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Brassica naponigra, a somatic hybrid resistant to *Phoma lingam*

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Summary. *Brassica napus* and *B. nigra* were combined via protoplast fusion into the novel hybrid *Brassica naponigra*. The heterokaryons were identified by fluorescent markers and selected by flow sorting. Thirty hybrid plants were confirmed by isozyme analysis to contain both *B. nigra* and *B. napus* chromosomes; of these, 20 plants had the sum of the parental chromosome numbers. A non-random segregation of the chloroplasts was found in the hybrids. Of 14 hybrid plants investigated, all had the *B. napus* type of chloroplast. The resistance to *Phoma lingam* found in the *B. nigra* cultivar used in the fusion experiments was expressed in 26 of the hybrid plants. The hybrids obtained in this study contain all of the three *Brassica* genomes (A, B and C) and have thus created unique possibilities for genetic exchanges between the genomes. Since most of the plants were fertile as well as resistant to *P. lingam*, they have been incorporated into conventional rapeseed breeding programs.

Key words: *Brassica napus* – *Brassica nigra* – Somatic hybrids – Resistance to *Phoma lingam*

Introduction

Introduction of genes for disease-resistance is one of several important goals in the breeding of our crops. In some cases genes for resistance are only available in species distantly related to the crop, and transfer of the genes to the crop plant can be severely restricted owing to sexual crossing barriers. The limitations can be overcome by using protoplast fusions, provided that regeneration and differentiation of plants from protoplasts of the crop is possible.

Phoma lingam has spread over the world and is parasitic on wild and cultivated genera of *Brassicaceae*, particularly different species of *Brassica, Sinapis* and *Raphanus*. Differences in susceptibility to *P. lingam* have been found in *B. napus* under field conditions, and tolerance to the disease at the adult stage has been incorporated into some French rapeseed cultivars by conventional plant breeding (Renard and Brun 1979). Complete resistance to *P. lingam*, however, has not been found in any of the species *B. napus, B. campestris* or *B. oleracea*. We have searched for sources of resistance to *P. lingam* and found several accessions of *B. nigra, B. juncea* and *B. carinata* with complete resistance to the pathogen (Sjödin and Glimelius 1988).

In this study, protoplasts of a susceptible cultivar of *B. napus* were fused with protoplasts from a resistant cultivar of *B. nigra* in order to transfer resistance to *P. lingam* from black mustard to rapeseed. The hybrid between *B. napus* and *B. nigra* implies a combination of the three *Brassica* genomes, A and C from *B. napus* and B from *B. nigra* (U 1935). In such a hybrid combination, several valuable traits may recombine, e.g. resistance against *P. lingam* may be transferred to rapeseed provided that crossing-over between the genomes takes place.

Materials and methods

Plant material

In the various fusion experiments, *Brassica napus* L. ssp. *oleifera* var. *annua* cv Hanna and *B. nigra* (L.) Koch cv Junius were used. Seeds of rapeseed were kindly provided by W. Weibull AB, Landskrona, Sweden and seeds of black mustard were provided by Institut National de la Recherche Agronomique (I.N.R.A.), Le Rheu, France.

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Protoplast isolation

Protoplasts of *B. napus* were isolated from 5-day-old hypocotyls. From *B. nigra*, protoplasts were isolated from leaves of in vitro grown plants. Plant material was grown and protoplasts isolated according to Glimelius (1984) with some modifications. The surface-sterilized seeds were germinated on MS-medium (Murashig and Skoog 1962) containing 1% (w/v) sucrose and 0.3% (w/v) Gellan Gum (Kelco). The seeds were placed in rows in Petri dishes and germinated in darkness at 25 °C in a slanting position, to promote straight growth of the hypocotyls, which facilitates the handling and cutting of the hypocotyls.

Fusion and selection

Protoplasts were fused using polyethylene glycol as described by Sundberg and Glimelius (1986). The protoplasts were stained, the fusion products pretreated and heterokaryons were selected by flow sorting according to Glimelius et al. (1986).

Culture of hybrid cells and plantlet regeneration

Cells were cultured in a modified 8 p medium (Glimelius et al. 1986) with the initial hormone concentrations of 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.2 μM 6-benzylaminopurine (BAP) and 0.5 μM 1-naphthylacetic acid (NAA). When cells began to divide after 3–6 days in culture, three times the original volume of fresh culture medium without hormones was added so as to dilute the hormone concentration. After about 2–3 weeks, small cell aggregates were plated in K_3 medium (Nagy and Maliga 1976) containing 0.13% agarose, 0.01 M sucrose, 1.13 μM 2,4-D, 0.55 μM BAP and 0.13 μM NAA. When small calli about 3 mm in size had developed, they were transferred for differentiation to K_3, a medium containing 0.0015 M sucrose, 0.01 M mannitol, 0.4% agarose and the hormone combinations and concentrations of indole-3-acetic acid (IAA), zeatin and BAP as reported in Table 2. After 15–20 days, the calli were transferred to solid K_3 medium with 0.03 M sucrose and the same hormones as in the differentiation step. Transfer to fresh regeneration medium was made every 3rd week. The hybrid cells were cultured under cool white light (215 W General Electric fluorescent tubes) of 2 W m^{-2}, and the plated cell aggregates in 20 W m^{-2} at 25 °C, with a daylength of 16 h. Shoots emerging from the calli were transferred to a hormone-free MS medium complemented with 0.03 M sucrose and 0.3% Gellan Gum. The shoots were cultured under sterile conditions in climate chambers with 20 °C, and 18 h day length under warm-white fluorescent light (Thorne Emi), 35 W m^{-2} and 40 W tungsten lamps (Asea Skandia). When roots had developed, the established plants were planted in the greenhouse, but duplicates were also stored under sterile conditions in the MS medium.

Isozyme, chromosome and ploidy level analysis

Isozyme analyses were performed using homogenized leaf tissue of hybrid shoots or plants and parental plants according to Sundberg and Glimelius (1986). The samples were electrophoresed in starch gels using the buffer G and N according to Shields et al. (1983), and the enzymes examined were leucine aminopeptidase (LAP), shikimate dehydrogenase (SHDH), glucose-6-phosphate dehydrogenase (G-6-PDH), phosphoglucomutase (PGM) and phosphoglucose isomerase (PGI).

From each hybrid plant, 5–10 cells from 2–4 root tips were stained by the Feulgen method and analysed for chromosome number according to Sundberg et al. (1987).

As a complement to chromosome counting, quantitative flow cytometric DNA analysis of the hybrid plants was performed. The DNA content was determined as the relative PI-fluorescence normalized to *B. campestris* and converted to pg DNA/nucleus according to Fahleson et al. (1988).

Hybrid fertility, pollen viability and morphology

For each hybrid plant, at least 50 mature flowers and 50 buds were self-pollinated. In addition, 50 flowers were pollinated with pollen from *B. napus* cv Hanna. The number of seeds obtained per pollination procedure was determined.

Pollen viability was studied using a fluorescein diacetate (FDA) staining method as described by Sundberg et al. (1987). Plant height of the hybrids was determined during seed set. Leaf shape, hairiness and flower morphology were determined on actively growing plants.

Chloroplast DNA analysis

Chloroplast DNA was isolated from leaves of young plants. The isolation procedure, restriction enzyme analysis and gel electrophoresis were performed according to Sundberg et al. (1987). The two restriction endonucleases *Bam*HI and *Hind*III were used for digestion of the chloroplast-DNA.

Resistance analysis

Resistance to *P. lingam* was tested in all hybrid plants by inoculation of adult leaves and stem bases with pycnospores from the pathogen according to Sjödin and Glimelius (1988). Three different virulent fungal isolates were used: 275.63 from Centraalbureau vor Schimmelcultures, Baarn, The Netherlands, and Lm 100 and Lm 479 which were provided by the Crucifer Genetics Cooperative, University of Wisconsin, Madison/WI.

Results

Selection and differentiation of fusion products

The results from the fusion experiments between *B. napus* and *B. nigra* are summarized in Tables 1 and 2. The hybrid cell frequency, after enrichment by the flow sorter, varied from 56% to 73% when calculated 1 day after sorting. Three days after fusion, about 30% of the cells had divided and after 5 days about 60% had passed the first division. Shoots were obtained from one experiment (Table 2). Of the 35 shoots obtained, 32 were successfully transferred to the greenhouse (Fig. 1).

Table 1. The percentage of fusion products in the cultures before cell sorting, and calli obtained from the number of sorted fusion products of *B. napus* (+) *B. nigra*

Experiment no.	Fusion[a] frequency (%)	No. of sorted cells	No. of plated calli
1	9.6	4.000	340
2	8.1	4.000	258
3	14.2	5.000	545
4	12.7	5.000	420

[a] Calculated at the day of fusion

Isozyme analysis

The hybrid character of the plants was verified using isozymes. Of five isozymes tested, phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM) had species-specific zymogram patterns. Out of the 32 plants obtained, 28 showed hybrid character for the isozymes PGI (Fig. 2) and PGM. Two plants showed hybrid character only for PGM (h2, h4) and *B. napus* character for PGI, indicating a preferential loss of *B. nigra* chromosomes, whereas two plants (h1, h26) showed only *B. napus* characters for both isozymes (Table 3).

Chromosome numbers in the hybrids

The chromosome numbers of the hybrid plants varied between 46 and 87 (Table 3).

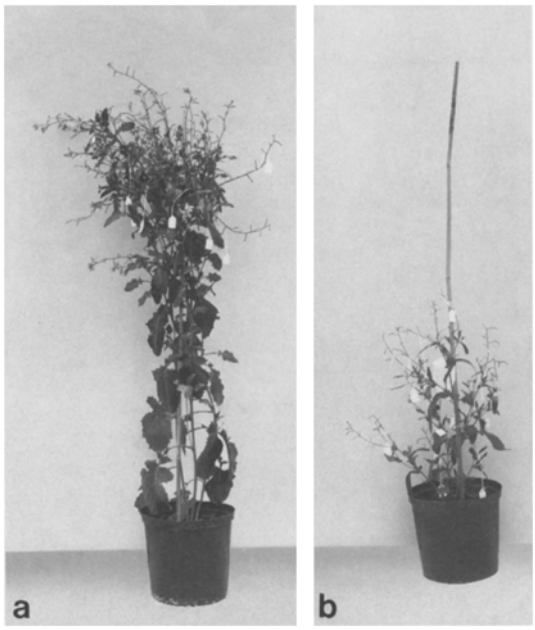

Fig. 1 a and b. Somatic hybrids at flowering, **a** h6 and **b** h10; both hybrids have 54 chromosomes

According to measurements of the DNA content, the hybrids with the sum of the parental genomes (54 chromosomes) had the expected value of 3.2–3.4 pg DNA/nucleus. One hybrid plant (h23) was found to be a chimera by the flow cytometric DNA analysis. When the chromosome number in root tip cells of the chimeric plant was examined, only cells with more than 54 chromosomes were found. Furthermore, the DNA-content in the three hybrids with less than 50 chromosomes and in the two hybrids with 80 or more chromosomes was in accordance with the low and high chromosome numbers, respectively.

Fertility and morphological assessment

Self-pollinated buds of the hybrid plants produced very few seeds in contrast to self-pollinated flowers. Seed set varied greatly. The fertility of the hybrids with the sum of the chromosomes from the two parents ranged from 0% to 18.1%, while seed set varied from 0% to 21% among the other hybrids (Table 3). An increase in seed set (41% as the highest value) was generally obtained when the hybrids were fertilized with pollen from *B. napus*.

In the hybrid plants with 54 chromosomes, pollen viability varied between 42% and 71%. Very little pollen was produced in the hybrid plant h10, although it had high viability (43%). The hybrids with fewer chromosomes than 54 had a pollen viability of 18%–45%,

Table 2. The effects of hormone concentrations on differentiation and shoot regeneration of hybrid calli after protoplast fusion between *B. napus* and *B. nigra*

Hormone combination	No. of calli	Hormone concentration (μM)			No. of shoots from the different calli
		IAA	Zeatin	BAP	
1	135	0.6	2.3	2.2	18
2	145	0.6	4.6	4.4	8
3	140	0.6	9.1	8.9	9

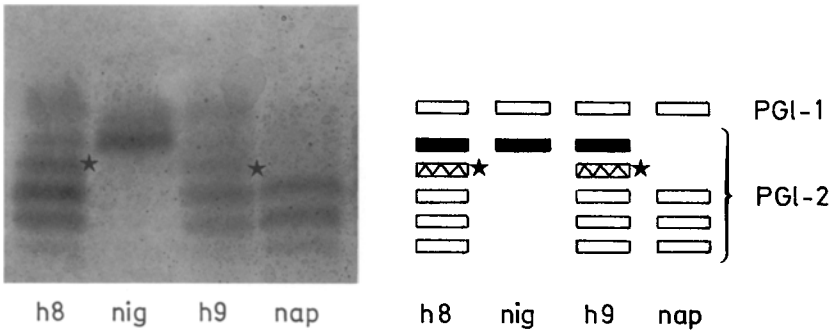

Fig. 2. Electrophoretic phenotypes of phosphoglucose isomerase (PGI) in a hybrid (h8), *B. nigra*, a hybrid (h9) and *B. napus*. The polypeptide components of the different isozyme bands are designated in the order of their migration. An *asterisk* indicates a combination of peptides from the two species, forming a heterodimeric band unique to the hybrid. The PGI-1 bands are clearly visible when using buffer N with pH 6.5. In the schematic drawing for PGI-2, *B. napus* bands are represented by unfilled rectangle, *B. nigra* by filled and crosshatched rectangles represent heterodimeric bands

Table 3. Chromosome number, isozymes, resistance to *P. lingam*, chloroplast genotype and fertility of *B. napus* (+) *B. nigra* hybrids

Hybrid no.	Chromosome no.	Isozymes		Resistance to *P. lingam*	Chloroplast genotype	Seed set[a] of selfed flowers
		PGI	PGM			
h 4	46	−	+	S	B. cam	1.5
h 2	47	−	+	R	B. cam	1.0
h17	48	+	+	R	B. cam	0.09
h25	50	+	+	R		9.8
h18	52	+	+	R		4.2
h 5	53	+	+	R	B. cam	7.0
h11	53	+	+	S	B. cam	21.0
h 3	54	+	+	R	B. cam	11.2
h 6	54	+	+	R		0.6
h 7	54	+	+	R	B. cam	4.9
h 8	54	+	+	R	B. cam	4.1
h 9	54	+	+	R	B. cam	7.0
h10	54	+	+	R	B. cam	0.5
h12	54	+	+	S		10.1
h13	54	+	+	R		18.1
h14	54	+	+	R		2.6
h15	54	+	+	R		4.3
h16	54	+	+	R		4.4
h19	54	+	+	R		2.4
h20	54	+	+	R		0
h21	54	+	+	R	B. cam	0.6
h22	54	+	+	R		5.3
h27	54	+	+	R	B. cam	2.6
h28	54	+	+	R		0.1
h30	54	+	+	R		8.3
h31	54	+	+	R	B. cam	1.7
h32	54	+	+	R	B. cam	3.1
h23	62	+	+	S/R		0.3
h29	80	+	+	R		0
h24	87	+	+	R		0.9
h 1	38	−	−	S	B. cam	0.9
h26	38	−	−	S	B. cam	7.5
Parental material						
B. napus cv Hanna	38			S	B. cam	8.3[b]
B. nigra cv Junius	16			R	B. nig	0.2[b]

[a] In % of *B. napus*

[b] Values represent the number of seeds obtained per pollinated flower

+ – hybrid isozyme pattern, − – *B. napus* isozyme pattern, R – resistant, S – susceptible, B. cam – *B. campestris*, B. nig – *B. nigra*

whereas the chimera and the two hybrids with more than 54 chromosomes had a very low pollen viability of 2%–8%. The pollen viability in the two plants with rapeseed characters (h1, h26) was 3% and 58%, respectively.

All hybrids between rapeseed and *B. nigra* with the expected chromosome number had a plant height between 105 and 150 cm, except one (h10), which had a low bush-like habit. The chimera (h23) and the two hybrids with extra chromosomes (h24, h29) as well as the two plants with *B. napus* characters were all short (25–76 cm). All the stunted plants had a diverging flower and leaf morphology, where h23, h24 and h29 were extremes with wrinkled, stiff leaves and abnormal flowers. Hairiness, a character typical for *B. nigra*, was found in all hybrid plants except h1 and h26, although a variation in density could be observed.

Chloroplast DNA

The chloroplast type in the hybrids was determined by RFLP (Table 3). Of 14 hybrids examined, all contained chloroplasts identical to *B. napus*, which for this cultivar has a DNA restriction pattern typical for *B. campestris* (Fig. 3).

Resistance

Resistance to *P. lingam* was expressed in all hybrids with a chromosome number of about 54, except one hybrid,

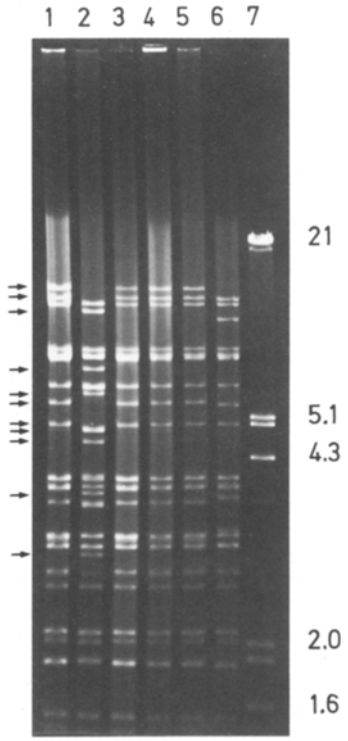

Fig. 3. Restriction pattern of chloroplast DNA from parental and somatic hybrid plants with *Bam*HI restriction endonuclease. *Lane 1 B. napus* cv Hanna; *lane 2 B. nigra* cv Junius; *lane 3* hybrid h27; *lane 4* hybrid h31; *lane 5 B. campestris; lane 6 B. oleracea; lane 7* lamda-DNA cut with *Eco*RI/*Hind*III. The differences in restriction pattern between the parentals are indicated with *arrows*

h12 (Table 3). The partial hybrids h4 and h11 proved to be susceptible. Susceptible and resistant areas could be detected in the chimera (h23), when inoculating the leaves with the pycnospores. The stem bases of h23, however, always gave a susceptible reaction.

Discussion

The alloploid plants obtained after fusion of protoplasts from *B. napus* and *B. nigra* are the first hybrids containing the sum of the chromosomes from these two species to be reported. Very few articles report attempts to cross *B. napus* with *B. nigra*. Jahier et al. (1987) obtained progenies with 27 chromosomes, but induction of chromosome doubling failed and aneuploids were produced. Furthermore, progenies with 27 chromosomes have been produced in the experiments where cytological studies of the meiotic chromosome pairing were the reason for the hybridization (Busso et al. 1987). According to Pearson (1928) and Sinskaia (1927), sexual crosses between the two species were unsuccessful without any seed set. *B. nigra* has in contrast, been successfully crossed with *B. oleracea* (Pearson 1972; Hinata et al. 1974) and with *B. campestirs* (Olsson 1960; Prakash 1973), in attempts to resynthesize *B. carinata* and *B. juncea,* respectively.

From meiotic analysis of sexual crosses between *B. campestris* (AA), *B. oleracea* (CC) and *B. nigra* (BB) and their amphidiploids, chromosome homology was assessed. A low level of chromosome pairing between the A and B genomes and B and C genomes, compared to pairing between A and C genomes, were found by Attia and Röbbelen (1986) and Attia et al. (1987). They proposed that the low pairing frequency between B and A or C genomes was due to structural chromosomal differences reflecting a more distant phylogenetic relationship of *B. nigra*. However, the low chromosome pairing ability could also be due to a genetic system in the B genome suppressing homoeologous pairing. This hypothesis has been tested by making haploid hybrids between *B. carinata* (BBCC) × *B. campestris* (AA), *B. napus* (AACC) × *B. nigra* (BB) and *B. juncea* (AABB) × *B. oleracea* (CC), all containing the A, B and C genomes of *Brassica*. From meiotic studies of these crosses, no genetic factor suppressing the chromosome pairing between the A and C genomes in the presence of the B genome could be revealed (Busso et al. 1987). These cytological studies reveal that meitoic pairing can occur in *Brassica* combinations where the B genome is included and chiasmata were formed. Thus, it can be concluded that a transfer of genes between the three genomes is possible.

According to Roy (1978), the gene(s) for resistance to *Phoma lingam* is located in the B genome. Thus, a recombination event like the one discussed above is required to be able to transfer the genes for resistance to *P. lingam* into *B. napus*. In the plants obtained from our experiments, resistance to *P. lingam* was expressed in all hybrids with 53 or 54 chromosomes, except two. These, h11, h12, and h4; with less than 50 chromosomes, must have lost resistance due to some mutational event such as loss of a small piece of a chromosome of due to changes in the nuclear constitution which repress expression of resistance to blackleg.

In spite of the fact that no cytoplasmic effects on the regulation of pairing between the three *Brassica* genomes were found (Busso et al. 1987), non-random chloroplast segregation was found in the *B. naponigra* plants. All hybrids investigated contained chloroplasts typical for *B. napus*.

Organelle segregation is often random following protoplast fusion (Chen et al. 1977); Sidorov et al. 1981; Sundberg et al. 1987), but non-random organelle segregation has also been reported (Flick and Evans 1982; Bonnett and Glimelius 1983). Segregation of chloroplast type may be due to chloroplast-genome incompatibility factors or various selective advantages due to the different culture regimes used (see review of Fluhr 1983). It has also been proposed that the larger number of chloro-

plasts present in mesophyll protoplasts compared to the number of proplastids in hypocotyl protoplasts may afford an advantage for chloroplasts of mesophyll origin during segregation (Evans and Flick 1983; Galun and Aviv 1986). In this case, however, the mesophyll protoplasts were always isolated from *B. nigra*, thus, other factors such as differences in replication rate of the chloroplasts or incompatibility between the genomes may be the reasons for the non-random segregation.

This report describes the production and evaluation of novel *B. naponigra* hybrids and clearly demonstrates the incorporation of *P. lingam* resistance from *B. nigra* into the somatic hybrids. The hybrid plants are fertile and a combination of all the three *Brassica* genomes (A, B, and C) has been obtained. Not only is the disease-resistance of great agronomic importance, but our results also demonstrate that protoplast fusion is a valuable tool where conventional breeding methods have failed.

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