Theor Appl Genet (2015) 128:119–130
DOI 10.1007/s00122-014-2416-6

ORIGINAL PAPER

Map-based cloning of a candidate gene conferring Fusarium yellows resistance in *Brassica oleracea*

Motoki Shimizu · Zi-jing Pu · Takahiro Kawanabe · Hiroyasu Kitashiba · Satoru Matsumoto · Yusuke Ebe · Monari Sano · Taketo Funaki · Eigo Fukai · Ryo Fujimoto · Keiichi Okazaki

Received: 20 August 2014 / Accepted: 11 October 2014 / Published online: 29 October 2014

Abstract
Key message **We identified the candidate gene conferring yellow wilt resistance (YR) in *B. oleracea*. This work will facilitate YR breeding programs for *B. oleracea* and its closely related species.**
Abstract Yellow wilt disease is one of the most serious diseases of cabbage worldwide. Type A resistance to the disease is controlled by a single dominant gene that is used in cabbage breeding. Our previous QTL study identified the *FocBo1* locus controlling type A resistance. In this study, the *FocBo1* locus was fine-mapped by using 139 recombinant F_2 plants derived from resistant cabbage (AnjuP01) and susceptible broccoli (GCP04) DH lines. As a result, we successfully delimited the location of *FocBo1* within 1.00 cM between markers, BoInd 2 and BoInd 11. Analysis of BAC and cosmid sequences corresponding to the *FocBo1* locus identified an orthologous gene of Bra012688 that was recently identified as an candidate gene that confers yellows resistance in Chinese cabbage. The candidate gene-specific DNA markers and phenotypes in F_1 cabbage cultivars and their selfed F_2 populations showed a perfect correlation. Our identification of the candidate gene for *FocBo1* will assist introduction of fusarium resistance into *B. oleracea* cultivars and contribute further understanding of interaction between Brassica plants and fusarium.

Communicated by Carlos F. Quiros.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2416-6) contains supplementary material, which is available to authorized users.

M. Shimizu · Z. Pu · Y. Ebe · M. Sano · T. Funaki · E. Fukai · K. Okazaki (✉)
Laboratory of Plant Breeding, Graduate School of Science and Technology, Faculty of Agriculture, Niigata University, 2-8050, Ikarashi, Nishi-ku, Niigata 950-2181, Japan
e-mail: okazaki@agr.niigata-u.ac.jp

Present Address:
M. Shimizu
Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami, Iwate 024-0003, Japan

T. Kawanabe · R. Fujimoto
Graduate School of Agricultural Science, Kobe University, Rokkodai, Nada-ku, Kobe 657-8501, Japan

H. Kitashiba
Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, Japan

S. Matsumoto
National Institute of Vegetable and Tea Science, Ano, Tsu, Mie 514-2392, Japan

Introduction

Yellow wilt disease, caused by the fungus *Fusarium oxysporum*, is one of the most serious diseases of Brassica crops worldwide (Beckman1987). The pathotypes of *Fusarium oxysporum* infecting Brassica crops are classified into four formae speciales based on specificity to the host: cabbage (*conglutinans*), radish (*raphani*), stock (*matthioli*), turnip, narinosa and other forms of *B. rapa* (*rapae*) (Puhalla 1985; Bosland and Williams 1987; Enya et al. 2008). The typical symptoms are vascular browning, leaf chlorosis, stunting, wilting, defoliation and plant death (Brown and Proctor 2013). The pathogen is soilborne and survives in soil as resting spores for long periods. Agricultural practices such as crop rotation, as well as use of agrochemicals, are insufficient to control fusarium wilt. Alternatively, use of resistance cultivars is effective for control of fusarium wilt (Dixon 2007). Since fusarium yellows is a warm temperature

cabbage disease, the increase of cabbage cropping during recent hot summers has made this disease a serious problem worldwide. Hence, there is an urgent need for development of cultivars that have durable resistance to this disease.

A cabbage breeding program for yellows resistance (YR) was conducted in the United States at the beginning of the 20th century (Walker et al. 1927; Anderson 1933). Initially, Type B resistance, which is under polygenic control, was incorporated into cultivars, but it is phenotypically unstable when temperatures rise above 24 °C (Walker and Smith 1930; Blank 1937; Walker 1953). Thereafter, Type A resistance, which is controlled by a single dominant gene and is stably expressed at temperatures lower than 26–28 °C, was introduced into cabbage cultivars (Walker 1930; Walker and Smith 1930; Blank 1937). At present, YR cabbage cultivars are commercially available, but all cultivars are not likely to contain Type A resistance. In *Brassica oleracea*, resistance to *F. oxysporum* f. sp. *conglutinans* (*Foc*) has been studied (Walker et al. 1927; Anderson 1933; Farnham et al. 2001; Pu et al. 2012; Lv et al. 2013). Pu et al. (2012) identified two quantitative trait loci (QTLs) for YR from the analysis using a type A resistant cabbage DH line. One of them, a major YR-QTL identified in the linkage group C7, behaved like a single dominant gene, and was named *FocBo1*.

Dominant fusarium wilt resistance genes have been identified in several crops such as tomatoes (Takken and Rep 2010). In tomatoes, three different physiological races within this forma specialis, named in order of discovery (race 1, race 2, and race 3), were distinguished by their differential pathogenicity (Rep et al. 2005; Houterman et al. 2008, 2009). Their corresponding R genes in tomato are *I-1, I-2*, and *I-3*, which recognize respective races in a gene for gene manner (Huang and Lindhout 1997; Simons et al. 1998). In *B. oleracea*, the type A resistance gene (*FocBo1*) has been durable for more than 80 years since its introduction (Walker et al. 1927), despite the fact that the presence of race 2 of cabbage yellows caused by *Foc* has been reported (Ramirez-Villupadua et al. 1985; Morrison et al. 1994). In *Arabidopsis thaliana*, which is closely related to Brassica crops, six YR loci have been identified by QTL analyses, and among them the three YR genes, *Resistance to Fusarium Oxysporum* (*RFO*)*1, 2* and *3*, which have been recently cloned; however, they only showed quantitative resistance to fusarium (Diener and Ausubel 2005; Diener 2013; Shen and Diener 2013; Cole and Diener 2013). In cruciferous plants, whether the YR resistance acts in the gene-for-gene manner as well as how many genes contribute to the YR response is not yet clear.

To develop durable YR cultivars and gain better understanding of host–pathogen interaction between *B. oleracea* and *Foc*, cloning of the Type A resistance gene is essential. To do this, we attempted the fine mapping of *FocBo1* by making several new PCR-based markers. The *FocBo1* region was delimited to a 360 kb region where a NBS-LRR type gene, which is a candidate of *FocBo1*, was found. The association analysis using the DNA markers detecting polymorphisms between resistant and susceptible alleles of the locus in F_1 cultivars and F_2 populations suggested that the locus contains the *FocBo1* gene.

Materials and methods

Plant materials

Two double-haploid (DH) lines, AnjuP01 and GCP04, were obtained from the fusarium wilt resistance cabbage cultivar 'Anju' (*B. oleracea* var. *capitata*) and the susceptible broccoli cultivar 'Green Comet' (GC) (*B. oleracea* var. *italica*) by microspore culture, respectively. F_1 plants were made by crossing AnjuP01 and GCP04, and a single F_1 plant was self-pollinated to produce F_2 seeds. To identify recombinants in the *FocBo1* region, 139 plants which had recombination between DNA markers KBrS012D09N1 (Nagaoka et al. 2010) and KBrH059N21F (Kato et al. 2012) were selected from 1,008 F_2 plants for fine mapping of the *FocBo1* locus. The 139 selected F_2 plants were self-pollinated to produce F_3 lines. F_3 plants were used for scoring the disease severity index (DI), and the means of F_3 lines were used as representative phenotypic data for each F_2 recombinant line. To validate association between allelic polymorphisms at the *FocBo1* locus and phenotypes, we tested fifty commercial F_1 cabbage cultivars as well as selfed F_2 progeny derived from four F_1 cultivars predicted to be heterozygous at the *FocBo1* locus. *B. oleracea* var. *alboglabra* A12 was also inoculated with *Foc* Cong: 1-1 strain. *B. rapa* spp. *pekinensis* inbred line T23 (Chinese cabbage) was used for sequencing Bra012688 that was recently identified as an candidate gene that confers yellows resistance in Chinese cabbage (Shimizu et al. 2014).

Pathogen and inoculation test

The Cong: 1-1 strain of *F. oxysporum* f. sp. *conglutinans* isolated from cabbage was provided by Dr. Kadota (National Agriculture Research Center for Tohoku Region, Japan), and was used to prepare inocula. Liquid inocula were prepared according to the method published by Pu et al. (2012); the isolate was cultured in potato sucrose broth medium containing 200 g/l potato extract and 20 g/l sucrose in distilled water at 130 rpm on a rotary shaker for 1 week, and thereafter incubated in healthy soil mixture (100 g) with liquid inocula (50 ml) at 25 °C for 2 weeks. The test for fusarium resistance was carried out in a growth room at 25 °C with the photoperiod of 16 h at light intensity

of 200 μmol m^{-2} s^{-1}. For the inoculation test, a ratio of 9:1 of healthy soil mix to inoculated soil medium was used. F_3 lines having recombination between the two *FocBo1*-linked DNA markers, KBrS012D09N1 and KBrH059N21F, were tested once or twice to evaluate susceptibility to Cong: 1-1; where 12–15 F_3 plants per F_2 line were used. Twelve-day-old seedlings were transplanted into plastic pots (8 cm in diameter) that contained the inoculated soil medium. Soil was kept moist throughout the test. A disease index (DI) of each inoculated plant was determined 28 days after transplantation. A DI scale of 0–2 was used (0: no symptoms, 1: plant yellowing, 2: plant death). The means of DIs in F_3 plants were used as represented values for F_2 plants. For commercial F_1 cultivars, inoculation testing was repeated twice to evaluate susceptibility to Cong: 1-1, using 12–15 plants per cultivar.

Detection of DNA polymorphism and construction of map

The *FocBo1* locus was delimited between BSA8 and KBrS003O10N1 in C7 of *B. oleracea* chromosome, which has colinearity to *A. thaliana* chromosome 4 (At4) (Pu et al. 2012). For choosing plants that had recombination between the markers close to *FocBo1*, recombinant plants were selected from 1,008 F_2 plants using KBrH059N21F (the same location of BSA8) and KBrS012D09N1 (upper location of KBrS003O10N1). Since *B. oleracea* genome information was limited at the beginning of this study, we were not able to design DNA markers at closer positions to *FocBo1*. Therefore, we used a comparative mapping-based approach for the fine mapping of *FocBo1*, based on the genome information of *A. thaliana* and *B. rapa*, species closely related to *B. oleracea*. To design the primers, the coding sequences (CDSs) were collected in the syntenic region of At4 (the Arabidopsis Information Resources, http://www.arabidopsis.org). Next, those CDSs were aligned with the *B. rapa* BAC sequences provided by DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp), using 'GENETYX Ver.10′ software (Genetyx, Tokyo, Japan). The sequences with >70 % similarity were represented as conserved regions among the two species. Using those sequences and Primer 3 software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi), primers named BoInd were designed to amplify ~1 kb fragments containing introns (Table S1). Of the newly designed markers, BoInd5 was most closely linked to the *FocBo1* locus, but amplified multiple bands. We therefore converted this marker to SCAR markers. The published CB and BoGMS SSR markers were also used to add more markers (Piquemal et al. 2005; Li et al. 2011). Total genomic DNA was isolated according to the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). Amplification and detection of DNA markers were conducted according to the report of Pu et al. (2012). Linkage analysis of markers was performed using AntMap ver 1.2 software (Iwata and Ninomiya 2006).

Interspecies synteny-based DNA marker establishment

To identify homologous regions between the *A. thaliana* genome and *FocBo1* locus in *B. oleracea*, PCR products amplified by primers closely linked to the *FocBo1* locus were sequenced. Next, BLASTN search using the sequences as queries and *A. thaliana* genome as a database was conducted using the web service of DNA Data Bank of Japan (DDBJ). Based on a threshold value of E^{-40}, we identified the syntenic regions. Regions in which at least two markers show homologies and colinearities were regarded as syntenic to each other.

BAC screening and sequence analysis

The JBo library, which consists of 33,792 clones made from *B. oleracea* var. *alboglabra* A12 (O'Neill and Bancroft 2000), were screened by PCR using the *FocBo1*-linked markers, BoInd4 and BoInd5 markers. The sizes of isolated BAC clones are estimated by pulse field gel electrophoresis (CHEF-DRII, Bio Rad, Laboratories, CA, USA). One of the isolated BAC clones (#5811) was sequenced by GS 454 Junior (Roche), and the sequence data were assembled by the GS Junior software ver.2.7. To close gaps between contigs, primers were also designed using the information on the terminal regions of the contigs as well as the *B. oleracea* GSS database (Brassica Genome Gateway, http://brassica.nbi.ac.uk). Thereafter, genes located on the assembled BAC sequences were predicted using Augustus, http://augustus.gobics.de (Stanke and Morgenstern 2005). The BAC sequence determined in this study was aligned to Chiifu-401 (BRAD, http://brassicadb.org) and Cabbage line 02–12 (Bolbase, http://ocri-genomics.org/bolbase).

Cosmid library construction and sequencing

A cosmid library for AnjuP01 was constructed. At first, high molecular weight (HMW) DNA was prepared according to the method used by Peterson et al. (2000), and was ligated into linearized and dephosphorylate PWE 15 vector (Stratagene). Partially *Bam*HI-digested HMW DNA was ligated to PWE15 vector. The ligation mixture was then packaged using an in vitro Packaging kit, LAMBDA INN (Nippon Gene Co., Ltd., Japan). The packaged cosmid clones were transformed into *E. coli* EPI300 (Epicentre, Madison, WI). Then the transfected cells were spread on LB agar containing 200 μg/ml ampicillin, and cultured overnight. Ten transformed clones were placed into

each cell of a 96-well plate. The plate was then cultured at 37 °C in an incubator overnight. Thereafter, 150 µl 25 % glycerol was added to each well, mixed by pipette, and stored at −80 °C. For library screening, the flanking markers closest to *FocBo1* were used as probes. The primer-walking method was used to determine candidate gene sequences detected in the AnjuP01-*FocBo1* region. The genomic (AB981181) and cDNA (AB981182) sequences of *FocBo1*(AnjuP01) were submitted to DDBJ.

cDNA analysis of *FocBo1*

Double-stranded cDNA was synthesized from leaf RNA of non-infected (2 weeks old) plants of YR Chinese cabbage inbred line T23, AnjuP01, GCP04, and *alboglabra* A12, using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan). 5′- and 3′-UTR sequences of Bra012688 and Bra012689 of Chinese cabbage inbred line T23 were amplified using 5′-Full RACE Core Set (Takara bio, Shiga, Japan) and 3′-Full RACE Core Set (Takara bio, Shiga, Japan), respectively, and sequenced. The internal sequence was determined using the primers listed in Table S2. Partial 5′-UTR, CDS and full 3′-UTR sequences of the *FocBo1* (AnjuP01) gene were amplified by PCR primer sets (Table S2) and sequenced. mRNA sequences of *focbo1* (GCP04), *focbo1* (A12), and Bra012689 ortholog of *B. olerecea* were partially determined using the internal primers. Double-stranded cDNAs, synthesized from leaf RNA of non-infected AnjuP01, GCP04, and A12, were used for determination of expression levels of candidate genes.

Association between allelic polymorphisms and phenotypes

Correlations between *FocBo1* genotypes and phenotypes were analyzed in fifty commercial F_1 hybrid cultivars (Table 1) and four F_2 populations (Table 2) derived from F_1 hybrid cultivars. Two DNA markers, MTK-C and MTK-1, were designed from the sequences of exon 9–3′-UTR of *FocBo1* and #11 gene (Bol037163) of A12, respectively (Table S2).

Results

Developing and mapping of DNA markers based on genomic information of *A. thaliana*

We tried to take advantage of the availability of genome information for the related species, *A. thaliana* and *B. rapa*; the *FocBo1* region of the *B. oleracea* genome is syntenic to the central region of At4 and the upper region of *B. rapa* A3 (Pu et al. 2012). More markers around the *FocBo1* locus were designed using highly conserved sequences between CDSs in the At4 and their orthologous *B.rapa* genes. The public CB and BoGMS SSR markers were also used to increase the number of markers. Of the 42 primer pairs tested in this study, 16 pairs (38.1 %) amplified clear polymorphic fragments between the two parental alleles, AnjuP01 and GCP04, and were designated as BoInd markers (Table S1). They were co-dominant markers between the two parental alleles. These BoInd primer sets were tested for their genetic linkage to *FocBo1* using the 94 F_2 plants previously studied (Pu et al. 2012). As a result, nine markers (BoInd1–5, 11, 14–16) were mapped to C7 (Fig. 1). Simultaneously, some BoInd markers were mapped to C1 (BoInd8, 9, 10, 12, and 15) and C8 (BoInd5, 6, 7, 13, and 15) (Fig. S1). BoInd5 and BoInd15 produced multiple bands and, consequently, BoInd5 were mapped to C7 and C8, and BoInd15 to C1, C7, and C8. Finding multiple locations for BoInd markers was consistent with the result that the regions of C1, C7, and C8 are homologous to the central region of At4 (Fig. S1).

Fine mapping of *FocBo1*

From 1,008 F_2 plants derived from the cross of AnjuP01 and GCP04, we selected 139 F_2 plants having recombination between DNA makers, KBrS012D09N1 and KBrH059N21F, which are located ca. 2.2 cM upstream and 3.4 cM downstream from *FocBo1* (Fig. 1). Those F_2 plants were then genotyped using the nine BoInd markers (this study), CB10634 (Piquemal et al. 2005), BoGMS1259 (Li et al. 2011), and three KBr markers (Nagaoka et al. 2010; Kato et al. 2012), and, consequently, a partial map of 5.6 cM was constructed (Fig. 2). Again, this region showed colinearity to the central region of At4 (Fig. 2). The region corresponds to the upper part of the U block and T block of ancestral crucifer blocks, and is syntenic to the bottom arm of At3, which corresponds to the N block.

F_3 seeds were then obtained by self-pollination of the selected F_2 plants. The inoculation test was carried out using thirty selected recombinants, which had the best spread of recombinations across the *FocBo1* region and produced enough seeds for test. Of thirty selected recombinants, the data obtained from the seventeen lines are depicted in Fig. 3. The recombination points of the remaining thirteen lines were almost identical to which of the seventeen lines and their data are omitted in Fig. 3 for simplicity. DI scores for the lines were clearly segregated into three categories based on ID scores: (1) resistant (DI < 0.07), (2) intermediate (0.38 < DI < 0.80), and (3) susceptible (DI > 1.93). Since *FocBo1* is a single dominant gene (Pu et al. 2012), these classes corresponded to

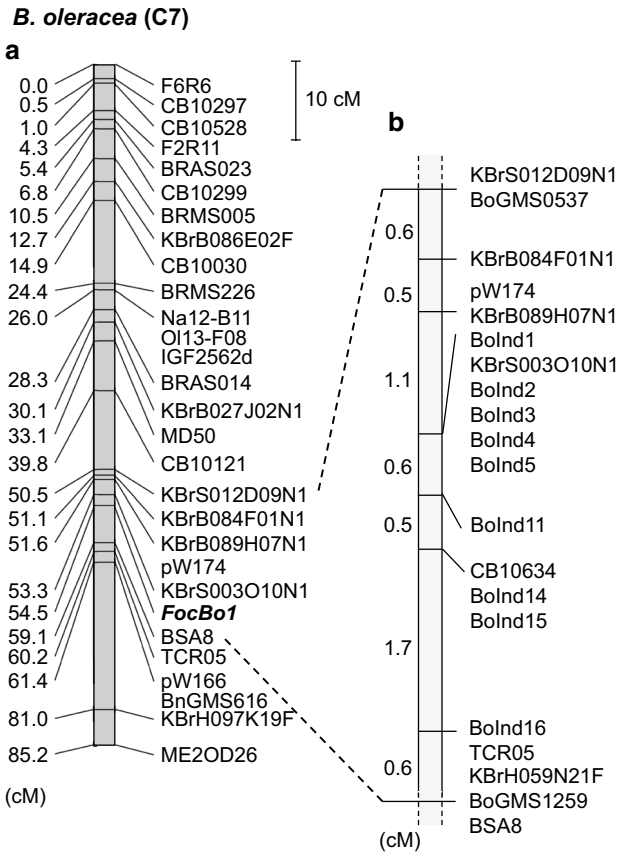


Fig. 1 Location of *FocBo1* on the map of *B. oleracea*. **a** Map of chromosome 7 (C7) containing the *FocBo1* locus in our previous study (Pu et al. 2012). Marker positions are indicated in centimorgans (cM) on the left side of the linkage group, and locus designations are provided on the right side. **b** Distances between *FocBo1* and the newly mapped markers are indicated in the enlarged genomic region containing *FocBo1*

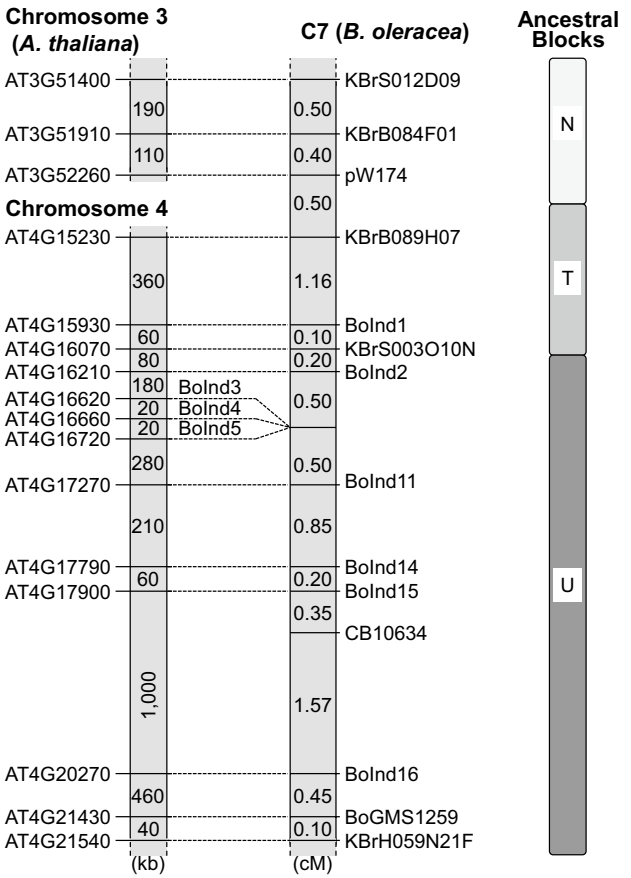


Fig. 2 A fine map of *B. oleracea* in the region of *FocBo1* (C7) based on an F_2 population ($n = 1{,}008$) and the syntenic region of *A. thaliana*. Gene loci of *A. thaliana* and marker names of the *B. oleracea* chromosome are shown at the *left* and *right sides* of the respective chromosomes. Approximate nucleotide distances between the loci of *A. thaliana* are shown in kb. Genetic distances between markers in the *B. oleracea* C7 map are given in cM. Three markers of BoInd3–5 were co-segregated in this mapping population

the resistant homozygote, the heterozygote, and the susceptible homozygote, respectively. When the DI scores and genotypes of the F_2 lines were compared, the genotypes between BoInd2 and BoInd11 (1.00 cM) showed correlation with the DI scores obtained. Therefore, the *FocBo1* locus is likely to be placed between BoInd2 and BoInd11 on C7.

Physical mapping of the *FocBo1* region and identification of a candidate gene

To know the physical structure of the *FocBo1* locus, we isolated BAC clones by screening the commercial BAC library of *B oleracea*, the JBo library derived from *B. oleracea* spp. *alboglabra* A12. Two isolated BAC clones, #5811 and #6611, were estimated as ca. 120 kb long and ca. 200 kb long, respectively, using pulse field gel electrophoresis. They made a contig spanning ca. 210 kb, which was determined by sequencing and physical mapping of the BAC clones (Fig. 4a). The end sequences of the BAC clone #5811 corresponded to *A. thaliana* genes, At4g16720 and At4g17030, whose region encompassed disease resistance gene cluster including RPP5 (between At4g16860 and At4g16960) on At4. At first, we sequenced the BAC clone #5811 by NGS technology (GS 454 Junior, Roche). The sequence data were assembled into contigs. The gaps between the resulting contigs were sequenced by the Sanger method. The contigs were overlapped and we recovered in a 120407-bp-long contig. AUGUSTUS (Stanke and Morgenstern 2005), a program that predicts genes, identified five transposable elements and twenty-four hypothetical genes in the #5811 clone (Fig. 4b; Table S3). In addition to information on the #5811 clone, we searched the genome sequence of Cabbage line 02-12, which became available in the middle of this study, in Bolbase (http://ocri-genomics.org/bolbase). The BLASTN search revealed that

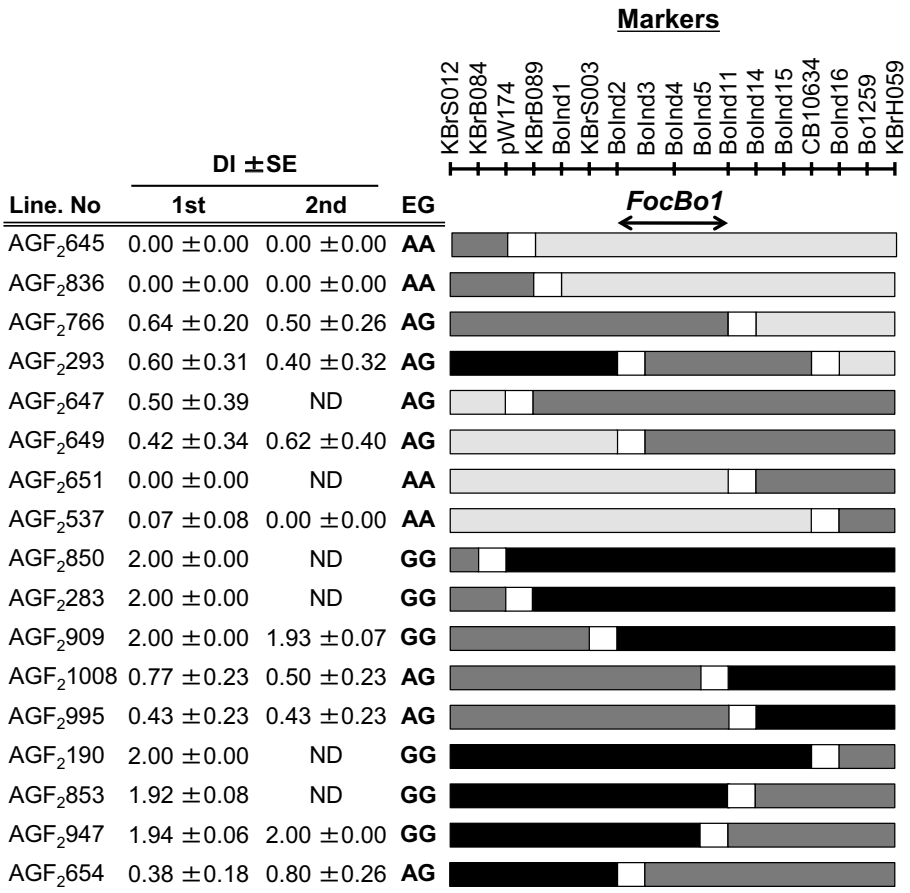


Fig. 3 Graphical genotype of the selected recombinants and their disease index (DI) in the F_3 progenies derived form F_2 plants. *Marker names* are indicated at the top of the column. Homozygotes of the resistant allele (AnjuP01), homozygotes of the susceptible allele (GCP04) and heterozygotes, are represented by *light gray*, *black*, and *dark gray boxes*, respectively. Regions of unknown genotype due to marker intervals are shown as *open boxes*. Name of lines and DIs are shown at the left side. *EG* shows the *FocBo1* genotypes estimated based on IDs. The putative location of the *FocBo1* gene is indicated with *arrows*

Scaffold000026 is highly syntenic to the region of *FocBo1* locus, and the sixty-one hypothetical genes, excluding the transposable elements, are encompassed between BoInd2 and BoInd11 in the Scaffold000026. In addition, the BLASTN search revealed that out of the hypothetical 24 genes predicted in the #5811 clone, 14 were common to genes predicted in Scaffold000026, and gene order was conserved. (Table S3). Altogether, the *FocBo1* locus delimited by the two DNA markers, BoInd2 and BoInd11 on C7, was ca. 361 kb where seventy-one genes (excluding transposable elements) were identified according to the information of #5811 and Bolbase.

Since *alboglabra* A12, used as the plant material for the construction of the JBo BAC library, was susceptible to *Foc* Cong: 1-1 isolate (data not shown), we designated the allele as *focbo1-a12* later on. In the *focbo1-a12* region, the genes predicted in AUGUSTUS and published in Bolbase (Table S3) suggested two types of disease resistance-related genes detected with *E*-value < E^{-40}, and furthermore, we did not find any other disease resistance-related genes in BLAST search against the BRAD database (http://brassicadb.org). One is the #5-gene (Bol037168), homologous to heat shock protein 70 (HSP70). Heat shock proteins are found to be critical for basal resistance: HSP70 for basal resistance against bacteria in *A. thaliana* (Jelenska et al. 2010), and HSP70 and HSP90 for INF1-mediated hypersensitive reaction and non-host resistance to *Pseudomonas cichorii* in *N. benthamiana* (Kanzaki et al. 2003). As mentioned above, *alboglabra* A12 was susceptible to *Foc* Cong: 1-1, and therefore should lack *FocBo1* or have mutational *FocBo1* with loss of resistance. The #5-gene (HSP70) of A12 includes a 2,610 nucleotide full sequence (869 amino acids), which shows 100 % similarity with that of AnjuP01. In addition, the HSP70 cDNA in both susceptible A12 and resistant AnjuP01 was detected in the non-infected plants at the same level by RT-PCR (data not shown). These results indicate that the #5-gene is not a candidate for *FocBo1*. The other gene detected is a disease resistance gene, predicted in the *focbo1-a12* region in #5811 BAC clone by AUGUSTUS, designated as #27-gene. The predicted #27-gene was classified into TIR-NBS-LRR class by Pfam software (http://pfam.sanger.ac.uk) (Fig. S2), and showed high sequence similarity to the disease resistance genes like RPP5 (Recognition of Peronospora Parasitica 5) (Table S3). In addition, the #27-gene showed high sequence similarity to TIR-NBS-LRR disease resistance genes Bra012688 and Bra012689, candidate genes conferring YR to Chinese cabbage T23 inbred line (*B. rapa*), which were found by differential

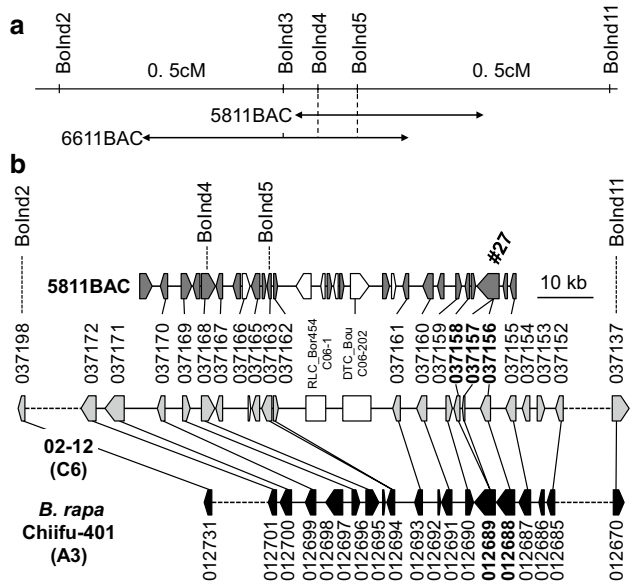
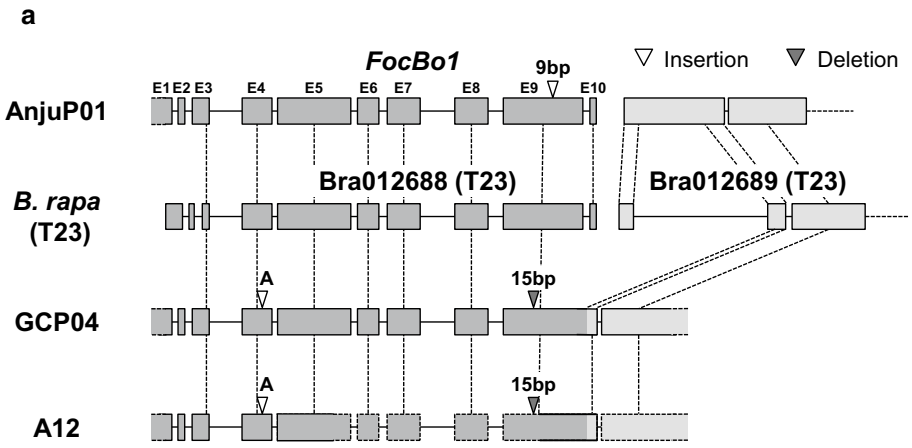


Fig. 4 Map-based cloning of *FocBo1* and comparisons of synteny and gene structures among *FocBo1* regions (CDSs) of different sources. **a** Genetic map derived from the F_2 population (AnjuP01×GCP04), and location of BAC clones obtained from JBo library of *B. oleracea* var. *alboglabra* A12. **b** Syntenic comparison of 5811 BAC, Scaffold000026 (C6) of Cabbage line 02–12 (cited from Bolbase) and the A3 region "Chiifu-401" (from BRAD). *Closed arrows on the horizontal lines* indicate structural genes. *Square boxes* and *open arrows* indicate transposable elements with and without information about transcript direction, respectively. The transcript directions of genes are indicated by the *direction of the arrow*. The locations of transposable elements are omitted in *B. rapa* (Chiifu-401)

expression analyses in RNA-seq conducted between resistant and susceptible Chinese cabbage inbred lines (Shimizu et al. 2014). The comparison between the hypothetical #27-gene and Bra012688 and Bra012689 suggested that the #27-gene was formed by the fusion of the two genes orthologous to each Bra012688 and Bra012689, respectively (Figs. 5, S2). A similarly structured sequence was found in susceptible GCP04 (later mentioned). In comparison to cabbage line 02–12 (Bolbase), the #27-gene showed high sequence similarity to the three CDSs in Scaffold000026 of cabbage line 02–12; Bol037156 (612 bp), Bol037157 (267 bp), and Bol037158 (1,422 bp), which contains conserved only LRR, TIR, and LRR domains, respectively (Fig. S2). These three CDSs having fragmented disease resistance gene motifs were likely to be nonfunctional. Otherwise, the gene prediction was thought to be wrong.

To determine the structure of the allelic gene for #27 in the resistant parent AnjuP01, we isolated the cosmid clones of AnjuP01 containing the region of #27 by screening the pooled library using #27 gene-specific primer sets (Table S2), resulting in the identification of the cosmid clone PWE#27-A7. The sequence of the region corresponding to the #27 gene in PWE#27-A7 was determined using the primer-walking approach. The sequence analysis revealed that the region contains two TIR-NBS-LRR type genes; the former part encompassed 4,095 bp ORF (1,364 amino acid) including ten exons, which showed higher homology to Bra012688 (*B. rapa* YR candidate gene), while the latter

Fig. 5 Comparison of *FocBo1* orthologs at the *FocBo1* region in *B. rapa* Chinese cabbage inbred line (T23), *B. oleracea* DH line AnjuP01, GCP04, *alboglabra* A12. **a** Schematic comparison of gene structure, **b** nucleotide length of exons and introns. The *boxes* indicate exons; the *solid-lined* exons determined by the alignments of the corresponding cDNA sequences, and the *dotted-lined* exons predicted by the *B. rapa* (T23) sequence. The *arrowheads* indicate indels, which were determined by alignment of the *B. rapa* (T23) sequence



b

	E1	I1	E2	I2	E3	I3	E4	I4	E5	I5	E6	I6	E7	I7	E8	I8	E9	I9	E10
***B. rapa* (T23)**	272	76	67	104	106	508	441	79	1084	92	309	138	480	494	483	248	1194	106	229
Anju	>15	75	68	101	245	511	441	90	1084	92	309	138	480	510	483	248	1185	101	232
GC	>15	76	68	101	245	489	442	89	1084	92	309	138	480	510	483	247	1329	98	>875
A12	>15	76	68	99	245	489	442	89	1084	92	309	138	480	510	483	248	1329	98	>875

half had no long ORF (Fig. S3), and fused exon 1 to exon 2 in contrast to Bra012689 (Fig. 5). Thus, it seemed that the latter half was a nonfunctional pseudogene. The predicted gene of 4,095 bp length showed 96.4 % homology to Bra012688 (T23). Another allelic sequence of the #27 gene in GCP04, the susceptible parent used for fine mapping of *FocBo1*, was sequenced and compared to that of AnjuP01 and A12. We found that GCP04 and A12 share similar nucleotide sequence identity in exon 1–10 (98.9 %) with similar nucleotide indels including a single nucleotide (A) insertion in exon 4 and deletion of exon 10, indicating that such structural changes induce malfunction of FocBo1 in both GCP04 and A12, while Bra012688 and the orthologous counterpart in AnjuP01 showed fully conserved structures (Fig. 5). These structural differences between resistant and susceptible alleles, together with the predicted function of the encoded protein, strongly suggest that the orthologous region of Bra012688 in AnjuP01 encodes *FocBo1*. Therefore, we designated the predicted gene in AnjuP01 as *FocBo1* thereafter.

5′- and 3′-RACE of *FocBo1* and Bra012688, and mutational genes

The complete mRNA sequence of Bra012688 (T23) including 5′- and 3′-UTR was determined by 5′- and 3′-RACE. The full length CDS, full 3′-UTR, and partial 5′-UTR sequences in *FocBo1* (Anju) were determined by sequencing RT-PCR products. Comparison of mRNA sequences and genomic sequence confirmed the location of ten exons and nine introns in Bra012688 (T23) and *FocBo1* (Anju) (Fig. 5), and also revealed that two small regions of 5′-UTR were spliced out in Bra012688 (T23) and *FocBo1* (Fig. S4), in contrast to CDS of Bra012688 (Chiifu-401), which has nine predicted exons. In Bra012688 (T23), *FocBo1* (Anju), and *focbo1-gc*, the start codon ATG was identified in the exon 3, and consequently, the CDS consisted of eight exons (exon 3–10). A single nucleotide (A) insertion in the exon 4 of *focbo1-a12* and *focbo1-gc* was also confirmed in both genomic and cDNA sequences. The *FocBo1* mRNA in both parents, susceptible GCP04, and resistant AnjuP01, was detected by RT-PCR in leaves of healthy plants (data not shown). The RT-PCR, using the forward primer in the exon 7 of *FocBo1* and the reverse primer in the Bra012689 ortholog of *B. olerecea*, produced a fragment in GCP04 and A12, thus indicating that *focbo1* and the flanking region were transcribed as one mRNA.

Association between the new markers and phenotypes in MAS

To confirm association between the candidate gene polymorphism and phenotypes, MTK-C primer set, designed

Table 1 Screening of commercial F_1 cabbage cultivars for yellow wilt susceptibility and genotype using the *FocBo1* specific primer set (MTK-C)

Cultivar	Phenotype	Genotype	Company
Haruhikari7gou	S	−	Takii & Co., Ltd
Jaenne	S	−	Takii & Co., Ltd
KogetusSP	S	−	Takii & Co., Ltd
Miharu	S	−	Takii & Co., Ltd
Shosyu	S	−	Takii & Co., Ltd
Terumisaki	S	−	Takii & Co., Ltd
Asashio	R	+	Takii & Co., Ltd
Ayahikari	R	+	Takii & Co., Ltd
Ayakaze	R	+	Takii & Co., Ltd
Ayasato	R	+	Takii & Co., Ltd
Hamamisaki	R	+	Takii & Co., Ltd
Harunokahori	R	+	Takii & Co., Ltd
Hoshimisaki	R	+	Takii & Co., Ltd
KoimisakiSP	R	+	Takii & Co., Ltd
Shokanokahori	R	+	Takii & Co., Ltd
Suzune	R	+	Takii & Co., Ltd
Wakamine	R	+	Takii & Co., Ltd
YR Haruzora	R	+	Takii & Co., Ltd
Yumegoromo	R	+	Takii & Co., Ltd
Satuski joou	S	−	Nippon Norin Seed Co
Ranpohikari	R	+	Nippon Norin Seed Co
YCR Rinen	R	+	Nippon Norin Seed Co
Oikaze	S	−	Mikado Kyowa Seed Co., Ltd
YR Delightball	S	−	Mikado Kyowa Seed Co., Ltd
Akizora	R	+	Mikado Kyowa Seed Co., Ltd
City	S	−	Watanabe Seed Co., Ltd
Delicious	S	−	Watanabe Seed Co., Ltd
Rakuen	S	+	Watanabe Seed Co., Ltd
Soen	R	+	Watanabe Seed Co., Ltd
Speedball	S	−	Watanabe Seed Co., Ltd
Kinkei201gou	S	−	Sakata Seed Co
Kinpo	S	−	Sakata Seed Co
Earlyball	R	+	Sakata Seed Co
Misaki	R	+	Sakata Seed Co
Seirin	R	+	Sakata Seed Co
Fuyusuruga	S	−	Ishi Seed Growers Co., Ltd
Ogosho	S	−	Ishi Seed Growers Co., Ltd
YR Haruiro	R	+	Ishi Seed Growers Co., Ltd
YR Fuyutaro	R	+	Masuda Seed Co
YR TaibyoST	R	+	Masuda Seed Co
YR Harunobu[a]	R	+	Snow Seed Co
YR Kiyomi[a]	R	+	Snow Seed Co
Irodori	R	+	Kaneko Seed Co., Ltd
Mikuni[a]	R	+	Kaneko Seed Co., Ltd
Newtop	S	−	Marutane Co., Ltd

Table 1 continued

Cultivar	Phenotype	Genotype	Company
Shinsei2gou	S	−	Sanyo Norin Seed Co.
Tamaki155	S	−	Nozaki Seed Co., Ltd
Hamanomai	S	−	Tokita Seed Co., Ltd
Fuyuki[a]	R	+	Toyohashi Seed Co., Ltd
YR SE	R	+	Nagano Prefecture

[a] indicates that their selfed progenies were used in the F_2 test

in the exon 9–3′ untranslated region of *FocBo1*, amplified a band (759 bp) in the resistant parent, AnjuP01, but not in susceptible GCP04. Association of phenotypes with genotyping data in fifty commercial cabbage F_1 cultivars is shown in Table 1. Twenty-nine (58 %) of the cabbage cultivars/inbred lines were resistant (DI = 0), whereas the remaining 21 (42 %) were susceptible (DI = 2) to Cong: 1-1. Since *FocBo1* is a single dominant gene, F_1 cultivars which have the homo- or heterozygous resistant *FocBo1* allele, demonstrated resistance to *Foc* in the inoculation test conducted at 25 °C. The genotyping using 49 cabbage cultivars was perfectly matched with the phenotyping data of cultivars, although the MTK-C set of primers could not distinguish between homo- and heterozygote in the *FocBo1* allele. An exception in this association analysis was the cultivar, Rakuen, which has the resistant allele band pattern, but is susceptible. The sequence analysis revealed that *FocBo1* in Rakuen contains an additional nucleotide in the exon 4, suggesting a malfunction of *FocBo1*. In addition, its structure was a recombinant derived from the functional *FocBo1* allele of AnjuP01 and the nonfunctional allele of *alboglabra* A12 (Fig. S4). Altogether, three susceptible haplotypes, i.e., *focbo1-a12* (spp. *alboglabra* A12), *focbo1-gc* (var. *italica*), and *focbo1-rakuen* (var. *capitata*) were identified in this study.

The MTK-1 primer set was designed from #11 gene (Bol037163) located on ca. 30 kb from *FocBo1* because primers designed from the internal sequence of *FocBo1* tended to amplify non-specific bands, which may have been derived from a common motif like NBS-LRR of other R genes. The MTK-1 amplified 484 and 506 bp band in AnjuP01 and GCP04, respectively. The aforementioned MTK-C primer set was also used for the segregation test. Four F_2 progenies derived from self-pollination of F_1 cultivars (Fuyuki, Mikuni, YR Harunobu, and YR Kiyomi) were investigated. As a result, the MTK-C and the MTK-1 markers were dominant and co-dominant, respectively. The MTK-1 marker distinguished between homo- and heterozygotes in *FocBo1* alleles with perfect matching between phenotype and genotype (Table 2). Those results indicated that our identified gene is the true *FocBo1* gene.

Table 2 Validation of association between allelic polymorphisms at the *FocBo1* locus and phenotypes in F_2 populations derived from commercial F_1 hybrid cultivars

Cultivars	No. of plants	Phenotyping		Genotyping	
		Resistant	Susceptible	MTK-C	MTK-1
Fuyuki	37	30	7	R_[a](30) rr (7)	RR (11) Rr (19) rr (7)
Mikuni	70	59	11	R_ (58) rr (11)	RR(15) Rr (43) rr (11)
YR Harunobu	54	43	11	R_ (43) rr (11)	RR(14) Rr (29) rr (11)
YR Kiyomi	32	19	13	R_ (19) rr (13)	RR(7) Rr (12) rr (13)

[a] R_ indicates that genotypes of resistant plants are RR or Rr

Discussion

Syntenic region and fine mapping of the *FocBo1* locus

To do fine mapping, we used gene colinearity between the genomes of *A. thaliana* and *B. oleracea*. The lower and upper parts of the *FocBo1* genome region corresponded to the *A. thaliana* genome regions spanned by At4g21540–At4g15230 and At3g52260–At3g51400, respectively. The BAC sequences corresponding to those Arabidopsis genes were collected from *B. rapa* and *B. oleracea* databases, and PCR primers were designed in the conserved exon sequences spanning intronic sequences. As a result of high conservation of exon sequences and high polymorphism in the intronic sequences, 17 pairs (37.8 %) per 45 primer pairs tested in this study amplified clear polymorphic fragments between the two parental alleles. Similarly, some of the authors designed primers for the targeted region in exons spanning introns, and, consequently, the PCR primer pairs demonstrated efficient polymorphic amplification: 28 % of the primer pairs (23/82) in *B. rapa* (Saito et al. 2006) and 32 % (383/1180) in *B. juncea* (Panjabi et al. 2008).

Linkage groups of *B. rapa* (AA), *B. oleracea* (CC), and *B. napus* (AACC) were numbered A1–A10, C1–C9, and N1–N19, respectively, based on syntenic relationship on chromosomes of each species, i.e., colinearity of marker order in A1, C1, N1, N11, and so on (Parkin et al. 2005). Recent comparative genomics of the family Brassicaceae showed that the current Brassica genome contains three subgenomes (LF, MF1, MF2) derived from the ancestor

genome of Brassicaceae, and representing 24 ancestral genomic blocks (Lysak et al. 2005; Schranz et al. 2006; Cheng et al. 2013). Using the 24 conserved block system, Panjabi et al. (2008) suggested that the current numbering of C6 and C7 should be designated as C7 and C6, respectively, because the ancestral genomic blocks composed in C6 and C7 revealed more similarity to A7 and A6, respectively. Bolbase actually follows this rule. Panjabi et al. (2008) and Cheng et al. (2013) reported that the U block replicates itself in the C genome of *B. oleracea* and is located on C1, C3, C6 (C7 of this study) and C8, which is consistent with our mapping results, except for the location of U block in C3. The obtained linkage markers on C1, C6 (C7) and C8 may be useful for future genome studies on *B. oleracea*, because, although we can easily collect DNA sequences from the genome database, it is still difficult to make PCR-based markers to identify each region of triplicated sequences in the C genome. In addition, the upper region of the U block contains several important disease resistance genes such as *FocBo1* and *PbBoAnju4* (Nagaoka et al. 2010; Pu et al. 2012), RPP gene cluster in *A. thaliana* (Van der Biezen et al. 2002), *CRa* (Ueno et al. 2012), and *Crr1*, *Crr2* (Suwabe et al. 2006; Hatakeyama et al. 2013).

The *FocBo1* candidate gene

In this study, we compared the physical structures of *FocBo1* locus in *alboglabra* A12 (#5811 BAC clone) and Cabbage line 02–12 (Bolbase) where most of the genes co-exist in the two ecotypes, and their gene order is completely conserved. In both genome regions, we identified the #5-gene (HSP70), which is found to confer disease resistance in Arabidopsis and *N. benthamiana*. This gene, however, is not considered to be a candidate for *FocBo1*, as mentioned in Results. On the other hand, an NBS-LRR motif gene (#27) was uniquely located in the *FocBo1* region in *alboglabra* A12 (Table S3), and was highly homologous to Bra012688 and Bra012689. These two genes, candidate genes conferring YR to Chinese cabbage, are tandemly linked, but not generated by a recent duplication event (Shimizu et al. 2014). In comparison, the #27-gene was thought to be formed by the fusion of the two genes, Bra012688 and Bra012689. In addition to this structure, the #27-gene contains an additional nucleotide in exon 4 and deletes exon 10. Those mutational changes could cause malfunction of this gene, which is consistent with the result that *alboglabra* A12 is susceptible. On the other hand, the allelic region of fusarium-resistant AnjuP01 seemed to contain a functional gene (1,364 amino acids) and a pseudogene, highly homologous to Bra012688 and Bra012689, respectively. This indicates that the former gene is a candidate for *FocBo1*. In *B. rapa* (Chinese cabbage), however, it is not clear which of the candidate genes, Bra012688 (T23) or Bra012689 (T23), confers resistance to Chinese cabbage, or if both of the genes are required for full expression of resistance (Shimizu et al. 2014). Since *FocBo1* alone is sufficient for full expression of YR in *B. oleracea* AnjuP01, Bra012688 (T23) could be sufficient for conferring YR in Chinese cabbage. Therefore, it was designated as *FocBr1*. A neighbor-joining tree showed that Bra012688 (*FocBr1*) and *FocBo1* are clustered in the same clade, suggesting that Bra012688 and *FocBo1* are orthologous (Fig. S5). Bra012689 (T23) contains long ORF and is normally expressed in the resistant Chinese cabbage inbred line (Shimizu et al. 2014), whereas the AnjuP01 ortholog of Bra012689 accumulates many mutations within the CDS. This feature may be indicative of a certain function of Bra012689 (T23) regarding disease resistance response.

Usefulness of the new markers in MAS

Pu et al. (2012) revealed that MAS using *FocBo1*-linked markers (KBrS003O10N1) was effective for identification of YR plants in the segregating population derived from the cross of resistant and susceptible parents, but misidentification occurred due to recombination between KBrS003O10N1 and *FocBo1*. In contrast, this time there was a perfect match between genotyping using the candidate gene markers and phenotyping by inoculation test in the F_1 cultivars as well as the F_2 segregating population.

It has been more than 80 years since the type A resistance gene was introduced into cabbage in the US. However, there is no paper reporting current status of use of Type A resistance in *B. oleracea* breeding, although old *B. oleracea* cultivars have been surveyed for resistance to yellows (Walker and Wellman 1928; Iijima 1971; Nomura et al. 1976). In this study, we found that about 60 % of cabbage varieties distributed in Japan have the *FocBo1* allele, indicating that current YR cabbage cultivars help control fusarium wilt disease. On the other hand, many susceptible cultivars without *FocBo1*, or YR cultivars with heterozygous alleles, have been distributed to the commercial market in Japan. To assess further the status of use of Type A resistance in *B. oleracea*, it is necessary to screen cabbage cultivars released worldwide and investigate how many *FocBo1* haplotypes are present in cultivars and wild allies. Since it is known that heterozygous plants are more disease-susceptible at high temperatures above 28 °C (Bosland et al. 1988; Pu et al. 2012), cabbage breeders need to produce cultivars with the homozygous-resistant *FocBo1* allele for use in the current hotter climate, especially in summer–autumn cabbage cropping patterns. To this end, MAS using the *FocBo1* markers identifying homo- and heterozygous alleles will be effective.

The association analyses using F_1 cultivars and F_2 segregating populations, and our discovery of the mutational

focbo1 genes in A12, Rakuen, and GCP04, were confirmed by perfect matches between the *FocBo1* polymorphism and phenotypes. The two different methods, map-based cloning (this study) and differential genes expression analysis (Shimizu et al. 2014), identified the orthologues as YR genes in the two different species, *B. oleracea* and *B. rapa*. That evidence suggested that *FocBo1* acts as a real YR gene in *B. oleracea*.

Author contributions MS delimited *FocBo1* locus by conducting the fine map, inoculation test, and sequencing BAC clones and cDNA clones, and was primary author. ZjP and TK constructed cosmid library, screened, and sequenced clones. HK screened BAC library. SM provided marker information and constructed the genetic map. YE and MSano determined cDNA sequences and did inoculation test for commercial cultivars. TF and EF conducted bioinformatics analysis. RF conducted experimental design and statistical analysis. KO contributed to markers for MAS, provided funding, and managed the whole project.

Acknowledgments The authors sincerely thank Dr. Y. Kadota at the National Agriculture and Food Research Organization/NARO Tohoku Agricultural Research Center, Japan, for kindly providing Cong: 1-1 strain of *F. oxysporum* f. sp. *conglutinans*. This work was supported in part by Research Fellowships of JSPS for Young Scientists for M. Shimizu, the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry, and the Adaptable and Seamless Technology Transfer Program through target-driven R&D from Japan Science and Technology Agency (JST) to K. Okazaki.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiment conducted complies with the laws of Japan.

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