Identification of candidate genes for fusarium yellows resistance in Chinese cabbage by differential expression analysis

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Abstract Fusarium yellows caused by *Fusarium oxysporum* f. sp. *conglutinans* is an important disease of Brassica worldwide. To identify a resistance (*R*) gene against Fusarium yellows in Chinese cabbage (*Brassica rapa* var. *pekinensis*), we analyzed differential expression at the whole genome level between resistant and susceptible inbred lines using RNA sequencing. Four hundred and eighteen genes were significantly differentially expressed, and these were enriched for genes involved in response to stress or stimulus. Seven dominant DNA markers at putative *R*-genes were identified. Presence and absence of the sequence of the putative *R*-genes, Bra012688 and Bra012689, correlated with the resistance of six inbred lines and susceptibility of four inbred lines, respectively. In F$_2$ populations derived from crosses between resistant and susceptible inbred lines, presence of Bra012688 and Bra012689 cosegregated with resistance, suggesting that Bra012688 and Bra012689 are good candidates for fusarium yellows resistance in Chinese cabbage.

Keywords Fusarium yellows · *R*-gene · RNA-sequence · NBS–LRR · Chinese cabbage

Introduction

Chinese cabbage (*Brassica rapa* var. *pekinensis*) is an important and widely cultivated vegetable in Asia. Its yield is decreased by various diseases and insects. Fusarium yellows (also called Fusarium wilt) is an economically important disease in warm, humid and semi-humid areas. The causal agent of fusarium yellows in Chinese cabbage is either soil born fungus *Fusarium oxysporum* f. sp. *conglutinans* or *F. oxysporum* f. sp. *rapae* (Daly and Tomkins 1995; Enya et al. 2008). Infection by this fungus leads to leaf yellowing, wilting, defoliation, stunted growth, and death of the host plant, resulting in detrimental effects on the quality and quantity of Chinese cabbage (Walker 1930; Daly and Tomkins 1995; Enya et al. 2008). Because this ubiquitous pathogen is able to survive in soil for many years even in the absence of hosts, measures such as crop rotation, improved sanitation, and fungicides are ineffective for its control. Once the disease is present, the most successful strategy is to use disease resistant/tolerant cultivars. Resistance to fusarium yellows in *Brassiceae* crops is studied in *Brassica oleracea* (Farnham et al. 2001; Pu et al. 2012), as well as in a model plant, *Arabidopsis thaliana* (Diener and Ausubel 2005; Diener 2013). A significant challenge in cabbage (*B. oleracea* var. *capitata*) breeding has been to introduce a durable fusarium yellows resistance gene into

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agronomically important cultivars (Walker et al. 1927). Few studies have been carried out on *B. rapa*, especially in Chinese cabbage.

There are two types of defense response of plants, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). In PTI plant defense responses are activated when pathogen molecules are recognized by the plant pattern recognition receptors, and in ETI plant defense responses are activated by the recognition of specific effectors [avirulence (Avr) proteins] by resistance (R) proteins. This recognition between R and Avr is termed 'gene-for-gene resistance' (Dangl and Jones 2001). Many *R*-genes that recognize pathogens have been identified in plants (Joshi and Nayak 2011). Most of these genes encode proteins with leucine-rich repeats (LRR), a central nucleotide binding site (NBS) domain, and in the N-terminus a domain that contains homology to cytosolic domains of the Drosophila Toll or animal interleukin-1 receptors (TIR) (termed TIR–NBS–LRR) or a potential coiled coil (CC) domain (termed CC–NBS–LRR) (Meyers et al. 2003; Joshi and Nayak 2011; Marone et al. 2013). There are 16 disease resistance gene families, which include 244 putative *R*-genes, in the recently released genome sequence of *B. rapa*, which has 41,174 protein coding genes (http://brassicadb.org/brad/) (Cheng et al. 2011; Wang et al. 2011).

cDNA or tiling arrays can be used to quantify the expression levels of target transcripts during development or under different conditions such as environmental or biotic stress. Transcriptome analysis has played a central role in unraveling the complexity of gene function and underlying biological processes in plants (Seki et al. 2002; Abe et al. 2011). Comprehensive analysis of the transcriptome in *B. rapa* is limited by a partial and biased knowledge of the transcriptome, as it is largely derived from gene prediction and limited expressed sequence tag (EST) evidence. The genome sequence of *B. rapa* together with RNA-sequencing (RNA-seq) allows accurate measurement of expression levels of genes and detection of allele specific expression and alternative splicing variants.

In this study, we examine the transcriptome of fusarium yellows resistant and susceptible lines using RNA-seq and focus on the expression of putative *R*-genes. We hypothesize that (1) a fusarium yellows resistance gene is differentially expressed between fusarium yellows resistant and susceptible lines before or after infection, or that (2) the resistance gene has lost its function by mutation without changing its expression level. RNA-seq could test these alternatives at the whole genome level. Sequences of two candidate *R*-genes with TIR–NBS–LRR motifs, Bra012688 and Bra012689, are present in resistant lines and absent in susceptible lines. The cosegregation of both candidate

R-genes with fusarium yellows resistance in two independent F_2 populations is compelling evidence that one of these candidate genes confers fusarium yellows resistance.

Materials and methods

Plant materials

Ten Chinese cabbage inbred lines (RJKB-T4, 13–15, 17, 21–25) and commercial cultivar 'Chiifu' were used as plant material. Fusarium yellows resistance lines, RJKB-T21 and RJKB-T23, were crossed to susceptible lines, RJKB-T22 and RJKB-T24, respectively, to generate the F_1 hybrid. F_1 plants were self-pollinated by bud pollination to generate F_2 progenies.

Resistance test for fusarium yellows

A field strain Cong; 1-1 (isolated from cabbage) of *F. oxysporum* f. sp. *conglutinans*, provided by Dr. Kadota (National Agricultural Research Center for Tohoku Region, Japan), was used to prepare inocula in this study. Primer set FIGS11 (5′-GTAAGCCGTCCTTCGCCTCG-3′) + FIGS12 (5′-GCAAAATTCAATAGTATGGC-3′) (Enya et al. 2008) was used to amplify the rDNA intergenic spacer (IGS) region from the strain. The sequencing results indicated that rDNA IGS region of Cong: 1-1 strain had 100 % sequence identity with that of *F. oxysporum* f. sp. *conglutinans* ATCC® 52557™, which was identified as race 1 (Data not shown).

An inoculation test was carried out following the method described by Pu et al. (2012). In the fusarium yellows inoculation test, the plants were grown in a well-controlled growth room at 25 °C under fluorescent lights and a 16 h/8 h (day/night) photoperiod. Twelve-day old seedlings were transplanted into inoculated soil, and the disease severity index (DI) was investigated 4 weeks after infection using the scale of 0–2 (0: no symptom, 1: plant yellowing, 2: plant death).

Sequence library preparation and sequencing

First or second true leaves in fusarium yellows resistant RJKB-T23 and susceptible RJKB-T24 lines were collected at 14 days after sowing. Total RNA was isolated with SV Total RNA Isolation System (Promega, WI, USA). For making libraries for RNA-seq, the first strand cDNA was synthesized by random hexamer primers using the short fragmented mRNA, which was purified by beads containing oligo (dT) from total RNA and then fragmented by fragmentation buffer. The second-strand cDNA was synthesized using buffer, dNTPs, RNase H, and DNA

polymerase I. Illumina sequencing adaptors were ligated to the short fragments and sequenced (read length; 50 nt, single sequencing) on an Illumina HiSeq™ 2000.

Mapping short reads to the Chinese cabbage genome

Tophat (http://bioinformatics.oxfordjournals.org/content/25/9/1105.abstract) was used to align RNA-seq reads against *Brassica* genome release 1.1. The multiply mapped reads were removed from further analyses. We used cuffdiff (http://www.nature.com/nbt/journal/v28/n5/full/nbt.1621.html) to identify differentially expressed genes between the fusarium yellows resistant (RJKB-T23) and susceptible (RJKB-T24) lines.

Gene ontology analysis

Analysis for enrichment of gene functional ontology terms was completed using the gene ontology (GO) tool agriGO (Du et al. 2010). The *B. rapa* genes were replaced with orthologous genes in *Arabidopsis thaliana* by BRAD and GO analysis was performed on the *A. thaliana* basis. The background reference was the list of genes that displayed expression above-background in early developmental vegetative tissues of *A. thaliana* (Fujimoto et al. 2012). Statistical tests for enrichment of functional terms used the hypergeometric test and false discovery rate (FDR) correction for multiple testing to a level of 5 % FDR.

Analysis of genomic DNA

All of the DNA markers used in our study were developed as EST markers. Total genomic DNA of each plant was isolated from leaves using the CTAB method (Murray and Thompson 1980). Using genomic DNAs as a template, the amplification of DNA markers by PCR was performed using EmeraldAmp MAX PCR Master Mix (Takara Bio, Shiga, Japan) according to the manufacture's instructions. The reaction mixture was incubated in the thermal cycler at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min or 30 s. PCR products were separated by electrophoresis using 1.5 or 2.0 % agarose gel. Sequences of all primers used are shown in Table S1.

Gene expression analysis

Total RNA was isolated from first and second true leaves using the SV Total RNA Isolation system (Promega) and different samples of total RNAs were used for quantitative real-time PCR (qPCR). From 500 ng total RNA, first strand cDNA was synthesized using random primers by Super Script III Reverse Transcriptase (Invitrogen, CA, USA) according to the manufacturer's specifications.

qPCR was performed using a Rotor-Gene 3000 Real-Time Cycler (Qiagen, Hilden, Germany). The cDNA was amplified using Platinum Taq DNA polymerase (Invitrogen). Sequences of all primers used are shown in Table S1. PCR conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Expression levels of genes of interest relative to the *actin* gene were calculated using the comparative quantification analysis method with Rotogene-6 (Qiagen) (Fujimoto et al. 2006). Data presented are the average and standard error (SE) from three biological and experimental replications.

Phylogenetic analysis

Proteins showing high sequence homology to Bra012688 or Bra012689 were searched for using BLASTp (http://blast.ncbi.nlm.nih.gov/). At4g16890 (NM_117792), At5g51630 (NM_001203587), Bra012688, and Bra012689 were aligned using DDBJ ClustalW (http://www.ddbj.nig.ac.jp). A phylogenetic tree was constructed by the neighbor joining method (Saitou and Nei 1987), and bootstrap probabilities of 1,000 trials were calculated.
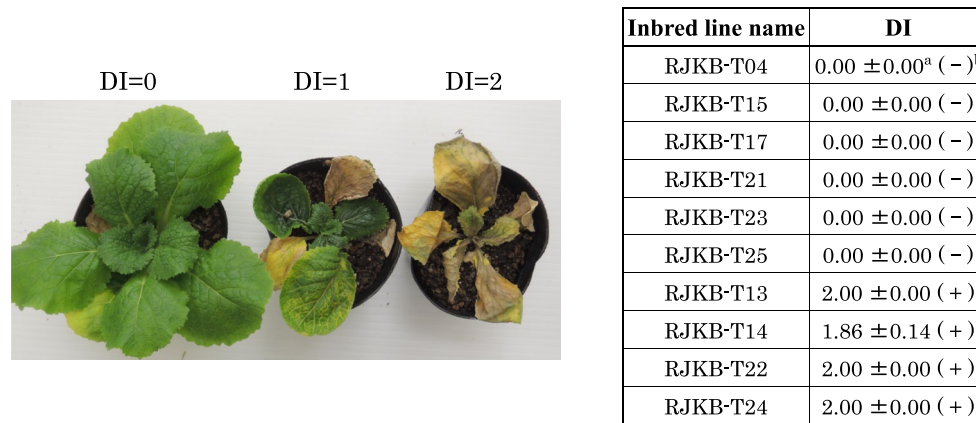
Results

Screening Chinese cabbage inbred lines for resistance to *Fusarium oxysporum* f. sp. *conglutinans*

Ten inbred lines of Chinese cabbage were examined for their response to fusarium yellows. The response to fusarium yellows was clearly divided into resistant (DI, 0.00) and susceptible (DI, 1.86–2.00) classes. Six of ten lines were resistant and the remaining four lines were susceptible (Fig. 1). One resistant (RJKB-T23) and one susceptible (RJKB-T24) line were chosen to perform further analyses for identifying the resistance gene to fusarium yellows.

Transcriptome analysis by RNA-sequencing

We examined the whole genome transcriptome by RNA-seq in fusarium yellows resistant (RJKB-T23) and susceptible (RJKB-T24) lines. Total RNA was isolated from true leaves at 14 days after sowing without fusarium yellows inoculation and RNA-seq was performed using Illumina Hiseq2000. As resistance genes of clubroot, which is soil bone fungus like fusarium yellows, show the same expression level in roots and leaves (Ueno et al. 2012; Hatakeyama et al. 2013), we used leaves for RNA-seq. About twelve million reads (approximately 600 Mb) were obtained (Table S2). 12,664 of 41,029 genes were comparable by Tophat/cuffdiff and four hundred and eighteen genes showed significantly different expression by two criteria,

Inbred line name	DI
RJKB-T04	0.00 ± 0.00^a (−)[b]
RJKB-T15	0.00 ± 0.00 (−)
RJKB-T17	0.00 ± 0.00 (−)
RJKB-T21	0.00 ± 0.00 (−)
RJKB-T23	0.00 ± 0.00 (−)
RJKB-T25	0.00 ± 0.00 (−)
RJKB-T13	2.00 ± 0.00 (+)
RJKB-T14	1.86 ± 0.14 (+)
RJKB-T22	2.00 ± 0.00 (+)
RJKB-T24	2.00 ± 0.00 (+)

Fig. 1 Inoculation test of fusarium yellows. [a]Average of disease index scores among 10 plants. Disease index score: DI = 0, no symptoms, DI = 1: plant yellowing, DI = 2: plant death. [b]Classification of fusarium yellows resistance; +, susceptible (DI ≥ 1.86); −, resistant (DI = 0.00)
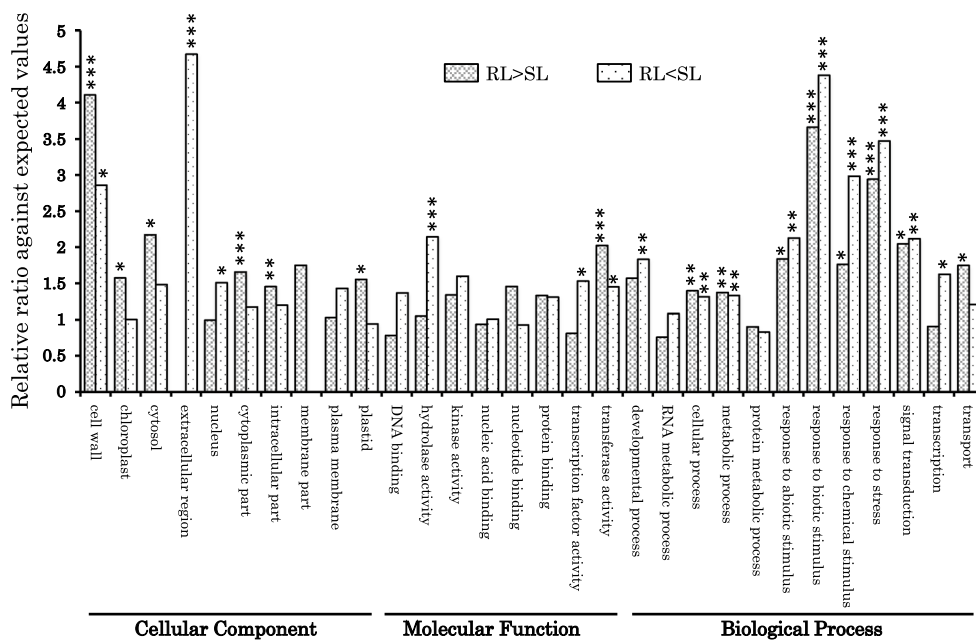
Fig. 2 GO classification of genes differentially expressed between fusarium yellows resistant line RJKB-T23 (RL) and susceptible line RJKB-T24 (SL). The y-axis is the relative ratio compared with the percentage of all annotated genes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. RL > SL, genes showing higher expression level in RJKB-T23 than in RJKB-T24. RL < SL, genes showing lower expression level in RJKB-T23 than in RJKB-T24

twofold difference (log2 ratio ≥1.0) and 95 % confidence (Figure S1). Of 418 genes, 183 (43.8 %) genes showed a higher expression level in the resistant line RJKB-T23 than in the susceptible line RJKB-T24 (RL > SL expression), and 235 genes (56.2 %) were more highly expressed in the susceptible line RJKB-T24 than in the resistant line RJKB-T23 (RL < SL expression) (Table S3).

We categorized the differentially expressed genes between resistant and susceptible lines into GO cellular component, GO molecular function, and GO biological process (Table S4). In the genes showing RL > SL

expression, genes categorized into 'Cell wall', 'Cytoplasmic part', and 'Intracellular part' in GO cellular component and 'Transferase activity' in GO molecular function were overrepresented ($p < 0.01$) (Fig. 2; Table S4). The genes categorized into 'Cellular process', 'Metabolic process', 'Response to biotic stimulus', and 'Response to stress' in GO biological process were overrepresented ($p < 0.01$) (Fig. 2; Table S4). In the category of 'Response to stress', genes categorized into subgroup 'Defense response' and 'Response to oxidative stress' were overrepresented. Not only the putative *R*-genes but also *ACD6* (Accelerated cell

death 6), *CCR2* (Cold, Circadian rhythm, and RNA biding 2), *CRK11* (Cysteine-rich RLK11), *ELI3-1* (Elicitor-activated gene 3-1), *ESP* (Epithiospecifier protein), *MBP2* (Myrosinase-binding protein 2), *MIPS2* (Myo-inositol-1-phostpate synthase 2), and *RLP54* (Receptor like protein 54) were involved in the category of 'Defense response'. In the category of 'Response to oxidative stress', *ALPHA-DOX2* (Alpha-dioxygenase 2), *APX1* (Ascorbate peroxidase 1), *CAT3* (Catalase 3), *CRK11*, *FER1* (Ferritin 1), *FER3*, *GSTU5* (Glutathione S-transferase TAU 5), and *MIPS2* were involved.

In the genes showing RL < SL expression, genes categorized into 'Extracellular region' in GO cellular component and 'Hydrolase activity' in GO molecular function were overrepresented ($p < 0.01$) (Fig. 2; Table S4). The genes categorized into 'Developmental process', 'Cellular process', 'Metabolic process', 'Response to abiotic stimulus', 'Response to biotic stimulus', 'Response to chemical stimulus', 'Response to stress', and 'Signal transduction' in GO biological process were overrepresented ($p < 0.01$) (Fig. 2; Table S4). In the category of 'Response to stress', genes categorized into subgroup 'Defense response' and 'Response to wounding' were overrepresented. In the category of 'Defense response', *AHK3* (Arabidopsis histidine kinase 3), *BGL2* (Beta-1, 3-glucanase 2), *ERF4* (Ethylene responsive element binding factor 4), *NDR1* (Non race-specific disease resistance 1), *PGIP1* (Polygalacturonase inhibiting protein 1), *PR-3* (Pathogenesis-related 3), and *SOBER1* (Suppressor of avrbst-elicited resistance 1) were involved as well as putative disease resistance (*R*) genes. The *NUDT8* (Nudix hydrolase homolog 8), *LDOX* (Leucoanthocyanidin dioxygenase), *PR-3*, *SEN1* (Senescence1), and *WR3* (Wound-responsive 3) were involved in the category of 'Response to wounding'.

Screening of the *R*-genes showing differential expression

We screened for putative *R*-genes showing differential expression between fusarium yellows resistant RJKB-T23 and susceptible RJKB-T24 lines. In the Brassica database (http://brassicadb.org/brad/), there are 244 putative *R*-genes that have NBS, LRR, TIR, or CC motifs and are sub-categorized into 16 families (Figure S2). Of 418 differentially expressed genes, nine genes were putative *R*-genes; six genes, Bra006556, Bra008053, Bra008055, Bra008056, Bra022071, and Bra027791, showed RL > SL expression, and three genes, Bra006146, Bra018834, and Bra018863, showed RL < SL expression (Figure S1, Table S3). We also included the putative *R*-genes showing only a twofold differential expression level without 95 % confidence or no expression in either line in further analyses, although lower expressed genes tended to be identified and statistical reliability is low. Sixty-one putative *R*-genes showed a twofold

differential expression level between resistant RJKB-T23 and susceptible RJKB-T24 lines (Figure S1, Table S5).

Our conclusions from RNA-seq analysis were verified using quantitative real time RT-PCR (qPCR) analysis on nineteen differentially expressed putative *R*-genes. Ten genes showed RL > SL and the remaining nine genes showed RL < SL expression (Table S3, S5). In RL > SL expressed genes, six genes, Bra008055, Bra008056, Bra012540, Bra012688, Bra012689, and Bra022071, showed no amplification of a PCR product in the susceptible line RJKB-T24 using genomic DNA as a template. The remaining four genes showed RL > SL expression by qPCR (Fig. 3). In RL < SL expression, one gene, Bra012116, was not amplified in the resistant line RJKB-T23 using genomic DNAs as a template. All 8 genes except for Bra012541 showed RL < SL expression by qPCR (Fig. 3). These results indicated that expression of most of the genes was consistent with the RNA-seq data.

Linkage analysis

We identified seven dominant DNA markers in the putative *R*-genes (Bra008055, Bra008056, Bra012116, Bra012540, Bra012688, Bra012689, Bra022071), which amplify PCR products in either RJKB-T23 or RJKB-T24. We tested these DNA markers on five more fusarium yellows resistant lines (RJKB-T4, 15, 17, 21, 25) and three more susceptible lines (RJKB-T13, 14, 22). PCR results using DNA markers termed Bra012688m and Bra012689m in Bra012688 and Bra012689, respectively, were consistent with inoculation tests as these markers were only amplified from fusarium yellows resistant lines (Table 1).

To test whether the two markers are linked to the disease resistance, we performed linkage analysis using F$_2$ populations derived from two hybrids between fusarium yellows susceptible and resistant lines, RJKB-T24 × RJKB-T23 and RJKB-T22 × RJKB-T21. The two RJKB-T24 × RJKB-T23 and RJKB-T22 × RJKB-T21 hybrids were tested by inoculation and both were resistant to fusarium yellows, indicating that fusarium yellows resistance is a dominant character. In the 102 plants of the F$_2$ population derived from RJKB-T24 × RJKB-T23, 74 and 28 plants were resistant and susceptible to fusarium yellows, respectively. In the 109 plants of the F$_2$ population derived from RJKB-T22 × RJKB-T21, 76 and 33 plants were resistant and susceptible to fusarium yellows, respectively. In both F$_2$ populations, resistance and susceptibility segregated in a 3:1 ratio (Table S6), indicating that fusarium yellows resistance is regulated by a single dominant gene. We tested the Bra012688m and Bra012689m in two F$_2$ populations, and 102 of 102 (RJKB-T24 × RJKB-T23 F$_2$) (100 %) and 108 of 109 (RJKB-T22 × RJKB-T21 F$_2$) (99 %) were consistent with the inoculation
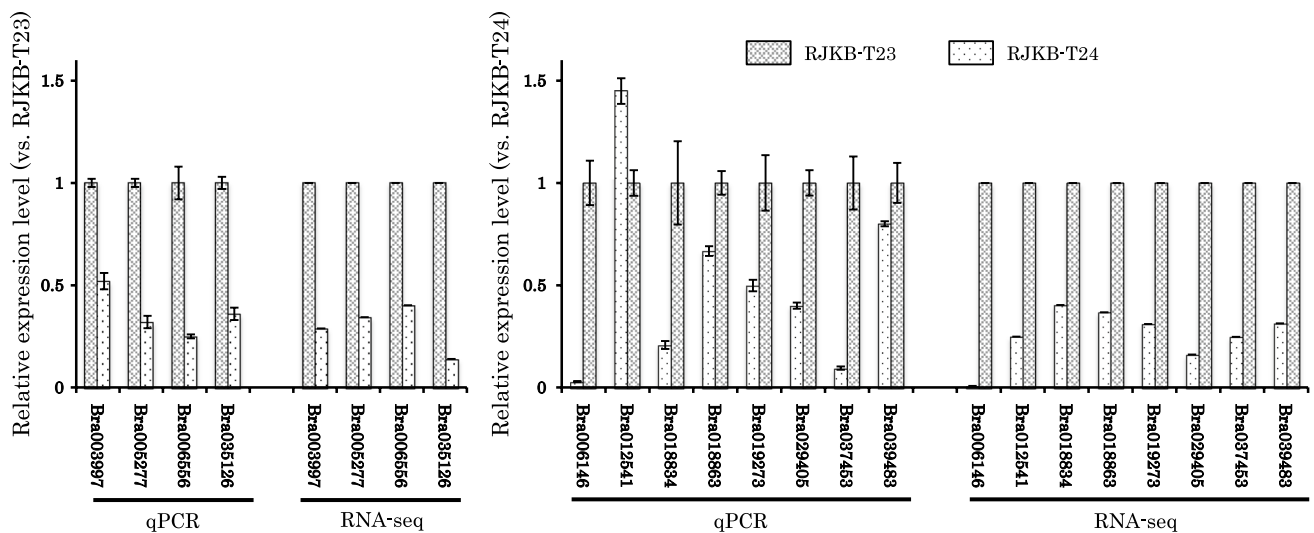
Fig. 3 The verification of RNA-seq data by quantitative real time PCR (qPCR). The y axis is the relative expression level compared with that of RJKB-T23 (*left panel*) and RJKB-T24 (*right panel*)

Table 1 Comparison between DNA-marker based genotype and fusarium yellows phenotype by inoculation test

	Phenotype	Bra008055m	Bra008056m	Bra012116m	Bra012540m	Bra012688m	Bra012689m	Bra022071m
RJKB-T04	R	+	+	−	+	+	+	−
RJKB-T15	R	+	+	−	−	+	+	+
RJKB-T17	R	+	+	−	+	+	+	+
RJKB-T21	R	+	+	−	+	+	+	+
RJKB-T23	R	+	+	−	+	+	+	+
RJKB-T25	R	+	+	+	+	+	+	+
RJKB-T13	S	+	+	+	−	−	−	−
RJKB-T14	S	+	+	+	−	−	−	−
RJKB-T22	S	+	+	−	−	−	−	−
RJKB-T24	S	−	−	+	−	−	−	−

R; resistance to fusarium yellows, S; susceptible to fusarium yellows

+ and − indicate amplification and no amplification of PCR product, respectively

tests, indicating that these two genes are strong candidates for the fusarium yellows resistance locus (Fig. 4).

In the database from Chiifu-401-42, Bra012688 and Bra012689 were arranged tandemly in the same orientation and encoded TIR–NBS–LRR proteins (Fig. 5). Amino acid identity between them was 45.2 %, and amino acid identities between Bra012688 and the putative orthologous At5g51630 and between Bra012689 and putative orthologous At4g16890 were 47.6 and 46.9 %, respectively, higher than that between Bra012688 and Bra012689. A neighbor-joining tree showed that Bra012688 and Bra012689 were not clustered in the same clade, suggesting that Bra012688 and Bra012689 were not generated by a recent duplication (Fig. 6). In the fusarium yellows resistant line RJKB-T23, Bra012688 and Bra012689 had 100 % amino acid identity

to the Bra012688 and Bra012689 in Chiifu-401-42, respectively. The commercial cultivar "Chiifu" showed fusarium yellows resistance by an inoculation test (data not shown). We tested PCR amplification using eight and seven primer sets in Bra012688 and Bra012689, respectively, and no amplification was seen in the fusarium yellows susceptible line RJKB-T24, while PCR products were detected with all primer sets in the fusarium yellows resistant line RJKB-T23 (Figure S3). No amplification of Bra012686m and Bra012687m was seen in the fusarium yellows susceptible lines. In contrast, Bra012685m and Bra012690m showed PCR amplification in the fusarium yellows susceptible RJKB-T24 line. All primer sets (Bra012685m–Bra012690m) were amplified in fusarium yellows resistance line RJKB-T23 (Fig. 5). These results suggest that the

Fig. 4 Linkage analysis of F$_2$ population derived from RJKB-T24 × RJKB-T23 hybrid using Bra012688m (*upper panel*) and Bra012689m (*lower panel*) markers. *R* and *S* show resistance and susceptibility to fusarium yellows, respectively
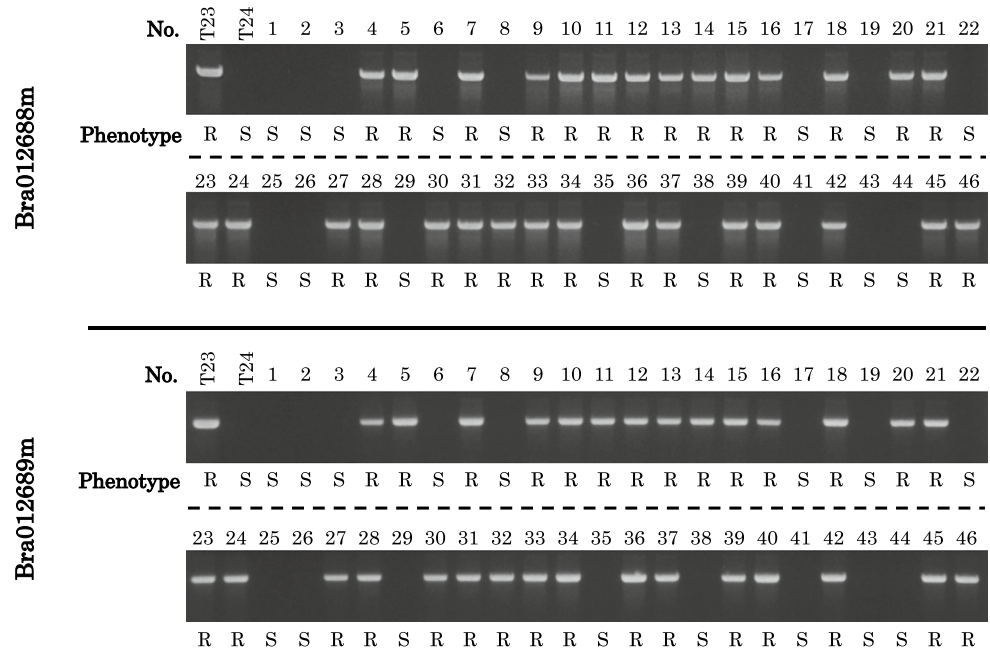
Fig. 5 Schematic view of the alignment of genes around Bra012688 and Bra012689 (*upper panel*). *Gray boxes* show exon regions. *Arrows* show the position of primers. The result of PCR using RJKB-T23 and RJKB-T24 using genomic DNAs as templates is shown in bottom panel. 685, 686, 687, 688, 689, and 690m are DNA markers in Bra012685, Bra012686, Bra012687, Bra012688, Bra012689, and Bra0126890, respectively
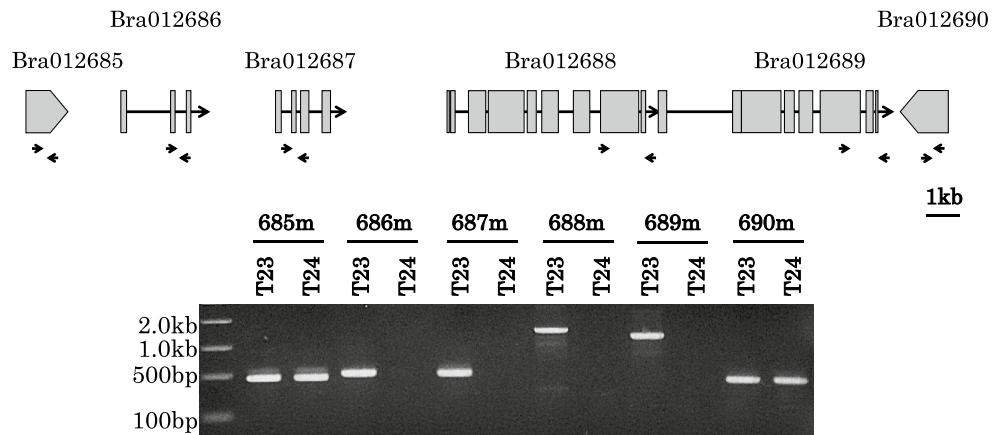
region from Bra012686 to Bra012689 has been deleted in the fusarium yellows susceptible line RJKB-T24 leading to the loss of fusarium yellows resistance.

Discussion

The category of 'Defense response' tended to be overrepresented in genes differentially expressed between fusarium yellows resistant and susceptible lines

To identify the fusarium yellows resistance gene, we performed differential expression analysis between resistant (RJKB-T23) and susceptible (RJKB-T24) inbred lines by RNA-seq. Although there are reports showing that infection with a disease agent causes activation of signal transduction pathways (Glazebrook 2005; Berrocal-Lobo and

Molina 2008; Zhu et al. 2013), we used non-inoculated tissues. One suggestion is that a fusarium yellows resistant line has already gained either higher expression level of a resistance gene or a functional gene relative to a fusarium susceptible line. We can also get information on the single nucleotide polymorphisms (SNPs) in coding regions, which are useful DNA markers for map based cloning.

GO analysis indicated categories in 'Cell wall', 'Cytoplasmic part', 'Intracellular part' and 'Transferase activity' were overrepresented in RL > SL expressed genes, while 'Extracellular region' and 'Hydrolase activity' were overrepresented in RL < SL expressed genes, suggesting that these genes may be involved in the physical or physiological difference between two lines. In contrast, the categories of 'Response to biotic stimulus' and 'Response to stress' were overrepresented in both RL > SL and RL < SL expressed genes, especially the subcategory of 'Defense response' in
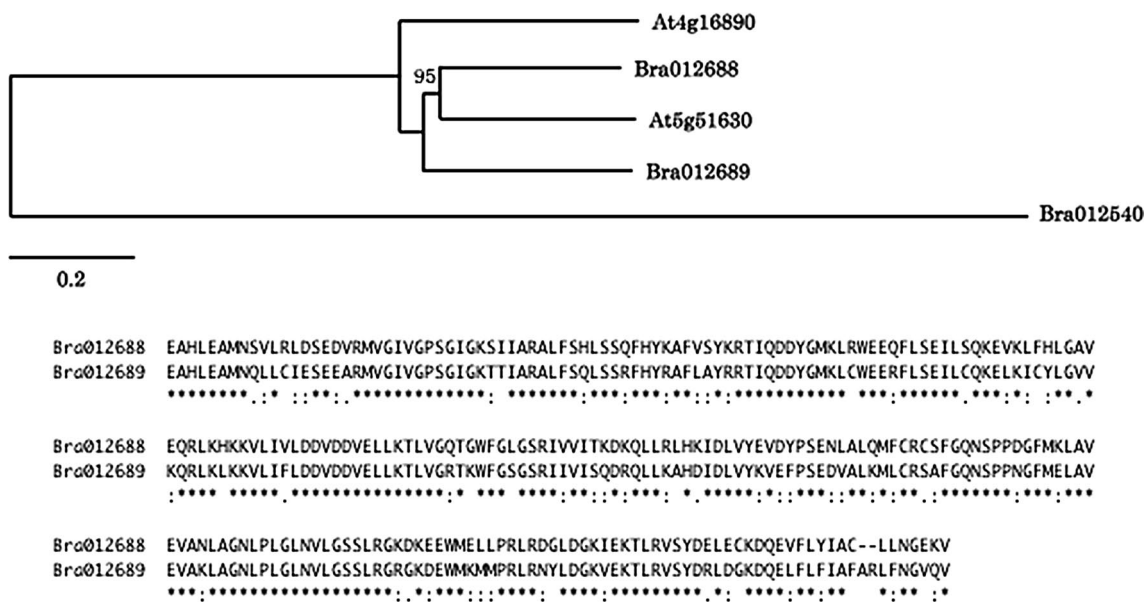
Fig. 6 Comparison of deduced amino acid sequences between Bra012688 and Bra012689. A neighbor-joining tree of amino acid sequences of Bra012540, Bra012688, Bra012689, At4g16890, and At5g51630. Bootstrap value with 1,000 repeats is indicated at the node of the neighbor-joining tree (*upper panel*). Alignment of amino acid sequences of NBS domain in Bra012688 and Bra012689 (*lower panel*)

'Response to stress'. In the category of 'Defense response', the genes involved in cell death, signal transduction (receptor-like kinase), or metabolizing glucosinolates showed RL > SL expression in addition to *R*-genes. A gene having sequence similarity to *ACD6* in *A. thaliana* showed RL > SL expression; an active allele of *ACD6* enhances resistance to a broad range of pathogens in *A. thaliana* (Todesco et al. 2010). In RL < SL expressed genes categorized into 'Defense response', the defense related genes, *PR* genes, and ortholog of *AtPGIP1*, which inhibits the function of polygalacturonase produced by pathogens, were found in addition to *R*-genes or genes encoding cytokine receptor or ethylene response factor (Ferrari et al. 2003, 2013). The *NDR1* ortholog also showed RL < SL expression, and overexpression of *NDR1* and *NDR1* homologs in *A. thaliana* and the *NDR1* ortholog in coffee and citrus increase disease resistance (Century et al. 1997; Varet et al. 2003; Cacas et al. 2011; Lu et al. 2013). The activation of genes categorized into 'Defense response' could be involved in disease resistance, but this category was overrepresented in both RL > SL and RL < SL expressed genes, suggesting that overrepresentation of 'Defense response' is independent from fusarium yellows resistance. These genes may be involved in the resistance to other pathogens.

Two candidate *R*-genes were identified by differential expression and segregation analysis

In *A. thaliana*, the fusarium resistance gene, *RESIST-ANCE TO FUSARIUM OXYSPORUM 1* (*RFO1*), has been identified by QTL analysis (Diener and Ausubel 2005). Bra035134 showed highest amino acid sequence homology to RFO1, but it is located on the end of chromosome 7 in *B. rapa*, indicating that the *RFO1* ortholog is not involved in fusarium yellows resistance in *B. rapa*. Most *R*-genes have been classified into NBS–LRR type genes (TIR–NBS–LRR or CC–NBS–LRR) (Joshi and Nayak 2011; Dodds and Rathjen 2010; Muthamilarasan and Prasad 2013). *R*-genes in tomato against *F. oxysporum* f. sp. *lycopersici race 2*, *I-2*, encode an NBS–LRR (non-TIR) protein (Simons et al. 1998), and the *R*-gene in melon against *F. oxysporum* f. sp. *melonis*, *Fom-1* and *Fom-2*, encode TIR–NBS–LRR and NBS–LRR proteins, respectively (Joobeur et al. 2004; Brotman et al. 2013). These results suggest proteins having these domains are candidates for fusarium yellows resistance genes in Chinese cabbage, although we could not find the proteins having sequence homology to known fusarium resistance genes, *I-2*, *Fom-1* and *Fom-2*. Thus we focused on putative *R*-genes to identify a fusarium yellows resistance gene. From the RNA-seq data, we identified putative *R*-genes differentially expressed between fusarium yellows resistant and susceptible lines. Nine *R*-genes showed a twofold difference with 95 % confidence limit and 61 genes showed a twofold difference without 95 % confidence (Figure S1). Of these 61 genes, 7 out of 8 showed differential expression by qPCR analysis. In total we found seven *R*-genes deleted from each parent among nineteen genes examined. Using 10 inbred lines of Chinese cabbage and seven dominant DNA

markers, we found deletion of Bra012688 and Bra012689 were related to susceptibility to fusarium yellows. Inoculation tests in two F_2 populations derived from hybrids between fusarium yellows resistant and susceptible lines, and the clear relationship between deletion of these two markers was likely to be associated with fusarium yellows resistance. The correlation was 100 % except for one plant derived from the RJKB-T22 and RJKB-T21 hybrid. This one plant showed a fusarium yellows resistant genotype but showed susceptibility by the inoculation test. Previously we have shown that some heterozygous *B. oleracea* plants show weakened fusarium yellows resistance at higher temperature (Pu et al. 2012), suggesting that this one exception might be due to an environmental effect and not to a cross over event. Taken together, we conclude that Bra012688 and Bra012689 are candidate genes for fusarium yellows resistance. Amino acid sequence identity between Bra012688 and Bra012689 was not high and neighbor-joining analysis including R proteins from *A. thaliana* showed that Bra012688 and Bra012689 were clustered with At5g51630 and At4g16890, respectively, indicating that Bra012688 and Bra012689 were not generated by a recent duplication event. Recently a single major effect QTL termed *RFO7* covering At5g51630 has been found using BC_1F_1 between Col-0 and Ty-0 accessions of *A. thaliana*, which show partial resistance to *F. oxysporum* f. sp. *conglutinans* race 1 (Diener 2013), suggesting that the ancestral genes of fusarium yellows were the same between two species. However At5g51630 has not been shown to function as a disease resistance gene, though At5g51630 encodes a TIR–NBS–LRR protein. By contrast, At4g16890 encodes a TIR–NBS–LRR termed *suppressor of npr1-1, constitutive 1* (*SNC1*) (Li et al. 2001; Zhang et al. 2003), identified as a suppressor mutant of *npr1-1* (Li et al. 2001), which blocks the induction of systemic acquired resistance by signals such as salicylic acid (Cao et al. 1997). *snc1* shows a dwarf phenotype, constitutive expression of *PR* genes, and enhanced disease resistance (Li et al. 2001). Transgenic cotton having *A. thaliana* SNC1 acquired significant resistance to *F. oxysporum* f. sp. *vasinfectum* (Lei et al. 2012), suggesting that SNC1 has a function in resistance against some pathogens. Recently a dual resistance gene system with a structure of two adjacent and head-to-head oriented NBS–LRR genes has been reported (Narusaka et al. 2009; Okuyama et al. 2011; Ashikawa et al. 2008; Lee et al. 2009; Brotman et al. 2013), but Bra012688 and Bra012689 are arranged tandemly in the same orientation. Further experiments will be needed to clarify which genes play a role in fusarium yellows resistance or whether both genes act together like in the dual gene resistance system.

Recently next-generation sequencing has been used for isolation of the causal mutation of a phenotype or for detection of quantitative trait locus (QTL) (Schneeberger

et al. 2009; Austin et al. 2011; Abe et al. 2012; Takagi et al. 2013). In this study, we succeeded in identifying the candidate *R*-genes of fusarium yellows using RNA-seq in a shorter time compared with a map-based cloning approach. The other putative *R*-genes showing differential expression between the two lines may be involved in resistance to other diseases. We also found differential expression of the clubroot resistance gene, *Crr1a* (Hatakeyama et al. 2013), and different level of clubroot resistance between RJKB-T22 and -T23 (Shimizu et al. unpublished data). Using RNA-seq, we found more than 100,000 SNPs in the exon regions of about 12,000 genes (Kawanabe et al. unpublished data), a quarter of the total genes in *B. rapa,* with a wide distribution over the whole genome. Thus we can use a map-based cloning approach using these SNPs. We found SNPs in putative *R*-genes, which may become direct DNA markers for resistance gene against other diseases.

The resistance locus in Chinese cabbage is syntenic to the resistance locus in cabbage

In this study, we showed that *F. oxysporum* f. sp. *conglutinans* can infect some lines of Chinese cabbage (*Brassica rapa*) and one locus covering Bra012688 and Bra012689 is sufficient for resistance. This result is similar to our previous study that one locus covering a fusarium resistance gene(s) located on O7 is sufficient for resistance to f. sp. *conglutinans* in cabbage (*B. oleracea*) (Pu et al. 2012). The fusarium yellows resistance locus in *B. rapa* has synteny with the central region of *A. thaliana* chromosome 4, and the resistance locus in *B. oleracea* has synteny with the same region in *A. thaliana*, suggesting that the ancestral resistance gene(s) against f. sp. *conglutinans* is the same between *B. rapa* and *B. oleracea*. However we could not find a deletion in a broccoli line, Greencomet, susceptible to f. sp. *conglutinans,* or in commercial F_1 hybrid cultivars in cabbage or broccoli. We did detect the deletion of Bra012688 and Bra012689 in some inbred or doubled haploid lines of other *B. rapa* varieties such as turnip and Komatsuna, suggesting that the deletion detected in susceptible lines of *B. rapa* occurred after speciation between *B. rapa* and *B. oleracea*. We should confirm that the ortholog of Bra012688 or Bra012689 or both is/are resistant gene(s) against f. sp. *conglutinans* in *B. oleracea*.

It has been reported that *F. oxysporum* f. sp. *rapae* and f. sp. *raphani* were also infectious to *B. rapa* (Enya et al. 2008). In radish (*Raphanus sativus*), fusarium resistance against f. sp. *raphani* is quantitative (Yu et al. 2013), suggesting that the mechanism of fusarium resistance is different between f. sp. *raphani* and f. sp. *conglutinans*. However a major QTL in *R. sativus* has been detected in the region having synteny to the region covering the resistance gene(s) against f. sp. *conglutinans* located on O7 in

B. oleracea (Yu et al. 2013), suggesting that the ortholog of the fusarium resistance gene against f. sp. *conglutinans* together with other genes may be involved in resistance against f. sp. *raphani* in *R. sativus*. There is no report of molecular identification of resistance genes against f. sp. *rapae* in Brassicacea. Further study will be required to clarify whether Bra012688 or Bra012689 are also involved in resistance against f. sp. *rapae* or f. sp. *raphani* in *B. rapa*.

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