threshold value of genome-wide 1% significance level for LOD score, which is obtained by a permutation test with 5,000 replications

[b] Predicted probability of plants being resistant to fusarium given QTL genotypes, QQ, Qq, and qq, where Q and q indicate QTL alleles derived from Anju and GC, respectively

QTL analysis

QTL analysis was performed using genotypic data from 94 F2 plants and DI data from 85 F2:3 plants. Because the DI distribution was a discontinuous rank with a significant gap and finite ends rather than a normal distribution, the interval mapping method for categorical traits was applied in QTL analysis. As described in "Materials and Methods", F2 plants were divided into two categories (resistance and susceptible) based on the gap in DI distribution. We adopted an empirical value of 2.01 that corresponded to the genome-wide 1% significance level obtained with permutation tests as a threshold value for LOD scores. Two QTL for fusarium wilt resistance were identified by our analysis. *QTL1* and *QTL2* were mapped to O4 and O7, respectively. The major QTL (LOD 19.5) affecting fusarium wilt resistance (*QTL2*) was located on O7 with the proximal marker of KBrS003O10N1 (Fig. 2b). This locus was also identified by linkage analysis, which mapped fusarium wilt resistance to the *Foc-Bo1* locus (30.7 cM on O7). Thus, *QTL2* could be identical to *Foc-Bo1*. As shown in Table 2, the allele from Anju at *Foc-Bo1* locus had a significant effect increasing the probability of fusarium wilt resistance. The probability of F2 individuals with the allele from Anju being classified as resistant was close to 1 (1.00 of QQ and 0.98 of Qq), while the probability of resistance was 0 (qq) for the plants without the Anju allele. Other than O7, a part of O4 showed a LOD score (2.06) slightly higher than threshold (2.01), and this locus was named *QTL1*. The probability of being resistant was higher for the plants with the GC allele, indicating that the resistance gene came from GC (Table 2). No other genomic region showed a LOD score higher than the threshold.

Associations between molecular markers and fusarium wilt resistance

KBrS003O10N1 was the closest marker to the gene *Foc-Bo1*, with only 1.2 cM between them (Fig. 2a). To validate the association between KBrS003O10N1 and *Foc-Bo1*, three F3 lines were selected for further analysis and tested using two temperature regimens (Fig. 3).

In F3 progeny tests at 25/28°C, when plants of each line (F2-37, F2-73) were classified either as disease resistant (DI $\leq$ 1) or susceptible (DI = 2), the segregation ratio of the populations fit the expected 3:1 ratio by χ^2 analysis (Table 1). This indicated that a single dominant gene controlled fusarium wilt resistance, which confirmed our F2 segregation analysis. For the 28°C test, although the F2-78 line fit the 3:1 resistant:susceptible segregation ratio, the segregation of the F2-73 line deviated from 3:1, due to the increase of infection of plants heterozygous for the KBrS003O10N1 locus. The mean DI of plants heterozygous for KBrS003O10N1 locus was significantly different between the 25/28°C test and the 28°C test on the same line (F2-73) by Duncan's new multiple range test ($P < 0.01$) (data not shown), indicating that the heterozygous genotypes have a lower level of resistance than the homozygous genotypes, especially at 28°C.

In F3 progeny tests, the genotype of each plant was also determined via PCR analysis (Electronic Supplementary Material Fig. S3). Most plants homozygous for the Anju KBrS003O10N1 locus were disease-resistant (DI = 0) (Fig. 3). Genotypes heterozygous for the KBrS003O10N1 locus exhibited variable levels of disease resistance, at 28°C in particular. Plants homozygous for the GC KBrS003O10N1 locus were mostly susceptible (DI = 2); however a small

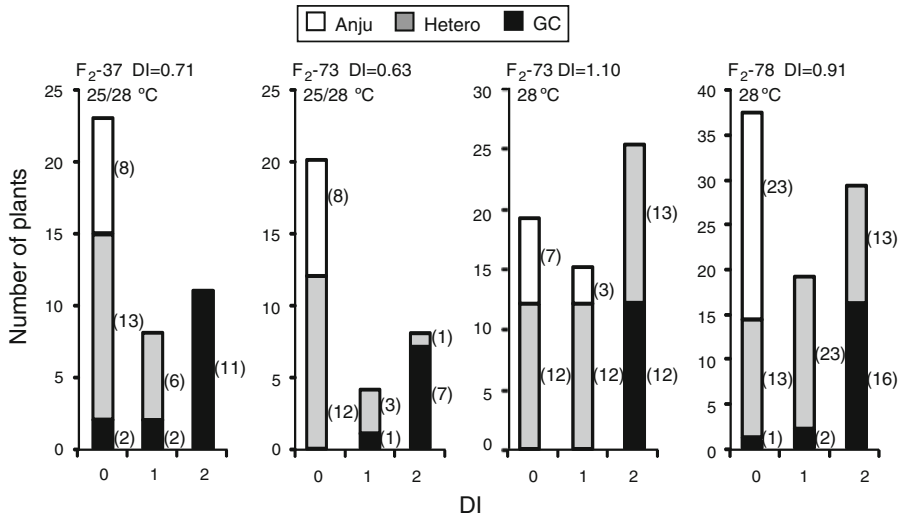

Fig. 3 Frequency distribution of disease severity index (DI) for three F3 populations. The name of each population is shown above the graph, followed by the mean DI in parentheses. Experimental temperatures are indicated. F3 plants homozygous for the Anju KBrS003O10N1 locus, homozygous for the GC KBrS003O10N1 locus, or heterozygous at the KBrS003O10N1 locus are indicated by *open*, *black*, or *gray bars*, respectively

number of plants were either moderately resistant (DI = 1, $n = 5$), or completely resistant (DI = 0, $n = 3$). Genotype analysis further indicated that resistant plants which were homozygous for the GC genotype at KBrS003O10N1 were heterozygous at BSA8 [the locus on the other side of *Foc-Bo1* (Fig. 2a)]. This indicated that a crossover event had occurred between the KBrS003O10N1 and BSA8 loci. Similar results were seen with the moderately resistant plants (three of five exhibited a crossover event). Therefore, recombinant plants that were homozygous for the GC genotype at the KBrS003O10N1 locus could be heterozygous at the *Foc-Bo1* locus. This may explain why some plants that were homozygous for the GC KBrS003O10N1 locus were not susceptible to fusarium wilt, though stochastic escape from infection of susceptible plants (i.e., environmental effects or experimental errors) might have contributed to these conflicting results.

Discussion

Inheritance of fusarium wilt resistance in *B. oleracea*

In a cross between fusarium wilt-resistant and fusarium wilt-susceptible plants, segregation analyses indicated that a single dominant gene controlled resistance against the *F. oxysporum* f. sp. *conglutinans* Cong: 1-1 strain in *B. oleracea*. In addition, disease resistance was seen at high environmental temperatures (28°C). These results are consistent with Type A resistance, which has been described in cabbage (Walker 1930; Walker and Blank 1934). The gene responsible for this resistance, *Foc-Bo1*, was closely linked to the genetic marker KBrS003O10N1 by both linkage and QTL analyses. *Foc-Bo1* was the major QTL that emerged from this analysis, of which the LOD score was 19.5. Combined with results from the marker validation test, these findings indicate that *Foc-Bo1* plays a major role in fusarium wilt resistance. To our knowledge, this is the first report that has successfully mapped a fusarium-resistance gene and verified the linkage relationship between the resistance gene and the closest genetic marker in *Brassica* species.

Blank (1937) reported that Wisconsin All Seasons cabbage cultivar carried the two types of resistance, Type A and Type B. With the expectation that fusarium resistance is a multiple trait in *B. oleracea*, QTL analysis was applied to an F2 population. Other than *Foc-Bo1*, a minor QTL, *QTL1*, was detected on O4. Although the LOD value of 2.06 on O4 was slightly higher than the 2.01 threshold, further analysis is required to determine whether *QTL1* represents a legitimate locus involved in fusarium wilt resistance.

Temperature effects on fusarium wilt resistance

Temperature plays an important role in fusarium wilt resistance. Type B resistance is polygenic and becomes phenotypically unstable at temperatures above 24°C. In contrast, Type A resistance is stable

at 26–28°C (Walker and Smith 1930; Blank 1937; Walker 1953). Whether Type A resistance becomes unstable above 28°C is unclear. Walker and Smith (1930) reported that for Type A resistance, a small number of diseased plants appear at 26°C, and disease incidence increases as the temperature rises to 28°C. At 26–28°C, the fusarium wilt symptoms in Type A-resistant cabbage are not typical of yellows. Instead, the plants become stunted, and exhibit a pale yellowing of the leaf parenchyma. In contrast, Farnham et al. (2001) observed no significant differences between Type A resistant cabbage (Bravo) under 25/25°C (day/night) and 30/25°C conditions. In our current study, plants homozygous for the resistant Anju genotype were stably resistant in both 25/28°C and constant 28°C growth chamber conditions, although a few Anju genotypes were infected at 28°C in the F3-73 line. Discrepancies between research groups may have resulted from different growth chamber conditions. Walker and Smith (1930) performed experiments in which the soil temperature in the incubation tank (26–28°C) was higher than the temperature of the air (15–17°C). In our study and Farnham et al. (2001), the soil and air temperatures were equivalent.

In this study, we identified plants that were heterozygous at the *Foc-Bo1* locus. Plants having only one copy of the disease-resistance allele were partially resistant to fusarium wilt and were more disease-susceptible at high temperatures. *Foc-Bo1*, therefore, acts in a dose-dependent manner to control fusarium wilt. This result is consistent with Walker and Smith (1930), who crossed resistant and susceptible parents to generate heterozygous progeny. These heterozygotes were disease-resistant at 24°C, but became stunted and yellow at 26°C. In contrast, homozygous resistant plants were quite healthy at 26°C. Stable resistance to clubroot disease also requires that clubroot-resistance genes are homozygous (Suwabe et al. 2003; Nomura et al. 2005; Nagaoka et al. 2010). Factors that regulate the cumulative effects of disease-resistance genes have not been identified.

Marker validation for MAS

In this study, we used the SSR marker KBrS003O10N1 to track the disease-resistance allele of *Foc-Bo1*. This method is both technically simple and inexpensive (Collard et al. 2005). During the F3 progeny test (Fig. 3), KBrS003O10N1 proved extremely effective in identifying the fusarium wilt-resistance genotype in *B. oleracea*. This method was not always accurate, however, as recombination events were detected between KBrS003O10N1 and BSA8, the markers that flank *Foc-Bo1*. As recombination between the KBrS003O10N1 and *Foc-Bo1* loci is clearly possible, genotyping using the two markers (KBrS003O10N1 and BSA8) would more accurately identify plants with the disease-resistance allele of *Foc-Bo1*. BSA8 is 4.6 cM away from *Foc-Bo1*, however, which allows for a high rate of recombination between these two loci. Therefore, to increase the accuracy of MAS, a novel marker that is more closely linked to *Foc-Bo1* must be generated to replace BSA8. Recently, *Brassica* genomics resources have been disclosed (http://www.brassica.info/; http://www.brassica-rapa.org/BRGP/geneticMap.jsp), and will facilitate fine mapping of the *FocBo1* region.

The artificial inoculation test could not distinguish between plants that were heterozygous or homozygous at the *Foc-Bo1* locus. As such, progeny tests were required to accurately assess the *Foc-Bo1* genotype, which were quite time-consuming. In contrast, using markers linked to the *Foc-Bo1* locus to identify heterozygous plants was technically practical and inexpensive. Moreover, KBrS003O10N1 analysis identified plants that carried the resistance allele in the F2 population. This is important because artificial inoculation tests on single plants within the F2 population were not reliable.

Race/species-specific resistance gene

Although *F. oxysporum* f. sp. *conglutinans* is the primary pathogen that causes fusarium wilt in *B. oleracea*, *F. oxysporum* f. sp. *raphani* can also infect *B. oleracea* (Garibaldi et al. 2006). In addition, Type A resistance in cabbage is race-specific; these plants are resistant to f. sp. *conglutinans* race 1, but can be infected by race 2 (Ramirez-Villupadua et al. 1985; Morrison et al. 1994). In *Arabidopsis thaliana*, *RFO1* (on chromosome 1) is non-race-specific, conferring resistance to f. sp. *matthioli*, f. sp. *conglutinans*, and f. sp. *raphani*, which are causal agents of fusarium wilt in Brassicaceae (Diener and Ausubel 2005). The syntenic regions in *A. thaliana* and *B. rapa*, corresponding to the *Foc-Bo1* region, are illustrated in Electronic Supplementary Material Fig. S4, based on the result of Nagaoka et al. (2010). In this study, we

revised the marker order to perform precise syntenic analysis. As a result, we confirmed that the central region of *B. oleracea* O7 was aligned with the corresponding region of chromosomes 3 and 4 of *A. thaliana* but not with chromosome 1 of *A. thaliana*. Besides, only one polymorphic band of a *RFO1* homologue was detected in GC and Anju. *BoRFO1* was then mapped to O2 in our map (data not shown). We therefore conclude that *RFO1* (on At chr1) is not a candidate gene for *Foc-Bo1*. Thus, it will be interesting to learn the reaction of *Foc-Bo1* against the related formae speciales and races, as well as the mechanism by which *Foc-Bo1* overcomes fusarium wilt.

This study showed that the central region of O7 in *B. oleracea* contains *Foc-Bo1*. Nagaoka et al. (2010) reported that this region contains the clubroot-resistance gene, *Pb-BoAnju4* (Electronic Supplementary Material Fig. S4). In addition, Nagaoka et al. (2010) suggested that the central region of O7 in *B. oleracea* might also correspond to the region harboring the clubroot-resistance gene (*CRb*) in R3 of *B. rapa* and chromosome 4 of *A. thaliana* that contains a disease-resistance gene cluster (Mayer et al. 1999; Young 2000). In this way, the central region of O7 of *B. oleracea* contains multiple disease-resistance genes, *Pb-BoAnju4* and *Foc-Bo1* at least, indicating the importance of this genomic region to the *B. oleracea* breeding program. Molecular cloning of *Foc-Bo1* and extensive analysis of the surrounding genomic region will be necessary to understand the molecular and evolutionary bases of disease resistance in *Brassica*.

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