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C. Dixelius · S. Wahlberg

Resistance to *Leptosphaeria maculans* is conserved in a specific region of the *Brassica* B genome

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Abstract Offspring from asymmetric hybrids between *Brassica napus* and the three B-genome species *Brassica nigra*, *Brassica juncea* and *Brassica carinata* were analysed for the presence of B-genome markers and resistance to the fungus *Leptosphaeria maculans*, the causal agent of blackleg disease. Twenty five plants from each species combination were analysed in the first back-cross (BC_1) generation, 30 plants in BC_2 and 60 plants in BC_3. The plants were analysed by 46 RFLP markers detecting 85 loci dispersed throughout the *B. nigra* genome. The plants with additional *B. carinata* DNA had a decrease in the presence of RFLP markers ranging from 59% in BC_1 to 36% in BC_2 and down to 11% in BC_3. Similar results were obtained in the lines with additional DNA from *B. juncea* where the 60% presence of RFLP markers in BC_1 was reduced to 33% in BC_2 and to 10% in BC_3. However presence of the markers were significantly lower in the *B. nigra*-derived material where BC_1 had 46%, BC_2 25% and BC_3 8%. Since at least two loci could be detected on each end of the eight linkage groups of the B genome, the degree of symmetry was estimated. After one back-cross between 0.5 and 1.25% intact chromosomes were retained, whereas in BC_2 this frequency was 0.21% for all three B-genome donor species. The maintenance of half-chromosomes ranged from 2.63% to 5.38% in BC_1 and between 0.73% and 1.15% in BC_2. No chromosome arms were found in any of the BC_3 plants. In total, four co-segregating markers for cotyledon and adult-leaf resistance to *L. maculans* were found which detected six loci located on linkage groups 2, 5 and 8. When the results from the three donor species were compared, one triplicate region in the B genome had preserved the resistance loci in all three species.

Key words *Brassica* species · *Leptosphaeria maculans* · Resistance · RFLP markers · B genome

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C. Dixelius (✉) · S. Wahlberg
Department of Plant Biology, The Genetic Centre,
Swedish University of Agricultural Sciences, Box 7080,
S-750 07 Uppsala, Sweden
Fax: + 46 18 673279
E-mail: christina.dixelius@vbiol.slu.se

Introduction

The survival of most organisms depends on the presence of specific genetic systems to maintain diversity in the face of a changing environment. Classical examples include antigenic variation in trypanosomes and immunoglobulin gene formation in mammals (Johnson et al. 1991; Plasterk 1992). Genetic studies have shown that many different plant-disease resistance genes occur in clusters and that two distinct arrangements may exist, either single genes with multiple alleles encoding different resistance specificities or a series of tightly linked genes forming complex loci (Pryor and Ellis 1993; Hulbert 1998). The recent cloning of several disease resistance genes (Baker et al. 1997) has made it possible to analyse the structural organisation of these loci. The results have shown that disease resistance loci are not randomly distributed in plant genomes; rather, clusters of disease- and defense-response genes exist. This clustering has been noted in *Arabidopsis* (Botella et al. 1997), barley (Jorgensen, 1994), lettuce (Hulbert and Michelmore 1985), flax (Ellis et al. 1995), tomato (Dixon et al. 1996; Jones et al. 1994) and maize (Richter et al. 1995).

Brassica nigra, *Brassica carinata* and *Brassica juncea* exhibit a high level of resistance to blackleg (Roy 1978; Sacristan and Gerdemann 1986; Sjödin and Glimelius 1988) caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not. [anamorph: *Phoma lingam* (Tode ex Fr.)]. *B. nigra* (BB) constitutes the B genome of the amphidiploid species *B. carinata* (BBCC) and *B. juncea* (AABB) as described by U (1935). By sexual crossings (Roy

1978, 1984; Jahier et al. 1989; Struss et al. 1996) in combination with embryo rescue (Sacristan and Gerdemann 1986), and by somatic hybridisation (Sjödin and Glimelius 1989a, b), blackleg resistance has been subsequently transferred to the *Brassica napus* genome (AACC) from these related *Brassica* species. It was initially hypothesised that blackleg resistance is located on the *Brassica* B genome (Roy 1984), which to-date has been shown to be the case for cotyledon resistance in *B. nigra* and *B. juncea* (Chèvre et al. 1996, 1997) and stem resistance in *B. nigra*, *B. juncea* and *B. carinata* (Struss et al. 1996). To examine the presence and the precise location of cotyledon and adult-leaf resistance in the three B-genome species, backcross generations of asymmetric somatic hybrids between *B. nigra*, B. *juncea*, *B. carinata* and *B. napus* were analysed using RFLP markers mapped to the *Brassica* B genome. The results show that one triplicated region in the B genome had preserved the resistance loci in all three species.

Materials and methods

Plant material

Asymmetric somatic hybrids were produced between *L. maculans*-resistant accessions of *B. nigra* (L.) Koch, *B. juncea* (L.) Czern, *B. carinata* (L.) Braun and the susceptible *B. napus* (L.) cultivar Hanna (Sjödin and Glimelius 1989 a). Additional unpublished material of asymmetric *B. napus* and *B. nigra* somatic hybrids from the same experiment were also included. Backcrosses (BCs) of the resistant asymmetric hybrid plants and their resistant offspring were conducted in three generations, with the same *B. napus* variety as used in the protoplast fusion being the male parent. All crosses were performed in the greenhouse. Seventy five plants deriving from five original hybrids between *B. napus* and *B. nigra*, *B. napus* and *B. juncea*, and *B. napus* and *B. carinata*, respectively, being both resistant and susceptible to *L. maculans*, were analysed after the first backcross. In BC_2 90 plants from all three species combinations were included in the study, while in BC_3 180 plants were included.

Screening of *L. maculans* resistance

Resistance to *L. maculans* was tested in all backcross generations by both inoculation of the cotyledons and the adult leaves, at the three-leaf stage, with pycniospores from the pathogen according to Sjödin and Glimelius (1988). The plants were grown in a culture chamber with a 16/8-h light/dark regime and a temperature of 21°/16°C. The humidity was raised to 90% for 2 h post-inoculation and then decreased to 65%. A highly virulent fungal isolate, Lm 1245 (PG 2), was used in all screening work. This isolate was kindly provided by the Crucifer Genetics Co-operative, University of Wisconsin, Madison, USA. The inoculated plants were scored, about 8–16 days after inoculation, either as resistant or as susceptible according to Delwiche (1980).

DNA analysis

Isolation of plant DNA, Southern blotting and hybridisation were all performed according to Sharp et al. (1988) with modifications as described in Forsberg et al. (1998). Forty six restriction fragment length polymorphism (RFLP) markers (Ferreira et al. 1994; Thormann et al. 1994) detecting 85 loci dispersed in the *B. nigra* genome (Lagercrantz and Lydiate 1995) were used as DNA probes.

Statistical analyses

For each backcross generation of a species, the mean percentage and standard deviation of retained RFLP loci was calculated. Student's *t*-test was used to determine significance levels regarding differences between the groups. All calculations and statistical tests were done using Microsoft Excel version 5.0.

Results

Genome configurations

A significant decrease of the *Brassica* B-genome RFLP markers was found between the three backcross generations (Fig. 1). The plants with additional *B. carinata* DNA had a decrease from 59% in BC_1 to 36% in BC_2, and which was further reduced to 11% of the RFLP markers present in BC_3. The situation was similar in plants where the additional DNA was derived from *B. juncea*, the 60% presence of RFLP markers in BC_1 being reduced to 33% in BC_2 and to 10% in BC_3. In the plants where *B. nigra* DNA was integrated into the *B. napus* background, the presence of RFLP markers was significantly lower than for the other species in all three generations ($P < 0.001$). In BC_1 46% of the markers were maintained, in BC_2 25%, and in BC_3 only 8% were present. Furthermore, no biased retention of large specific B-genome regions could be found when the RFLP analyses of the different plant materials were compared.

Since at least two loci could be detected at each end of the eight linkage groups of the B genome, the degree of symmetry could be estimated. The extent of retained

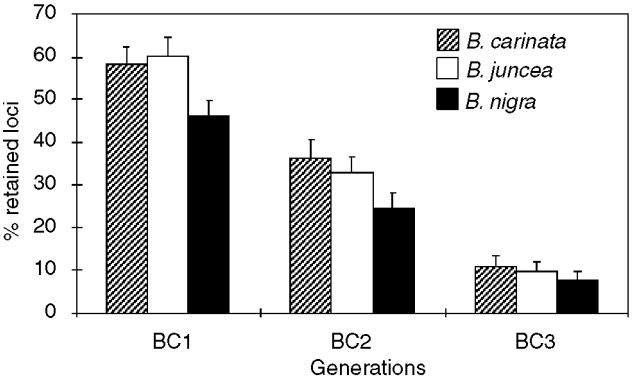

Fig. 1 The bars represent the mean frequency of retained B-genome loci in three different backcross (*BC*) generations. The standard deviation is added to all bars. The mean frequencies of the *B. nigra* material is in all three cases significantly lower than those of *B. carinata* and *B. juncea* ($P < 0.001$)

intact and half-chromosomes, respectively, in each back-cross generation is shown in Table 1. The frequencies represent the ratio of the total number of chromosomes maintained in each generation as compared to the total possible number of maintained chromosomes (i.e. all chromosomes maintained in all plants). After one backcross generation between 0.5 and 1.25% of the intact chromosomes were retained, whereas in the second backcross generation this frequency was 0.21% for all three B-genome donor species. The maintenance of half-chromosomes ranged from 2.63% to 5.38% in BC_1 and between 0.73% and 1.15% in BC_2. No chromosome arms were found in any of the BC_3 plants.

Co-segregating RFLP markers

When the RFLP analyses of the plants were compared with the phenotypic results of the *L. maculans* tests on cotyledons and adult leaves, a total of four co-segregating markers were found to be located on linkage groups 2, 5 and 8. In the backcrossed generations with additional *B. carinata* DNA, two cotyledon resistance loci were found on linkage groups 5 and 8 and one adult-leaf locus was found on linkage group 2. In the material derived from the *B. napus* and *B. juncea* hybrids, a cotyledon resistance locus was found on linkage group 5 and two adult-leaf resistance loci were found on linkage groups 2 and 8. In the third case, where *B. nigra* DNA was added to the *B. napus* background, one cotyledon resistance locus was found on linkage group 2 and two adult-leaf resistance loci on linkage groups 5 and 8. When this marker information from the three populations was compared, one single sub-region located on the three linkage groups 2, 5 and 8 in the B genome could be significantly linked to resistance (Fig. 2).

Table 1 Frequency of retained intact and half-chromosomes in the first and second backcross (BC) generations. All plants of one generation were grouped together. The frequency represents the ratio of the total number of chromosomes retained within the generation as compared to the total possible amount, i.e. all chromosomes retained in all plants

Generation	B-genome species	% Retained whole chromosomes	% Retained half-chromosomes
BC_1	*B. carinata*	1.25	4.25
	B. juncea	1.0	5.38
	B. nigra	0.5	2.63
BC_2	*B. carinata*	0.21	0.73
	B. juncea	0.21	1.15
	B. nigra	0.21	0.83

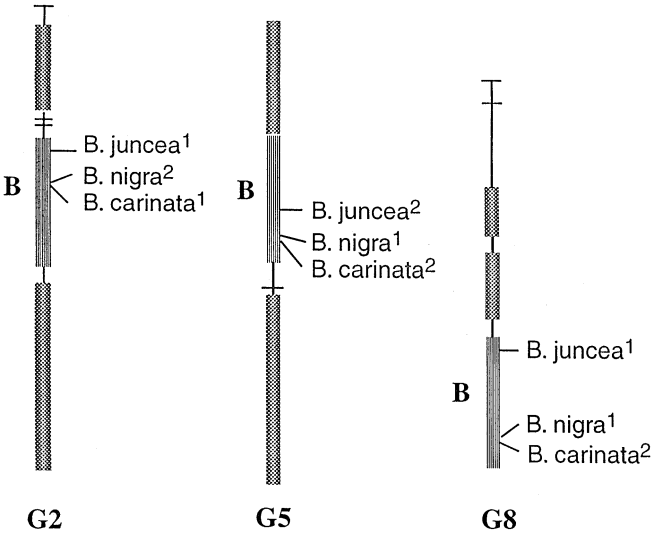

Fig. 2 Location of cotyledon and adult-leaf resistance loci derived from *B. nigra*, *B. juncea* and *B. carinata*, on three linkage groups in the *Brassica* B genome. *1* adult resistance, *2* cotyledon resistance. Modified genetic map from Lagercrantz and Lydiate (1996)

Discussion

Somatic hybridisation is an extensively used technique to transfer agronomically important characters from alien or related species into different crops where normal sexual crossings are restricted by incompatiblity barriers (Waara and Glimelius 1995). This technique has also been a tool to improve disease resistance, through the transfer of resistance traits to crop species from related, mostly wild, species (Dixelius and Glimelius 1995). When utilising somatic hybridisation as a genetic bridge, this approach usually produces asymmetric somatic hybrids. This was also the case with the hybrid material employed in this investigation, where X-irradiation had been used to fragment the donor genomes of *B. nigra*, *B. juncea* and *B. carinata* before hybridisation with *B. napus* (Sjödin and Glimelius 1989 a). Since somatic hybrids never develop cotyledons, five asymmetric hybrids of each species combination showing resistance on the adult leaves were chosen to be parents of the investigated progeny. However, when cytological analyses were performed the chromosome numbers varied between 44 and 54 in the selected original *B. napus* (+) *B. nigra* hybrid plants (Sjödin and Glimelius 1989 a). The *B. napus* (+) *B. juncea* plants had between 42 and 66 chromosomes and the chromosome numbers varied between 44 and 66 in the *B. napus* (+) *B. carinata* plants. This information can be compared with the RFLP analyses of the backcross generations. After one backcross the frequency of retained chromosomes was 0.5–1.25% corresponding to, at the most, one chromosome in five individuals. The presence of complete linkage groups was further reduced in the subsequent generation and in BC_3 only

DNA fragments of the B genome were left in the *B. napus* background. These results can be compared to information from asymmetric hybrids between *B. napus* cv Hanna and *Arabidopsis thaliana* (Bohman et al. 1999). Here, after one backcross the chromosome number was reduced to 38.4 and only 3.8% of the analysed plants had two *A. thaliana* RFLP loci maintained on each chromosome. This implies that one or two whole *A. thaliana* chromosomes were present in a few individual plants. Moreover, only 16% of the *A. thaliana* RFLP markers were present in BC_1 progeny from the asymmetric hybrids between *B. napus* and *A. thaliana.* In contrast, the amount of RFLP markers retained in the plant material of the present investigation was rather large in the first and second backcross generations. This was especially the case for the two amphidiploid donor species where the presence of the B-genome markers was significantly higher compared to *B. nigra*-derived lines even after three backcrosses. Whether this slow decrease of the B genome reflects some chromosome events which had taken place between the rather closely related amphidiploid *Brassica* species or is the effect of selection pressure can only be speculated upon. However, evidence that selection can give large effects was found in oilseed rape hybrids with *A. thaliana*, where it was possible to retain a chromosome pair after three backcrosses with *B. napus* in combination with *L. maculans* screenings (Bohman and Dixelius, in preparation).

The RFLP analyses resulted in the identification of a of four *L. maculans* resistance co-segregating markers which mapped to different loci on linkage groups 2, 5 and 8 of the *Brassica* B genome. Cotyledon and adult-leaf resistance co-segregated as different loci in all three species, as earlier proposed in *B. juncea* (Salisbury and Ballinger 1993). A comparative analysis of synteny groups extracted from the B-genome species *B. nigra*, *B. juncea* and *B. carinata* was conducted by the addition of these chromosomes to *B. napus* by Struss et al. (1996). By making consensus synteny groups of the three species, the stem resistance to *L. maculans* was localised on synteny groups 1, 3 and 4. These results correspond very well to those obtained in the present investigation where the adult-leaf resistance of the three B-genome species was found to be located on three different linkage groups. However, the individual designations of the various linkage groups differ because different linkage maps have been used in different investigations. This is also the case when comparing our results with studies of cotyledon resistance in *B. nigra* (Chèvre et al. 1996), which was found to be localised on linkage group 4, and cotyledon resistance of *B. juncea*, which was found on linkage group 8 (Chèvre et al. 1997). Furthermore, different parental material has been used as resistance gene donors in these three investigations; thus, whether the number of resistance genes is constant within the *B. nigra*, *B. juncea* and *B. carinata* species is unknown. It is also possible that some genes were initially present in the original hybrid plants but were subsequently lost during the backcrossing procedure. This was indicated by one marker located on linkage group 6 which in a few cases co-segregated with resistance but did not show a consistent performance in the following generations.

All markers found to co-segregate with the *L. maculans* resistance in the three B-genome species were localised to a specific sub-region of the *Brassic*a B genome, designated B by Lagercrantz and Lydiate (1996). This region is present in three copies in the B genome and has probably evolved by intragenomic duplication events. Both cotyledon and adult-leaf resistance from the three B-genome species was represented in each sub-region, indicating that these characters must have been present in the common ancestral hexaploid genome. Previous studies have indicated that natural *B. juncea* may have changed substantially, and that complex genome duplications and rearrangements occurred after its initial formation (Cheung et al. 1997). However, more recent investigations based on RFLP maps of both natural and synthetic *B. juncea* indicate that the A- and B-genome components of *B. juncea* have remained intact since the formation of the amphidiploid (Axelson et al. 1998). These latter results point to a strong conservation of genetic information between the two *Brassica* genomes, though additional events like gene duplications, gene conversions or deletions may have played a role in creating the differentiated resistance genes. Only sequence information on these resistance genes can shed more light on how they have been generated in the three B-genome species throughout evolution.

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