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Development and characterisation of *Brassica napus-Sinapis arvensis* addition lines exhibiting resistance to *Leptosphaeria maculans*

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Abstract Blackleg caused by *Leptosphaeria maculans* is one of the most important diseases affecting oilseed rape worldwide. *Sinapis arvensis* is valuable for the transfer of blackleg resistance to oilseed rape (*Brassica napus*) because this species contains high resistance against various aggressive isolates of the blackleg fungus. These include at least one Australian isolate which has been found to overcome resistance originating from species with the *Brassica* B genome, until now the major source for interspecific transfer of blackleg resistance. Backcross offspring from intergeneric crosses between *Brassica napus* and *S. arvensis* were subjected to phytopathological studies and molecular cytogenetic analysis with genomic *in situ* hybridisation (GISH). The BC_3S progenies included fertile plants exhibiting high seedling (cotyledon) and adult plant resistance associated with the presence of an acrocentric addition chromosome from *S. arvensis*. In addition, some individuals with adult plant resistance but cotyledon susceptibility were observed to have a normal *B. napus* karyotype with no visible GISH signals, indicating possible resistant introgression lines. Phytopathological analysis of selfing progenies from 3 different highly resistant BC_3 plants showed that seedling and adult plant resistance are probably conferred by different loci.

Key words *Brassica napus* · *Sinapis arvensis* · *Leptosphaeria maculans* · Intergeneric hybrids · Blackleg resistance · Genomic *in situ* hybridisation

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Introduction

Leptosphaeria maculans (Desm.) Ces. et De Not. [anamorph *Phoma lingam* (Tode ex Fr.) Desm.], the causal agent of blackleg, induces severe damage worldwide to basal parts (stem canker) of oilseed rape (*Brassica napus* L., genome AACC, 2n=38) and other susceptible plants of the family Brassicaceae. Moreover, it can also provoke lesions and necroses on leaves, pods and seeds. The genetic basis of blackleg resistance in *B. napus* in Europe is narrow and originates for the most part from the French cultivar Jet Neuf, which possesses a partial, polygenically controlled adult plant resistance not expressed at the seedling stage (Cargeeg and Thurling 1980). In contrast, all *Brassica* species containing the B genome exhibit an absolute and stable resistance to most of the aggressive pathogen isolates studied to date. B-genome resistance is mono- or oligogenically controlled (see Rimmer and van den Berg 1992; Dixelius 1999) and efficient from the seedling stage onwards. Thus, B-genome donors like *B. nigra* (L.) Koch (BB, 2n=16) and *B. juncea* (L.) Czern (BBCC, 2n=36) are often used as a genetic pool for the development of resistant oilseed rape (Roy 1978; Sacristán and Gerdemann 1986; Sjödin and Glimelius 1989; Chèvre et al. 1996a; Struss et al. 1996; Plieske et al. 1998; Dixelius 1999).

Some aggressive isolates of the pathogen have recently been shown to overcome the resistance of *B. juncea* (Purwantara et al. 1998; Winter et al. 1999). *L. maculans* exhibits a broad variation in virulence, giving it the potential to adapt quickly to a given resistance (Kuswinanti et al. 1999). The generation of a durable resistance therefore necessitates the application of a broad spectrum of resistance sources in oilseed rape breeding. For this reason, interspecific and intergeneric transfer of blackleg resistance from wild crucifers like *Sinapis arvensis* L. (wild mustard, genome SarSar, 2n=18) is becoming increasingly important. *S. arvensis* possesses resistance in all developmental stages to various *L. maculans* isolates (Plümper 1995), including one from Australia that overcomes the resistance of *B. juncea* (see Winter et al. 1999).

Table 1 Scales for evaluation of blackleg symptoms on cotyledons and adult plants

Score	Symptoms	Class[a]
Cotyledon		
1	Punctiform necrosis at inoculation site	R_C
2	Minor chlorotic lesion (< 2 mm diameter)	R_C
3	Moderate chlorotic lesion (2–5 mm)	S_C [b]
4	Major chlorotic lesion (> 5 mm), usually with pycnidia formation at later stages	S_C
Adult plant		
1	Limited necrosis around inoculation site	R_A
2	Minor damage to the basal parts of the stem (≤ 25% girdling)	R_A
3	Moderate damage on the basal parts of the stem (>25% and ≤50% girdling)	S_A
4	Major damage on the basal parts of the stem (>50% and <100% girdling)	S_A
5	Severe damage on the basal parts of the stem (100% girdling)	S_A
6	Plant collapse	S_A

[a] Classes: R = resistant, S = susceptible, C = cotyledon, A = adult plant.
[b] Classified as R_C when lesion develops only at late stage

Sexual hybridisation between *S. arvensis* and *B. napus* has been reported by various authors (Kerlan et al. 1993; Plümper 1995; Bing et al. 1996; Chèvre et al. 1996b; Lefol et al. 1996). Although such hybrids between *Brassica* species and their close relatives are generally easy to produce, until now they have been used in only a few cases for the breeding of new rapeseed varieties with traits of agronomical importance (see Friedt and Lühs 1998). The generation of new lines containing the character of interest in the desired genetic background is accelerated considerably when the transfer of donor chromatin can be effectively monitored (Jiang and Gill 1996). Classical cytogenetic investigations are difficult in *Brassica*, however, due to a lack of cytological markers for a reliable identification of chromosomes. Molecular cytogenetic techniques like genomic *in situ* hybridisation (GISH), on the other hand, provide an effective alternative for identifying chromosome additions and introgressions in hybrid offspring without the need for time-consuming and expensive molecular genetic investigations. The suitablility of GISH for the characterisation of intergeneric *Brassica* hybrids has been demonstrated (Fahleson et al. 1997; Sharzhinskaya et al. 1998; Snowdon et al. 1998). Despite this, genetic investigations of hybrids carrying introduced blackleg resistance have until now been mainly limited to molecular marker or classical cytogenetic analyses in interspecific crosses between *Brassica* species (Chèvre et al. 1996a, 1997; Plieske et al. 1998; Dixelius 1999).

In the study reported here, selfing progenies (BC_3S) of resistant BC_3 plants from hybrids between *B. napus* and *S. arvensis* were characterised with respect to their resistance behaviour, at different developmental stages, to the aggressive *L. maculans* isolate W4. Molecular cytogenetic analysis using GISH was applied to investigate the genomic composition of resistant and susceptible individuals. Preliminary results have been reported by Snowdon et al. (1999) and Winter et al. (1999).

Materials and methods

Plant material

Offspring from intergeneric crosses between *B. napus* cv. Madora (winter oilseed rape) and *S. arvensis* (origin: Biologische Bundesanstalt Braunschweig, gene bank accession No. 22529) were backcrossed three times with winter oilseed rape cv. Ceres. Following blackleg resistance tests (see below) of backcross offspring, BC_3 plants exhibiting both cotyledon and adult plant resistance were selfed to generate BC_3S progenies. Plants were grown in a greenhouse at a minimum temperature of 18°C, with additional illumination during the months October-April. The material was vernalised for 6–8 weeks at 4°C.

Crosses, ovule and ovary cultures (embryo rescue), along with classical mitotic chromosome analyses of the original hybrids and plants of the backcross generations, have been described by Plümper (1995), who used methods modified from Sacristán and Gerdemann (1986). In some cases, 3- to 6-mm-long styles from young flower buds were used instead of root tips for chromosome counts, as suggested by Wu et al. (1997).

In addition to the resistance tests on different BC_3 and BC_3S progenies, susceptible control genotypes (*B. napus* cvs Madora, Ceres and Lesira) and plants of the resistance donor *S. arvensis* were also included as positive and negative infection controls, respectively. Lesira was integrated in the tests because it is one of the most susceptible oilseed rape cultivars.

Resistance tests

Plants were inoculated with a pycnidiospore suspension of the aggressive German *L. maculans* isolate W4 (Tox^+, Hassan et al. 1991) obtained from cultures grown on V8-agar medium (Sacristán 1982). Cotyledon and adult plant resistance tests with double inoculation were carried out in the greenhouse as follows. Cotyledons of 5- to 9-day-old plants were punctured centrally and inoculated with 5 µl of a standard spore suspension (10^7 spores/ml). Plants were incubated for 3 days in a transparent tent under high-humidity conditions. Maximum lesion sizes were scored at 2-day intervals between the 14th and 24th day after inoculation using a scale (see Table 1) modified from Sacristán (1982). Plants with scores 1, 2 and only late occurrence of 3 were classified as cotyledon resistant (R_C); the remaining cases were classified as cotyledon-susceptible (S_C).

At the age of 4–5 weeks the same plants were inoculated a second time. A cellulose pad, soaked with 100 µl of the standard spore suspension, was applied with a strip of parafilm to a wound at least 1 cm long and 1–2 mm deep that had been inflicted with a lancet across the border between the hypocotyl and epicotyl. The pads were removed after 10 days. External blackleg symptoms on adult plants (see Table 1) were evaluated 6–7 weeks after stem in-

Table 2 Chromosome counts and blackleg resistance response frequencies in hybrid plants and negative (-) and positive (+) infection controls. *Sinapis arvensis* was the resistance donor, while Ceres, Madora and Lesira are blackleg susceptible *B. napus* cultivars

Genotype	Chromosome number (2n)		Plants tested	Resistance[a] response frequencies							
				R_C	S_C	R_A	S_A	$R_{C/A}$	S_C/R_A	R_C/S_A	$S_{C/A}$
BC_3 9.10	38, 39	BC_3S offspring	19	6	13	11	8	4	7	2	6
BC_3 13.6	37, 39, 41		19	4	15	12	7	4	8	0	7
BC_3 16.3	40–43[b]		25	6	19	17	8	5	12	1	7
BC_3 31.2	36, 38		19	0	19	0	19	0	0	0	19
*S. arv*ensis (-)	18	Control genotypes	25	25	0	25	0	25	0	0	0
Ceres (+)	38		21	0	21	3	18	0	3	0	18
Madora (+)	38		17	0	17	2	15	0	2	0	15
Lesira (+)	38		19	0	19	0	19	0	0	0	19

[a] Resistance classes are described in Table 1; C/A = combined cotyledon and adult plant resistance or susceptibility, respectively
[b] Exact determination of BC_3 chromosome number was not possible

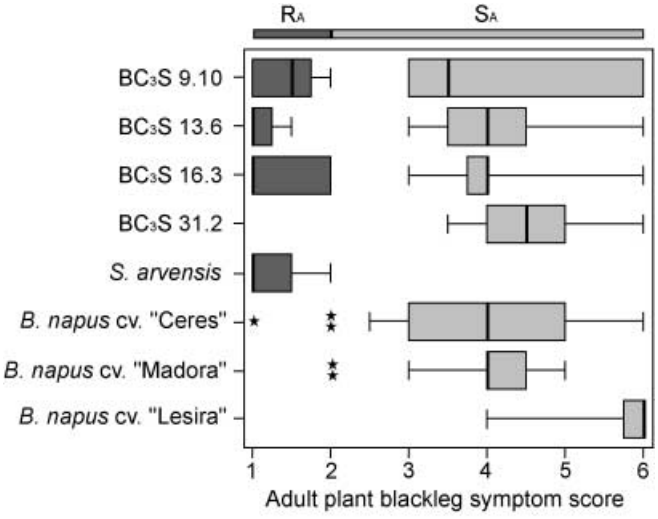

Fig. 1 Boxplot (SPSS) showing adult plant blackleg symptom scores of resistant (R_A) and susceptible (S_A) plants in 4 BC_3S progenies from the interspecific cross *B. napus* × *S. arvensis*, along with control genotypes. *Boxes* cover the interquartile range, with median scores shown by the *thick vertical lines*. *Whiskers* cover the remaining variation, with the exception of 5 resistant *B. napus* individuals that are represented by *stars*

oculation. Selected plants with no or only small external lesions were chosen as candidates for resistance. These plants were vernalised and evaluated once more during seed ripening to give a post-vernalisation score. Plants with a maximum external score of 2 or less were classed as adult plant resistant (R_A); others were grouped as adult plant susceptible (S_A). Distributions of adult plant blackleg symptom scores in different BC_3S progenies and control genotypes were compared with a boxplot generated by the software SPSS.

For the majority of the plants, internal lesions were scored simultaneously, by cross-sectioning of the basal stem parts, using a scale based on that of Hammond and Lewis (1987). Because the internal lesion scores usually reflected the external symptoms, this data is omitted here.

Genomic *in situ* hybridisation

Genome composition in resistant and susceptible backcross offspring was analysed by GISH following methods described previously (Snowdon et al. 1997). Chromosome spreads were produced from protoplast suspensions from root tips of young plants or cuttings, then fixed with formaldehyde and denatured using standard techniques. For the GISH probe, genomic DNA from *S. arvensis* was directly labelled with the fluorochrome Cy3 by nick translation and resuspended in hybridisation solution with a 50-fold excess of unlabelled, sheared *B. napus* competitor DNA. The probe was pre-annealed for 20 min at 37°C prior to hybridisation. Following overnight *in situ* hybridisation (see Schwarzacher et al. 1994) and a stringent wash for 10 min in 0.2 ´ SSC at 42°C, chromosomes were counter-stained in DAPI and examined using a Leica DM 3 fluorescence microscope. At least ten metaphases were examined for each hybridisation.

Results

Out of 222 tested BC_3 plants, with chromosome numbers from 2n=38–43, 15 exhibited both cotyledon and adult plant resistance; 98 were classed as susceptible on both levels. Results of phytopathological tests on BC_3S progenies derived from 3 double resistant BC_3 plants (9.10, 13.6 and 16.3) and 1 double susceptible BC_3 individual (31.2), respectively, along with positive and negative control genotypes, are outlined in Table 2 and Figs. 1 and 2. Tested offspring from the BC_3S individual 31.2 were all double susceptible, and plant collapse occurred in some instances, although the average adult plant susceptibility was not as high as for the very susceptible control *B. napus* cv. Lesira (Figs. 1, 2a, 2e). BC_3S progenies derived from the BC_3 plants 9.10, 13.6 and 16.3 exhibited cotyledon and/or adult plant resistance (Figs. 2a, 2d, 2e). The majority of BC_3S offspring from these 3 individuals were adult plant resistant but cotyledon susceptible. Only a small number of plants showed the reciprocal resistance behaviour.

Two out of seventeen tested plants from the original cross *B. napus* parent Madora and 3 out of 21 plants from the backcross parent *B. napus* cv. Ceres were scored as adult plant resistant (see Discussion), while most of the others were only moderately adult plant susceptible, similar to the majority of adult plant susceptible BC_3S progeny (Figs. 1, 2c, 2e left). All *S. arvensis* individuals showed a clear cotyledon (Fig. 2a) and adult plant resistance phenotype (Figs. 1, 2b).

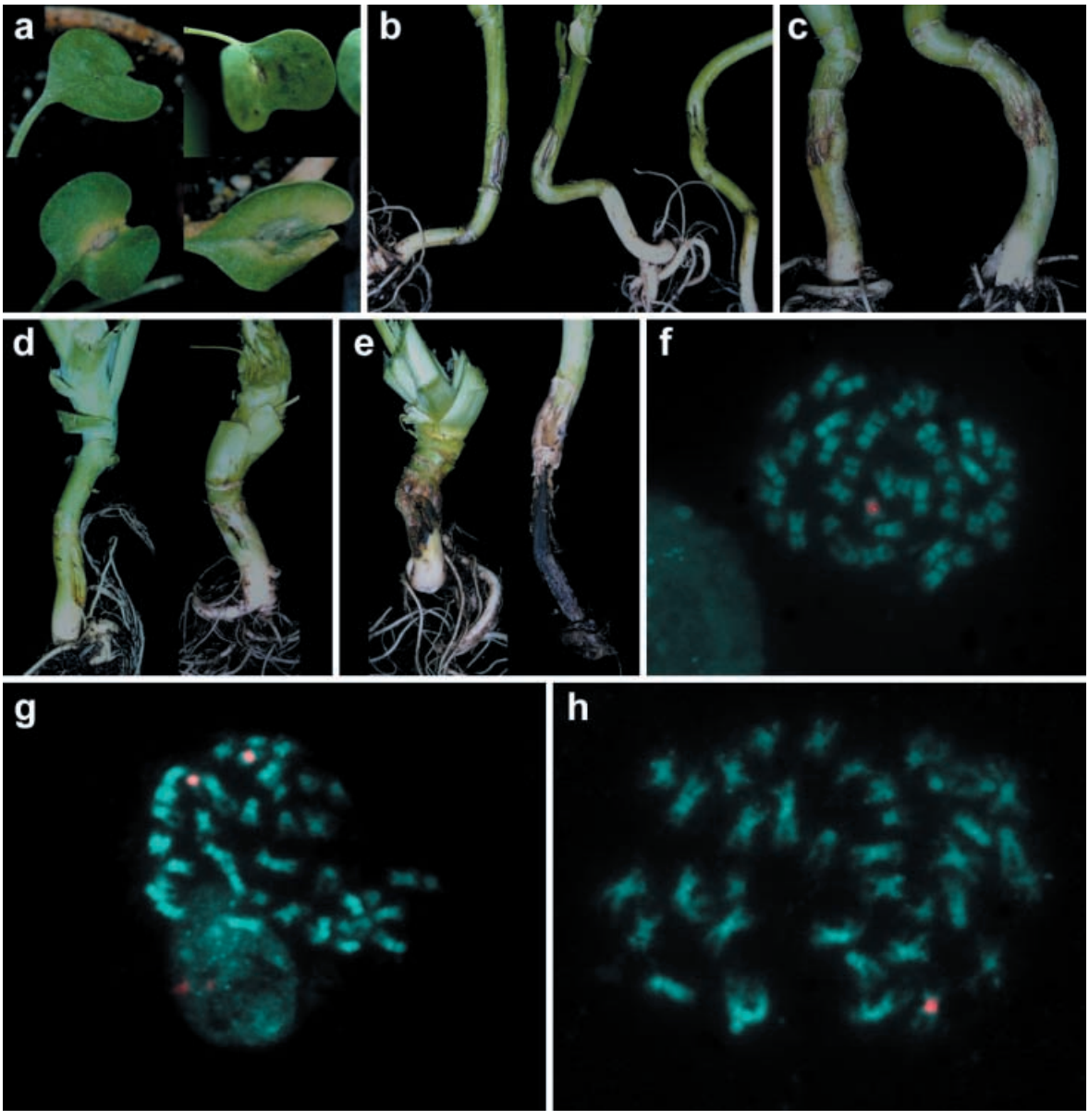

Fig. 2a–h Phytopathological and molecular cytogenetic characterisation of hybrid plant material. **a** Cotyledon resistance (*above*) and susceptibility (*below*) phenotypes in BC_3S progeny. The *above-left* phenotype is typical for *S. arvensis*, while the *below-right* symptoms are typical for the *B. napus* cultivars Ceres, Madora and Lesira. **b** Adult plant resistance of *S. arvensis*. **c** Moderate adult plant susceptibility of *B. napus* cv. Ceres. **d** Adult plant resistance phenotypes in BC_3S progeny. **e** Adult plant susceptibility phenotypes in BC_3S progeny. The phenotype on the *left* is representative for the majority of adult plant susceptible BC_3S offspring, while the stem on the *right* shows plant collapse, typical for *B. napus* cv. Lesira. **f-h** Genomic *in situ* hybridisation results in three BC_3S offspring plants. *S. arvensis* chromosomes are labelled *red* with Cy3, while *B. napus* chromosomes show no hybridisation signals and are stained *blue* with DAPI. **f, g** Individuals showing both cotyledon and adult plant resistance and corresponding respectively to the *left* and *right* plants in **d**, with **f** a monosomic, acrocentric addition, and **g** one acrocentric and a second, metacentric *S. arvensis* addition chromosome. **h** An adult plant sensitive individual with a monosomic, metacentric addition chromosome, corresponding to the *right-hand* plant in **e**

Results from GISH with selected BC_3S offspring are given in Table 3, while Fig. 2 (f–h) shows examples of GISH images from resistant and susceptible BC_3S plants with monosomic or double addition chromosomes. Two individuals showed adult plant resistance but cotyledon susceptibility and a stable chromosome number of 2n=38 with no visible GISH signals, indicating the possible presence of small introgressions carrying the resistance gene(s). A further plant with the same resistance behaviour and no GISH signals had variable chromosome numbers (2n=36–38). The BC_3 parents studied also showed somatic variations in mitotic chromosome number (Table 2), however chromosome counts in the BC_3S offspring were generally more uniform than in the BC_3. All remaining adult plant resistant individuals tested contained an acrocentric addition chromosome, and in 3 plants that were also cotyledon resistant this addition was monosomic (Fig. 2f). One adult plant resistant individual and 2

Table 3 Karyotpyes of BC_3S plants investigated by GISH detection of *S. arvensis* chromatin, grouped according to their respective resistance response (see Table 1)

		Chromosome number (2n)					
GISH karyotype:		36–37 No GISH signal	38 No GISH signal	38 Monosomic metacentric substitution chromosome	39 Monosomic metacentric addition chromosome	39 Monosomic acrocentric addition chromosome	40 Metacentric plus acrocentric addition
Resistance response	R_A	1[a]	3[a]			1	1
	$R_{C/A}$					3	2
	$S_{C/A}$		5	1	1		

[a] Variable chromosome number within one plant

plants also exhibiting cotyledon resistance had both an acrocentric and a metacentric addition (Fig. 2g). Plants that possessed only a metacentric addition (Fig. 2h), or in one case a metacentric substitution chromosome, were fully susceptible. All other susceptible plants tested had 38 chromosomes and no visible GISH signals.

Discussion

This study describes the characterisation of blackleg resistant backcross offspring from a cross between *B. napus* and *S. arvensis*. The use of the GISH method provides what is to the best our knowledge the first clear evidence of monosomic and double addition chromosomes in crosses between species of the genera *Brassica* and *Sinapis*. Moreover, the results presented here indicate the possible occurrence of intergenomic recombination, resulting in plants with a normal *B. napus* karyotype (2n=38; no *S. arvensis* chromatin detected by GISH) exhibiting resistance introgressed from the donor genome.

In intergeneric hybrids between *S. arvensis* and the three diploid *Brassica* species containing the A, B and C genomes, respectively, Mizushima (1950) reported three allosyndetic bivalents in ASar and CSar hybrids and seven in BSar hybrids. This is in agreement with data published by Kerlan et al. (1993) and Chèvre et al. (1996b) who found few paired chromosomes in ACSar hybrids. Investigations using chemotaxonomic markers (Tsukamoto et al. 1993) and on the DNA level (Song et al. 1988; Warwick and Black 1991; Kapila et al. 1996) also showed the relatively close relationship of *S. arvensis* to *Brassica* species containing the B genome.

S. arvensis is particularly interesting for oilseed rape breeding because of its high level of blackleg resistance to various *L. maculans* isolates. Furthermore, its resistance response differs largely from that of *Brassica* species with the B genome with respect to the timing of phytoalexin induction after inoculation with the pathogen (Storck and Sacristán 1995), making it a potentially useful complementation to existing resistance sources. This study confirms the suitability of *S. arvensis* as a donor plant for blackleg resistance transfer into oilseed rape and reinforces the value of GISH for the investigation of intergeneric hybrids between *B. napus* and its close relatives.

Blackleg resistance tests like those used in this study, involving a double inoculation with the same isolate, are the method of choice for accurate comparative resistance evaluation at both the seedling (cotyledon) and adult plant stages. Because double inoculation induces a very severe phytopathological response, it widely prevents escapes and levels differences in the reaction within one genotype. It is possible with this test, however, to overlook specific resistances; for example in the lamina of the cotyledons or leaves, or in the petioles (Hammond and Lewis 1987; Pang and Halloran 1996). Nevertheless, in comparison with tests of adult plants inoculated only on cotyledons or basal parts of the stem, enhanced effects were observed in tests with double inoculation (data not shown), especially in susceptible or only moderately resistant genotypes. This indicates that systemic acquired resistance, as described by Mahuku et al. (1996) for co-infection of weakly and highly virulent *L. maculans* isolates, does not occur in this system.

The oilseed rape cultivars Madora and Ceres, used as the respective cross and backcross parents in the present study, showed a certain degree of adult plant resistance in comparison to the other positive control, *B. napus* cv. Lesira (Fig. 1). The resistance behaviour of these genotypes, which both derive from the cultivar Jet Neuf, demonstrates that adult plant tests with double inoculation are preferable to clearly differentiate a significant resistance response. Moreover, the results presented here confirm previous reports on oilseed rape, which showed that the cotyledon response is not a suitable indicator for adult plant resistance behaviour (Cargeeg and Thurling 1979; Sacristán 1982; Pang and Halloran 1996).

The segregation pattern in selfing progenies derived from 3 different, highly resistant BC_3 individuals suggests that adult plant resistance is inherited more readily than cotyledon resistance. Probably, more gene loci conferring adult plant resistance are present in the *S. arvensis* genome than genes for cotyledon resistance. Alternatively, it is also possible that the genes for adult plant and cotyledon resistance in the hybrid material might be carried on addition chromosomes with variable transmission rates. It is, however, impossible to draw firm conclusions regarding the

number of resistance genes or their inheritance because aneuploids do not show Mendelian segregation.

The addition chromosomes of the different BC_3S plants could not be reliably compared due to the small size of the chromosomes and the lack of cytogenetic markers in these species. Nevertheless, chromosome morphology provides strong evidence that some or all of the genes for adult plant resistance are found on a single, acrocentric *S. arvensis* chromosome. With the exception of plants containing 38 chromosomes or less, all BC_3S individuals with adult plant resistance were found to possess a similar acrocentric *S. arvensis* addition chromosome. A second, metacentric addition chromosome was sometimes also present, however plants with only a metacentric addition showed no resistance. Of course it cannot be ruled out that the resistance might in each case be carried on one or more translocations, independent of the addition chromosome(s), that cannot be detected by GISH.

The BC_3 plants used in this study, along with 1 BC_3S individual, showed indications of mitotic instability leading to somatic variations in chromosome number. This phenomenon, probably caused by different parental cell cycles influencing the respective *B. napus* and *S. arvensis* chromosomes, can result in incongruous chromosome count results, particularly in earlier backcross offspring with more alien chromosomes where more mitotic disturbances might be expected. This could make a cytogenetic selection of resistant individuals with a minimal number of donor chromosomes difficult. The BC_1 plant from which all the BC_3 plants in the present study originated were originally scored, using classical cytogenetic techniques, as having chromosome numbers from 2n=37–39 (Plümper 1995). The higher chromosome counts in some later backcross offspring could be explained by the mixoploid nature of the BC_1 plant.

While GISH has been shown, both in this study and previously (e.g. Fahleson et al. 1997; Sharzhinskaya et al. 1998; Snowdon et al. 1998), to be very effective for the detection of addition chromosomes in intergeneric hybrids between *Brassica* species and close relatives, its utility for localising small translocations in such hybrids is perhaps questionable. Chromosome arms in *Brassica* and species of related genera contain unusually low copy numbers of dispersed repeat sequences (Heslop-Harrison and Schwarzacher 1996). Because such dispersed repeats generally form the basis of chromosome ''painting'' and GISH signals, GISH in *Brassica* is therefore normally characterised by strong signals at centromeric heterochromatin and only very weak hybridisation on chromosome arms (cf. Fig. 2f-h). A translocation in a backcross individual from a *B. napus*-*Raphanus sativus* hybrid has been successfully detected by GISH (Snowdon et al. 1999), however translocations in non-heterochromatic regions may be beyond the resolution limits of this technique in *Brassica*.

Because of the two phenomena described above, it cannot be stated with absolute certainty that the individuals observed in this study with adult plant resistance, but no visible GISH signals, indeed contain introgressed blackleg resistance genes. Alternatively, the resistance might be carried on one or more addition chromosomes that were not seen due to mitotic instability. This appears unlikely, however, because for the plants in question a large number of metaphases and interphases were scored with no variation in the GISH results. Conclusive evidence and characterisation of the chromosome introgressions, where present, will be obtained by detailed molecular genetic analysis of the plant material.

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