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ORIGINAL PAPER

Identification of genomic regions involved in resistance against *Sclerotinia sclerotiorum* from wild *Brassica oleracea*

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Abstract The lack of resistant source has greatly restrained resistance breeding of rapeseed (*Brassica napus*, AACC) against *Sclerotinia sclerotiorum* which causes severe yield losses in rapeseed production all over the world. Recently, several wild *Brassica oleracea* accessions (CC) with high level of resistance have been identified (Mei et al. in Euphytica 177:393–400, 2011), bringing a new hope to improve *Sclerotinia* resistance of rapeseed. To map quantitative trait loci (QTL) for *Sclerotinia* resistance from wild *B. oleracea*, an F2 population consisting of 149 genotypes, with several clones of each genotypes, was developed from one F1 individual derived from the cross between a resistant accession of wild *B. oleracea* (*B. incana*) and a susceptible accession of cultivated *B. oleracea* var. *alboglabra*. The F2 population was evaluated for *Sclerotinia* reaction in 2009 and 2010 under controlled condition. Significant differences among genotypes and high heritability for leaf and stem reaction indicated that genetic components accounted for a large portion of the phenotypic variance. A total of 12 QTL for leaf resistance and six QTL for stem resistance were identified in 2 years, each explaining 2.2–28.4 % of the phenotypic variation. The combined effect of alleles from wild *B. oleracea* reduced the relative susceptibility by 22.5 % in leaves and 15 % in stems on average over 2 years. A 12.8-cM genetic region on chromosome C09 of *B. oleracea* consisting of two major QTL intervals for both leaf and stem resistance was assigned into a 2.7-Mb genomic region on chromosome A09 of *B. rapa*, harboring about 30 putative resistance-related genes. Significant negative corrections were found between flowering time and relative susceptibility of leaf and stem. The association of flowering time with *Sclerotinia* resistance is discussed.

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Introduction

Sclerotinia stem rot, caused by the fungal pathogen *Sclerotinia sclerotiorum*, infects more than 400 plant species including several important crops such as sunflower, chickpea and rapeseed (Boland and Hall 1994, Purdy 1979). The pathogen usually infects plants as mycelia or airborne ascospores (Jamaux et al. 1995). Although a few fungicides are available to manage this disease, the low efficiencies, the environmental contaminations caused by the chemicals and the economic costs cannot be ignored (del Río et al. 2007). Thus, breeding resistant varieties is the best strategy to control this disease (Zhao et al. 2004).

Rapeseed (*Brassica napus*, AACC), which is the second most important oilseed crop in the world after soybean, suffered seriously from *S. sclerotiorum* (Koch et al. 2007; Pope et al. 1989). Although partial resistance was reported

in some genotypes of *B. napus* (Falak et al. 2011; Li et al. 1999; Wang et al. 2004; Zhao and Meng 2003), completely or highly resistant lines of rapeseed are not available. The lack of a resistant source has greatly restrained the resistance breeding of *B. napus*, introducing little practical benefit from the *Sclerotinia* resistance-related researches in rapeseed (Bradley and Hamey 2005; Yang et al. 2007; Zhao and Meng 2003; Zhao et al. 2004, 2006, 2007).

Considering the wide genetic diversity in Brassicaceae, resources with high levels of resistance against *S. sclerotiorum* may exist in the relatives of *B. napus*. Some efforts have been made to identify resistance resources from wild crucifers such as *Erucastrum cardaminoides* (Garg et al. 2010), *E. abyssinicum* (Garg et al. 2010), *E. gallicum* (Lefol et al. 1997; Seguin-Swartz and Lefol 1999) and *Capsella bursa-pastoris* (Chen et al. 2007). More recently, we have identified resources with high level of resistance against *S. sclerotiorum* from wild *B. oleracea* (Mei et al. 2011), one of the parental species of rapeseed (U 1935). This finding brings a new hope to improve *Sclerotinia* resistance of rapeseed.

Thereby, a resistant accession of wild *B. oleracea* (*B. incana*) was employed to cross with a susceptible cultivar, and an F2 population, with several clones of each F2 genotype, was developed from one individual F1 plant. The objective of this study was to map the quantitative trait loci (QTL) for *Sclerotinia* resistance from wild *B. oleracea*. Meanwhile, the association of *Sclerotinia* reaction with flowering time was investigated.

Materials and methods

Parental materials and population construction

According to our previous research (Mei et al. 2011), a wild *B. oleracea* accession ('C01', *B. incana*) with high level of resistance against *S. sclerotiorum* and late flowering time was employed to pollinate to a cultivated accession ('C41', *B. oleracea* var. *alboglabra*) with low level of resistance and early flowering time. An F2 population, consisting of 149 F2 genotypes, was developed from one individual F1 plant. Clones were developed for each F2 genotype by asexually reproducing the stem according to Luo et al. (2000).

The F2 population was transplanted in the experimental field of Southwest University, Chongqing (China) in 2009 and 2010 together with the F1, the two parents and a control, 'Zhongyou 821' which is recognized as a partial resistant rapeseed cultivar in China (Li et al. 1999). A randomized complete block design was adopted, with two replications. Each plot consisted of 10 clones, with 30 cm between rows and 25 cm within rows.

Phenotypic measurements

The *S. sclerotiorum* isolate used in our previous study (Mei et al. 2011) was maintained and cultured on potato dextrose agar (PDA) medium (20 % potato, 2 % dextrose and 1.5 % agar) in the dark at 22 °C and 6-mm-diameter mycelia agar plugs punched from the growing margin of 3-day-old culture of *S. sclerotiorum* were used as inoculums.

The Sclerotinia resistance of each plant was evaluated according to Mei et al. (2011, 2012). In brief, the fourth leaves at nine-to-twelve-leaf stage and stems at flowering stage were detached to evaluate resistance under controlled conditions. Differing with leaf inoculation, stems were treated prior to inoculation as follows: stem segments of 30 cm length were excised at a height of 10 cm above the ground, and the two ends of each stem segment were wrapped with polyethylene film to keep fresh. Two wounds with a 10-cm interval were artificially created on each stem by a 4-mm-diameter puncher. The detached leaves or injured stems were placed on a platform (2 m × 2 m) which was covered with moist towels and filter papers, and the 6-mm-diameter mycelia agar plugs were then placed above the leaves or the wounds of stems. A 50-cm-height frame was then placed above the platform and sealed together with the platform by plastic film. The infection temperature was maintained at 22 °C. Lesion size of inoculated leaves 3 days after inoculation (DAI) and lesion length of inoculated stems 4 DAI were recorded.

The resistance was evaluated under controlled condition for two times in each year, and there were slight differences in the development of tissues. In order to reduce such interference, relative susceptibility (*S*) compared to 'Zhongyou 821' was calculated based on the equation $S = V/V_{\text{control}}$, where V is the value of the accession tested for leaf (lesion size) or stem reaction (lesion length), while V_{control} is that of 'Zhongyou 821'.

Flowering time was recorded as the interval from transplanting to the day when the first flower emerged on half of the plants in a plot. The flowering time of 5 % of F2 genotypes which did not flower before stem resistance evaluation was scored as the time of stem resistance identification plus 30 days.

Statistical analyses

Analyses of variance (ANOVA) were performed using Proc GLM in SAS software (SAS Institute 1992). The broad-sense heritability (h^2) was estimated as described by Hallauer and Miranda (1988):

$$h^2 = \sigma_g^2 / \left(\sigma_g^2 + \sigma_{ge}^2 / e + \sigma^2 / r \times e \right),$$

where σ_g^2, σ_{ge}^2 and σ^2 are estimates of genotypic, genotype by environment interaction and error variances, respectively,

e and *r* represent the number of environments and replications per environment. Pearson's simple correlations were calculated between traits of interest (SAS Institute 1992).

Molecular markers assays

Total DNA was extracted from young leaves of each line using the CTAB method (Saghai-Maroof et al. 1984) for molecular marker polymorphism analyses such as simple sequence repeats (SSR), sequence-related amplified polymorphism (SRAP) and amplified fragment length polymorphism based on resistance gene analogs (AFLP-RGA). SSR-primers were derived from references (Gao et al. 2011; Iniguez-Luy et al. 2009; Li et al. 2011) and www.brassica.info, except for the 'CEN-' and 'SWUC-' primers which were explored according to genome sequences of *Brassica* species (see Online Resource 1). PCR products of SSR and SRAP were separated on 10 % polyacrylamide gels and stained with silver nitrate. Degenerate RGA primers were designed according to Fourmann et al. (2001) and AFLP-RGA reaction was performed according to Niu et al. (2011) and Zhang et al. (2007). The PCR products were separated on a 7 % polyacrylamide gel in a Licor 4300 DNA analyzer and distinct bands with fragment sizes from 100 to 600 bp were scored.

Linkage map construction, QTL mapping and interaction analysis

A linkage map was constructed using the software package JoinMap3 (Stam 1993) at a LOD threshold of 8.0 and assigned to published maps of *B. oleracea* (Handley et al. 2005; Li et al. 2011) based on common markers. The composite interval mapping (CIM) procedure in the software WinQTL Cartographer 2.5 was used to scan QTL (Wang et al. 2011b). A 1,000-permutation was performed to estimate a significance threshold of the test statistics for a QTL based upon a 5 % experiment-wise error rate. Linkage groups and QTL were visualized using the MapChart software (Voorrips 2002). Epistatic interactions were estimated using QTLnetwork V2.0 software (Yang et al. 2008), in which a 1,000-permutation test was conducted to calculate critical *F* value, and significance level of 0.05 was set for putative QTL detection and interaction analyses.

Identification of synteny on the reference genome of *B. rapa*

In order to identify the synteny of resistant QTL region(s) on the reference genome of *B. rapa*, the sequence of molecular markers in the QTL intervals was assembled to the reference genome of *B. rapa* 'Chiifu-401' (http://brassicadb.org/brad/). The most significant hits returned by 'Blastn' were used to infer the putative physical positions of these markers on the *B. rapa* genome. To estimate putative resistance-related genes against *S. sclerotiorum* in *B. oleracea*, the function of genes in the syntenic genomic region of *B. rapa* was annotated according to those of *Arabidopsis thaliana*.

Results

Performance of parents and the F2 population

The *Sclerotinia* reactions of leaf and stem among F2 population and the two parents were evaluated for two times in each year, with two replications of each time. The two parents exhibited obvious differences in both leaf and stem reaction against the pathogen *S. sclerotiorum* (Fig. 1), being consistent with the previous observations (Mei et al. 2011). In the F2 population, the lesion size on leaves ranged from 5.0 to 12.3 cm^2 in 2009 and from 3.6 to 10.2 cm^2 in 2010. The lesion length on stems ranged from 2.8 to 10.4 cm in 2009 and from 2.5 to 7.0 cm in 2010. In order to compare resistance over years, the relative susceptibility in comparison to 'Zhongyou 821' was calculated. A continuous segregation for relative susceptibility in both leaf and stem was found in the F2 population and the resistance level of F1 was intermediate between the two

Fig. 1 Leaf and stem reactions of parents and F2 plants after infection by *S. sclerotiorum*. **a** The resistant parent 'C01', three F2 plants, the susceptible parent 'C41' and the partial resistant control 'Zhongyou 821' are shown from *left* to *right* (3 DAI); **b** 'Zhongyou 821', 'C01', two F2 plants and 'C41' are shown from *top* to *bottom* (4 DAI). *Bar* 1 cm

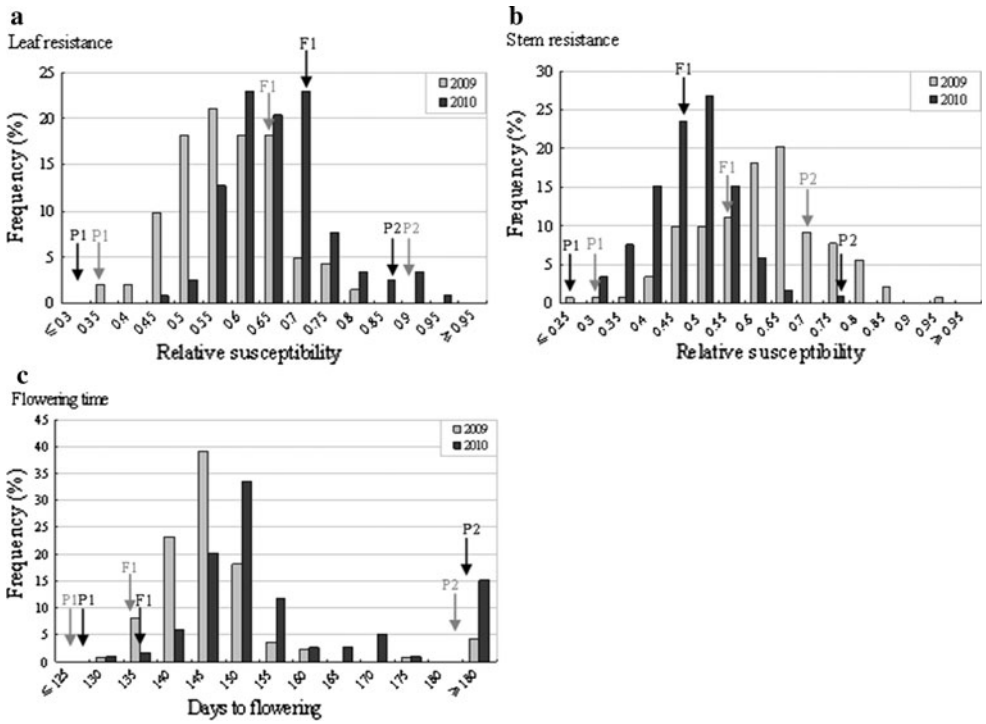

Fig. 2 Frequency distributions for *Sclerotinia* leaf and stem reaction and flowering time of the F2 population in 2009 and 2010. **a**, **b** and **c** show frequency distributions of leaf reaction, stem reaction and flowering time, respectively. The positions of P1 (resistant parent), P2 (susceptible parent) and F1 are marked with *arrows*

Table 1 Analysis of variance for flowering time (FT), leaf (LR) and stem reaction (SR) against *S. sclerotiorum* in the F2 population of *B. oleracea*

Source	Mean square (MS)		
	LR	SR	FT
Year	1.133*	1.643*	8,036.8*
Genotype	0.025*	0.018*	406.3*
Year × Genotype	0.008*	0.007	177.6*

* Significance at $P = 0.01$ level

parents (Fig. 2), suggesting multi-genic control of resistance in leaf and stem.

ANOVA for leaf and stem resistance in the F2 population is shown in Table 1. Significant differences were found among genotypes for leaf and stem reaction, while no significant genotype by environment interaction was detected for stem resistance, indicating that genetic variance accounted for a major portion of the phenotypic variance of *Sclerotinia* resistance especially stem resistance. The finding was in accordance with the high heritability estimated for leaf (83.1 %) and stem reaction (77.9 %). A low positive correlation was found between leaf and stem reaction in the 2 years (Table 2), indicating that the genetic control of leaf and stem resistance is probably different, but common genetic factors may be involved in the *Sclerotinia* reaction of the two tissues.

The distribution of flowering time among F2 genotypes was generally continuous (Fig. 2). Significant negative corrections of flowering time with relative susceptibility in leaf and stem were found in each year (Table 2).

Genetic map construction and QTL identification

Data of 440 segregating loci of the F2 population were used to construct the linkage map of *B. oleracea*, among which 267 loci were placed into nine linkage groups, spanning a genetic distance of 1,087.6 cM with an average distance of 4.1 cM between adjacent markers (see Online Resource 2). All the nine linkage groups were assigned to the respective chromosomes of *B. oleracea* according to published maps (Iniguez-Luy et al. 2009; Li et al. 2011).

The CIM procedure of WinQTL Cartographer was used to screen resistant QTL with a 1,000-permutation test. The significance threshold was estimated with LOD of 3.6 and 3.5 for leaf resistance and 3.5 and 4.5 for stem resistance in 2009 and 2010, respectively. The QTL for resistance were listed in Table 3. Six QTL of leaf resistance were identified each year, jointly explained 62.1 and 69.0 % of the phenotypic variation in 2009 and 2010, respectively. One QTL on C01 (*qLR09*-3) and two on C09 (*qLR09-5* and *qLR09-6*) identified in 2009 were repeatedly detected in 2010 (*qLR10-1*, *qLR10-5* and *qLR10-6*). Two and four QTL for stem resistance were identified in 2009 and 2010, totally explaining 32.8 and 31.9 % of the phenotypic variation, respectively. Two QTL for stem resistance on C09 were detected in both years (*qSR09-1* and *qSR09-2* in 2009, *qSR10-3* and *qSR10-4* in 2010), and had overlapping 1-LOD confidence intervals with QTL for leaf resistance

Table 2 Correlations between *Sclerotinia* leaf (LR) and stem reaction (SR) and flowering time (FT) of the F2 population of *B. oleracea* in 2009 and 2010

Trait	LR	SR	FT
LR	0.60**[a]	0.18*[c]	−0.26**[c]
SR	0.07[b]	0.41**[a]	−0.39**[c]
FT	−0.38**[b]	−0.33**[b]	0.71**[a]

[a] Correlation coefficient for one certain trait between year 2009 and year 2010

[b] Correlation coefficient between different traits in year 2009

[c] Correlation coefficient between different traits in year 2010

* and ** Significance at $P = 0.05$ and 0.01 level, respectively

(Fig. 3). The combined effect of alleles from the resistant donor parent reduced the relative susceptibility by 21 and 24 % in leaves and 13 and 17 % in stems in 2009 and 2010, respectively. No significant epistatic interaction was identified for stem resistance, while a few epistatic interactions were detected for leaf resistance, jointly explaining about 5 % of the total phenotypic variation of leaf resistance.

A total of 26 QTL concerning flowering time were detected in the 2 years, including 19 QTL in 2009 and 7 in 2010, jointly explaining 13.6 and 37.6 % of the total phenotypic variation, respectively (see Online Resource 3). Complex epistatic interactions were tested for flowering time, including a (additive) × a, a × d (dominant), d × d, a × a × e (environment), a × d × e and d × d × e, totally explaining about 26 % of the phenotypic variation of this trait. Three QTL were repeatedly identified in 2 years (*qFT09-2*, *qFT09-8* and *qFT09-19* in 2009, *qFT10-1*, *qFT10-3* and *qFT10-6* in 2010). It is interesting to note that one genetic region on C09 harbors QTL for flowering time, leaf resistance and stem resistance in both years.

Synteny on the *B. rapa* genome

In order to survey putative resistance genes, the sequences of 12 'SWUC-'markers in the region on C09 that colocalized two QTL for both leaf and stem resistance were blasted to the reference genome of *B. rapa* (http://brassicadb.org/brad/). Six of the 12 markers showed the most significant hits on A09 and exhibited the same order as those on C09 of *B. oleracea*, although the other six showed the most significant hits on different chromosomes such as A02, A03, A05 and A06 (see Online Resource 4). Thus, a 2.7-Mb genomic region on A09, starting from 1.6 Mb and ending at 4.3 Mb to the top of this chromosome, was delimited corresponding to the 12.8-cM genetic region on C09 in which two QTL for both leaf and stem reaction were colocalized. Almost 700 genes are assumed to be present in this genomic region of A09, among which about 30 genes encode putative resistance-related proteins such as those with the structure of zinc finger, leucine-zipper, LRR (leucine-rich repeat), CC (coiled-coil)-NBS (nucleotide binding site) or CC-NBS-LRR, and defense-associated proteins such as 1,3-beta-glucan synthase, pectinesterase inhibitor, peroxidase, proline-rich family proteins and oxidative-stress-responding proteins (see Online Resource 5). The information of sequences and candidate genes from the

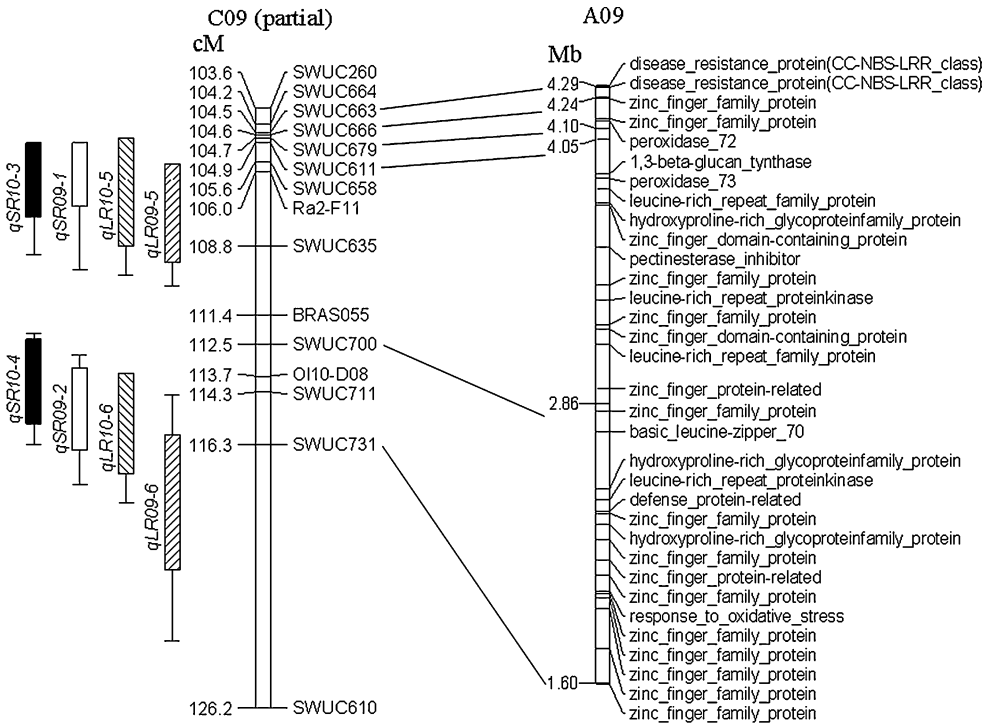

Fig. 3 Comparison of the *Sclerotinia*-resistance-associated region on C09 of *B. oleracea* with the reference genome of *B. rapa* by blasting the sequence of adjacent markers. The 12.8-cM genetic region on C09 located two major QTL for both leaf and stem resistance, while the corresponding 2.7-Mb genomic region of A09 harbors about 30 genes putatively encoding resistance-related and defense-associated proteins

Table 3 Putative QTL for resistance against *S. sclerotiorum* detected in the F2 population of *B. oleracea*

QTL[a]	LOD[b]	LG[c]	Position[d]	Marker[e]	Flanking marker	IL_LOD1[f]	Var %[g]	Add[h]	Dom[i]
2009LR									
qLR09-1	4.8	C01	90.8	E01M06/290	E01M06/290 ~ E01M04	1.3	9.3	0.0305	−0.0795
qLR09-2	4.5	C01	98.2	E03M18/380	E03M18/380 ~ SWUC277/550	0.2	7.2	0.0302	−0.0632
qLR09-3	4.7	C01	121.9	SWUC59/170	SWUC59/170 ~ Na12-C08	10.1	3.5	0.0243	0.038
qLR09-4	3.6	C03	163.6	E02M25/200	M32-RGA13R/320 ~ E02M25/200	2.1	7.0	0.0279	0.0874
qLR09-5	3.6	C09	106	Ra2-F11	SWUC658 ~ SWUC635	3.7	6.7	0.0334	−0.0071
qLR09-6	6.6	C09	118.3	SWUC731	SWUC700 ~ SWUC731	5.1	28.4	0.0671	−0.0025
2010LR									
qLR10-1	5.2	C01	123.5	SWUC150	SWUC59/170 ~ Na12-C08	8.5	13.6	0.0473	0.0071
qLR10-2	4.7	C03	165.2	FITO114	E02M25/190 ~ FITO114	1.2	10.8	0.0367	−0.0566
qLR10-3	4.4	C06	17.1	E07M09	SWUC177 ~ BoGMS1032	5.3	10.3	0.0378	−0.0473
qLR10-4	6.1	C06	25.2	E06M23	E06M23 ~ SWUC81	6.1	13.3	0.0404	−0.0611
qLR10-5	4.6	C09	104.9	SWUC611	SWUC679 ~ SWUC635	4.1	12.5	0.0452	−0.0242
qLR10-6	4.5	C09	116.3	SWUC731	Ol10-D08 ~ SWUC731	3.8	8.6	0.0352	−0.0024
2009SR									
qSR09-1	5.2	C09	105.7	SWUC658	SWUC611 ~ Ra2-F11	2.4	16.1	0.0644	−0.0312
qSR09-2	6.9	C09	113.7	Ol10-D08	Ol10-D08 ~ SWUC731	3.1	16.7	0.0623	−0.0127
2010SR									
qSR10-1	4.6	C04	82.7	E01M03/610	E01M03/610 ~ Ra2-E04	1.2	2.2	0.0156	0.2822
qSR10-2	5.3	C07	34.8	FITO398	FITO398 ~ BoGMS0545	5.7	3.4	−0.021	−0.0303
qSR10-3	6.0	C09	105.7	SWUC658	SWUC611 ~ Ra2-F11	2.8	13.6	0.0444	0.0002
qSR10-4	5.3	C09	112.6	SWUC700	SWUC700 ~ SWUC711	3.2	12.7	0.0401	0.0013

[a] QTL were designated using the initials of 'q' and the abbreviate of the trait, evaluation year, and a '−' followed by a number distinguishing from others

[b] Peak effect of the QTL (LOD, limit of detection)

[c] Linkage groups were designated according to Iniguez-Luy et al. (2009) and Li et al. (2003)

[d] Position (cM) of the closet marker of the peak effect of the QTL

[e] The closet marker of the peak effect of the QTL

[f] Length of 1-LOD score confidence interval (cM)

[g] Proportion of the phenotypic variation explained by the QTL

[h] Additive effect. Negative values indicate alleles from the susceptible parent 'C41' enhance resistance, positive values indicate alleles from the resistant parent 'C01' increase resistance

[i] Dominant effect. Positive values indicate alleles from the susceptible parent 'C41' are dominant, negative values indicate alleles from the resistant parent 'C01' are dominant

B. rapa reference genome will facilitate fine mapping and cloning of resistance genes against *S. sclerotiorum* from wild *B. oleracea*.

Discussion

In this study, the resistant parents and its progenies exhibited higher level of *Sclerotinia* resistance than 'Zhongyou 821' which is a recognized partial resistant accession in *B. napus* (Li et al. 1999). Although only part of the disease cycle (the expansion of lesion after infection) was tested in the present study, this finding is in accordance with our previous observation under the field condition that this wild *B. oleracea* accession possessed higher level of *Sclerotinia* resistance in comparison with several recognized partial resistant accessions of *B. napus* including 'Zhongyou 821' (Mei et al. 2011).

In the present study, we identified two important resistance-related regions on C09 which affected both leaf and stem resistance, whereas several independent studies on mapping of *Sclerotinia* resistance QTL in rapeseed (Yin et al. 2010; Zhao and Meng 2003; Zhao et al. 2006) did not find major QTL on N19 which corresponds to C09 of *B. oleracea*, although Zhao et al. (2006) detected two minor QTL on the same linkage group. These findings seem to indicate that this wild *B. oleracea*, differing from *B. napus*, is a unique source of resistance against

S. sclerotiorum. However, due to the different marker systems and accessions used in these studies, additional efforts are needed to compare these resistant QTL in different genetic backgrounds.

In the present study, significant negative correlations were observed between flowering time and relative susceptibility in both leaves and stems, and several regions were detected to have colocalizing QTL for flowering time and resistance, such as those on C01, C06 and C09 (Online Resource 2). Zhao et al. (2006) also found that *Sclerotinia* resistance QTL on N02 and N12 were in the same genomic regions as QTL for flowering time. It was reported that QTL for flowering time colocalized with those of resistance to southern leaf blight, gray leaf spot and northern leaf blight in maize (Zwonitzer et al. 2010) which are caused by necrotrophic fungal pathogens as well as Sclerotinia stem rot. The relative smaller lesions in later flowering individuals observed in this study were not related to leaf age, since the leaves used for resistance evaluation were all of the same age in the vegetative phase. However, our data do not provide an answer for stem resistance, where age varied slightly. More research, thus, is required to determine physiological effects on resistance. Moreover, although several genes were reported to simultaneously regulate plant defense against disease and flowering time in *Arabidopsis* (Wang et al. 2011a), it is unclear whether the locus that colocalized QTL for both flowering time and *Sclerotinia* resistance has pleiotropic effects or the locus is composed of closely linked genes that separately controlled these two traits. Nevertheless, the association between flowering time and *Sclerotinia* resistance would not affect the earliness and resistance breeding of rapeseed, since flowering time is controlled by many genetic factors, and the effects of late flowering time genes linked to *Sclerotinia* resistance can be cancelled out by some early flowering time genes in other loci.

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