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Connected populations for detecting quantitative resistance factors to phoma stem canker in oilseed rape (*Brassica napus* L.)

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Abstract Improvement of effectiveness and durability of disease resistance in crops most often relies on the use of quantitative resistance, with the hypothesis that a wide range of quantitative resistance factors (QTL) makes the overcoming of the resistance by the pathogen more difficult. For an optimum use of these QTL in effective and durable strategies of resistance deployment, there is a need to precisely know their localization but also their stability/specificity and their allelic effects in various genetic backgrounds. Stem canker caused by the fungus *Leptosphaeria maculans* is one of the most important diseases in oilseed rape. In this *Brassica napus- L. maculans* pathosystem, QTL were previously identified by linkage analysis using populations derived from biparental crosses that were analyzed separately. In this study, we explored new quantitative resistance factors using a multi-cross connected design derived from four resistant lines crossed with a single susceptible line. Independent and connected mapping analyses revealed to be complementary to get an overview of QTL organization. We validated different QTL across different years and genetic backgrounds and identified novel QTL which had not yet been mapped. Population-common and population-specific QTL were identified. Knowledge of QTL organization and effects should help in the rational choice of relevant factors in breeding resistant genotypes to be integrated with other control means such as cultural practices and rotations for durable management of the disease.

Keywords Oilseed rape · *Brassica napus* · *Leptosphaeria maculans* · Quantitative resistance · QTL · Multi-cross design

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Introduction

Phoma stem canker caused by the fungus *Leptosphaeria maculans* is one of the most damaging diseases in oilseed rape (*Brassica napus*, AACC, 2n = 38), causing serious losses in Europe, Australia

and North America (Fitt et al. 2006; West et al. 2001). The intensification of oilseed rape production and increased areas of cultivation have favored the development of this disease in recent years, leading to economical losses worldwide, which are estimated at more than US$900M per growing season (Fitt et al. 2008). The severity of phoma stem canker could potentially increase in the future due to global warming (Fitt et al. 2008). Deployment of resistant cultivars is an effective and environmentally friendly method for phoma stem canker control that can be combined with cropping practices or deployment strategies.

Both qualitative and quantitative resistances were identified in *B. napus* or in related species (Delourme et al. 2006a; Rimmer 2006; Hayward et al. 2012; Raman et al. 2012). Qualitative resistance is based on a gene-for-gene interaction, which is expressed from the seedling stage. More than ten specific resistance genes have been identified in *B. napus* and related Brassica species *B. rapa*, *B. juncea* and *B. nigra* (*Rlm*1-11; *LepR1-4*) (Delourme et al. 2006a; Rimmer 2006; Balesdent et al. 2013), some of which are organized in clusters on *B. napus* chromosomes (Delourme et al. 2004, 2006a). Since this resistance is total, it exerts strong selection pressure on the fungal populations that rapidly adapt. Varieties whose resistance is based solely on such major genes lose their effectiveness only 3–4 years after their release (Brun et al. 2000; Li et al. 2003; Rouxel et al. 2003). Quantitative adult-plant resistance, which is a partial, polygenic resistance mediated by QTL (Quantitative Trait Loci), is considered as non-race specific (Delourme et al. 2006a). Polygenic quantitative resistance is also considered to be more durable than qualitative resistance (Boyd 2006; Poland et al. 2009), but its effectiveness varies between cropping seasons due to environmental conditions. Thus, the combination of qualitative resistance with a high level of quantitative resistance in cultivars is a way to maximize the effectiveness and durability of the resistance (Brun et al. 2010). When combined with quantitative resistance, the qualitative *Rlm6* resistance provided effective control of phoma stem canker until the 7th year, 4 years longer than when it was deployed in a susceptible background. In addition, with the presence of quantitative resistance, the disease is maintained at a less severe level even after the fungus population has adapted to the major gene (Brun et al. 2010; Delourme et al. 2014). Similar results were obtained for the pepper–Potato virus Y interaction (Palloix et al. 2009). Quenouille et al. (2013) showed that the frequency of resistance overcoming was negatively correlated with the level of quantitative resistance of the genetic background. These findings highlight the interest and need to strengthen quantitative resistance studies in order to help breeding and improve management of disease resistance.

To date, few studies focused on the search for QTL for stem canker resistance. One French winter oilseed rape source of resistance, 'Darmor,' was studied in two genetic backgrounds (Jestin et al. 2012; Pilet et al. 1998, 2001). 'Darmor,' derived from 'Jet Neuf,' was deployed over a large acreage in Europe in the 1970s and the 1980s, and its resistance remained effective even after many years of wide cultivation. The varieties 'Jet Neuf' and 'Darmor' were used as sources for resistance breeding in winter and spring *B. napus* (Roy et al. 1983). Genetic analyses revealed a total of 16 QTL, and Pilet et al. (2001) showed that the genetic background had a strong influence on QTL detection. Indeed, only four QTL were common to the two crosses. A study was also performed in Australia on four biparental populations derived from five different *B. napus* cultivars (Kaur et al. 2009). One to four QTL were detected in each population. However, none of the QTL was found to be common between the different environments for a given population, and few QTL were common between the populations at a trial site (Kaur et al. 2009). Raman et al. (2012) studied another biparental population derived from Australian cultivars and detected seven and one QTL in a 2-year experiment, but no QTL were common to the 2 years.

Stem canker resistance QTL were so far identified by linkage analysis using populations derived from biparental crosses that were analyzed separately (Pilet et al. 1998, 2001; Kaur et al. 2009). Linkage mapping based on a joint analysis on multiple connected populations has been suggested as a promising strategy in many species (e.g., Blanc et al. 2006; Christiansen et al. 2006; Pierre et al. 2008; Cuesta-Marcos et al. 2008; Paulo et al.2008; Billotte et al. 2010; Negeri et al. 2011; Steinhoff et al. 2011; Pauly et al. 2012; Schwegler et al. 2013; Lee et al. 2014). Indeed, the combination of several connected information sources provides many advantages: (1) This approach increases the probability that a polymorphic allele is present, at least, in one population; (2) the connected

multi-cross design increases the power of QTL detection (Rebai and Goffinet 1993; Schwegler et al. 2013) and also contributes to reduce the QTL support interval (Blanc et al. 2006; Pierre et al. 2008; Negeri et al. 2011; Steinhoff et al. 2011); (3) in addition, this method makes it possible to simultaneously estimate allelic effects at each QTL, which facilitates their comparison in different genetic backgrounds (Blanc et al. 2006; Steinhoff et al. 2011). Overall, a multi-parental approach would increase the potential for marker-assisted selection (MAS) as shown in a simulation study based on the results of a QTL detection experiment in maize (Blanc et al. 2008).

In this study, our objective was to explore the diversity of quantitative stem canker resistance in *B. napus* using a connected multi-cross design. Four mapping populations, connected by the susceptible variety 'Bristol,' were used. The parental lines were chosen to maximize the likelihood of obtaining a more global picture of resistance QTL organization in winter oilseed rape compared with the variety 'Darmor,' which was used in our previous studies. The benefits and pitfalls of analyzing independent and connected populations were examined. Locations and effects of QTL across the populations were compared and discussed, which gave information on the most favorable allele or allele combination at each common QTL. Since few QTL studies have been conducted in this pathosystem, our results are discussed in light of those reported in previous studies, to provide information for the choice of relevant QTL in breeding.

Materials and methods

Plant material

Different criteria were applied to choose the parental lines used to generate the mapping populations. The material was chosen among a set of varieties described in Jestin et al. (2011). To increase the likelihood of identifying a diverse range of QTL, the varieties were genetically divergent from the variety 'Darmor' used in the previous studies and as divergent as possible from each other. In addition, the varieties were representative of the material used in winter oilseed rape (WOSR) breeding programs. Another selection criterion was the absence of any effective specific resistance genes conferring total resistance in our field trial conditions in order to only evaluate the level of quantitative resistance. This was deduced from cotyledon tests using isolates carrying known *AvrLm* genes (Balesdent et al, pers. comm.). Finally, the varieties known to have a high level of quantitative resistance to *L. maculans* were chosen. Thus, four biparental crosses were performed with the varieties 'Aviso,' 'Canberra,' 'Darmor' and 'Grizzly,' as the female parents and the variety 'Bristol' as the male parent. The specific resistance genes carried by these varieties are as follows: 'Aviso' (*Rlm9*), 'Canberra' (*Rlm1, Rlm9*), 'Darmor' (*Rlm9*), 'Grizzly' (*Rlm2, Rlm3*) and 'Bristol' (*Rlm2*). *Rlm1* is partially effective and *Rlm2, Rlm3* and *Rlm9* are not effective to control *L. maculans* population in our field conditions, taking into account the frequency of the corresponding *AvrLm* genes. A single F1 plant was self-pollinated to derive $F_{2:3}$ populations, with around 120 individuals for each population. The 'Bristol' variety is susceptible to *L. maculans* whereas the other parents show a high level of resistance to this fungus. We adopted the following abbreviations to designate mapping populations in our study: AB ('Aviso' × 'Bristol'), CB ('Canberra' × 'Bristol'), DB ('Darmor' × 'Bristol') and GB ('Grizzly' × 'Bristol').

Experimental design

The F3 progenies were assessed for stem canker resistance in the field at one location (Le Rheu, France) in 2008, 2009 and 2010. Experimental design followed an alpha design with two replicates. In each plot (3 m × 1.5 m), individual F3 families were sown at 60 seeds on average per m^2. Control lines as well as the parental lines were included in each replicate. The controls were winter-type *B. napus* cultivars showing different levels of *L. maculans* resistance: 'Jet Neuf' (highly resistant), 'Goeland' (partially resistant), 'Falcon' (intermediate) and 'Eurol' (moderately susceptible). The spring variety 'Yudal' (very susceptible) was also used in the GB and AB trials. Infected rapeseed stubble collected from the previous year's trial (Le Rheu, France) was scattered through the field when the plants were at the three-leaf stage in autumn to increase inoculum pressure.

Stem canker severity was evaluated before harvest (mid June) for each line using a method modified from Pilet et al. (1998) as proposed by Aubertot et al.

(2004). Forty plants per plot were uprooted, and crown canker was assessed on a 1–6 scale as follows: 1 = no disease, 2 = 1–25 %, 3 = 26–50 %, 4 = 51–75 %, 5 = 76–100 % of crown section cankered. An additional disease score category of 6 was used to indicate plants broken at the crown from severe canker. All crown canker data were transformed to a standardized 0–9 disease severity scale using the formula: G2 index = [(N1 × 0) + (N2 × 1) + (N3 × 3) + (N4 × 5) + (N5 × 7) + (N6 × 9)]/Nt, where N1, N2,…,N6 = the number of plants with a canker score of 1, 2,…6, respectively, and Nt = the total number of plants assessed.

Genotyping

Genomic DNA was extracted from young leaf tissue using a modified CTAB extraction protocol from Doyle and Doyle (1990), from each F_2 and parental line. A set of different types of markers including simple sequence repeat (SSR), sequence-related amplified polymorphism (SRAP), sequence characterized amplified region (SCAR) and physical functional markers (PFM) was first screened on the parental lines and then used on the F_2 plants.

SSR amplification

The SSR markers were amplified in a 5 μL reaction mixture consisting of 20 ng DNA, buffer 1X, 2 mM $MgCl_2$, 5 % DMSO, 75 μM dNTP, 0.15 U/μL Taq DNA polymerase (Promega), 0.04 μM forward primer appended with the M13 tail 5′-CACGACGTTG-TAAAACGAC-3′, 0.4 μM reverse primer, 0.36 μM of the fluorescently (blue, green, red or yellow) labeled universal M13 primer.

PCR cycling conditions for the SSR developed by the Celera AgGen consortium (SSR primer pairs prefixed 'BRAS' and 'CB') and the Institute of Agronomy and Plant Breeding of the University of Göttingen (SSR primer pairs prefixed 'MR' or 'MD') (Piquemal et al. 2005; Radoev et al. 2008), as well as for the marker designated 'BN' (EST-SSR markers) developed by INRA (C. Falentin and G. Lassalle, IGEPP, Rennes) and the markers designated 'NMS' (Trait Genetics, Germany), were as follows: 94 °C for 4 min, 12 cycles of [94 °C for 30 s; 65 °C with a decrease of 1 °C in each subsequent cycle for 1 min; 72 °C for 30 s], 25 cycles of [94 °C for 30 s; 53 °C for 1 min; 72 °C for 30 s], and a 10 min extension at 72 °C. The PCR cycling conditions for the UK SSR markers (http://www.ukcrop.net/perl/ace/search/BrassicaDB) were the same as previously described except for the touchdown PCR cycle: 10 cycles of [94 °C for 30 s; 60–51 °C with −1 °C/cycle; 72 °C for 30 s]. The PCR cycling conditions for the Agriculture Canada SSRs, prefixed 'sN' or 'sR' or 'sS' (http://brassica.agr.gc.ca/index_e.shtml), were as follows: 94 °C for 10 min, 8 cycles of (94 °C for 15 s; 50 °C for 15 s; 72 °C for 30 s), 27 cycles of (89 °C for 15 s; 50 °C for 15 s; 72 °C for 30 s), 72 °C for 10 min. PCR cycling conditions for the markers derived from a microsatellite-enriched genomic library and obtained from the National Institute of Vegetable and Tea Science Japan (Suwabe et al. 2002) (SSR primer prefixed 'Brms') and for the FITO designated SSR markers (http://www.osbornlab.agronomy.wisc.edu/research/maps/ssrs.html) (Iniguez-Luy et al. 2008) were as follows: 94 °C for 4 min, 37 cycles of (94 °C for 30 s; 50 °C for 1 min; 72 °C for 30 s), 72 °C for 5 min. PCR cycling conditions for the markers developed by the National Key Laboratory of Crop Genetic Improvement and National Centre of Plant Gene Research in China (designated 'BnGMS') (Cheng et al. 2009) were performed as described by Cheng et al. (2009).

Amplification of other PCR markers

PFM markers, obtained through a Génoplante project in collaboration with INRA-Evry France (coll. B. Chalhoub), were amplified as follows: 94 °C for 5 min, 13 cycles of (94 °C for 45 s; 55 °C with a decrease of 1 °C in each subsequent cycle for 45 s; 72 °C for 1 min), 29 cycles of (94 °C for 45 s; 55 °C for 45 s; 72 °C for 1 min), 72 °C for 7 min. The reaction mixture was the same as for the SSR markers. SRAP (Li and Quiros 2001; Sun et al. 2007) was also used in some mapping populations. The SA7 and Me2 labeled primers (6-FAM and VIC, respectively) were used in combination with the Bg68, em3 and em2 unlabeled primers (markers designated as 'SA7Bg68', 'Me2em2', 'Me2Bg68' or 'Me2em3' according to the primer combination used). The PCR was the same as for the SSR markers. The SCAR marker ScJ14 was amplified as described by Delourme et al. (2008).

Electrophoresis

DNA fragments (SSR, SRAP and PFM) were fractioned using capillary electrophoresis on an ABI PRISM® 3130xl automatic DNA sequencer. The internal LIZ 500 size standard was used. The alleles were analyzed and scored with GENEMAPPER® 3.7 (Applied Biosystems®) software.

Amplification products for the SCAR, RAPD and some PFM (PFM327-380-566-576-577-632) markers were separated on 2 % agarose gels buffered with 0.5X TAE and visualized by ultraviolet illumination after ethidium bromide staining.

Genetic map and Linkage Group (LG) nomenclature

All markers were tested for Mendelian segregation ratios (1:2:1 or 3:1 with codominant or dominant markers, respectively) using a Chi-square test ($\alpha = 0.05$) in each population. Genetic maps were built using Mapmaker/Exp 3.0 (Lincoln et al. 1992). LGs were established with a maximum LOD threshold of 6.0, and then, the markers not assigned were tested with a minimum LOD of 3.0. Genetic distances expressed in centimorgan (cM) between markers were estimated with Haldane function (Haldane 1919). We built a genetic map for each population, and then, a consensus map was generated from the four populations connected by the parental line 'Bristol' with Mapmaker/Exp 3.0. In addition, the markers were positioned on the reference *B. napus* sequence obtained from 'Darmor-*bzh*' (Chalhoub et al. 2014) through BLASTn alignment of the sequence of the SSR genomic clones with an *E* value threshold of 10^{-6}.

LGs were assigned using the common markers from mapping data published in the literature and the reference genetic map 'Damor-*bzh*' × 'Yudal' used in our laboratory (Delourme et al. 2006b, 2008; Lombard and Delourme 2001).

We have used the LG nomenclature based on the diploid progenitors of *B. napus,* as proposed by the Multinational Brassica Genome Project Steering Committee. We refer to LGs A1–A10 which correspond to *B. rapa* (genome A) and C1–C9 which correspond to *B. oleracea* (genome C). LGs derived from different populations are identified by the abbreviations used to name the mapping populations. For example, LG A1 in the 'Aviso' × 'Bristol' (AB) population is named A1–AB. No supplementary abbreviation was used in the consensus map. An alphabetical suffix (a, b, c, …) was used when one LG was represented by different fragments (e.g., A1a–AB and A1b–AB are assigned to the A1 LG).

Statistical analysis

Disease data

G2 disease index data in 2008, 2009 and 2010 were analyzed for each population by a generalized linear model. Within each year, the analysis of variance (ANOVA; proc GLM of statistical Analysis System, SAS, SAS Institute Inc. 1989) partitioned total variation into line, replicate and error effects ($P_{ij} = \mu + L_i + R_j + e_{ij}$ where P_{ij} is the G2 disease index of the *i*th line located in the *j*th replicate, μ the mean of all data, L_i the line *i* effect, R_j the replicate *j* effect and e_{ij} the residual). The year effect and the genotype × year interaction were also tested on these data. The PROC UNIVARIATE procedure (SAS 1989) was used to determine the normality of each raw and residual distribution. From means of the G2 disease index, a Kendall correlation coefficient was calculated with the PROC CORR procedure (SAS 1989) between the 3 years. Heritability (h^2) was estimated with the formula: $h^2 = \sigma_g^2/[\sigma_g^2 + (\sigma_e^2/n)]$ with σ_g^2 the genetic variance, σ_e^2 the environmental variance and n the number of replicates.

QTL detection

QTL were identified using G2 data means in each population and year. Firstly, QTL analysis was conducted in each population using composite interval mapping (CIM) implemented in Windows QTL Cartographer 2.5 (Wang et al. 2007). A forward–backward stepwise regression analysis was used in the CIM procedure, with five cofactors, a 10 cM window size (on either side of the markers flanking the test site) and $P_{in/out} = 0.05$. The LOD thresholds estimated after 1000 permutation tests (Churchill and Doerge 1994) ranged between 3.2 and 3.7, but all the QTL revealed with a LOD higher or

equal to 2.5 were examined. We also detected QTL by regression interval mapping using the software package MCQTL (Jourjon et al. 2005). An iterative method (iQTLm) based on genetic cofactors was used (Charcosset et al. 2001). Markers were chosen as cofactors through a forward selection procedure (window of 10 cM). The F threshold for the cofactor selection was set up at 2.5, and F threshold for QTL detection was estimated between 3.2 and 3.5 according to year and population, after 1000 permutations with a global alpha risk of 10 %. Then, multi-population QTL mapping was performed with MCQTL taking into account the connection between the populations (connected by the variety 'Bristol'). The F threshold for the cofactor selection was set at 3.5, and the F threshold for QTL detection in the connected population was estimated at 4.2, 4.7 and 4.2 for 2008, 2009 and 2010, respectively, after 1000 permutations with a global alpha risk of 10 %. Both additive and dominance models were examined in independent and connected population analyses.

Average levels of dominance were calculated as the DR ratio = D/A (expressed in absolute value with D and A, the dominance and additive effects, respectively). Genetic effects at the QTL were estimated employing the criteria of Stuber et al. (1987) from the DR ratio: additive (A) if DR = 0–0.2; partial dominance (PD) if DR = 0.21–0.80; total dominance (D) if DR = 0.81–1.20; and over-dominance (OD) if DR >1.20.

A LOD support interval was constructed for each QTL on the basis of a 1.5 unit fall, which is more appropriate for a 95 % confidence rate in an F_2 population (Vanooijen 1992; Lynch and Walsh 1998). From common markers between the genetic maps, QTL identified in the independent populations were projected on the consensus map using the BioMercator 2.1 software (Arcade et al. 2004). The schematic of each LG carrying QTL was generated with MAPCHART 2.2 software (Voorrips 2002). We compared QTL detection between years, populations and the different analyses, considering that two QTL were potentially similar if their confidence interval overlapped. The QTL were named according to their location on each LG as in Delourme et al. (2008), i.e., *QlmA9* for QTL of resistance to *L. maculans* located on the LG A9.

Results

Stem canker resistance assessment

ANOVA revealed a significant ($P < 0.05$) genotype × year interaction effect in all the populations. Thus, data from each year were analyzed separately for all populations. The genotypic effect was highly significant ($P < 0.001$) for every year and population. The replicate effect was significant or not depending on the population and the year. Residues obtained after ANOVA were normally distributed for every year and population, according to the Shapiro–Wilk statistics. Kendall correlations between years for the G2 disease index were highly significant for each population and ranged from 0.20 ($P = 0.002$) and 0.24 ($P = 0.0002$) for 2008/2010 and 2009/2010, respectively, in the AB population to 0.31–0.43 ($P < 0.0001$) in all other cases.

The continuous distribution of the mean disease index of the F3 families for each population and year confirmed the quantitative nature and polygenic control of the resistance in these different genetic backgrounds (Fig. 1). Leaf lesions were present at the autumn on all the F3 families, confirming the absence of major genes conferring total resistance to *L. maculans* and, thus, that mainly quantitative resistance was segregating within the different populations. Overall, the disease was the most severe in 2010 and the least severe in 2009, as indicated by the phenotypic means of controls (Table 1) and the phenotypic distribution of the F3 families (Fig. 1). The broad-sense heritability estimates for stem canker resistance varied from 0.29 (DB population in 2009) to 0.74 (DB population in 2010) (Table 1). For all populations and years, transgressive F3 families were observed toward both resistance and susceptibility.

Genetic maps

Each F2 individual was genotyped in order to generate four independent genetic maps and a consensus genetic map. The independent genetic maps contained between 143 and 199 marker loci mapped onto 17 to 23 LGs depending on the population (Table 2). Some LGs were completely missing (such as A2 in the CB and GB populations, A4, A6 and C9 in the AB

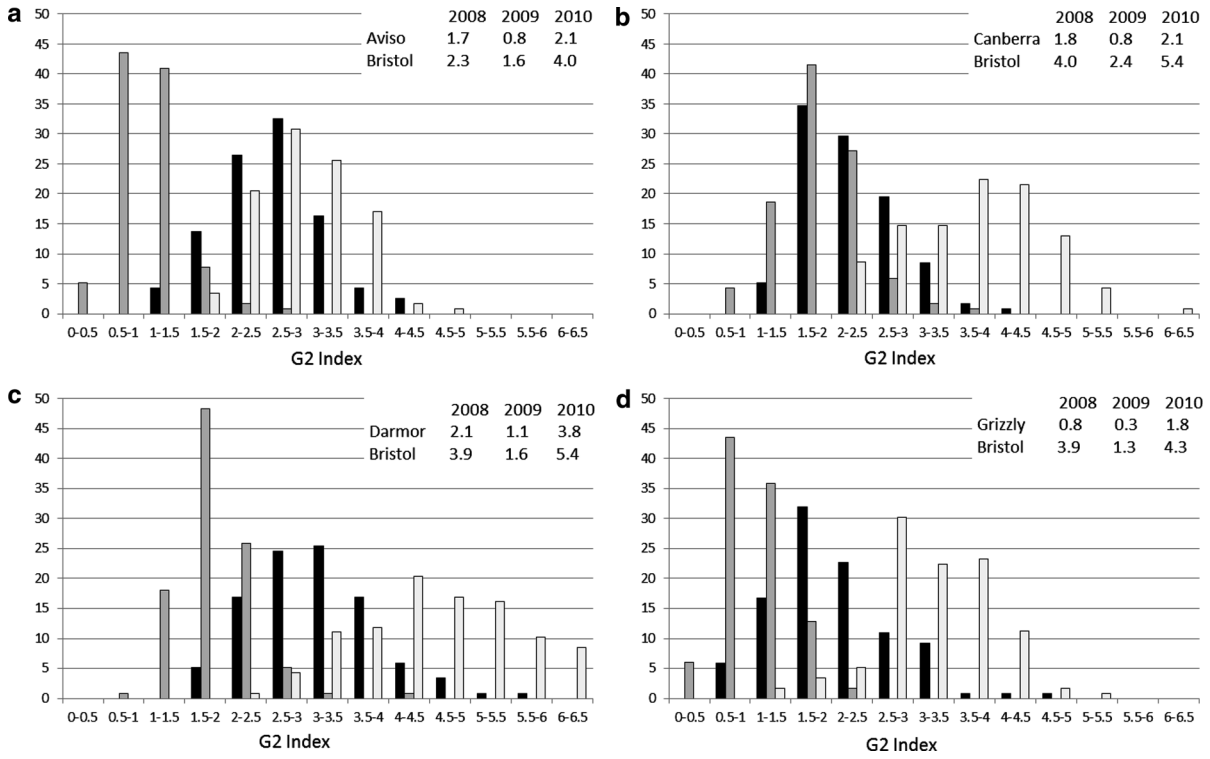

Fig. 1 Frequency distribution (%) of mean G2 Disease Index (G2 Index) estimated in 2008 (*black bars*), 2009 (*gray bars*) and 2010 (*white bars*) in the F3 populations derived from the crosses between the susceptible variety 'Bristol' and the resistant varieties 'Aviso' (**a**), 'Canberra' (**b**), 'Darmor' (**c**) and 'Grizzly' (**d**). The G2 index for the *parental lines* is indicated on each *graph*

populations and A7 in the CB population) or showed poor coverage, but all the LGs were assigned to one of 19 *B. napus* reference LGs (Supplementary Figure 1). The map lengths were estimated at 1119, 1115, 1421 and 1627 cM with an average inter-marker distance of 7.2, 7.9, 7.5 and 8.2 cM for the AB, CB, DB and GB populations, respectively. Out of the set of mapped markers, 7.1, 6.4, 8.8 and 5.3 % showed distorted segregation ratios ($\alpha = 0.05$) in these same populations, respectively. The order of marker loci was well conserved between the different genetic maps, which allowed the construction of a consensus map (Supplementary Figure 1). It was composed of 20 LGs built from the 366 marker loci (since LG A2 was still divided into two sections). The consensus map length was estimated at 2647 cM with one locus per 6.1 cM on average. The number of common marker loci between the populations varied between four and 19 depending on the LGs [if we disregard A2a and A2b which carry only three markers) (Supplementary Table 1)]. Among all mapped markers, 7 % were segregating in all four populations and 53% of the markers segregated in at least two populations. The coverage of the consensus genetic map was estimated at 85 % of the 'Darmor-*bzh*' × 'Yudal' map published in Wang et al. (2011). Only 159 out of the 366 loci from the consensus map provided non-ambiguous positions on the reference *B. napus* sequence. Globally, a good consistency was found between the genetic and physical positions of these loci (Supplementary Table 2).

QTL detection

QTL identification in the single populations

Since genotype × environment interactions were significant for most populations, QTL were mapped for each year. Overall in the single populations, 12 to 22 QTL were detected depending on the year and software used (Table 3; Supplementary Tables 3 and 4). When the results determined with both QTL

Table 1 Summary of G2 disease index for the 'Aviso × Bristol' (AB), 'Canberra × Bristol' (CB), 'Darmor × Bristol' (DB), 'Grizzly × Bristol' (GB) populations, the parental and control lines and heritability estimated for each trial in 2008, 2009 and 2010

	AB			CB			DB			GB		
	2008	2009	2010	2008	2009	2010	2008	2009	2010	2008	2009	2010
Population mean	2.57	1.05	2.97	2.22	1.86	3.72	3.09	1.85	4.56	2.02	1.04	3.2
Range	1.2–4.41	0.29–2.77	1.77–4.77	1.26–4.05	0.63–3.78	2.2–6.35	1.66–5.85	0.71–4.26	2.62–6.4	0.7–4.57	0.18–2.07	1.31–5.3
Parental means												
Aviso	1.74	0.80	2.10	–	–		–	–	–	–	–	–
Canberra	–	–	–	1.79	0.76	2.04	–	–	–	–	–	–
Darmor	–	–	–	–	–	–	2.09		1.14	3.80	–	–
Grizzly	–	–	–	–	–	–	–	–	–	0.81	0.30	1.78
Bristol	2.30	1.57	3.97	4.02	2.45	5.39	3.92	1.60	5.44	3.91	1.29	4.33
Control means												
Eurol	3.85	1.84	5.30	2.30	4.24	5.55	4.06	2.55	6.52	3.44	2.54	5.29
Falcon	3.90	2.06	3.88	1.91	2.55	5.19	3.46	2.33	6.20	3.19	1.45	5.38
Goeland	1.83	1.16	3.49	1.93	1.56	4.69	2.95	1.93	4.90	1.81	1.38	3.29
Jet Neuf	2.01	0.44	2.17	1.20	0.69	2.30	2.21	0.88	3.00	1.46	0.43	2.10
Yudal	8.65	6.71	6.83	–	–	–	7.64	6.68	7.05	–	–	–
Heritability	0.55	0.48	0.58	0.66	0.66	0.52	0.71	0.44	0.74	0.71	0.29	0.44

Table 2 Description of the genetic maps for the 'Aviso × Bristol' (AB), 'Canberra × Bristol' (CB), 'Darmor × Bristol' (DB), 'Grizzly × Bristol' (GB) and connected populations (consensus)

Mapping population	AB	CB	DB	GB	Consensus
No. of mapped marker loci	154	143	190	199	366
No. of linkage groups	17	18	23	20	20
Map length (cM Haldane)	1119	1115	1421	1627	2247
Mean distance between marker loci (cM)	7.2	7.9	7.5	8.2	6.1
Percentage of distorted marker loci	7.1	6.4	8.8	5.6	–
Percentage of codominant marker loci	65	61	71	64	–

Table 3 Number of QTLs identified in the 3 years of experimentation and number of regions and linkage groups (LG) carrying QTL in the single and the connected populations

Type of analysis	Number of QTL			Number of regions	Number of LGs
	2008	2009	2010		
Single population					
AB	6/4 (7)	1/1 (1)	5/4 (6)	9/**7** (11)	8/7 (10)
CB	4/4 (6)	4/5 (6)	3/5 (5)	9/12 (15)	5/9 (10)
DB	6/8 (8)	5/3 (5)	8/10 (14)	14/15 (19)	13/14 (15)
GB	5/5 (6)	3/3 (4)	2/3 (4)	7/8 (10)	5/7 (8)
Total	21/21 (27)	13/12 (16)	18/22 (29)	26/26 (31)	16/17 (17)
Connected multi-population	7	4	5	13	10

For the single populations, first and second numbers indicate the number of QTL detected with QTLCartographer and MCQTL, respectively, and the numbers in parenthesis indicate the total number of QTL/regions/LGs overall identified with the two softwares. For the connected population, QTL were detected with MCQTL

detection softwares were considered, 27, 16 and 29 QTL were detected in 2008, 2009 and 2010, respectively. These were located in a total of 31 genomic regions on 17 LGs. From one to 14 QTL were detected within a single population and year. Ten QTL were detected over two years, and four QTL were detected over the three years (*QlmC1.1_AB*, *QlmC1.2_GB*, *QlmC3.1_DB*, *QlmC4.3_DB*) (Supplementary Tables 3 and 4). Each individual QTL explained on average 12 % (range 3–31 %) of the phenotypic variation. The global phenotypic variation (as estimated with QTL Cartographer) explained by the QTL detected for one population and year ranged from 35 % (GB population in 2010) to 62 % (DB population in 2010). Most resistant alleles were derived from the resistant lines ('Aviso,' 'Canberra,' 'Darmor' or 'Grizzly') with only a few exceptions (Supplementary Table 4). Additive or over-dominance effects at QTL were identified in the different independent mapping populations. The estimated genetic effects in the regions that were detected across different years were consistent (e.g., for *QlmC1.1_AB*, *QlmC1.2_GB*, *QlmC3.1_DB,* *QlmC4.1_CB*, *QlmC4.2_AB*, *QlmC4.2_CB* *QlmC4.3_DB* (except in 2010)) or inconsistent (e.g., for *QlmA6_DB*). When over-dominance was observed, it led to an increase or decrease in the G2 index depending on the QTL and population (Supplementary Table 4).

QTL identification in the connected multi-population design

QTL mapping on the connected multi-population design revealed seven, four and five QTL in 2008, 2009 and 2010, respectively (Supplementary Table 5). They were located on LGs A2a, A6, A9, C1, C2, C3, C4, C5, C6 and C9. They explained from 4.9 to 17.2 % of the total variation, with a global R^2 equal to 36.9,

28.2 and 30.7 % in 2008, 2009 and 2010, respectively. Three QTL on LGs A9, C3 and C4 co-localized in 2 years within the connected population. The consensus QTL identified on LG C4 were located at different positions depending on the year. Most of the QTL effects were estimated to be additive except for *QlmA2* in 2008 and *QlmC4* in 2009 in the DB genetic background.

Multiple comparison of means showed that the additive effect of the 'Bristol' allele was only significantly higher than that of the 'Aviso,' 'Canberra,' 'Grizzly' and 'Darmor' alleles for *QlmC4* in 2008 and *QlmC2* in 2009. This suggests that the 'Bristol' allele confers higher disease index and thus leads to more susceptible plants. For these two QTL, no significant difference was found between the other parental alleles. This means that either the resistant parental lines share the same allele or a lack of power in the comparisons did not allow the allelic effects between parents to be differentiated. For all the other QTL, the additive effect of the 'Bristol' allele was significantly higher than one, two or three of the other parental alleles, and significant differences were found or not between the other parental alleles (Supplementary Table 5).

Comparison of QTL identified in the different populations (independent and consensus)

The QTL identified in each single population and in the connected design were projected onto the consensus map, to compare them between populations (Supplementary Figure 2). Most QTL-carrying genomic regions were identified in one or two single populations. Nineteen regions (61 %) were detected in more than one population. Six or seven regions were common to the different pairs of populations (Table 4). Three regions on A9, C4 and C8 were detected across three populations, and three regions on C2, C4 and C5 were detected in the four populations. Many LGs (A5, A6, A9, C1, C3, C4, C6 and C8) carried more than one QTL with non-overlapping support intervals.

The QTL identified from the connected design always co-localized with QTL detected either in the four single populations (on C2, C4 and C5), three single populations (on A9 and C4), two single populations (on A2a, A6, A9, C3, C4 and C6) or one single population (on C1 and C9). The QTL support intervals were either reduced compared with the single populations (for A2a_2008, A6_2010, C2_2010, C4_2008, C4_2009, C4_2010, C6_2008, C6_2010), equivalent to the one detected in the single populations (for A9_2008, C1_2008, C3_2009, C4_2009, C5_2008, C9_2010), or included all the regions detected in the single populations (for A9_2009, C3_2008). In general, the genetic effects estimated in the single *versus* connected populations were similar to those made from the analyses of the single population.

Table 4 Number of common regions identified between the four single populations

	AB	CB	DB
CB	2/4 (6)		
DB	3/4 (6)	4/4 (7)	
GB	3/4 (6)	2/6 (7)	1/4 (6)

First and second numbers indicate the number of common QTL detected with QTLCartographer and MCQTL, respectively, and the numbers in parenthesis indicate the total number of common QTL overall identified with the two softwares

Discussion

This aim of this study was to gain insight into the genetic architecture of quantitative resistance to *L. maculans* in different winter oilseed rape genetic backgrounds. In this context, the parental choice was focused on their genetic dissimilarity with the variety 'Darmor' which was studied previously. For the first time, the trait was analyzed not only in independent populations, but also in a connected multi-population design. We identified common QTL across the years and the populations as well as QTL specific to some populations.

Genetic maps

Among the four crosses, no map covered the entire genome, or even represented all the LGs. Despite the parental lines were chosen to optimize their genetic dissimilarity, the narrow genetic diversity of WOSR varieties led to a low number of polymorphic markers in some genomic regions. However, the consensus map allowed us to overcome the limits of low coverage of the independent genetic maps by

combining their information, for easier comparison of all QTL detected in this study. The uneven coverage of LGs between independent populations was still taken into account to compare the QTL. For example, LG A2 was only identified in the AB and DB populations and with poor coverage. This LG often showed lower marker coverage in other *B. napus* maps mainly based on SSR markers (e.g., Delourme et al. 2006b; Suwabe et al. 2008) as well as in *B. rapa* maps (e.g., Kim et al. 2009).

Disease assessment

Trait distribution across the 3 years reflected the polygenic nature of stem canker resistance in the four crosses. The genetic origin of the parental lines explains the narrower range of trait variation compared with Pilet et al. (1998)'s study. Indeed, those authors used varieties showing an extreme phenotype for stem canker resistance. In our study, the magnitude of variation observed for the disease index fluctuated over the 3 years, possibly due to different environmental conditions which might have been less favorable for the development of the pathogen in 2009. The weather and especially the temperature during the cropping season have a strong impact on the severity of stem canker (Evans et al. 2008). These inter-year differences influenced the number of QTL detected and led to inconsistencies in the QTL observed in each population. As the level of disease was lower in 2009 than in the other 2 years, as reflected in the G2 disease index from the control varieties and the populations, fewer QTL were identified in 2009. The low level of disease in 2009 led to a low heritability for the resistance trait and thus decreased the power of QTL detection.

The presence of transgressive lines, as previously observed in this *B. napus–L. maculans* pathosystem (Pilet et al. 1998), can be explained by an accumulation of positive or negative alleles coming from both parents (e.g., Barchi et al. 2009), by over-dominance or by epistasis in synergic or antagonistic ways (e.g., Tanksley and McCouch 1997; Rowe et al. 2008).

Consistency of QTL from the different independent crosses

Sixty percent of the QTL-carrying regions were detected in more than one population. In the other regions, the presence of specific QTL alleles involved in stem canker resistance in some parental lines might have led to population-specific QTL. However, the incomplete coverage of the genetic maps, which differed depending on the populations and the LGs, could also have led to an overestimate of the number of specific QTL. In genomic regions that were not covered by markers, it is possible that QTL exist but could not be detected (e.g., A2 was only identified in the AB and DB populations, A6 was absent in the AB population or some regions on A5 and C1 which were absent from AB and CB, respectively). An uneven coverage of LGs between the populations could also cause a bias in overall QTL detection. Inter-population differences can also be related to the low size of each population. By reducing the LOD threshold, it was possible to identify more common QTL between the populations, or between the years, but with a higher risk of taking into account false QTL (Lander and Botstein 1989). All these elements could also explain the relatively low global R^2 obtained in some populations.

QTL mapping in the connected population

By combining information from multiple populations, we could identify four to seven QTL, depending on the year. This inter-year difference is directly in connection with possible differences in environmental conditions, as observed in single populations. Only three QTL on A9, C3 and C4 were detected across 2 years. Thus, by using multi-cross analysis we have clearly increased the power of QTL detection. For example, the allelic effects at consensus QTL *QlmC2_2010* and *QlmC4.2_2008* were revealed in all populations in the connected design, whereas they were only significant in three populations when they were analyzed independently. Similarly, the allelic effects at consensus QTL *QlmA6_2010*, *QlmC1_2008* and *QlmC6_2008* were revealed in three populations in the connected design, whereas they were only significant in two populations when they were analyzed independently. The QTL located on the A2 LG was identified only in the AB independent population, but in the connected analysis with the sR12095a marker, which was common to DB and AB populations, the QTL could be detected in both these populations. This gain in detection power in connected designs was demonstrated theoretically by Rebai and Goffinet (1993) who

compared six related F2 versus six independent populations and experimentally by Blanc et al. (2006) using six maize populations. As previously observed (Blanc et al. 2006; Pierre et al. 2008; Larièpe et al. 2012; Schwegler et al. 2013), the position and support interval of some QTL could also be localized more precisely from the analysis of the connected population. Many population-specific QTL were not detected in the connected population. This observation can be explained by the relatively small contribution of specific QTL to trait variation within a multi-cross design. Such a dilution effect was also observed in studies performed on Medicago (Pierre et al. 2008), potato (Danan 2009) and ryegrass (Pauly et al. 2012). Independent and connected analyses should thus be performed in a complementary way to get a complete overview of QTL organization.

QTL were identified at different positions on many LGs. In a previous study carried out on a biparental population, Jestin et al. (2012) already raised the issue that QTL could be identified at different places on some LGs (A9 and C4) depending on the size and the sampling used. It is possible that two or more QTL exist on the same LG, whose detection could be biased by the sample size, the environment or epistasis with the genetic background.

It was possible to simultaneously estimate the allelic effect for each parental line, allowing an overall comparison of their effects in the different genetic backgrounds. Such a comparison highlights the interest of this multi-population approach because it is possible to determine the most favorable allele for stem canker resistance at each QTL. The 'Bristol' allele most often increased susceptibility compared with other parental line alleles. The other parental allele comparisons identified significant differences between the alleles of the resistant parents for the consensus QTL *QlmA6_2010*, *QlmA9_2009*, *QlmC1_2008*, *QlmC3_2009*, *QlmC4_2009*, *QlmC6_2010* and *QlmC9_2010*. The results also suggest that, for the consensus QTL *QlmC2_2010* and *QlmC4_2008*, the resistant alleles from the different resistant parents had a similar effect on resistance. For the region carrying *QlmC4_2008* and *QlmC4_2010*, the presence of identical alleles at the common markers between the resistant parents strongly suggests that a similar allele is present in all the resistant varieties at this QTL. The same comparison could not be carried out precisely for each common QTL because the number of markers was too low. Additive, dominance and over-dominance effects were observed in the different populations. Dominance and over-dominance effects are important criteria to take into account when selecting hybrid varieties. While QTL with a positive over-dominance would be favorable, the opposite would require fixing favorable alleles in each parental line. Pilet et al. (2001) also demonstrated similar effects in another segregating population. However, the sample size and/or the poor coverage of genetic maps could have biased the results. Negative over-dominance effects are often observed when a QTL is located in a region with not very many markers, confirming the potential bias in estimating genetic effect QTL.

Comparison of QTL for stem canker resistance with previous studies and implication in breeding

Previous studies on quantitative resistance of oilseed rape focused on the biparental populations: 'Darmor-*bzh*' × 'Yudal (DY) (Pilet et al. 1998; Jestin et al. 2012) and 'Darmor' × 'Samourai' (DS) (Pilet et al. 2001) at Le Rheu (France). Another study was also published on the search for QTL in four biparental mapping populations between 'Westar' (susceptible) and three resistant varieties 'Caiman' (CmW), 'Canberra' (CbW) and 'Sapphire' (SW) and between two resistant lines 'Rainbow' × 'Sapphire' (RW) in Australia (Kaur et al. 2009). Jestin et al. (2011) and Fopa Fomeju et al. (2014) used an alternative approach based on association mapping on an oilseed rape collection.

In the present study, stable QTL derived from the 'Darmor' variety in at least two genetic backgrounds among the three studied (DS, DY and DB) were observed on A1, A2, A4, A6, A8, C1, C2, C4, C5 and C8 LGs. QTL present on A1, A2, A6, C2, C4 and C8 were also identified in some other resistance sources in our study. These co-localizations highlight the fact that potentially many QTL present in 'Darmor' or its parent 'Jet Neuf' were introduced into current varieties through breeding. In addition, new QTL on A5, C1, C3, C5 and C6 were detected in different populations in the present study. For many of these QTL, markers were also found to be associated with quantitative stem canker resistance by association mapping in a WOSR collection (Jestin et al. 2011; Fopa Fomeju et al. 2014), which reinforces the interest of these QTL in breeding.

The QTL in this study were revealed from a single location in France. The Kaur et al. (2009)'s study in Australia used a parental line in common with our study, the variety 'Canberra.' The presence of common markers reveals the presence of only one potential common QTL (on A5 LG) between the CB and 'CbW' populations whose resistance allele is derived from 'Canberra.' However, the 'CbW' population was very small (76 DH) and few QTL were detected. More exhaustive studies are needed to know which QTL could be useful in different countries.

Resistance alleles derived from susceptible lines were observed in our study ('Bristol') and the literature data ('Yudal,' 'Samourai' and 'Westar'). This is the case for example of the QTL in the DS (Pilet et al. 2001), SW (Kaur et al. 2009) and DY (Jestin et al. 2012) populations that was located approximately at the same position on the C1 LG. This suggests that there is a common resistance allele shared by these susceptible varieties. Thus, the contribution of alleles deriving from susceptible varieties should not be neglected in breeding.

In the DS population, Pilet et al. (2001) found a QTL that co-localized with the specific resistance gene *Rlm2*, with both the resistance gene and QTL allele derived from 'Samourai.' In our study, LG A10 was not well represented in the GB population where *Rlm2* is segregating. Thus, this co-localization could not be investigated further. LG A7 carrying *Rlm1*, *Rlm3* and *Rlm9* is only well mapped in the DB and GB populations, where only *Rlm9* and *Rlm3* are segregating, respectively. No QTL was found on this LG, which could be related to the absence of *AvrLm9* and *AvrLm3* in the French *L. maculans* populations (Balesdent et al. 2006). The only specific resistance gene that could have been partially effective in our field trial is *Rlm1*. It was segregating in the CB population, but LG A7 was not represented in this population, so its effect could not be studied.

Conclusion

We explored new quantitative resistance factors in the *B. napus–L. maculans* pathosystem using four resistant lines crossed with a single susceptible line. We validated different QTL across different years and genetic backgrounds and identified novel QTL which had not yet been mapped. Using a connected multi-cross approach, the first on this trait, we compared the different allele effects at each QTL and obtained information on QTL location, which is essential for the introduction of these factors by MAS. Nevertheless, improved coverage of the genetic maps will be needed in order to correctly estimate all information related to QTL (location, allelic and genetic effect). The contribution of epistatic interactions to quantitative stem canker resistance should also be determined. Knowledge of common and novel QTL and their effects should help in the rational choice of relevant factors in breeding and the construction of resistant genotypes to be integrated with other control means such as cultural practices and rotations for durable management of the disease.

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