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ORIGINAL PAPER

Identification of quantitative trait loci for resistance to *Xanthomonas campestris* pv. *campestris* in *Brassica rapa*

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Abstract Resistance to six known races of black rot in crucifers caused by *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson is absent or very rare in *Brassica oleracea* (C genome). However, race specific and broad-spectrum resistance (to type strains of all six races) does appear to occur frequently in other brassica genomes including *B. rapa* (A genome). Here, we report the genetics of broad spectrum resistance in the *B. rapa* Chinese cabbage accession B162, using QTL analysis of resistance to races 1 and 4 of the pathogen. A *B. rapa* linkage map comprising ten linkage groups (A01–A10) with a total map distance of 664 cM was produced, based on 223 AFLP bands and 23 microsatellites from a F_2 population of 114 plants derived from a cross between the *B. rapa* susceptible inbred line R-o-18 and B162. Interaction phenotypes of 125 F_2 plants were assessed using two criteria: the percentage of inoculation sites in which symptoms developed, and the severity of symptoms per plant. Resistance to both races was correlated and a cluster of highly significant QTL that explained 24–64% of the phenotypic variance was located on A06. Two additional QTLs for resistance to race 4 were found on A02 and A09. Markers closely linked to these QTL could assist in the transference of the resistance into different *B. rapa* cultivars or into *B. oleracea*.

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Introduction

Black rot of crucifers is a bacterial disease caused by *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson. The pathogen invades the xylem and colonizes the mesophyll. The symptoms of black rot include marginal leaf chlorosis, necrosis and darkening of leaf veins and vascular tissue within the stem. Wilting and necrosis also occur as the disease advances. The disease has a wide geographical distribution and is particularly destructive to *Brassica oleracea* L. vegetables causing reduction in yield and quality (Williams 1980), but it can also attack other *Brassica* spp. and cruciferous weeds and ornamentals. In *B. rapa*, the disease can be serious in turnip and turnip greens (Pammel 1895; Vicente 2004) and it has also been reported in Chinese cabbage crops (Schaad and Thaveeschai 1983; Ignatov et al. 2000).

Black rot control is difficult and usually attempted through the use of disease-free planting material (seeds or transplants) and the elimination of other potential inoculum sources such as infected crop debris and cruciferous weeds (Taylor et al. 2002).

Disease resistance could potentially provide low-cost and sustainable means of controlling black rot in vegetable brassicas. However, breeding black rot resistant cultivars is complicated by the existence of at least six races of the pathogen (Vicente et al. 2001). Worldwide, races 1 and 4 appear to be the most important in causing disease in *B. oleracea* crops; therefore resistance to both of these two races is a minimum requirement to

be of value in controlling black rot. Following extensive screening of *B. oleracea* accessions, Taylor et al. (2002) concluded that resistance to races 1 and 4 was either non-existent or very rare, whereas resistance to less important races (2, 3 and 6) was frequently found. In contrast, race specific resistance to races 1 and/or 4 is relatively common in other species of the genus *Brassica* (Bain 1952; Guo et al. 1991; Westman et al. 1999; Taylor et al. 2002) and potential broad spectrum quantitative resistance has been found at a low frequency in some accessions of *B. rapa*, *B. nigra* and *B. carinata* (Taylor et al. 2002). These accessions represent potential sources of resistance for breeding programmes, so we have initiated a programme to introgress quantitative resistance to races 1 and 4 of *X. campestris* pv. *campestris* into *B. oleracea* from a *B. rapa* source of resistance.

The *B. rapa* Chinese cabbage accession B162 was previously reported to possess quantitative resistance to *X. campestris* pv. *campestris* (Guo et al. 1991). This was confirmed by Taylor et al. (2002) who found that this accession had potential broad-spectrum resistance to type isolates of five races of the pathogen. The aims of the present study were to study the inheritance of resistance of B162 to the two most important races of *X. campestris* pv. *campestris*, to produce a genetic linkage map and to locate QTL for resistance, determine their relative importance and identify molecular markers of potential use for marker assisted selection for this quantitative resistance.

Materials and methods

Plant material and disease resistance assay

Seeds of *B. rapa* accession B162 were obtained from the Genetic Resources Unit at Warwick HRI and the resistance was 'fixed' by producing individual plant progeny by bud selfing following selection of resistant plants. R-o-18 is a highly inbred *B. rapa* Yellow–Sarson line and was originally obtained from the John Innes Centre, Norwich, UK. In previous experiments it was shown to be susceptible to all races of X. *campestris* pv. *campestris*. The cross R-o-18 × B162 was made by bud pollination following emasculation and an F_2 mapping population was produced by bud pollination of an individual F_1 plant.

The resistance of parental, F_1 and F_2 plants to *X. campestris* pv. *campestris* was assessed in a glasshouse experiment. Seeds were sown in 7 cm plastic pots filled with Levington M2 compost (The Scotts Company, Ipswich, UK) and pots were placed in a glasshouse with a minimum temperature of 20/15°C (day/night), venting at 22/17°C. Six R-o-18, 21 B162, 20 F_1 and 125 F_2 plants were assessed for resistance. One-month-old plants were inoculated with an isolate of race 1 (HRI 3811) and an isolate of race 4 (HRI 1279A) (Vicente et al. 2001). Four leaves were inoculated per plant, two with each isolate and 10–12 point inoculations were made per leaf, following the method described by Taylor et al. (2002). Three other host genotypes were also included in the experiment as controls: *B. oleracea* cv. Wirosa (previously described as susceptible to both races), *B. napus* Cobra 14R (resistant to race 4) and a selection of *B. carinata* PI 199947 (resistant to races 1 and 4) (Vicente et al. 2001).

Results were recorded for each leaf, 2 and 3 weeks after inoculation. Two traits were recorded as 'measures of resistance': the percentage of inoculation points from which symptoms developed (% successful infection) and the severity of symptoms that developed (symptom score). The latter was assessed on a visual six-point scale from 0 to 5 based on the relative lesion size (0 no symptoms, 1 small necrosis or chlorosis surrounding the infection point, 2 small V-shaped lesion with black veins, 3 lesion half way to the middle vein, 4 lesion progressing to the middle vein and 5 lesion reaching the middle vein). The highest symptom score between the two inoculated leaves was recorded; this reflects the fact that a larger lesion at the initial site of infection is more likely to lead to infection of the whole plant i.e. it provides a measure of the potential for the disease to develop further in the plant.

Correlation coefficients were calculated between the two traits for each race and between races for each trait with the procedure PROC CORR of SAS (2000).

DNA marker analysis

Leaf samples of approximately 100 mg were taken from R-o-18, B162, F_1 and each plant of the F_2 population before the resistance assay. DNA was extracted from the samples with DNeasy® Plant Mini and 96 Kits (Qiagen Ltd., UK) using the manufacturer's protocols.

Amplified fragment length polymorphism (AFLP) analysis was carried out on 114 F_2 plants as described by Vos et al. (1995) with minor modifications. Preamplification was performed with *Eco*RI and *Mse*I (E & M) primers each containing one selective nucleotide (E + A, M + C). Selective amplification was carried out with a total of 15 primer combinations, using E primers that contained 2 or 3 selective nucleotides, each combined with an M primer that contained 3 selective nucleotides (Table 1). Amplified fragments were separated

Table 1 List of the names and codes given to AFLP primer pairs used for selective amplification with individuals from the F_2 population

*Eco*RI primer	*Mse*I primer	Code
E + AA	M + CAT	E11M50
	M + CTA	E11M59
E + AC	M + CAC	E12M48
	M + CAA	E12M47
	M + CTA	E12M59
	M + CTG	E12M61
E + AAC	M + CAC	E32M48
E + AAG	M + CAA	E33M47
	M + CAC	E33M48
	M + CTT	E33M62
E + ACA	M + CAA	E35M47
	M + CTT	E35M62
E + AGC	M + CAA	E40M47
	M + CAT	E40M50
E + ACG	M + CAT	E37M59

on a 6% denaturing polyacrylamide gel and visualised by autoradiography. Segregating AFLP bands were scored dominantly and labelled following the method of Vuylsteke et al. (1999) with the primer codes plus a number indicating the estimated fragment size and a suffix of 'r' for R-o-18 or 'b', for B162, according to which parent possessed the band. Fragments larger than the upper range of the size marker (330 bp) were given a suffix of a number plus '>330'.

Thirty-three primer pairs were screened to identify microsatellites in the same F_2 individuals used for AFLP analysis. The primer sequences used were taken from Hee Kim et al. (1999), Suwabe et al. (2002), Lowe et al. (2004) and the AAFC Brassica/Arabidopsis Genomics Initiative (http://www.brassica.agr.gc.ca/). Following PCR in a 10 µl volume, products were separated in a 3% agarose gel and stained with ethidium bromide or in Spreadex® gels (Elchrom Scientific) and stained with SYBR® gold (Cambridge BioScience, UK). The majority of the microsatellites were scored co-dominantly and labelled according to their source (prefix SSR, Hee Kim; BRMS, Suwabe; Na, Ni, OL, Ra, Lowe; s, AAFC). Microsatellites, which gave an amplification product in only one parent were scored as dominant and labelled with a suffix _b or _r to denote which parent gave the band.

Linkage analysis

The Joinmap 3.0 software package (Van Oijen and Voorrips 2001) was used to test for segregation distortion, to assign markers to linkage groups and to calculate the most probable order and distances of the markers within each linkage group, with the parameters set for a F_2 population. The threshold for assigning markers to linkage groups was a LOD score of 4.0. Map distances in centimorgans (cM) were calculated from recombination frequencies using all pairwise recombination estimates smaller than 0.45, LOD scores larger than 0.01, and the Kosambi mapping function (Kosambi 1944). Two rounds of mapping were run and loci were excluded if they caused a jump in goodness-of-fit >4.00. Loci were excluded if they were involved in a large proportion of suspect linkages (recombination fraction >6.00). Individuals with large numbers of missing genotype scores were also excluded. Any data points involved in improbable double-recombination events were re-scored against the original data.

Autoradiographs from the R-o-18 × B162 mapping population were compared with autoradiographs from the Chinese cabbage mapping population produced by Hee Kim et al. (1999). AFLP bands produced in both populations from the same pair of primers were considered homologous loci if the fragments migrated to the same position on a gel (Waugh et al. 1997). Map positions of microsatellites were compared with data from Suwabe et al. (2004) and Lowe et al. (2004) to assist in the identification and alignment of linkage groups with those in other *B. rapa* maps. The genetic map was drawn using MapChart (Voorrips 2002).

QTL analysis

QTL analysis was performed separately for the two measures of resistance for each race. The logit transformation of the % successful infection and the highest symptom score between the two inoculated leaves were used in the QTL analysis. Interval mapping was used to calculate the presence of a QTL for each position on the map using the software MapQTL 4.0 (Van Ooijen et al. 2002). The LOD threshold value used to determine the genome-wide significance of a putative QTL was obtained empirically by permutation tests with MapQTL 4.0 following Churchill and Doerge (1994). The threshold value for a chromosome-wide significance level of 5% ['suggestive linkage' as proposed by Lander and Kruglyak (1995)] was chosen following Van Ooijen (1999). In the region of the putative QTL, loci were chosen as co-factors for multiple-QTL mapping (MQM) by the backward elimination technique (Jansen 1993; Jansen and Stam 1994). When LOD values in other regions reached a significant level, the MQM was repeated by adding new markers as cofactors until a stable LOD profile was reached. A two-LOD support interval was calculated for each QTL to obtain a 95% confidence interval (Van Ooijen et al. 2002).

Results

Disease resistance test

When inoculated with race 1, B162 was resistant with symptoms developing in only 2.6% of the inoculation sites and a low mean symptom score of 0.5. In contrast, R-o-18 plants were very susceptible with 90.8% of infection points developing symptoms and a mean symptom score of 5. When inoculated with race 4, 10.9% of infection points on B162 developed symptoms with an average symptom score of 2.4, whereas for R-o-18, 86.4% of infection points developed symptoms with a mean score of 5. Plants of the susceptible control *B. oleracea* cv. Wirosa had high scores and very high percentages of infected points with both races. Plants of *B. napus* line Cobra 14R were resistant to race 4 and partially susceptible to race 1 (very low percentages of infected points, but high scores); whereas plants of the *B. carinata* selection of PI 199947 were resistant to both races (very low scores and percentages).

The R-o-18 × B162 F_1 plants were intermediate between the two parents with 15.0% successful infections and an average symptom score of 3.3 when inoculated with race 1 and 16.8% successful infections and an average symptom score of 2.5 when inoculated with race 4. The F_2 segregated for both measures of resistance for both races showing a continuous variation. The F_2 had average % successful infection values of 28.6 and 37.3% for race 1 and 4 respectively and average symptom scores of 3.2 and 3.8, respectively. Resistance to both races was significantly correlated ($P < 0.005$) with correlation coefficients of 0.59 for % successful infection and 0.45 for symptom scores. The two measures of resistance were also significantly correlated ($P < 0.005$), with a correlation coefficient of 0.81 between symptom score and % successful infection for race 1 and 0.58 for race 4.

R-o-18 × B162 map

A genetic linkage map of *B. rapa* was generated based on recombination frequencies between 287 AFLP bands and 29 microsatellites in 114 F_2 individuals. Of these markers, 223 AFLP bands and 23 microsatellite loci were mapped into ten linkage groups (A01–A10) corresponding to the chromosome number of *B. rapa*, varying in length from 46 to113 cM (Fig. 1). The total map distance was 664 cM and the average distance between loci was 2.9 cM. The spacing between adjacent markers ranged from 0 cM in several linkage groups to 18 cM in A10. A small number of mapped loci (16% of the total) had segregation ratios which deviated significantly ($P < 0.05$) from the Mendelian ratio expected for an F_2 population. Several adjacent loci with segregation distortion in the direction of the B162 parent were observed on A02 and at the top of A07, while distortion in the direction of the R-o-18 parent was observed at the bottom of A05.

Comparison of the relative position of markers common to this map and the maps of Hee Kim et al. (1999) and Suwabe et al. (2004) allowed the order and orientation of each linkage group to be established. Nine of the linkage groups contained at least two markers in common with other *B. rapa* maps (Hee Kim et al. 1999; Suwabe et al. 2004), in the same relative positions (Fig. 1). The remaining linkage group (A02) contained a single marker in common with the corresponding linkage group on the CK map (Hee Kim et al. 1999). Common markers are shown in bold text in Fig. 1. The linkage groups were numbered according to the A genome linkage groups of *B. napus* (Parkin et al. 1995), therefore linkage groups A01–A10 in the map derived from the R-o-18 × B162 cross correspond to the N01–N10 A genome groups in *B. napus*.

QTL analysis

Four highly significant QTL were detected for resistance to *X. campestris* pv. *campestris* on A06 (Table 2; Fig. 1). The QTL for resistance to race 1 as measured by symptom score (*XccR1d-1*) and % successful infection (X*ccRli-1*) were located 5 cM apart, while the QTL for the two measures of resistance against race 4 (*XccR4d-1* and *XccR4i-1*) were placed at the same position (Table 2). An additional suggestive QTL for % successful infection by race 4 was found on A02 (*XccR4i-2*) and on A09 a suggestive QTL with a LOD peak just below the significance threshold of 2.7 was found (*XccR4i-3*).

The QTL for symptom severity caused by each race on A06 (*XccR1d-1* and *XccR4d-1)* were located 13 cM apart and for % successful infection (*XccR1i-1* and *XccR4i-1)* 18 cM apart. Apart from *XccR4i-3* the resistant allele for the QTL came from the B162 parent (Table 2). The size of the significant QTL effects ranged from 24% of the variance observed for % successful infection for race 4 (*XccR4i-1*) to 63.9% for symptom score for race 1 (*XccR1d-1*). For the four significant QTL, the average score of the susceptible parent genotype was greater than the average score of the resistant one, indicating that the beneficial (i.e. resistant) allele was present in the resistant parent B162. Dominance effects were calculated as the estimated value of the heterozygote minus the average of the estimates of both parents; they were positive except for *XccR1i-1* and *XccR4i-2* (Table 2).

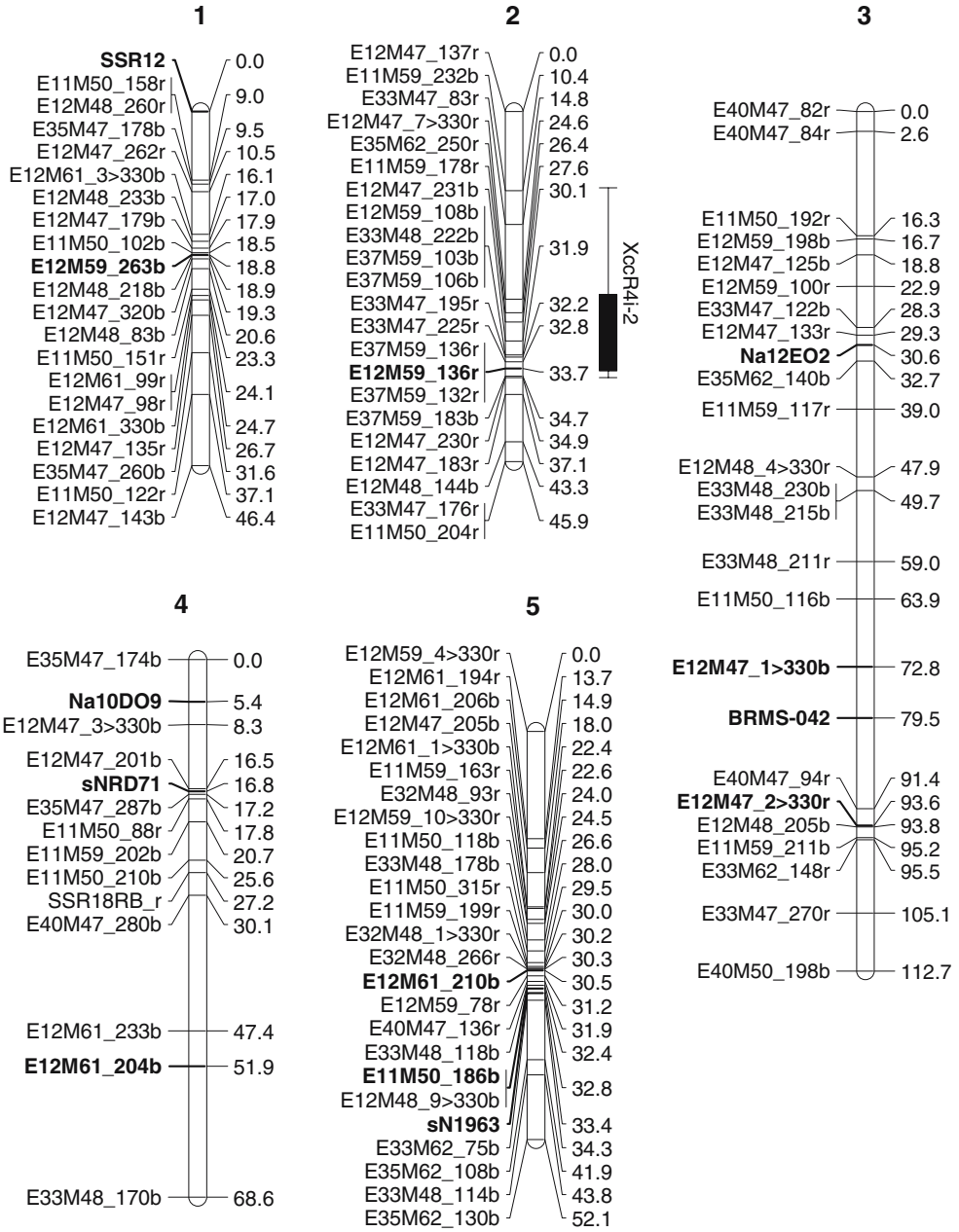

Fig. 1 Genetic linkage map for a *Brassica rapa* F_2 population derived from a cross between R-o-18 × B162. Linkage group numbers correspond to the A genome linkage groups of *B. napus*. Locus names are listed on the left and map distances in centiMorgans on the right. Joint markers used for map alignment are shown in *bold text*. QTL positions are indicated to the right of the appropriate linkage groups: the 1-LOD confidence intervals are shown as *bars* and the 2-LOD intervals as lines. QTL for race 1 resistance are shown as *hatched bars* and those for race 4 resistance as *solid bars*. AFLP loci are labelled with the primer pair and marker band size. Other labels indicate SSR loci. Markers scored as dominant have the suffix 'b' or 'r' to indicate which mapping parent gave an amplification product

Discussion

The inheritance of potential broad-spectrum quantitative resistance of *B. rapa* to *X. campestris* pv. *campestris* has been elucidated. A *B. rapa* linkage map has been constructed and we report for the first time the identification of QTL that control resistance to the two most important races (1 and 4) of the pathogen in *B. rapa*.

The R-o-18 × B162 linkage map comprises ten linkage groups, corresponding to the ten chromosomes of *B. rapa*. It has been anchored to the maps of Hee Kim et al. (1999) and Suwabe et al. (2004). The map length of 664 cM was similar to the map length (735 cM) of

Fig. 1 continued

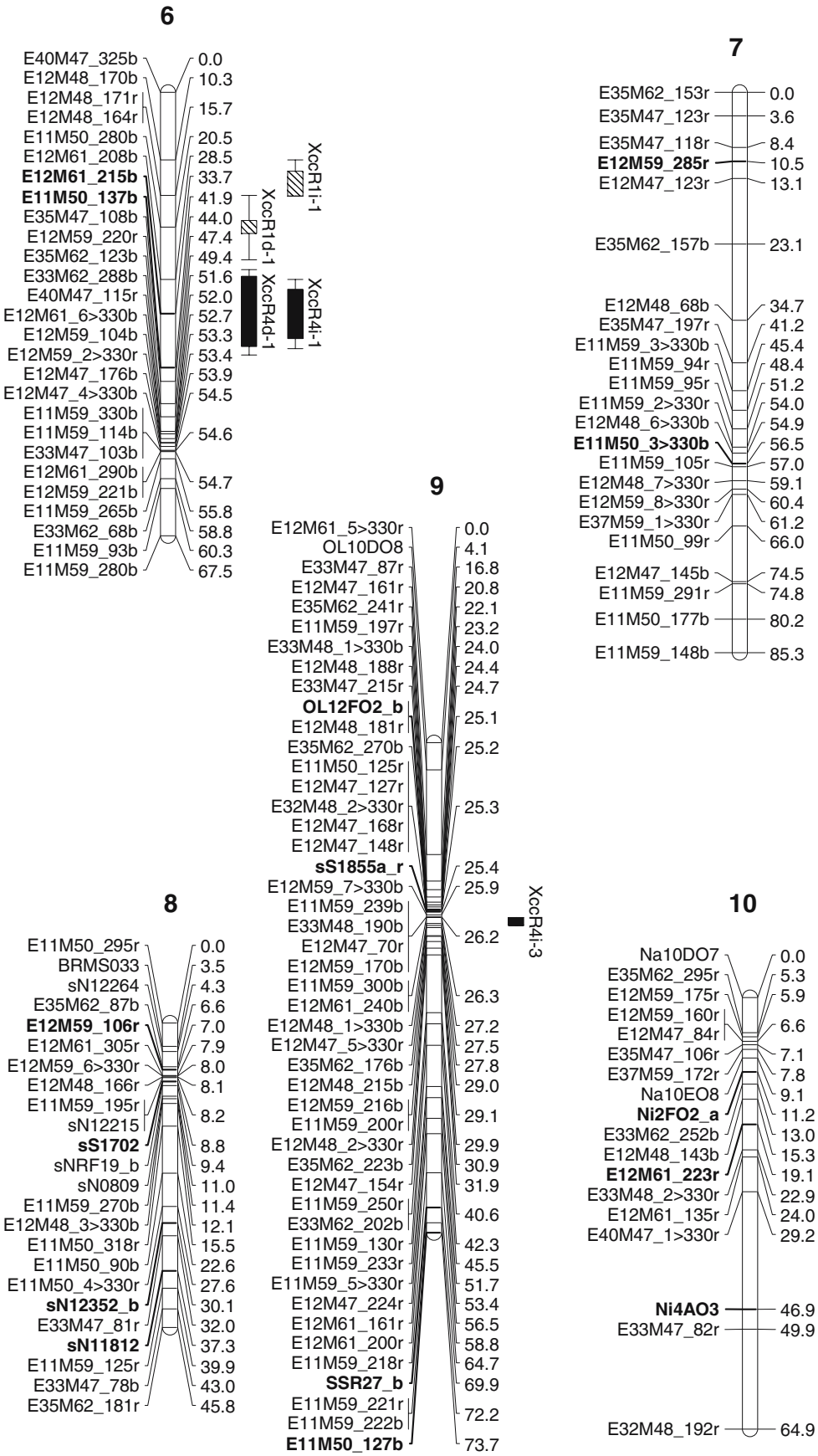

Table 2 Significant and suggestive *Brassica rapa* QTL for traits related to resistance to *Xanthomonas campestris* pv. *campestris* races 1 and 4, LOD scores, position of the QTL on the map, percentage of variance explained, additive and dominant effects

Character	QTL	Linkage Group	LOD Score[a]	Map position (cM)[b]	% variance explained[c]	Additive effect	Dominant effect	Closest marker	Resistant allele
Race 1 symptom score	*XccR1d-1*	A06	19.48	20.5 (15.7–25.5)	63.9	1.80	0.92	E11M50_280b	B162
Race 1 % successful infection	*XccR1i-1*	A06	18.8	15.7 (10.3–15.8)	51.0	2.10	−0.28	E12M48_171r	B162
Race 4 symptom score	*XccR4d-1*	A06	10.55	33.5 (27.0–40.0)	58.0	1.33	1.02	E12M61_215b	B162
Race 4 % successful infection	*XccR4i-1*	A06	6.45	33.7 (28.5–39.0)	24.0	1.29	0.34	E12M61_215b	B162
Race 4 % successful infection	*XccR4i-2*	A02	2.94	27.6 (10.0–34.9)	12.6	2.50	−0.83	E11M59_178r	B162
Race 4 % successful infection	*XccR4i-3*	A09	2.65	27.2 (26.3–27.5)	6.5	−0.62	0.28	E12M48_1 > 330b	R-o-18

[a] Peak LOD score in the QTL. LOD confidence threshold (5%) = 3.5, suggestive linkage threshold = 2.7

[b] Position of peak LOD score (2-LOD support interval in parentheses)

[c] Percentage of variance explained at peak LOD score

Matsumoto et al. (1998) and shorter than the map lengths of 1,850, 1,785 and 1138.1 cM of Song et al. (1991), Teutónico and Osborn (1994) and Nováková et al. (1996), respectively. The average distance and space between markers were lower than in several previously published *B. rapa* maps (Song et al. 1991; Teutónico and Osborn 1994; Nováková et al. 1996; Matsumoto et al. 1998). The R-o-18 × B162 map is therefore smaller and more saturated than previously published *B. rapa* maps. In addition the percentage of markers showing segregation distortion is lower than reported by Teutónico and Osborn (1994) and Chyi et al. (1992) for *B. rapa* maps constructed using RFLP markers.

The phenotypic assay confirmed that Chinese cabbage line B162 was resistant to races 1 and 4 of *X. campestris* pv. *campestris* and that line R-o-18 was susceptible to both races. Both the F_1 and F_2 means for % successful infection by both races are below the mid point between the two parents indicating that resistance is dominant (i.e. dominance for reduced % successful infection). Dominance effects for symptom scores (severity of the disease) caused by race 1 were negligible with both the F_1 and F_2 means close to the mid point between the parental means. For race 4 the F_1 data indicated dominance for resistance (i.e. reduced symptom scores) but this was not found in the F_2.

Resistance to races 1 and 4 in the F_2 was significantly correlated suggesting linkage between the genetic factors for resistance to the two races. This was confirmed by the QTL analysis. Although the QTL analysis suggested that resistance to race 4 may involve QTL on A02 and A09, the significant QTL for resistance to both race 1 and 4 were clustered together on A06. The confidence intervals of the QTL on A06 for resistance to race 1 and 4 do not overlap, indicating that resistance to each race is determined by different but closely linked QTL. Similar clusters of QTL for resistance to different races of the same pathogen have been reported previously in *Brassica* (Manzanares-Daulex et al. 2000; Kole et al. 2002).

The two measures of resistance were correlated and the QTL analysis showed that the two traits were largely determined by the same or linked QTL on A06. This may be explained partly by the fact that symptoms could only develop where infection was successful (i.e. the two measures of resistance may be confounded). However, the level of correlation for race 4 was relatively small and this, together with the identification of suggestive QTL for % successful infection by race 4 on two other linkage groups, indicates that there may be a degree of separate genetic control for the two traits. QTL for two different measures of resistance to brassica diseases have been reported previously by Zhao and Meng (2003) and Pilet et al. (1998). Ignatov et al. (2000) identified some RAPD markers linked to strong race-specific resistance to race 4 in *B. rapa* and Vicente et al. (2002) located the locus *Xca4* that controls race-specific resistance to race 4 on linkage group N5 in a *B. napus* map. The linkage group N5 corresponds to A05 in our map, where we did not find any QTL for resis-

tance. The resistance studied by Ignatov et al. (2000) in *B. rapa* and the resistance mapped by Vicente et al. (2002) were due to single dominant genes and are probably not related to the resistance found in this study.

The quantitative, potential broad-spectrum disease resistance studied here could be transferred into other economically important *B. rapa* cultivars (e.g. to turnips) or could be introgressed into *B. oleracea* cultivars through a programme of interspecific hybridisation. The introgression of resistance genes to *X. campestris* pv. *campestris* from the A or B genomes into *B. oleracea* has been previously proposed (Hansen and Earle 1995; Taylor et al. 2002; Tonguç and Griffiths 2004). A dominant resistance to black rot found in a *B. carinata* line was transferred into *B. oleracea* by means of protoplast fusion (Hansen and Earle 1995), but the inheritance was unstable and segregation analysis revealed a deviation in expected ratios of resistant and susceptible plants. Tonguç and Griffiths (2004) introduced resistance to races 1 and 4 from two *B. juncea* accessions into *B. oleracea* and all hybrids and backcrosses were reportedly resistant to both races. However, no information on the genetic control of this resistance has been reported. The broad-spectrum resistance from *B. rapa* on its own or in combination with strong race-specific resistance could contribute to the durable control of the disease.

We have located QTL in *B. rapa* for resistance to the two most important races of *X. campestris* pv. *campestris* and we are now in the process of identifying more tightly linked markers for the QTL on A06. By identifying QTL determining each component of resistance we have been able to 'dissect' the complex trait of a plant resistance to two races of a pathogen and will be able to provide plant breeders with tools to breed for resistance to these economically important races using marker-assisted selection.

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