Theor Appl Genet (2000) 100:810–819

J.P. Ren · M.H. Dickson · E.D. Earle

Improved resistance to bacterial soft rot by protoplast fusion between *Brassica rapa* and *B. oleracea*

Received: June 1999 / Accepted: 29 July 1999

Abstract Erwinia soft rot is a destructive disease of *Brassica rapa* vegetables. Reliable sources of resistance and control methods are limited, so development of highly resistant breeding lines is desirable. Protoplasts from *B. rapa* and *B. oleracea* genotypes selected for resistance to soft rot were fused in order to combine different sources of resistance. Twelve somatic hybrids (synthetic *B. napus*) were obtained and confirmed by morphology, nuclear DNA content, and RAPD analysis. They were normal looking plants that easily set seeds following self-pollination and backcrossing to *B. rapa*. Assays of detached leaves or seedlings inoculated in a mist-chamber showed that most somatic hybrids had lower disease severity ratings than the *B. rapa* fusion partner and a commercial variety of *B. napus*. Some progeny from selfing or backcrossing of somatic hybrids to *B. rapa* showed much more resistance than either fusion partner. The offspring populations of the somatic hybrids (F_1–S_1 and F_1–BC_1) clearly moved to the resistant direction compared to the parents; the percentage of resistant plants increased from 21% (average of parents) to 36% (F_1–S_1) and 48% (F_1–BC_1). These results suggest that it may be possible to obtain highly resistant *B. rapa* lines by further backcrossing and selection.

Key words Bacterial soft rot · Disease resistance · Chinese cabbage · Protoplast fusion · *Brassica rapa* · *B. oleracea* · *B. napus* · *Erwinia carotovora* subsp. *carotovora*

Communicated by Yu. Gleba

J.P. Ren · M.H. Dickson
Department of Horticultural Sciences,
New York State Agricultural Experimental Station, Geneva,
NY 14456, USA

J.P. Ren · E.D. Earle (✉)
Department of Plant Breeding, Cornell University, Ithaca,
NY 14853-1901, USA
Fax: +1-607-255-6683
e-mail: ede3@cornell.edu

Introduction

One strategy for combining desirable characters into a crop is interspecific hybridization by either sexual crosses or protoplast fusion. Protoplast fusion allows the transfer of organelle characters as well as agriculturally desirable nuclear traits between sexually incompatible species. Many successful protoplast fusions within the *Cruciferae* family have been reported (e.g., Fosberg et al. 1994; Gerdemann-knorck et al. 1994; Hansen and Earle 1997). *Brassica napus* has been resynthesized by protoplast fusion between *B. rapa* and *B. oleracea* (e.g., Ozminkowski and Jourdan 1994), producing some lines with economically important traits, such as seed characters (Heath and Earle 1995), cytoplasmic male sterility and triazine resistance (Jourdan et al. 1989), heat tolerance (Hossain and Asahira 1992), and disease resistance (Yoshikawa et al. 1989).

Bacterial soft rot, caused by *Erwinia carotovora* subsp. *carotovora*, is a serious disease in most vegetable crops including potato, carrots, radish, and Brassicas, especially Chinese cabbage, *B. rapa* (syn. *campestris*) subsp. *pekinensis*. The bacteria usually produce large amounts of pectic enzymes which degrade plant cell walls and cause soft rot disease with no host specificity. Severe losses occur not only in fields but also during storage and in transit. The incidence of disease can be reduced by cultural practices such as delaying the planting date, raising the planting bed, and reducing plant density, but these are not always effective. Chemical treatments are not highly effective, and bactericides and biocontrol are not available. The best means for controlling this disease is by host resistance. Differences in resistance level among genotypes of *B. rapa* in naturally infected fields and artificially inoculated plots have been reported (Qiu et al. 1955; Shimizu et al. 1958; Togashi 1981; Ren 1998), but no highly resistant materials were found.

Efforts have been made to introduce resistance from other *Brassica* species into Chinese cabbage. A resistant Chinese cabbage cultivar Hiratsuka No. 1 was produced by backcrossing *B. rapa* to a sexually obtained interspe-

cific hybrid between Chinese cabbage and cabbage (*B. oleracea* L. var 'capitata') (Shimizu et al. 1962). A somatic hybrid and its progeny were produced by protoplast fusion between Chinese cabbage and wild kale (*B. oleracea* L. var 'acephala') (Yoshikawa et al. 1989). Similarly, a soft rot-resistant potato material was produced by fusion between non-tuber-bearing wild species and potato cultivars and backcrossed to the cultivars (Austin et al. 1988).

In the study reported here, somatic hybrids (resynthesized *B. napus*) were obtained by fusion of genotypes of Chinese cabbage and broccoli previously identified as resistant. The somatic hybrids and their progeny from selfing and backcrossing were tested for resistance using seedling mist-chamber inoculation and detached leaf inoculation methods (Ren 1998).

Materials and methods

Plant material

Five Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) genotypes identified as having resistance genes were used. PI germplasm G30444 and G30449 were obtained from USDA-ARS, Plant Genetic Resources Unit (PGRU), Geneva, N.Y.; AVRDC2837(B126) originally came from the Asian Vegetable Research and Development Center (AVRDC); and C3–27 and C3–28 were breeding materials produced by three cycles of recurrent selection by J.P. Ren (1998). 'CC25', a pak choi (*B. rapa* subsp. *chinensis*) cultivar from China, was used as a susceptible control.

Six genotypes of broccoli (*B. oleracea* L. var 'italica') and two of cauliflower (*B. oleracea* L. var 'botrytis') were tested in protoplast culture. Commercial cultivars 'Shogun', 'Marathon', 'Arcadia' and 'Everest' came from Sakata Seed America; PIs were from USDA-ARS, PGRU, Geneva, N.Y.; and the self-compatible breeding line 2393 was from M.H. Dickson. 'Shogun' broccoli was selected as the *B. oleracea* fusion partner because it had high levels of resistance (Ren, unpublished data) and high regenerability in protoplast culture.

Surface-sterilized seeds were germinated and grown on LS (Linsmaier and Skoog 1965) medium with no growth regulators and 3% sucrose (LS-0, 3%) at 25°C under cool-white fluorescent lights (60 µE m^{-2} s^{-1}), 16 h photoperiod. Plants were subcultured every 1–2 months.

Protoplast isolation, pretreatment, culture, and fusion

Protoplasts were isolated from newly expanded young leaves of 1- to 5-month-old plants in vitro following the procedure of Hansen and Earle (1994). The enzyme treatment for *B. rapa* was about 12 h long, while that for *B. oleracea* lasted 18 h. For culture, protoplasts at a concentration of 3×10^5 protoplasts/ml were plated on membranes (Millipore, type AA, 0.8 µm) over a feeder layer of *B. napus* cell suspension on solid medium B (Walters and Earle 1990). Later culture steps used the series of media (B, C, E, F) developed by Pelletier et al. (1983) as described by Hansen and Earle (1994). Regenerated shoots were transferred to LS-0 1% sucrose medium solidified with 2.2 g/l Gelrite (Chemical Dynamics Corp). In the fusion experiment, 'Shogun' protoplasts were treated with 3 m*M* iodoacetate (IA) for 15 min to prevent the division of unfused protoplasts. No pretreatment was used for the *B. rapa* protoplasts because of their poor regenerability. Protoplasts of both partners were adjusted to 3×10^6 per milliliter, mixed in a 1:1 ratio, and placed in droplets on the bottom of plastic petri dishes. Fusion was induced with a polyethylene glycol (PEG, 3350) solution at pH 10.5 as described by Sigareva and Earle (1997). After fusion, protoplasts were cultured in liquid medium B for 2–3 days in the dark before transfer to Millipore filters and subsequent culture as described above.

Morphology and fertility

Morphological characteristics of the fusion-derived plants were observed and compared to those of the parents. Pollen viability was determined by staining with 1% acetocarmine. Three flowers from each plant and ten microscope fields (400×) per flower were sampled. Average percentage pollen viability was calculated. The fusion-derived plants were selfed and backcrossed to the *B. rapa* C3–28 parent by bud pollination.

Nuclear DNA content

The nuclear DNA content of leaves of parental plants and regenerated hybrids was estimated by flow cytometry. Samples were prepared and analyzed with an EPICS PROFILE cell cytometer (Coulter Electronics, Hialeah, Fla.) as described by Arumuganathan and Earle (1991). Nuclei from rice (cv. Taipei 309, 2C=0.87 pg) prepared within each sample were used as an internal standard.

Randomly amplified polymorphic DNA (RAPD) analysis

Nuclear DNA was extracted from young leaves of parental and fusion-derived plants according to Hu and Quiros (1991). Five random ten-base oligonucleotide primers (OPA-1, -2, -3, -17, and -18; Operon Technologies, Alameda, Calif.) were used for amplification of DNA. Amplification conditions were as described by Hu and Quiros: 3 min of denaturation at 94°C followed by 45 cycles of 92°C/30 s, 35°C/1 min, 72°C/2 min. The PCR products were separated on 2% agarose gels and photographed with Polaroid 667 film.

Analysis of resistance to bacterial soft rot

A USA local isolate (Geneva no. 1) of *Erwinia carotovora* subsp. *carotovora* was obtained from M.H. Dickson, Geneva, N.Y. The bacteria were grown on CPG medium (Williams 1981) for 2 days before inoculation. Resistance was tested both on plants (in a mist-chamber) and on detached leaves. The somatic hybrids (F_1 plants), selfed and backcrossed progenies (F_1–S_1 and F_1–BC_1 plants), the parents, commercial *B. napus* var 'Westar', and susceptible check *B. rapa* CC25 were included in the tests. Also included were lines 921100–1, 921110–1, and 921110–2, selfed progeny of resynthesized *B. napus* in which the fusion partners had not been selected for soft rot resistance (Heath and Earle 1995).

The somatic hybrids were tested for resistance to soft rot at different times after they were transplanted into soil. In the plant test, a needle was dipped into the bacterial culture, and two petioles per plant were pricked. Inoculated plants were incubated in a mist-chamber under conditions of 23°C and 100% humidity. Two days after inoculation, plants were rated using a 1–9 scale (Ren 1998): 1=no disease, and 9=plant dead. In the detached leaf test, three leaves from each plant were sampled. Petioles were pricked 1 cm from the end with a needle dipped into the bacterial culture. Inoculated petioles were placed in a plastic box and sprayed with sterile water to keep them moist. Four days after inoculation, the disease lesion length and severity ratings were recorded individually, 1=no disease, and 9=entire petiole rotten. The mean disease severity ratings were calculated.

To test the progeny of the somatic hybrids, we planted ten F_1–S_1 lines and five F_1–BC_1 lines, plus the fusion partners, 'CC25', 'Westar', 921100–1, 921110–1, and 921110–2, with three replications and six plants per replication. The tests were conducted by two different methods: mist-chamber inoculation of 4-week-old seedlings (needle prick of two petioles per plant) and detached

Table 1 Regenerability of *B. oleracea* and *B. rapa* genotypes from protoplast culture

Species	Genotype	Experiment 1			Experiment 2		
		Number of calli[a]	Number of shoots[b]	Frequency (%)	Number of calli	Number of shoots	Frequency (%)
B. oleracea	Broccoli						
	Shogun	158	83	52.5	178	77	41.6
	Marathon	109	36	33.0	191	14	7.3
	Arcadia	20	2	10.0	145	6	4.1
	2393	30	3	10.0	213	3	1.4
	Everest	–	–	–	203	0	0.0
	Cauliflower						
	PI372585 (B-18)	240	187	77.9	65	51	78.5
	PI372858 (B-19)	103	87	84.5	113	25	22.1
B. rapa	Chinese cabbage PIs						
	AVRDC 2837	43	0	0	–	–	–
	G30444 (C3)	83	0	0	121	0	0
	G30449 (C4)	60	0	0	114	0	0
	Cycle 3 selections						
	C3–27	150	0	0	252	0	0
	C3–28	120	0	0	173	0	0

[a] Calli isolated from the membranes and moved to medium E
[b] Shoots regenerated from individual calli

leaf inoculation of 2-month-old plants. Selected F_1–S_1 and F_1–BC_1 lines were repeatedly tested using seedling mist-chamber inoculation. The data from the seedling test were analyzed using Minitab 10.5 Xtra Power Statistical Package. Fisher's LSDs were used for multiple comparisons.

Results and discussion

Plant regenerability

Protoplasts were released readily from leaves of all genotypes of both species, and calli formed easily from the protoplasts. The ability to regenerate shoots from protoplast-derived callus varied greatly both between *B. rapa* and *B. oleracea* and among genotypes of *B. oleracea* (Table 1), as has been reported in other studies (Jourdan and Earle 1989). No shoots developed from calli of any of the five genotypes of *B. rapa*. The calli grew vigorously on regeneration medium (medium E) for about 3 weeks and then rapidly turned brown. Shoot regeneration from calli of *B. oleracea* genotypes varied from 0% to 84%. 'Shogun' was the best of the broccoli, and the cauliflower PIs had even better regenerability. These cultivated genotypes may be more suitable fusion partners than the model genotype of rapid cycling *B. oleracea* (Crucifer Genetics Cooperative no. 3–1); the latter has often used as a fusion partner because of its good performance in protoplast culture (e.g., Hansen and Earle 1994, 1997) but lacks an agriculturally useful phenotype.

Recovery of fusion-derived plants

Differences in calli formation and shoot regeneration were seen when different fusion partners were used. Of the 1040 calli from the combination of 'Shogun'+ G30449, 9 formed shoots, a frequency of 0.77%. Shoots were also recovered from 2 of 421 calli of 'Shogun'+ G30444 (0.48%) and 2 of 1015 calli of 'Shogun'+C3–28 (0.19%). The combinations of 'Shogun'+AVRDC2837 and 'Shogun'+C3–27 produced no shoots despite the recovery of 47 and 645 calli, respectively. The 13 independent shoots regenerated from the three successful fusion combinations were expected to be somatic hybrids since no shoots were recovered from the unfused control protoplasts: IA-treated 'Shogun' and *B. rapa*. The low frequency of regeneration in the fusion experiments is not surprising because most calli probably developed from *B. rapa* protoplasts rather than fusions between the two partners. All fusion-derived shoots (except 'Shogun'+ G30444–1, which became contaminated) formed many subshoots, rooted easily, and produced normal vigorous plants after transfer into soil.

Analysis of the fusion-derived plants

Morphological characters

All fusion-derived plants had normal vegetative and floral morphology generally intermediate between the two parents. Some traits, such as leaf trichomes, dark-yellow flowers, and inflorescence type, were more like *B. rapa*. Similar results have been reported by Ozminkowski and

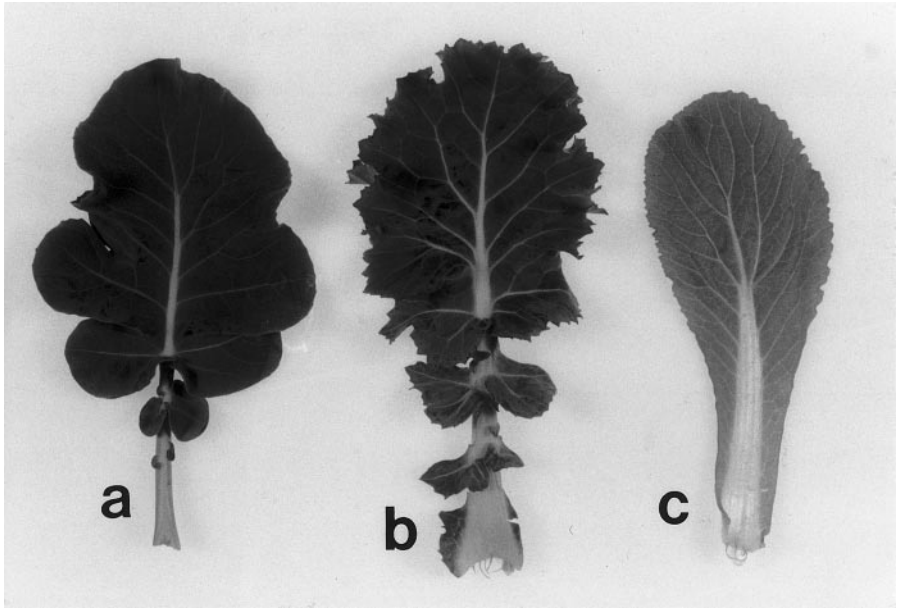

Fig. 1a–c Comparison of leaves of a somatic hybrid (**b**) and its fusion partners (**a** *B. oleracea* and **c** *B. rapa*)

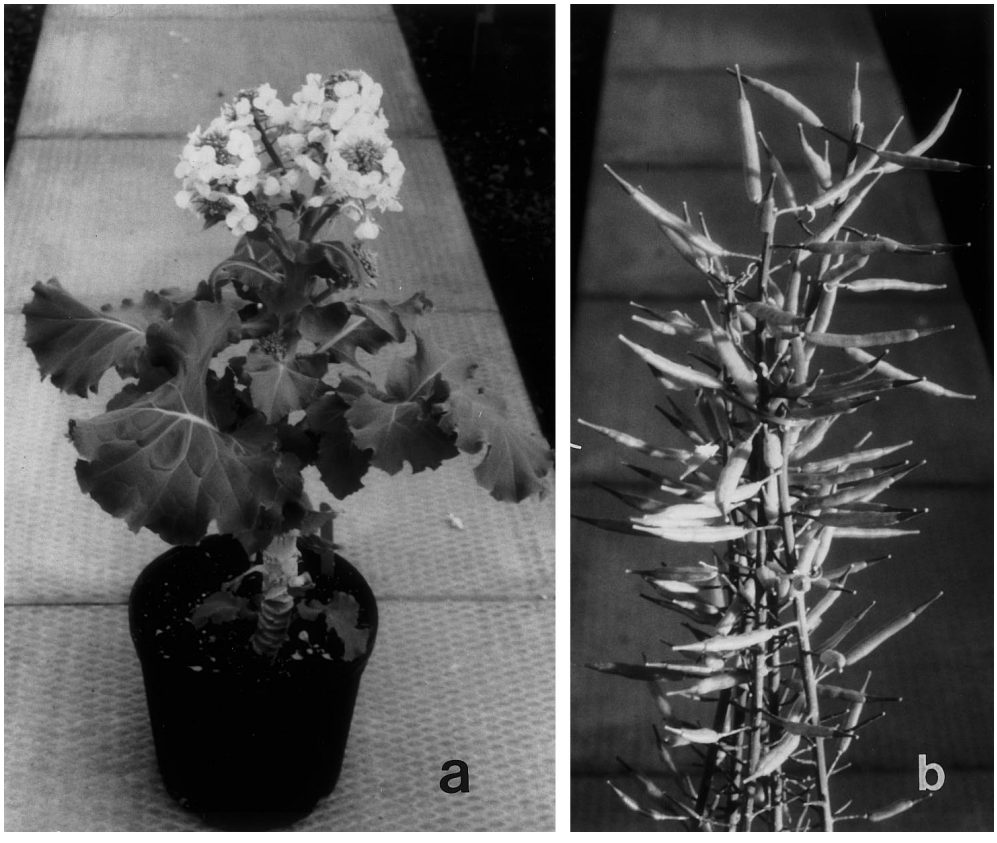

Fig. 2a, b A somatic hybrid derived from protoplast fusion between *B. rapa* and *B. oleracea* shown flowering (**a**) and setting seeds (**b**)

Jourdan (1994). Leaves from a somatic hybrid with its fusion partners are compared in Fig. 1. A somatic hybrid with normal flowers and pods is shown in Fig. 2. The somatic hybrids grew vigorously and had larger leaves and flowers than the parents and *B. napus* 'Westar'. Some flowered 1–2 months after transfer into soil without vernalization, while others flowered only after exposed to lower temperature. ('Shogun'+G30449)- 4 required almost 2 months of treatment at 4°C to induce flowering. Accurate times to flower were difficult to establish as regenerated plants were transferred from in vitro conditions to soil at different times, but all somatic hybrid plants eventually reached the flowering stage. They had uniformly normal yellow flowers with high pollen viability (86–96%) and easily set seeds, except ('Shogun'+G30449)- 4 which had lower pollen viability (52%) and lower seed set.

Table 2 Nuclear DNA content and male fertility of fusion partners and fusion-derived plants

Plant material	Shoot no.	Number of plants analyzed	DNA (pg/2c)±SD	Pollen viability (%)
Fusion partners & *B. napus*				
B. rapa	G30449	1	0.98	95
	C3–28	2	1.00±0.04	97
B. oleracea	Shogun	3	1.32±0.03	94
	B-19	2	1.39±0.01	–
	B-18	2	1.22±0.00	–
B. napus	Westar	2	2.53±0.07	96
Fusion combination				
Shogun+G30449	1	11	2.46±0.17	93
	2	2	2.44±0.02	90
	3	9	2.39±0.20	91
	4	4	3.53±0.06	52
	5	4	2.42±0.06	–
	6	4	2.38±0.13	–
	7	4	2.72±0.60	96
	8	3	2.38±0.04	89
	9	4	2.55±0.15	86
Shogun+G3044	1	–	–	–
	2	4	2.34±0.09	90
Shogun+C3–28	1	6	2.47±0.15	93
	2	4	2.32±0.04	96

Nuclear DNA content

The average nuclear DNA content (2c) of seed-grown Chinese cabbage G30444 and C3–28 was 0.99 pg/2c (aa genome) and that of seed-grown 'Shogun' broccoli was 1.32 pg/2c (cc genome) (Table 2). Of the 12 fusion-derived plants 11 had a DNA content ranging from 2.32±0.04 pg/2c to 2.72±0.60 pg/2c. This is about the sum of the parental genomes (aacc) and is comparable to the DNA content of 'Westar' (2.53 pg/2c). ('Shogun'+ G30449)-4 had a DNA content of 3.53 pg/2c and probably came from fusion of three protoplasts: two of 'Shogun' (cccc), and one of Chinese cabbage (aa) to produce a hexaploid (aacccc). This probably accounted for its low pollen viability.

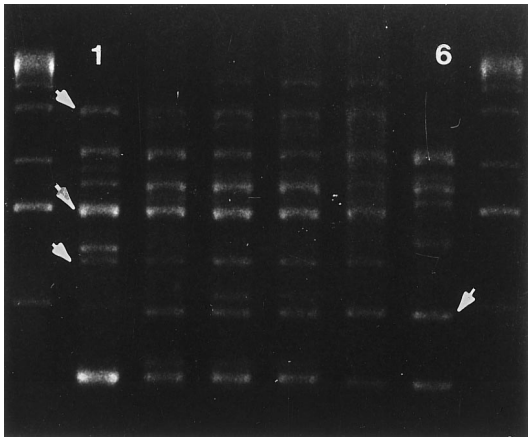

Fig. 3 An example of RAPD profiles of fusion partners and somatic hybrids generated using primers OPA-01. *Lane 1 B. rapa*, *lane 6 B. oleracea*, *lanes 2–5* somatic hybrids. *Arrows* indicate partner-specific bands

RAPD analysis

The somatic hybrids were confirmed by RAPD analysis. Twelve independent hybrids were analyzed using five primers. All of the primers showed at least one polymorphism between the parents. The somatic hybrids contained species-specific bands from both fusion partners. Primers OPA-1 and OPA-18 were the most informative. An example of RAPD profiles is shown in Fig. 3.

Tests for bacterial soft rot resistance

Bacterial soft rot resistance is a quantitative trait which exhibits continuous variation from resistant to susceptible (Yamagishi et al. 1990; Ren 1998). The objective of this experiment was to combine resistance genes from different species by protoplast fusion with the hope of obtaining somatic hybrids or their progeny with a higher resistance than one or both of the parents and than commercial cultivars of *B. napus*. The fusion partners used were relatively resistant genotypes selected in previous work (Ren 1998). Both partners exhibited similar levels of resistance in mist-chamber seedling tests, while *B. oleracea* 'Shogun' showed higher levels in detached leaf tests. It was suspected that the *B. rapa* and *B. oleracea* parents contain different resistance genes. If the somatic hybrids recovered were more resistant than the *B. rapa* parent, the *B. oleracea* resistance genes might be introduced into Chinese cabbage by repeated backcrossing and selection.

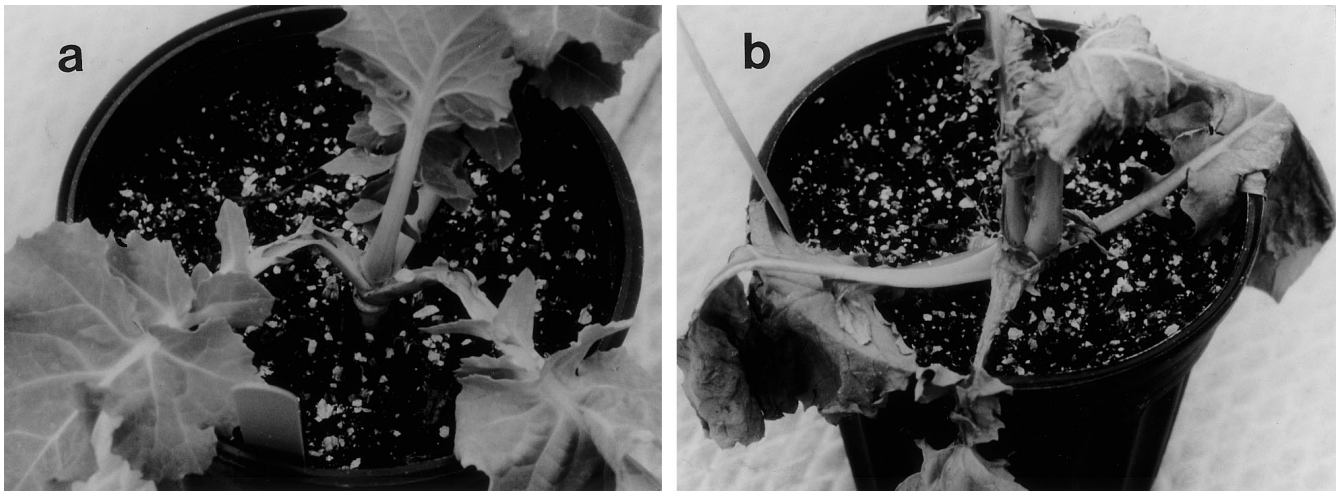

Fig. 4a, b Resistant (**a**) and susceptible (**b**) reaction of somatic hybrids inoculated at the plant level

Table 3 Disease severity ratings and lesion length of somatic hybrids tested by the detached leaf method at different stages[a]

	Callus that produced plants	Two months mean[a]		Six months mean	
		MDSR[b]	LL[c] (cm)	MDSR	LL (cm)
Fusion combination					
Shogun+G30449	1	4.8	6.4	1.3	1.3
	2	7.0	9.8	1.5	1.5
	3	4.6	6.4	0.5	0.9
	4	7.0	11.9	4.0	4.3
	5	6.3	9.8	1.1	1.4
	6	3.8	5.2	0.1	0.3
	7	5.3	7.5	0.1	0.2
	8	5.8	8.7	0.0	0.0
	9	5.5	8.7	2.4	2.0
Shogun+G30444	2	5.3	7.9	2.1	2.3
Shogun+C3–28	1	5.3	8.3	1.0	1.1
	2	5.8	9.3	1.3	1.8
Fusion partners					
Shogun		4.0	5.8	0.9	0.6
G30449		7.8	13.7	–	–
G30444		8.0	12.2	–	–
C3–28		6.2	9.5	6.0	6.9
Controls					
CC25		9.0	15.2	–	–
Westar (*B. napus*)		9.0	15.8	4.4	4.8

[a] Experiments were conducted at 2 and 6 months after transplanting. Each experiment was repeated three times. Each time, two to three leaves per plant were sampled, and the data are averages of leaves
[b] Mean disease severity rating from 0 to 9, where 0=no disease and 9=disease in entire petiole
[c] Lesion length

Resistance of somatic hybrids to soft rot

Test at plant level

Plants grown in soil for only 1 month were more susceptible than those grown in soil for 2 months or longer (data not shown). Resistance tests of somatic hybrids should therefore be conducted at least 2 months after transfer out of culture. The seed-grown fusion partners showed little variation when tested at 2 or 4 months, with most disease ratings between 3 and 4. Similarly, the susceptible *B. rapa* control ('CC25') consistently had ratings of 7–8. In contrast, there was large plant-to-plant variation among the somatic hybrids (data not shown). Even plants derived from the same fusion-derived callus varied from 1 to 7 when tested at 2 months. This variation in resistance may be caused by the different physiological status of the plants since they were

transplanted into soil and tested at different times of the year. Some hybrids, such as (‘Shogun’+G30449)-1–1, (‘Shogun’+G30449)-3–7, and (‘Shogun’+G30449)-8–1, had low disease ratings at both 2 and 4 months. Hexaploid hybrids (‘Shogun’+G30449)-4 were highly susceptible, with disease ratings from 6 to 8 at both time periods. Figure 4 shows the difference between highly resistant and highly susceptible somatic hybrids.

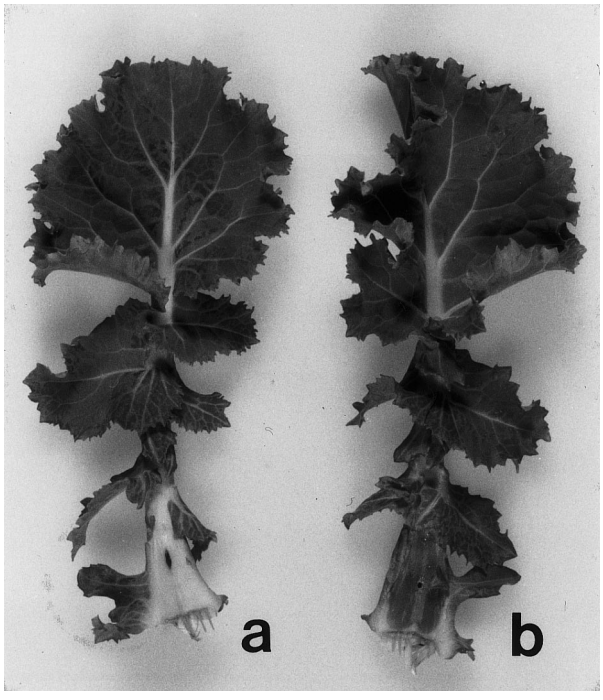

Fig. 5a, b Resistant (**a**) and susceptible (**b**) reaction of somatic hybrids inoculated at the detached leaf level

Test at detached leaf level

Detached leaves from additional plants regenerated from somatic hybrid calli were scored for disease severity and lesion length at 2 months after transfer to soil (Table 3). The parental plants were grown from seeds and were at a similar stage. The *B. oleracea* fusion partner had a lower mean disease severity rating than any of the *B. rapa* parents, as seen in previous work. Among the *B. rapa* partners, C3–28 had lower disease ratings than G30444 and G30449, but all three were more resistant than the susceptible check ‘CC25’. A comparison of the somatic hybrids with the fusion partners showed that all hybrids had less disease than the *B. rapa* parent but more than ‘Shogun’. Somatic hybrids (‘Shogun’+ G30449)-2, -4, and -5 were more susceptible than the others. However, all somatic hybrids had lower disease ratings than *B. napus* ‘Westar’. Figure 5 shows differences in resistance of detached leaves of somatic hybrids.

Results from similar assays with plants 6 months after transfer into soil are also shown in Table 3. *B. rapa* C3–28 had the highest disease score (6.0) with a value close to that at 2 months. By this time ‘Westar’ had started to flower and showed more resistance than when it was younger. ‘Shogun’ and the somatic hybrids also exhibited better resistance at 6 months than in the earlier assay. Except for (‘Shogun’+G30449)-4, all somatic hybrids had similar low disease ratings and lesion length; they were not different from ‘Shogun’, but were more resistant than the C3–28 and ‘Westar’. Hexaploid (‘Shogun’+G30449)-4 was again more susceptible than the diploid somatic hybrids. Figure 6 compares a resistant somatic hybrid with its fusion partners and *B. napus* ‘Westar’ in detached leaf inoculation.

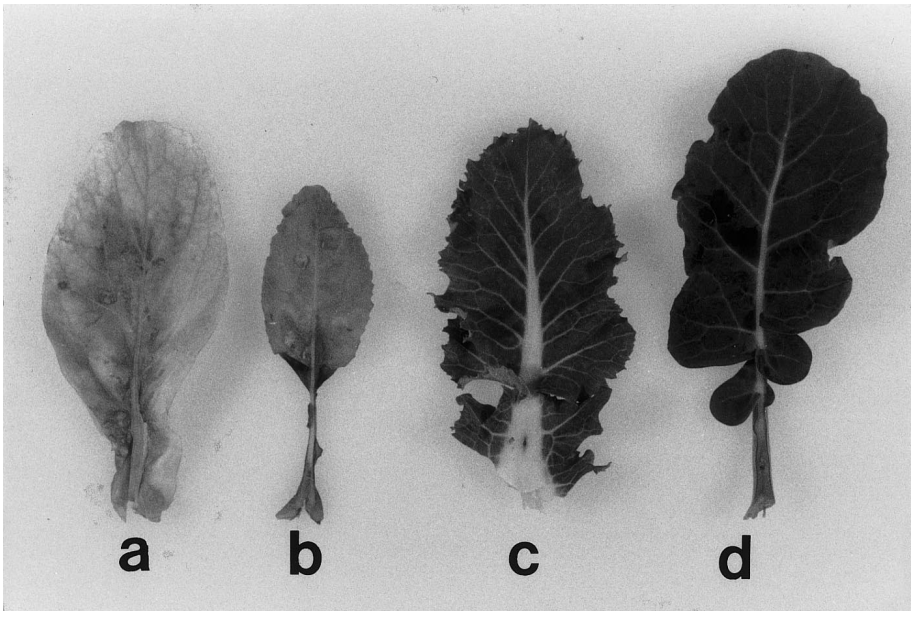

Fig. 6a–d Comparison of a resistant somatic hybrid (**c**) with its fusion partners (**a** *B. rapa*, **d** *B. oleracea*) and *B. napus* ‘Westar’ (**b**) in detached leaf inoculation

Table 4 Bacterial soft rot resistance in the progeny of the somatic hybrids

Plant material	Line	F_1–S_1 or F_1–BC_1	Mist-chamber (1) MDSR[a]	Mist-chamber (2) MDSR[a]	Detached leaf[b]	
					MDSR	LL[c] (cm)
Fusion partners						
B. rapa	G30449		6.0	5.0	8.0	15.0
	G30444		6.3	4.3	–	–
	C3–28		4.8	4.2	5.0	7.0
B. oleracea	Shogun		6.4	6.2	4.0	5.5
Somatic hybrids						
Shogun+G30449	1–1	S_1	5.4	5.2	3.0	4.3
		BC_1	2.7	4.4	2.0	2.3
	1–2	S_1	4.6	4.4	5.4	6.6
		BC_1	5.7	4.8	3.6	4.0
	2	S_1	5.5	–	5.0	6.8
	3	S_1	5.5	–	4.4	5.5
	5	S_1	5.5	–	3.6	4.6
	7	S_1	6.9	–	5.0	7.1
		BC_1	5.1	–	4.4	5.3
	8	S_1	4.4	4.4	5.0	6.8
		BC_1	–	3.0	–	–
	9	S_1	4.4	–	3.4	4.2
Shogun+G30444	2	S_1	4.4	–	4.0	4.7
Shogun+C3–28	1	S_1	5.7	4.6	6.6	12.3
		BC_1	3.7	4.3	2.6	3.2
	2	S_1	5.5	–	5.4	6.0
		BC_1	5.3	–	4.0	4.6
Controls						
B. rapa	CC25		7.3	7.1	–	–
B. napus	Westar		5.1	6.6	9.0	20.0
Re-synthesized						
B. napus	921100-1	S_1	7.1	–	7.6	11.3
	921110-1	S_1	7.9	–	9.0	20.0
	921110-2	S_1	9.0	–	9.0	19.0
$LSD_{0.05}$			2.15	–	–	–

[a] Mean disease severity rating
[b] Two leaves per plant were sampled; the data are the average of three plants from the same line
[c] Lesion length

Resistance of F_1–S_1 and F_1–BC_1 progeny to soft rot

Mist-chamber seedling inoculation

The analysis of variance from the first mist-chamber seedling test indicated significant differences in mean disease severity rating among lines (Table 4). There were large variations in disease severity ratings (from 1 to 9) among individual plants within each F_1–S_1 or F_1–BC_1 line, as reported by Yamagishi et al. (1990) in their study using detached leaf inoculation. It is likely that the segregation and recombination of resistance genes caused these variations. Although the mean disease severity ratings of selfed progeny lines were not statistically lower than those of both parents because of the heterogeneous population, many individual F_1–S_1 plants had much lower ratings (data not shown). Some F_1–S_1 lines showed lower ratings than both parents, especially ('Shogun'+G30449) -1–2, -8, and –9 and ('Shogun'+G30444)-2 with disease ratings of 4.6 or 4.4. Almost all of the selfed progeny lines had lower disease ratings than lines 921100–1, 921110–1, and 921110–2 (selfed progeny of somatic hybrids in which the fusion partners had not been selected for soft rot resistance and probably did not contain resistance genes). The results indicated that the fusion partners used in this study possessed resistance genes that could be combined in somatic hybrids and transferred to their offspring.

All F_1–BC_1 lines, except ('Shogun'+C3–28)-2×C3–28, had lower disease ratings than both parents. Again, many individual F_1–BC_1 plants showed high resistance. Notably, ('Shogun'+G30449)-1–1×C3–28 and ('Shogun'+C3–28)-1×C3–28 had significantly lower disease severity ratings (2.7 and 3.7) than most other lines. Repeated tests gave similar results (Table 4).

Almost all of the F_1–BC_1 lines had lower disease ratings than their corresponding F_1–S_1 lines. This may indicate that additional resistance genes were added by the backcrosses of the *B. rapa* parent to the somatic hybrids. It is known that additive gene effects are significantly more important than non-additive gene effects in soft rot resistance (Ren 1998). The resistant parents used in the fusion experiments were not homozygous. By backcrossing, more resistance genes from C3–28 were probably added, resulting in homozygosity in certain resistance loci in some plants of F_1–BC_1 which had much lower disease ratings. The fact that F_1–BC_1 lines were more resistant than the corresponding F_1–S_1 lines probably also in-

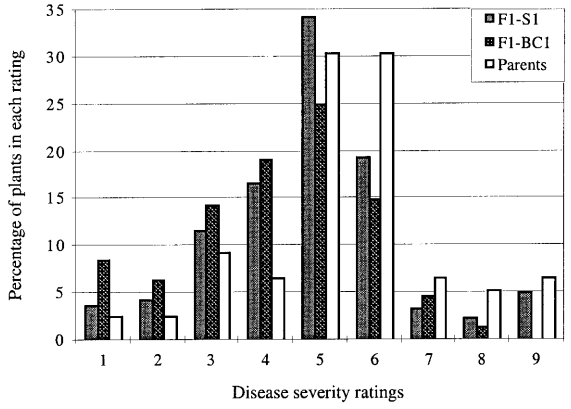

Fig. 7 Frequency distribution of disease severity ratings in populations of parents and offspring of somatic hybrids between Chinese cabbage and broccoli (mist-chamber inoculation method)

dicates that the *B. rapa* parents contain some recessive resistance genes. After backcrossing, some progeny may contain both dominant and recessive resistance genes and therefore exhibit a much higher level of resistance (ratings of 1 or 2) than progeny that were heterozygous for these alleles. In contrast, Yamagishi et al. (1990) reported that F_1–BC_1 plants were more susceptible than F1-S1 plants. This was probably due to differences in the genetic background of the *B. rapa* used as fusion partners and recurrent parents in their experiment and ours. In their study, a Chinese cabbage cultivar, 'Nozaki No. 2', a susceptible cultivar, was the *B. rapa* partner and backcross parent. Information about the susceptibility of 'Nozaki No. 2' compared to the genotypes used in this study is not available. Since there was a wide variation in resistance in the F_1–BC_1 populations, and susceptibility was not correlated with leaf morphology (Yamagishi et al. 1990), it should be possible to obtain more resistant Chinese cabbage by further backcrossing and selection.

The disease severity ratings in the tested parents and F_1–S_1 and F_1–BC_1 populations showed continuous variation from 1 to 9 with a normal distribution, a typical quantitative character (Fig. 7). The data were organized by combining both mist-chamber tests for parental lines, F_1–S_1 lines, and F_1–BC_1 lines. The mean disease severity ratings of parents, F_1–S_1 and F_1–BC_1 were 5.4, 5.0 and 4.5, respectively. In the parental population, 14.4%, 21%, and 51.3% of the plants had disease ratings lower than 3, 4, and 5, respectively. The percentage of plants in each category increased to 19.5%, 36.2%, and 70.5% in the F_1–S_1 population, and to 29.3%, 48.4%, and 73.4% in the F_1–BC_1 population. Thus, the offspring populations of the somatic hybrids moved in the direction of resistantance, especially the backcross population.

Detached leaf inoculation

The mean disease severity rating and lesion lengths of F_1–S_1 and F_1–BC_1 lines after detached leaf inoculation are also shown in Table 4. The results were similar to those of the mist-chamber seedling test, but even more encouraging. In terms of both disease severity rating and lesion length, most F_1–S_1 lines had less disease than the *B. rapa* fusion partner (G30449 parent). All F_1–BC_1 lines had much less severe disease than both fusion partners and their corresponding F_1–S_1 lines. In addition, all F_1–S_1 and F_1–BC_1 lines had much less disease than 'Westar', 921100–1, 921110–1, and 921110–2. Yamagishi et al. (1990) also reported that the selfed offspring of their somatic hybrids showed more resistance than a cultivar of *B. napus*.

In conclusion, combining of resistance genes from different species by protoplast fusion and backcross techniques is a promising way to increase the level of resistance to bacterial soft rot in the somatic hybrids and their offspring populations. Seeds of an additional backcross generation have already been harvested from several F_1–BC_1 lines, and others are being obtained. Further inoculation and selection will be conducted soon. Resynthesized *B. napus* and their progeny provide a basic breeding population for improving the level of resistance to bacterial soft rot in *B. rapa*. The somatic hybrids also expand the genetic base of *B. napus*.

Acknowledgments This work was supported in part by a grant to E.D.E. from the Vegetable and Flower Seed Permanent Research Fund.

References

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9: 221–231

Austin S, Lojkowaka E, Ehlenfeldt MK, Kelman A, Helgeson JP (1988) Fertile interspecific somatic hybrids of *Solanum*: a novel source of resistance to *Erwinia* soft rot. Phytopathology 78:1216–1220

Fosberg J, Landgren M, Glimelius K (1994) Fertile somatic hybrids between *Brassica napus* and *Arabidopsis thaliana*. Plant Sci 95:213–223

Gerdemann-knorck M, Sacristan MD, Braatz C, Schieder O (1994) Utilization of asymmetric somatic hybridization for the transfer of disease resistance from Brassica nigra to *B. napus*. Plant Breed 113:106–113

Hansen LN, Earle ED (1994) Regeneration of plants from protoplasts of rapid cycling *Brassica oleracea* L. Plant Cell Rep 13: 335–339

Hansen LN, Earle ED (1997) Somatic hybrids between *Brassica oleracea* L. and *Sinapis alba* L. with resistance to *Alternaria brassicae* (berk.) Sacc. Theor Appl Genet 94:1078–1085

Heath DW, Earle ED (1995) Synthesis of high erucic acid rapeseed (*Brassica napus* L.) somatic hybrids with improved agronomic characters. Theor Appl Genet 91:1129–1136

Hossain MM, Asahira T (1992) Development of heat tolerant somatic hybrids by PEG-mediated protoplasts fusion between *Brassica oleracea* L. and *B. campestris* L. Plant Tissue Cult 2: 61–69

Hu J, Quiros CF (1991) Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Rep 10:505–511

Jourdan PS, Earle ED (1989) Genotypic variability in the frequency of plant regeneration from leaf protoplasts of four *Brassica* ssp. and of *Raphanus sativus*. J Am Soc Hortic Sci 114: 343–349

Jourdan PS, Earle ED, Mutschler MA (1989) Synthesis of male-sterile, triazine-resistant *Brassica napus* by somatic hybridization between cytoplasmic male-sterile *B. oleracea* and atrazine-resistant *B. campestris*. Theor Appl Genet 78:445–455

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Ozminkowski RH, Jourdan P (1994a) Comparing the resynthesis of *Brassica napus* L. by interspecific somatic and sexual hybridization. I. Producing and identifying hybrids. J Am Soc Hortic Sci 119:808–815

Ozminkowski RH, Jourdan P (1994b) Comparing the resynthesis of *Brassica napus* L. by interspecific somatic and sexual hybridization. II. Hybrid morphology and identifying organelle genomes. J Am Soc Hortic Sci 119:816–823

Pelletier G, Primard C, Vedel F, Chétrit P, Rémy R, Renard M (1983) Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191:244–250

Qiu WF, Zhang JZ, Tao GH (1955) Variation of resistance to bacterial soft rot (*Erwinia aroideae*) in Chinese cabbage cultivars. China Phytopathol 1 61–70

Ren JP (1998) Resistance to bacterial soft rot (*Erwinia carotovora* subsp. *carotovora*) in *Brassica rapa* vegetables. PhD thesis, Cornell University, Ithaca, N.Y.

Shimizu S, Kanazawa K, Kobayashi T (1958) Studies on the breeding of Chinese cabbage for resistance to soft rot. 1. Difference of resistance among varieties under field conditions. Bull Natl Inst Agric Sci Ser E 6:75–108

Shimizu S, Kanazawa K, Kobayashi T (1962) Studies on the breeding of Chinese cabbage for resistance to soft rot. 3. The breeding of the resistant variety 'Hiratsuka No. 1' by interspecific crossing. Bull Hortic Res Stn A1:157–174

Sigareva MA, Earle ED (1997) Direct transfer of a cold-tolerant Ogura male-sterile cytoplasm into cabbage (*Brassica oleracea* ssp. *capitata*) via protoplast fusion. Theor Appl Genet 93: 1242–1250

Togashi J (1981) Studies on relationship between variety difference of Chinese cabbage in resistance to the soft rot disease and growth of the causal organism (*Erwinia carotovora*) in rhizosphere soils. Bull Yamagata Univ Agric Sci 8:1–7

Walters TW, Earle ED (1990) A simple, versatile feeder layer system for *Brassica oleracea* protoplast culture. Plant Cell Rep 9:316–319

Williams PH (1981) Workshop on screening crucifers for multiple disease resistance. University of Wisconsin, Madison, Wis

Yamagishi H, Yoshikawa H, Yui S (1990) Leaf morphology and soft rot resistance in offspring of a somatic hybrid between Chinese cabbage and kale (*Cruciferae*). Euphytica 47:215–221

Yoshikawa H, Sawada T, Yamagishi H, Yui S (1989) Soft rot resistance of progenies of somatic hybrid: Chinese cabbage+Kale. J Jpn Soc Hortic Sci 58 [Suppl 1]:190–191