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Transfer of resistance to *Xanthomonas campestris* pv *campestris* into *Brassica oleracea* L. by protoplast fusion

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Abstract Black rot caused by the bacterium *Xanthomonas campestris* pv *campestris* is one of the most serious diseases of *Brassica oleracea*. Since sources of resistance to the disease within *B. oleracea* are insufficient and control means are limited, the development of resistant breeding lines is extremely desirable. Certain lines of *B. napus* contain very high resistance controlled by a dominant gene, but crossing the two species sexually is very difficult. Therefore, somatic hybrids were produced by protoplast fusion between rapid cycling *B. oleracea* and a *B. napus* line highly resistant to *X. campestris* pv *campestris*. Hybrid identity was confirmed by morphological studies, flow cytometric estimation of nuclear DNA content, and analysis of random amplified polymorphic DNA (RAPD). Inoculations with the pathogen identified four somatic hybrids with high resistance. The resistant hybrid plants were fertile and set seed when selfed or crossed reciprocally to the bridge line '15' (Quazi 1988). Direct crosses to *B. oleracea* were unsuccessful, but embryo rescue facilitated the production of a first-backcross generation. The BC_1 plants were resistant to the pathogen. Progeny from the crosses to 'line 15' were all susceptible. Embryo rescue techniques were not obligatory for the development of a second-backcross generation, and several resistant BC_2 plants were obtained.

Key words Somatic hybridization · Black rot · Disease resistance

Introduction

Xanthomonas campestris pv. *campestris* (Pammel) Dowson causes black rot in *Brassica oleracea* L. It is considered the most important disease of crucifers worldwide (Williams 1980), especially devastating in regions where warm and humid conditions are present during the growing season. Control measures are limited and include only seed treatment, crop rotation, and general crop hygiene. Resistance to *X. campestris* pv *campestris* is therefore a highly desirable character in the vegetable *Brassicas*.

Several papers report on the identification of resistance to the pathogen in *B. oleracea* (Bain 1952; Hunter et al. 1987; Ferreira et al. 1993); however, none of these genotypes have very high resistance. In addition, studies have often been done only at the adult stage (Staub and Williams 1972; Williams et al. 1972; Ferreira et al. 1993), and genotypes showing resistance as mature plants may be very susceptible as seedlings, at which stage the pathogen is capable of causing considerable damage (Hunter et al. 1987).

Guo et al. (1991) identified very high resistance to *X. campestris* pv *campestris* in certain lines of *B. napus*. No, or only trace, symptoms developed in these lines, even when a very severe wound inoculation method was used, and the resistance was expressed at all plant developmental stages. The introduction of this resistance to *B. oleracea* would therefore be very valuable.

Although sexual hybridization between *B. oleracea* and *B. napus* has been achieved in a few cases (U 1935; Grabiec 1971; Honma and Summers 1976; Chiang et al. 1977; Ayotte et al. 1987; Quazi 1988), this cross is extremely difficult to produce, and success is dependent on the genotypes involved in the cross and the use of in vitro techniques.

Protoplast fusion offers an alternate method of gene transfer between the two species. Somatic hybridization between *B. oleracea* and *B. napus* has been accomplished a number of times; however, the primary interest has been in cytoplasmic characters. Segregation and recombination of organelles have been studied (Sundberg and Glimelius 1991; Sundberg et al. 1991; Kao et al. 1992), and a transfer of cytoplasmic traits from *B. napus* to *B. oleracea* has been successfully carried out (Jourdan et al. 1989; Yarrow et al. 1990).

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In the present study, rapid cycling *B. oleracea* was fused to *B. napus* as a first step toward the transfer of a nuclear trait found in *B. napus*: the high, stable resistance to *X. campestris* pv *campestris*. Somatic hybrids expressing high resistance were regenerated and backcrossed to *B. oleracea* using embryo-rescue techniques. Two backcross generations were developed.

Materials and methods

Plant material and bacterial cultures

Rapid cycling *B. oleracea* (CrGC 3-1), for which a good regeneration system is available (Hansen and Earle 1994a), and a line of *B. napus* (PI 199947) highly resistant to *X. campestris* pv *campestris* were used in the protoplast fusion. Rapid cycling *B. oleracea*, a white-flowered broccoli (*B. oleracea* ssp. *italica*) selection from the variety 'Packman', and a Chinese kale (*B. oleracea* ssp. *alboglabra* NVRS02,006707) with high resistance to *Alternaria* spp. were used in greenhouse pollinations to the hybrids. The resistant *B. napus* line and two isolates of *X. campestris* pv *campestris* were provided by Dr. M. H. Dickson, New York State Agricultural Experimental Station, Geneva. The bacterial cultures were maintained on YDCP medium (yeast, dextrose, calcium, phosphate) (Shelton and Hunter 1985) at 4°C.

Protoplast isolation and pretreatments

Protoplast isolation was carried out as previously described (Hansen and Earle 1994a). Prior to fusion, the *B. oleracea* partner was inactivated by treating the protoplast solution with 5 mM iodoacetate for 30 min before processing to prevent division of unfused protoplasts. Since the *B. napus* fusion partner has low regenerability under the present culture conditions, somatic hybrids could be produced and selected without any pretreatment of these lines. The densities of the two fusion partners were adjusted to 2×10^6 protoplasts/ml W5 medium (Menczel et al. 1981).

Protoplast fusion and culture

Protoplast fusions were performed as described (Hansen and Earle 1994b). The concentration of protoplasts in the fusion mixture was adjusted to 8×10^4 intact protoplasts per ml, and the protoplasts were plated on membranes (Millipore, type AA, 0.8 μm) placed over a 2-ml feeder cell suspension of *B. napus* on solid medium B (Pelletier et al. 1983) lacking Tween 20, according to Walters and Earle (1990). Approximately 1 ml of protoplast solution was plated per plate. Protoplasts were cultured on a series of media (B, C, E, F) (Pelletier et al. 1983) as previously described for rapid cycling *B. oleracea* (Hansen and Earle 1994a).

Plant regeneration

Shoots excised from regenerating calli on medium F were transferred to MS medium containing 3% sucrose and no growth regulators (MS-3,0) (Murashige and Skoog 1962) for rooting. Upon root formation, plantlets were transferred to soil and covered with plastic bags for gradual adaptation to conditions out of culture. When well established, the plants were moved to the greenhouse, where they were grown to maturity.

Nuclear DNA content

Estimation of nuclear DNA content was conducted using flow cytometry. Samples were prepared and analyzed with an EPICS Profile Analyzer (Coulter Electronics, Hialeah, Fla.) as described by Arumuganathan and Earle (1991). The standard consisting of chicken red blood cells was run separately, because the peak of chicken red blood cells coincides with the G_0/G_1 peak of *B. napus*. Chicken red blood cells have a DNA value of 2.33 pg according to Galbraith et al. (1983).

Analysis of RAPDs

DNA was isolated from 0.1–0.2 g of leaf tissue by the procedure described by Hu and Quiros (1991). Each somatic hybrid and several individual plants for each parental species were tested with primers from Operon Technologies (Alameda, Calif.), kit A. Polymerase chain reactions were done according to Hu and Quiros (1991), except denaturation was for 30 s rather than 1 min. The program was initiated by 30 s at 92°C followed by 45 cycles of 92°C/30 s, 35°C/2 min, 72°C/1 min. The program was terminated with 5 min at 72°C and soaking at 10°C. Samples were separated in 2% agarose gels at 90 V for 4–6 h.

Pollinations

Somatic hybrids grown under greenhouse conditions were selfed using bud-pollination, and the pollinated clusters were covered with bags to prevent cross-pollination. For cross-pollinations, all buds were emasculated, unless no seed set was observed following self-pollination. They were pollinated immediately with freshly collected pollen and covered with bags for 1 week.

Ovary and embryo culture

Ovaries were removed from the plants 2, 3, 4, 5, 7, 8 and 10 days after pollination (DAP). They were surface sterilized in 10% Chlorox, 0.1% Tween 20, rinsed three times in sterile water and cultured in MS-medium containing 5% sucrose, 0.25 mg/l BA, 0.25 mg/l NAA, 400 mg/l casein hydrolysate (enzymatic grade from milk) and 2.6 g/l of Gelrite (Chemical Dynamics Corp.), pH 5.7. The ovaries were cultured for 1–3 weeks, while being examined for swelling. They were then cut open, and enlarged ovules were slit open opposite to the chalazal end and transferred to 5 ml liquid embryo culture medium (Quazi 1988) in 60-mm Petri dishes placed on a rotary shaker (35–50 rpm) at 25°C. Depending on how well the embryo developed, it was transferred after 10–12 days to solid medium E (Pelletier et al. 1983) for shoot proliferation, solid embryo culture medium consisting of MS medium with 2% sucrose, 0.25 mg/l BA, 0.025 mg/l NAA, 2.2 g/l Gelrite, pH=5.7, or MS medium with 3% sucrose and no growth regulators.

Inoculation with *X. campestris* pv *campestris*

Plants were screened for resistance to *X. campestris* pv *campestris* by inoculating young, mature leaves with a pin dipped in 2-day-old bacterial cultures grown on YDCP medium at room temperature. Two leaves of each plant were inoculated, both with three inoculation sites for each of two bacterial isolates. The two different isolates were applied on separate sides of the mid-vein. Control sites were created with a clean pin. Plants were incubated at 28–30°C for 7 days. Disease severity was evaluated on a scale from 1 to 4, with 1=symptomless or nearly so; 2=some susceptibility with medium size chlorosis; 3=large chlorosis; and 4=chlorosis of entire leaf.

Fertility studies

As a measure of pollen viability, pollen stainability by aceto-carmine (1%) was examined in newly opened flowers. From each flower, hundreds of pollen grains were observed under the microscope. Pollen germination and pollen-tube growth on stigmas following self-pol-

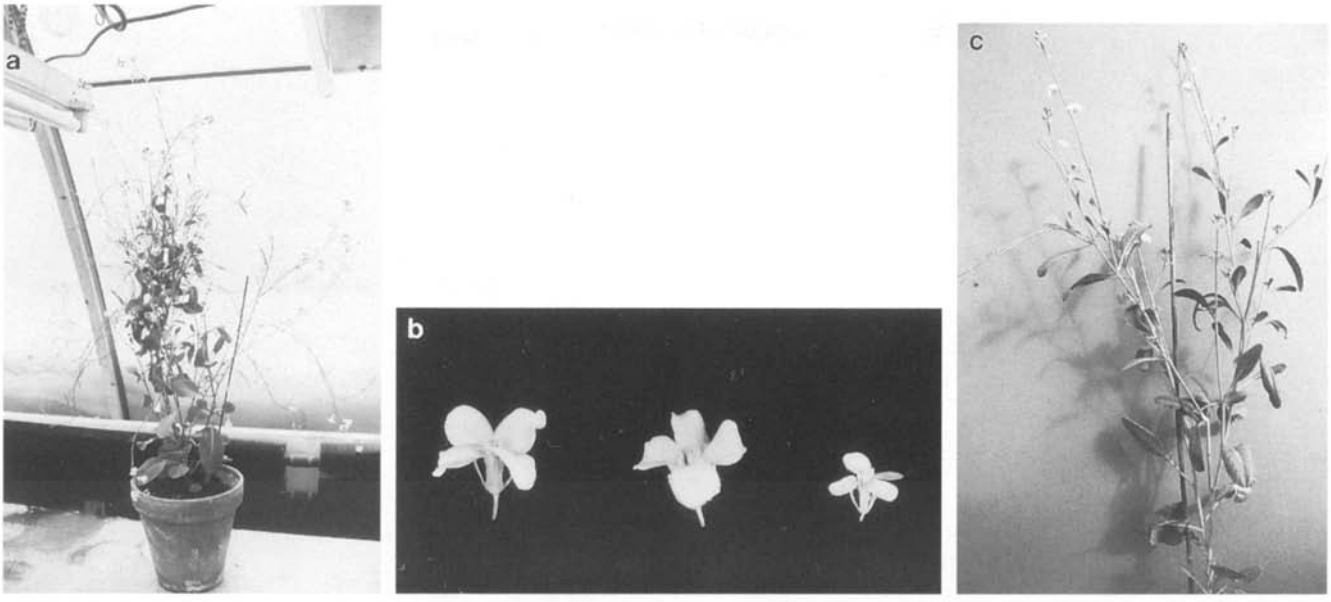

Fig. 1 **a** Somatic hybrid. The plant height is approximately 1.25 m. **b** Flowers of (from left to right) rapid cycling *B. oleracea*, somatic hybrid, black rot-resistant *B. napus* (1×). **c** BC_1 plant generated by embryo rescue in a cross between a somatic hybrid and a white-flowered broccoli line. The plant height is approximately 1 m

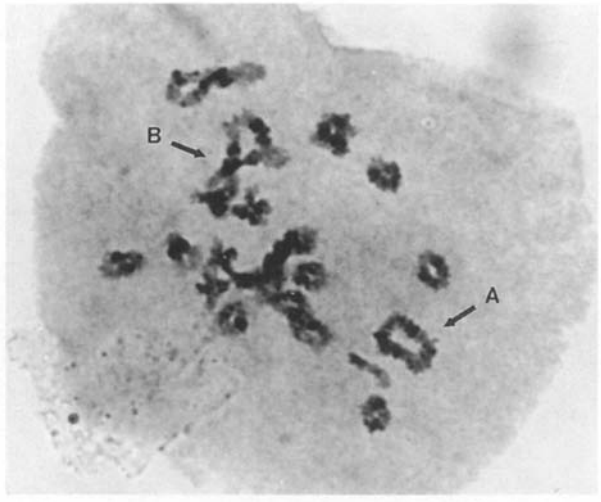

Fig. 2 Meiotic chromosome pairing in one of the somatic hybrids. Bivalent formation is most commonly seen. Quadrivalents (**A**) and pairings of more than four chromosomes (**B**) are also present (3500x)

lination and crosses between hybrids and genotypes of *B. oleracea* were studied using aniline-blue staining. The stigmas were fixed in acetic acid and 70% ethanol 1:3 for 15 min 5–6 hours after pollination. They were then softened in 1 N NaOH overnight, squashed onto a microscope slide in a drop of aniline blue (2 g/l) in 10 mM K_3PO_4 and observed under the fluorescence microscope. Meiotic behaviour was studied in the somatic hybrid that was successfully backcrossed to the broccoli line. Floral buds were fixed in Carnoy's solution (ethanol, chloroform, acetic acid 6:3:1) for 48 h and stored in 70% ethanol at 4°C. Anthers were squashed in 1% aceto-carmine solution, and pollen mother cells undergoing division were observed in the microscope.

Results

Protoplast division, callus growth and plant regeneration

Protoplast yield for *B. napus*, PI 199947, varied between 6×10^6 and 2×10^7 protoplasts/g leaf tissue. Unfused controls divided well and grew rapidly into light-green, healthy looking calli. No shoot regeneration was seen. The rapid cycling *B. oleracea* genotype yielded protoplasts as previously found: $2–12\times10^6$ protoplasts/g leaf tissue (Hansen and Earle 1994a). The iodoacetate treatment was very efficient, i.e., no division of iodoacetate-treated protoplasts was observed.

Calli from fusion treatments were transferred from the membranes on medium C to regeneration medium (E) starting 3 weeks after fusion. Six calli regenerated shoots and were moved to medium F 9 to 16 weeks after fusion. Eleven shoots were excised, and transferred to MS-3,0 medium for rooting. However, one shoot was lost, and ten plants originating from five of the calli were established in soil after 2–6 weeks on rooting medium.

Morphology and fertility of somatic hybrids

Five plants originating from two calli had a morphology similar to the black rot-resistant *B. napus* and were presumed to be escapes. The *B. napus* parent has purple stems, hairy leaves and abundant small light-yellow flowers. One fusion-derived plant was abnormal with thick, crisp, wrinkled leaves, purple stems and male sterile flowers. The remaining four plants, one from one callus and three from another, had a similar morphology. They resembled the *B. napus* parent, but were larger and more branched (Fig. 1a). Plant height was approximately 1–1.25 m. The flowers were large and dark yellow like the flowers of rapid cycling *B. oleracea* (Fig. 1b). They were perfect and shed

large amounts of healthy appearing pollen. Pollen viability was estimated to be approximately 95% in three of the hybrids and 87% in one hybrid. In black rot-resistant *B. napus* and in rapid cycling *B. oleracea*, the pollen viabilities were estimated to be 98% and 94%, respectively. Observations of 25 pollen mother cells undergoing division indicated that meiosis was normal. Few univalents were seen, while bivalent and quadrivalent formation were frequent (Fig. 2). Quadrivalents are to be expected, since pairing will occur between the C-genomes coming from each of the two parents. Pairing with more than four chromosomes involved was also seen, indicating that recombination may occur between the A-genome from *B. napus* and the C-genomes (Fig. 2).

DNA analysis

Flow cytometric estimation of nuclear DNA content supported the belief that the five plants with *B. napus* morphology were escapes. They all had 2C DNA contents of 2.68–2.71 pg, which is similar to the *B. napus* parent (2.67±0.16 pg). The abnormal plant had a high DNA content of 7.52 pg. The four putative somatic hybrids all had DNA contents similar to the sum of the two parents, although a bit higher, from 3.94 to 4.25 pg, when estimated as young plants at a height of approximately 20 cm. The DNA content appeared to increase a little as the plants grew older (up to 4.32 pg). The 2C DNA content of rapid cycling *B. oleracea* was estimated to be 1.29±0.07 pg in ten individual plants. The flow cytometric profile of one of the four normal somatic hybrids mixed with nuclei of the two parental species is shown in Fig. 3.

Hybrid identity was further confirmed by analysis of random amplified polymorphic DNA (RAPD). Primer OPA-18 was selected for analysis of the fusion products based on consistent polymorphism between parents. Bands of approximately 0.9 kb and 1.7 kb were present only in the *B. oleracea* parent, and bands of approximately 1.1 kb and 1.8 kb were present only in the *B. napus* parent. Bands specific to the two parents were all found in the four symmetric somatic hybrids, the abnormal plant had a complex banding pattern, and a presumed escape had the banding pattern of black rot-resistant *B. napus*. Figure 4 shows the RAPD profiles of rapid cycling *B. oleracea* (lanes 2–5), black rot-resistant *B. napus* (lanes 11–15) and two of the normal somatic hybrids (lanes 7–10).

Reactions to inoculation with *X. campestris* pv. *campestris*

B. napus PI 199947 shows no, or only trace, symptom development following inoculation with *X. campestris* pv *campestris*, even with the severe inoculation method applied in this study. As expected, the five escapes were completely resistant to the pathogen. By contrast, the abnormal plant was very susceptible. It showed severe chlorosis around the inoculation sites and was given a disease severity rating of 3. The four symmetric somatic hybrids were exposed to inoculation as young plants (plant height approximately 20 cm) and again later as mature plants, at which time four cuttings were taken from each plant to participate in the inoculation experiment. As young plants,

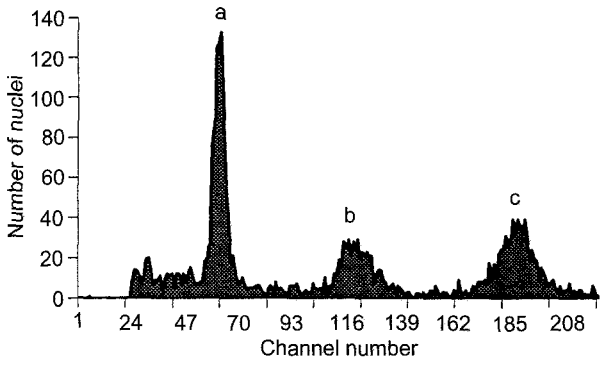

Fig. 3 Flow cytometric profile of a somatic hybrid mixed with nuclei of the two parental species. Peaks are: **a** rapid cycling *B. oleracea*. 2C= 1.29 pg; **b** black rot-resistant *B. napus*, 2C= 2.67 pg. **c** Somatic hybrid, G_0/G_1, 2C=4.14 pg

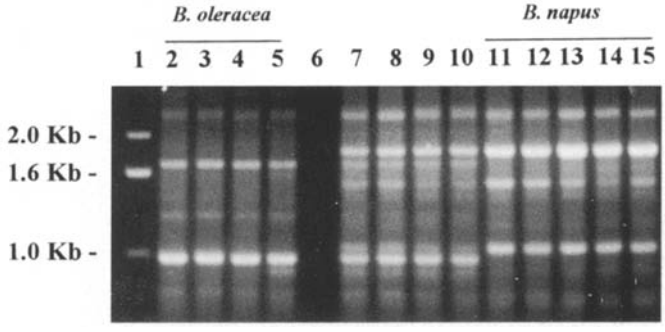

Fig. 4 RAPD profiles of fusion partners and two somatic hybrids generated using primer OPA-18. *Lane 1*: 1-kb DNA ladder. *Lanes 2–5*: rapid cycling *B. oleracea*. *Lane 6*: blank. *Lanes 7–8*: somatic hybrid, 922010-3-1. *Lanes 9–10*: somatic hybrid, 922010-1-1. *Lanes 11–15*: black rot-resistant *B. napus*

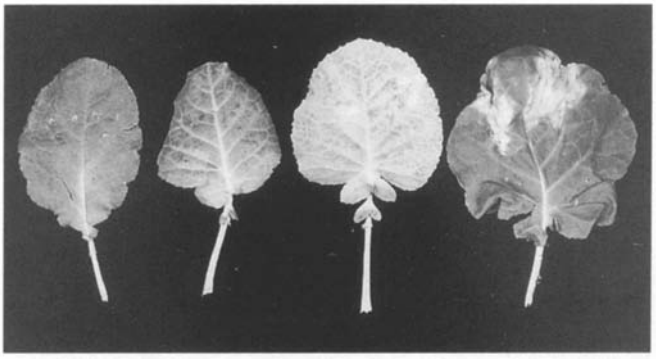

Fig. 5 Reaction to inoculation with *X. campestris* pv *campestris* in (from left to right) black rot-resistant *B. napus*, somatic hybrid with no symptom development, somatic hybrid with slight symptom development, and susceptible control, *B. napus* cv 'Westar'

Table 1 Summary of the analysis of regenerants

Type of plants	Number of plants	Morphology	DNA content (pg/nucleus)	RAPD analysis	Disease rating
Parents					
B. napus	–	–	2.67	–	1
RC[a] *B. oleracea*	–	–	1.29	–	3–4
Regenerants					
Somatic hybrids	4	Intermediate	3.94–4.25	Hybrid	1–1.5
Abnormal plants	1	Abnormal	7.52	Complex	3
B. napus escapes	5	*B. napus*	2.68–2.71	*B. napus*	1

[a] Rapid cycling

Table 2 Sexual crosses[a] for transfer of black rot-resistance to *B. oleracea* and self pollination of the resistant somatic hybrids

Female parent	Male parent	Number of pollinations	Normal seeds	Shrivelled seeds	Normal seeds per pollination
Somatic hybrid	Line 15	144	9	47	0.06
Line 15	Somatic hybrid	60	98	11	1.6
Somatic hybrid	RC[b] *B. oleracea*	40	0	0	0
RC *B. oleracea*	Somatic hybrid	42	0	0	0
RC *B. oleracea*	BRR[c] *B. napus*	18	0	0	0
Somatic hybrids selfed	–	162	219	190	1.4

[a] No embryo-rescue techniques were applied
[b] Rapid cycling
[c] Black rot-resistant

three somatic hybrids showed no symptom development, while one plant showed weak chlorosis around the inoculation sites (disease severity rating 1–1.5) (Fig. 5). Plants grown from cuttings from mature plants showed similar results. Although some plants received a disease severity rating of 2, most plants were rated symptom-free or nearly so (score 1). Plants derived from the same somatic hybrid were given different disease severity ratings, and there was no apparent difference between somatic hybrids. A summary of the results from the analysis of regenerants is given in Table 1.

Crosses to somatic hybrids and progeny analysis

Reciprocal sexual crosses were done between the somatic hybrids and the bridge line '15' (Quazi 1988), and between the somatic hybrids and rapid cycling *B. oleracea*. In addition, the somatic hybrids were selfed. The results of these pollinations are summarized in Table 2. The crosses between the somatic hybrids and 'line 15' gave rise to seeds in both directions; however, using 'line 15' as the female parent was more successful. No crosses between the hybrids and rapid cycling *B. oleracea* yielded seeds. A few crosses were done directly between rapid cycling *B. oleracea* and black rot-resistant *B. napus*. As expected, no seeds were obtained. The somatic hybrids set seeds very well following self pollination.

The progeny obtained from the crosses between the somatic hybrids and 'line 15' all had a similar morphology, which resembled the somatic hybrids except for white flowers like 'line 15'. The nuclear DNA content (2C) estimated by flow cytometry varied from 2.38 to 3.87 pg in 31 plants tested. The average DNA content was 3.18±0.49 pg. The DNA content of 'line 15' was estimated to be 2.51±0.21 pg, so the expected DNA contents in the sexual hybrids between 'line 15' and the somatic hybrids would be 3.2–3.3 pg, which is within the range of what was actually found. All plants showed some susceptiblity in inoculation experiments with *X. campestris* pv *campestris*. They were given disease severity ratings of 2, 3, and 4, and were therefore not used in further sexual crosses.

Selfed progeny of the somatic hybrids had a morphology similar to the parents, with large yellow flowers and purple stems. The nuclear DNA content was estimated in ten plants. Nine had a DNA content close to the somatic hybrids (3.89–4.33pg), while one plant was estimated to have 5.10 pg. Sixteen plants were analyzed for resistance to *X. campestris* pv *campestris*. Most showed only very slight symptom development (disease severity rating 1), while five plants were rated 2–3.

Ovary culture and embryo rescue

Since all plants obtained from the crosses between the somatic hybrids and 'line 15' were susceptible, more crosses were done directly to *B. oleracea* followed by ovary culture and embryo-rescue attempts. Crosses to the somatic hybrids as well as to the black rot-resistant *B. napus* were

Table 3 Ovary culture and embryo-rescue attempts after crossing *B. oleracea* with black rot-resistant somatic hybrids or *B. napus*

Female parent	Male parent	DAP[a]	Ovaries cultured	Embryos obtained
B. oleracea	Somatic hybrid	2, 3, 4, 5, 7, 10	78	0
Somatic hybrid	*B. oleracea*	2, 3, 4, 5, 8	90	1[c]
B. oleracea	BRR[b] *B. napus*	2, 3, 4, 7	48	0
BRR *B. napus*	*B. oleracea*	2, 3, 4, 7	50	0

[a] Days after pollination
[b] Black rot-resistant
[c] Five plants were obtained from this embryo after transfer to medium E

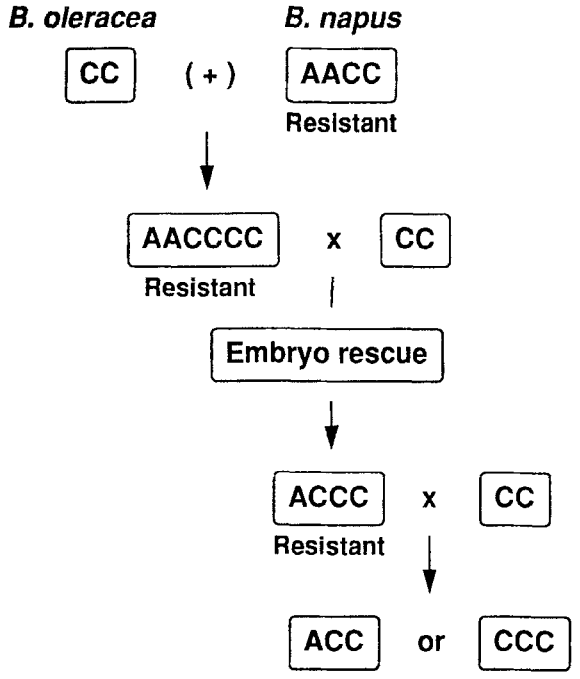

Fig. 6 Diagram showing somatic and sexual hybridizations to the second backcross generation (ACC or CCC), including the initial protoplast fusion between rapid cycling *B. oleracea* and the black rot-resistant *B. napus*, with the following crosses and genetic constitutions of parents and offspring. '*A*'=genome of *B. rapa*. '*C*'=genome of *B. oleracea*

carried out. Two different genotypes of *B. oleracea* were used: rapid cycling *B. oleracea* and the white-flowered broccoli line. The results of these efforts are summarized in Table 3. Even though swellings were seen in the pods in ovary culture, almost all ovules were shrivelled at the time the ovaries were opened. Collecting the siliques shortly after pollination (2–3 days) appeared to give the most promising results. However, only two well-developed embryos were isolated, and only one of these proliferated shoots upon transfer to medium E. This embryo was obtained from a cross between a somatic hybrid as the female parent and the white-flowered broccoli line as the male parent. The ovary had been harvested 3 days after pollination and cultured for 13 days. Five shoots were excised from the embryo and rooted on MS-3,0. All five plants were successfully transferred to soil.

Analysis of the first backcross generation

Four of the five plants obtained in the first-backcross generation were normal with a morphology intermediate to the two parents (Fig. 1c). They had the branched growth habit and the purple stems of the somatic hybrids and intermediate sized and shaped leaves (roundish oval with a length of 10 cm or less). The flowers were white and the leaves bluish waxy like the broccoli parent. They produced large amounts of pollen, of which 25–50% stained with acetocarmine. The fifth plant was abnormal with thick, wrinkled leaves and short growth habit. The nuclear DNA content of the broccoli line was estimated to be 1.23±0.05 pg. The DNA content of the backcross offspring was intermediate to the two parents in all five plants: 2.48–2.62 pg. In inoculation experiments, the four normal plants showed good resistance to *X. campestris* pv *campestris*. They all received the disease severity rating 1.5. The abnormal plant was very susceptible and was given the rating 3.

Second backcross generation

In order to identify crosses with a good chance of generating BC_2 seeds, pollen-tube germination was studied using aniline-blue staining and fluorescence microscopy. The BC_1 plants were crossed reciprocally to three different *B. oleracea* genotypes: rapid cycling *B. oleracea*, white-flowered broccoli and Chinese kale. Buds, as well as open flowers, were pollinated. In addition, the hybrids and all three *B. oleracea*s were selfed using buds and open flowers to study self-incompatibility. Good germination of pollen tubes was observed only in the reciprocal cross between the hybrids and Chinese kale and was best when open flowers were pollinated. On the plants, pods appeared to develop best when the hybrids were used as the female parents. Pollen germination was also seen in crosses with rapid cycling *B. oleracea* as the male parent but was not as good as in the crosses with Chinese kale. The hybrids were self-incompatible. Even when bud pollinations were applied, no germination was observed. Pollen germination was seen following selfing of rapid cycling *B. oleracea* and the white-flowered broccoli at the bud stage. No pollen germination was seen in the Chinese kale after selfing. Based on these observations, the primary cross that was chosen for production of a second-backcross generation used the

hybrids as the female parent and the Chinese kale as the male parent. In this cross, the pollen grains germinated rapidly and grew into the style in fairly straight lines. In incompatible crosses, some germination was seen, but pollen tubes were thick and curled without a sense of direction. Also, heavy callose formation was observed in the stigma papillae in the incompatible crosses.

Because the hybrids did not set seed without pollination, and no pollen germination was seen upon self-pollination, cross-pollinations could be done without emasculation of the flowers. Some siliques were harvested for ovary culture and embryo rescue 2, 3, 5, 7 and 20 days after pollinations with Chinese kale. In other cases, the siliques were left on the plants for in vivo development of the seeds. Thirty ovaries were cultured in vitro, and two embryos were successfully rescued. One embryo, obtained from a silique harvested 5 days after pollination, was excised from a well-developed ovule and transferred directly to medium E and later to solid embryo-culture medium, where multiple shoots developed. Two shoots were excised and transferred to MS-3,0 medium for rooting and later to soil. The other embryo was isolated from a silique harvested 7 days after pollination. It germinated in liquid culture and was transferred to soil after 2 weeks of growth on MS-3,0. For in vivo seed development, 628 pollinations were performed. From these crosses, 149 normal looking seeds were harvested. Germination of the seeds was best achieved in vitro. Of 18 plants so far examined for resistance, seven were highly resistant.

Figure 6 shows the hybridizations up to the second-backcross generation (ACC or CCC), including the initial protoplast fusion, the following crosses and the genetic constitutions of parents and offspring. 'A' and 'C' designate the *B. rapa* and the *B. oleracea* genomes, respectively. It should be noted that the second-backcross generation may be of the constitution CC+partial A and/or partial C due to irregular meiosis of the BC_1 plants.

Discussion

Protoplast fusions between rapid cycling *B. oleracea* and black rot-resistant *B. napus* produced highly resistant, self-fertile somatic hybrids. For further transfer of the trait towards *B. oleracea*, the hybrids were succesfully backcrossed to *B. oleracea* through embryo rescue, and resistant BC_1 plants were generated. A second backcross generation of resistant plants was developed.

Regeneration of fusion products was low compared to the non-treated regenerating parent (Hansen and Earle 1994a). Although much higher regeneration frequencies have been reported in the protoplast fusions between *B. oleracea* and *B. napus* (Sundberg and Glimelius 1991; Sundberg et al. 1991), these results were obtained using a different system for selection of the fusion products. Sundberg et al. (1991) found regeneration frequencies of 8–18%, and Sundberg and Glimelius (1991) regenerated shoots from 9% of the calli following flow sorting of the fusion products, which reduces the proportion of unfused protoplasts. In the present study, the fusion products were mixed with unfused donor protoplasts with very low regenerability, which naturally reduces the calculated regeneration frequency substantially.

The symmetric somatic hybrids seemed to be slightly less resistant than the *B. napus* parent, and the BC_1 plants were not quite as resistant as the somatic hybrids. Guo et al. (1991) found that the resistance in the *B. napus* parent is controlled by a single dominant gene. However, the F_1 plants were not consistently as resistant as the *B. napus* parent, indicating that either the resistance is not as strong in the heterozygous condition, or it is influenced by modifier genes. These two possibilities could both explain the observations in the present study. In *B. oleracea,* modifier genes have been found to influence the expression of resistance to *X. campestris* pv *campestris* (Williams et al. 1972; Jamwal and Sharma 1986; Dickson and Hunter 1987), so it is possible that modifiers in the *B. oleracea* fusion partner have weakened the resistance from *B. napus*.

The BC_1 plants had lower male fertility than their parents (25–50% pollen stainability), which is to be expected from the genetic constitution (ACCC) (Fig. 6). Gametes would have the constitution C+(1–9) C and/or (1–10) A, giving rise to non-viable pollen in some cases. In sexual crosses between *B. napus* and *B. oleracea*, Ayotte et al. (1987) obtained tetraploid hybrids with the genetic constitution ACCC. The hybrids had pollen stainabilities of 24–40% (Ayotte et al. 1988a) and could only be backcrossed to *B. oleracea* through embryo rescue (Ayotte et al. 1988b), unlike the BC_1 plants in the present study. Embryo rescue was also needed for production of the second- but not for the third-backcross generation (Ayotte et al. 1989). Chiang et al. (1977) generated tetraploid hybrid plants (ACCC) in crosses between *B. napus* and tetraploid cabbage. Three hybrids were obtained from 3061 pollinations, a frequency of only 0.10%. Studies of meiosis in the tetraploid hybrids (Chiang et al. 1978) showed that quadrivalents were formed in variable numbers, which seemed to be influenced by the genotypes involved. Pentavalents, hexavalents and even octavalents were seen in one of the hybrids. These findings support the cytogenetic observations in one of the somatic hybrids in this study, and indicates homology and possible crossing over between the A and the C genomes. Therefore it is possible that even if the resistance trait is located on the A genome, it could still be transferred to the C genome of *B. oleracea* by intergenomic recombination. The tissue-culture phase may have increased the crossing-over frequency, making gene transfer more likely. The resistant selfed progeny of the somatic hybrids have a genetic constitution similar to their parents; however, the additional meiotic step most likely involved recombination, so these plants could also be useful for sexual crosses for transfer of the resistance to *B. oleracea*. The incorporation of this very high resistance to *X. campestris* pv *campestris* into *B. oleracea* would be a significant improvement for the management of disease in *B. oleracea* vegetable crops.

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