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RFLP mapping of resistance to the blackleg disease [causal agent, *Leptosphaeria maculans* (Desm.) Ces. et de Not.] in canola (*Brassica napus* L.)

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Abstract We report the tagging of genes involved in blackleg resistance, present in the French cultivar Crésor of *B. napus*, with RFLP markers. A total of 218 cDNA probes were tested on the parental cultivars Crésor (resistant) and Westar (susceptible), and 141 polymorphic markers were used in a segregating population composed of 98 doubled-haploid lines (DH). A genetic map from this cross was constructed with 175 RFLP markers and allowed us to scan for specific chromosomal associations between response to blackleg infection and RFLP markers. Canola residues infested with virulent strains of *Leptosphaeria maculans* were used as inoculum and a suspension of pycnidiospores from cultures of *L. maculans*, including the highly virulent isolate Leroy, was sprayed to increase disease pressure. QTL mapping suggested that a single chromosomal region was responsible for resistance in each of the four environments tested. This QTL accounted for a high proportion of the variation of blackleg reaction in each of the assays. A second QTL, responsible for a small proportion of the variation of blackleg reaction, was present in one of four year-site assays. A Mendelian approach, using blackleg disease ratings for classifying DH lines as resistant or susceptible, also allowed us to map resistance in the region of the highly significant LOD scores observed in each environment by interval mapping. Results strongly support the presence of a single major gene, named $LmFr_1$, controlling adult plant resistance to blackleg in spring oilseed rape cultivar Crésor. Several RFLP markers were found associated with $LmFr_1$.

Key words *Brassica napus* · DNA mapping · *Leptosphaeria maculans* · Quantitative trait loci · Restriction fragment length polymorphism

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Introduction

Blackleg disease, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [anamorph=*Phoma lingam* (Tode ex Fr.) Desm.], is widespread throughout the canola-growing areas of the western Canadian prairies and causes significant yield losses (Gugel and Petrie 1992).

Two types of blackleg resistance, early (seedling) and late (adult plant), referring to the plant growth stage when the resistance to the pathogen is expressed, have been identified in Brassica (Rimmer and van den Berg 1992). The phenotype of adult plant resistance in *B. napus* is measured in the stem of the plant at maturity. Genetic resources for adult plant resistance are very limited; most are derived from the French cultivar Jet Neuf (Wittern 1984). Late resistance was attributed to the A genome of *B. juncea* in progenies of an interspecific cross between *B. juncea* and *B. napus* (Roy 1978). Species carrying only the C genome of *Brassica*, such as *B. oleracea* and wild species of the *B. oleracea* group, are suceptible to blackleg (Mithen et al. 1987; Monteiro and Williams 1989; Sjödin and Glimelius 1988).

Genetic studies of seedling resistance to blackleg in *B. napus*, showed that resistance was controlled by a single dominant gene (*Lm1*) in the French breeding line R39, which later became the cultivar Jet Neuf. A second dominant gene (*Lm2*) was identified in the German winter rape cultivar Girita. The genes appeared to be linked (Delwiche 1980). Recessive monogenic inheritance of seedling resistance was observed in two French spring rape breeding

lines (Sawatsky 1989). Monogenic inheritance patterns was also observed in Australian spring canola cultivars (Stringam et al. 1992). Polygenic control of field resistance has been reported and adult plant resistance was primarily recessive and polygenic in a genetic study with Canadian and European breeding lines (Cargeeg and Thurling 1980; Sippell et al. 1991). Other results showed that adult plant resistance was under the control of two dominant genes, designated *Bl-1* and *Bl-2* (Sawatsky 1989). Genetic studies of adult plant resistance to blackleg, therefore, still remain inconclusive. It is becoming clear that DNA markers linked to adult plant resistance would allow reliable and rapid indirect screening of large segregating populations at a very early plant growth stage. Here, we report the tagging of RFLP markers to a blackleg resistance gene that confers a high level of adult plant resistance to the pathogenic fungus *L. maculans* in *B. napus*.

Materials and methods

Plant materials for DNA analyses

Seeds from *B. napus* spring cultivars Crésor and Westar, and the doubled-haploid (DH) progeny derived from the F_1 of this cross, were obtained from the Agriculture and Agri-Food Canada Research Centre in Saskatoon. Crésor is a French cultivar with low erucic acid, and a high glucosinolate seed content, carrying adult plant resistance to blackleg under Western Canadian conditions. Westar is a Canadian canola (oilseed rape low in erucic acid and glucosinolate) cultivar fully susceptible to the disease. The segregating population consisted of 98 DH lines derived from the microspores of the F_1 of this cross. Parental and DH lines were grown in the greenhouse at St-Jean-sur-Richelieu Research Centre under conditions described previously (Landry et al. 1991). Leaves of each line were removed from flowering plants and stored at –80°C until DNA extraction.

Genomic DNA isolation, Southern blots and probe preparations, and hybridization conditions

Plant genomic DNA was isolated and digested with the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII. Extractions, enzyme digestions and Southern transfers of DNA were carried out as described by Landry et al. (1991). Hybridizations were performed at high stringency in a 50% formamide hybridization solution at 42°C, as previously described, except that 10% dextran sulfate was added in the hybridization solution to enhance hybridization signals (Wahl et al. 1979). The labeling of probes was done with either RANDOM PRIMER kits (Amersham) or the QUICK PRIME labeling kits (Pharmacia). Probes labeled with ^{32}P were eluted on BIOGEL P-60 using chromatography columns (POLY-PREP, Bio-Rad) or centrifuge spin-columns (SPIN-X Costar).

The probes used in this study were previously mapped cDNA clones from the *B. napus* genetic map and new cDNAs from the same library (Landry et al. 1991). Some of the probes have been previously characterized as seedling-specific genes (Harada et al. 1988). Cruciferin cDNA (pC1) was provided by Martha L. Crouch, Indiana University. Two probes were obtained from the amplification of genomic DNA with arbitrary primers. Probes C2 and G2R were obtained using the 10-bp-long oligonucleotides OPC02 and OPG02 (Operon Technologies) as primers. Amplification products C2 ($OPC02_{1150}$) and G2R ($OPG02_{550}$) had been cloned in the PCR II vector using the TA cloning system (Invitrogen Corporation). RAPD analysis leading to C2 and G2R products has been previously described (Delourme et al. 1994).

Evaluation of the parents and segregating lines for blackleg resistance

Single-row plots of parental and DH lines were sown in blackleg field nurseries at Saskatoon and Melfort, Saskatchewan 1985. In 1986, two replicate rows of the parental and DH lines were sown at Saskatoon. The blackleg nurseries contained abundant canola residues infested with virulent *L. maculans*. In 1986, a suspension of pycnidiospores from cultures of *L. maculans* growing on V8-juice agar was sprayed on the plants at the 4–5 leaf stage to increase disease pressure. Blackleg severity was assessed when the plants were fully matured and just beginning to ripen. Twenty five plants from each row were uprooted and scored using the following scale: 0=no visible lesion; 1=basal stem < 1/2 girdled; 2=basal stem>1/2 girdled, plant still green; 3=basal stem completely girdled, plant prematurely ripened, seed plump; 4=basal stem completely girdled, plant prematurely ripened, seed shrivelled. Mean blackleg severity in each replicate was calculated for each entry.

For the purpose of mapping blackleg resistance as a Mendelian trait, each DH line was classified as "resistant" or "susceptible" on the basis of a mean separation test using the disease ratings from all replicates and years. Year was the main-plot treatment and DH line was the sub-plot treatment. The error term for the separation of means was the residual of the sub-plot. The resistant lines were defined as those showing the smallest disease ratings and forming a group within which means were not significantly different according to the Waller-Duncan k-ratio t-test multiple range mean separation test (k=100).

Linkage analysis

Linkage analysis and mapping of DNA markers were performed with MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992a). Localization of blackleg resistance by an interval mapping approach was done with MAPMAKER/QTL 1.1 under Apple Unix system 3.0 (Paterson et al. 1988; Lincoln et al. 1992a, b). Interval mapping analysis was performed on squared disease ratings in order to normalize the data.

Results

Identification of useful clones

A high proportion of the expressed DNA sequences used in the construction of a map of *B. napus* with the parent Topas and Westar were also polymorphic between the parents Crésor and Westar (Landry et al. 1991). The percentage of useful markers in this group was greater than 92% (79/85), whereas the percentage was lower (52%, 39/75) in the case of cDNAs monomorphic between parents Topas and Westar. An additional 58 new cDNAs were also tested; 37 (63%) detected polymorphism between Crésor and Westar. This supports our previous hypothesis that DNA markers that detect polymorphisms between closely related lines are most likely to be polymorphic in several crosses. A total of 218 cDNA probes were tested for the presence of polymorphisms between Crésor and Westar and yielded 155 informative cDNAs, of which 141 were used on the DH segregating population to reveal 175 loci.

Construction of linkage groups

Linkage analysis of the 175 RFLP loci allowed the construction of a detailed genetic map for the Crésor×Westar

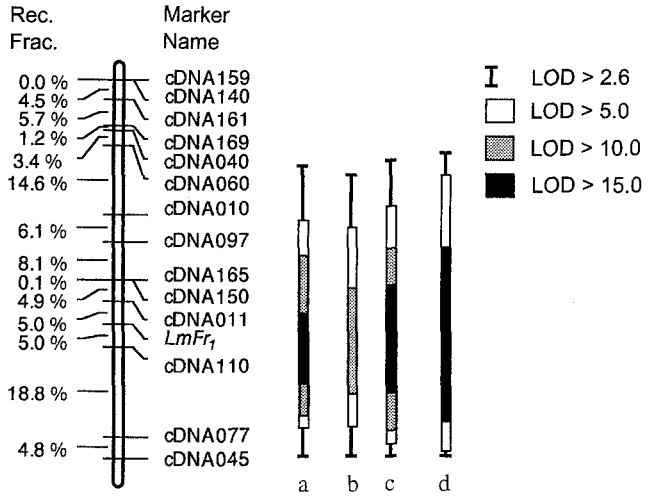

Fig. 1 RFLP map of the linkage group showing the location of *LmFr1*. The map of this linkage group was developed with RFLP markers segregating in a DH population derived from plants regenerated from the microspores of a F_1 produced from the cross of cultivars Crésor and Westar. Distances are given on the left in recombination units. The name of the RFLP marker is given on the right. The *continuous line or segment* indicates a significant QTL effect according to the LOD thresholds indicated in the figure at **(a)** Saskatoon 1985 **(b)** Melfort 1985 **(c)** Saskatoon 1986, replicate 1 **(d)** Saskatoon 1986, replicate 2

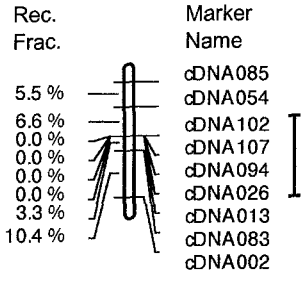

Fig. 2 RFLP map of the linkage group showing the minor QTL related to late resistance in *B. napus*, in Saskatoon 1985. The map of this linkage group was developed with RFLP markers segregating in a DH population derived from the microspores of a F_1 produced by the cross of cultivars Crésor and Westar. Distances are given on the left in recombination units. The name of the RFLP marker is given on the right. The *continuous line or segment* indicates a significant QTL effect over the LOD threshold of 2.6

cross. The markers were first grouped by two-point mapping into groups using a LOD (log of the odds ratio) score of 4.0 and a maximum recombination fraction (RF) of 0.3 as linkage criteria. The best order within each group was then obtained using multipoint mapping. The best order of small groups (seven loci and less) was directly obtained by multipoint mapping, while linkage groups composed of many markers were each constructed from a reliable frame composed of a subset of highly informative markers. These markers were identified by the two-point "big lods" command, under the restrictive criteria of recombination fractions between 0.15 and 0.30 and LOD scores≥6.0. Linkage group frames were then constructed and markers were ordered using the multipoint "compare" and "ripple" commands. The remaining markers were then sequentially added with the multipoint "try" command. The resulting order was finally compared with the one obtained by automated multipoint mapping ("order" command). The sequence of markers showing the highest log-likelihood was selected as the best order for each linkage group. The markers fell into 23 linkage groups. Five markers, however, could not be assigned to any of the linkage groups under these criteria.

Tagging of blackleg resistance

QTL mapping allowed the blackleg resistance trait, showing continuous variation, to be dissected into discrete Mendelian factors. At regular intervals on linkage groups, the method of maximum likelihood was used to estimate the phenotypic effect and a LOD score was computed to test the presence of the phenotypic effect at any particular location (Lander and Botstein 1989). The genomic localization of putative QTLs responsible for blackleg resistance was determined using the disease ratings recorded on the DH lines in each environment (i.e., Saskatoon 1985, Melfort 1985, and replicates 1 and 2 in Saskatoon 1986). Interval mapping was performed at a LOD threshold of 2.6, above which the effect was considered to be significant.

For each environment, QTL LOD score reached a maximum value within the same linkage group and was located between markers cDNA011 and cDNA110 (Fig. 1). The maximum LOD scores were 16.6 for Saskatoon 1985, 12.1 for Melfort 1985, 17.8 for replicate 1 in Saskatoon 1986, and 27.0 for replicate 2 in Saskatoon 1986. This major QTL was located 0.07 recombination units downstream from marker cDNA011 for Saskatoon 1985 and Melfort 1985, and 0.05 recombination units downstream from marker cDNA011 for replicates 1 and 2 of Saskatoon 1986. In addition to this major peak, a significant effect was observed on a duplicated region elsewhere in the genome for each combination of replicate and year. LOD scores of this QTL were 7.0, 9.8, 8.8, and 9.6 for Saskatoon 1985, Melfort 1985, replicates 1 and 2 of Saskatoon 1986, respectively. In order to validate the presence of this QTL and to look for other minor QTLs with increased sensitivity, the first major QTL was fixed at its most likely position and all the linkage groups were re-scanned. Except for Saskatoon 1985, residual LOD scores dropped below the threshold and none of the models tested supported the presence of QTLs other than the major QTL located between markers cDNA011 and cDNA110. The variance explained by the single major QTL was 57.0% for Melfort in 1985, 68.0% for replicate 1 of Saskatoon 1986, and 83.9% for replicate 2 of Saskatoon 1986. For Saskatoon 1985, the interval mapping with the major fixed QTL revealed a second, small but significant, QTL located between markers cDNA083 and cDNA002 on linkage group 2 (Fig. 2). This second significant QTL was located 0.09 recombination units downstream from marker cDNA083 (Fig. 2).

A significant increase (+4.3) of the LOD score was obtained when using a model that included both the major and the minor QTLs compared with the model defined by the major QTL alone. This indicates that two QTLs act additively and explains independent portion of the variation in response to blackleg infection. Therefore, we conclude that two QTLs explain 80% of the variation in blackleg reaction in Saskatoon 1985. The major QTL, common to all environments, accounted for most of this variation (72%), while the minor QTL, specific for Saskatoon 1985, explained only 8% of the variation.

A second approach was also used to map blackleg resistance as a Mendelian trait. The DH lines were categorized as "resistant" or "susceptible" as described in Materials and methods. The disease ratings were then mapped as a Mendelian trait. This phenotypic marker was added to the RFLP markers segregating in the DH population. Although the observed segregation ratio of this marker did not fit the model for single-gene inheritance (39 R: 59 S ; χ^2=11.44), it mapped between RFLP markers cDNA011 and cDNA110 in all environments at the same position found for the major QTL. We called it *LmFr1*.

Discussion

Our results indicate the presence of a major gene and an environmentally-dependent minor gene responsible for the expression of blackleg resistance in the DH progeny derived from the resistant cultivar Crésor and the susceptible cultivar Westar. The single major resistance gene was observed in all environments tested but the environmentally dependent gene was detected in only one environment. Both QTL-and Mendelian-mapping approaches pointed to the same chromosomal region. This resistance gene is effective against isolates of the virulent strains of blackleg prevalent in Western Canada, including the Leroy isolate. Our results support a previous report on the efficiency of resistance present in Jet Neuf against several strains of blackleg (Salisbury and Ballinger 1993). Our results also provide support to findings of a major gene controlling resistance against a PG2 isolate of *L. maculans* (Ferreira et al. 1993). The high proportion of the variance explained by the major QTL identified in this study, however, contrasts with previous reports of polygenic inheritance of resistance (Thurling and Venn 1977; Cargeeg and Thurling 1980; Sippell et al. 1991), although the identification of a second minor, environmentally-dependent, gene may explain their findings. The genetic background of each DH line that accounts for non-specific resistance (horizontal resistance) may also have an important cumulative effect on the phenotypic expression of resistance to blackleg (Cargeeg and Thurling 1980). Finally, environmental factors such as plant age, temperature, and inoculum density, could affect the disease incidence (Hall 1992). The use of a DH population in our study, combined with field assays performed over several years and locations, clearly helped to elucidate the Mendelian nature of this trait.

A second minor QTL, however, was identified from the data obtained from Saskatoon in 1985. This second QTL explained only a small, but significant, part of the variation (8%). One possible explanation for the presence of this minor QTL is the presence of specific isolate(s) and specific host-pathogen interactions occuring under this environment. Studies of host-pathogen interactions in adult plants indicate the existence of an isolate-specific expression of resistance genes (Salisbury and Ballinger 1993). It is likely that the natural inoculum, which was prevalent in the blackleg nursery plot in Saskatoon in 1985, contained both highly and weakly virulent isolates. The differences in development of the fungus and the important polymorphisms observed between the highly and weakly virulent strains has led to the hypothesis of the existence of different species of the fungus (Hammond and Lewis 1987; Morales et al. 1993; Williams 1992; Goodwin et al. 1993). Therefore, the resistance response to these different isolates may be under the control of different resistance genes. Finally, the unexplained portion of the variance of the trait could be attributed to minor resistance genes, the overall genetic background of our population, or environmental factors.

In conclusion, both Mendelian and QTL approaches strongly support the presence of a single major gene responsible for most of the adult plant resistance to blackleg present in the French Spring rape cultivar Crésor. This resistance represents true field resistance and is effective against highly virulent fungal isolates like Leroy, and possibly other fungal isolates present in Western Canada. We designate this major gene as *LmFr1*.

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