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Phenotypic variation in progenies from somatic hybrids between *Brassica napus* and *Sinapis alba*

Aimin Li · Cunxu Wei · Jinjin Jiang · Yongtai Zhang · Rod J. Snowdon · Youping Wang

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Abstract A number of variant phenotypes, such as long siliques, high density of pods, increased seed number per pod and yellow seed color resembling *Sinapis alba*, were selected from intergeneric somatic hybrids between *Brassica napus* and *Sinapis alba* through successive backcrosses. Resistance to *Sclerotinia sclerotiorum* among 24 BCF_4 lines was also tested by inoculation with mycelia, and four lines showed stronger resistance than the resistant rapeseed variety 'Zhongshuang 9'. Comparative anatomy studies on mature seed revealed that the seed coat pigments were mainly distributed in the palisade layer, which is considerably thinner in *S. alba* compared to *B. napus*. The area index of protein bodies in cotyledon cells was highest in *S. alba*, lowest in *B. napus* and intermediate in the intergeneric progenies. This study demonstrates that wide hybridization can enable exploitation of valuable trait diversity from *S. alba* for broadening the genetic basis for rapeseed breeding.

Keywords *Brassica napus* L. · Intergeneric somatic hybrid · *Sclerotinia sclerotiorum* · *Sinapis alba* L. · Yellow seed

A. Li · C. Wei · J. Jiang · Y. Wang (✉)
College of Bioscience and Biotechnology,
Yangzhou University, 225009 Yangzhou, China
e-mail: wyouping@yahoo.com

A. Li · Y. Zhang
Jiangsu Institute of Agricultural Science in the Lixiahe District, 225009 Yangzhou, China

R. J. Snowdon
Institute for Plant Breeding and Crop Science I,
Justus Liebig University, Heinrich-Buff-Ring 26-32,
35392 Giessen, Germany

Introduction

The genetic basis of *Brassica napus* L., one of the most important oilseed crops worldwide, is quite narrow. The species originated in Europe and was introduced into China as an oil crop in the 1950s (Liu 1984; Wang 2005). Intergeneric hybridization with related crucifer species is often used to broaden genetic variability in *B. napus* (Snowdon et al. 2006), however, sexual incompatibility limits the use of such approaches (Wang et al. 2005a, b, c). On the other hand, somatic hybridization allows not only the creation of hybrid and cybrid combinations of species that are sexually incompatible, but also recombination and transfer of cytoplasmic genes from both parents, with the aim of improving the characteristics controlled by cytoplasmic genes (Ge and Chen 2004; Liu et al. 2005; Prakash et al. 2009). Protoplast fusion was also used by Ren et al. (2000) and Hu et al. (2002) to recover novel rapeseed germplasm resistant to bacterial soft rot and *Leptosphaeria maculans*.

Sinapis alba L., a member of the Brassicaceae, possesses desirable agronomic characteristics such as yellow seed color, tolerance to drought stress, reduced pod shattering (Downey 1987), resistance to virus diseases, blackleg disease, black spot and beet cyst nematodes (Bodnaryk and Lamb 1991; Lelivelt et al. 1993; Brown et al. 1997; Hansen and Earle 1997). Intergeneric hybrids resistant to black spot were produced by ovary culture of *B. rapa* × *S. alba* sexual hybrids (Gong et al. 1994). Somatic hybrids of *B. napus* and *S. alba* obtained by electrofusion have been described previously (Wang et al. 2005b, c). For creation of high yielding rapeseed lines with improved disease resistance and seed quality, these hybrids were subsequently backcrossed with *B. napus* and self-pollinated to obtain a BCF_4 generation with valuable agronomic characteristics derived from *S. alba*. This material contains various interesting phenotypes that may be useful for rapeseed breeding. The objective of this study was to characterize phenotypic variants observed in the BCF_4 progenies.

Materials and methods

Materials

Intergeneric somatic hybrids (AACCSS, $2n = 62$) between *B. napus* and *S. alba* obtained by electrofusion were subsequently backcrossed with *B. napus* cv. 'Yangyou 6' (AACC, $2n = 38$) and self-pollinated. Figure 1 shows the backcrossing scheme for the development of BCF_4 progeny during 2005 and 2008.

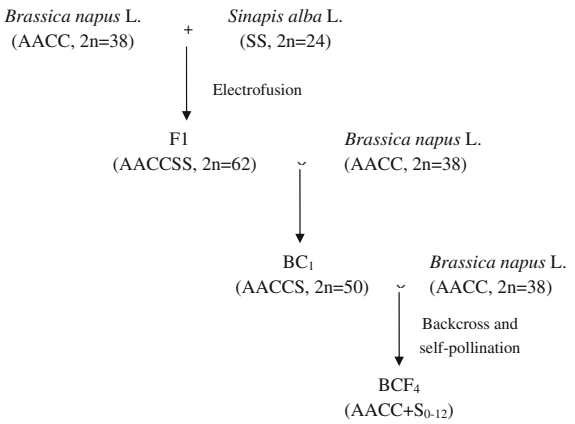

Fig. 1 Backcrossing scheme for the development of BCF_4 progenies

Observation of agronomic characteristics

Field tests of the somatic hybrid backcross progeny were carried out at the experimental station of Jiangsu Lixiahe Region Agricultural Research Institute, China. Morphological variations observed in BCF_4 included main axis length, number of pods per plant, number of seeds per silique and pod length, as well as the beak length of pods. Pod density was described by the ratio between the number of pods and the length of the main raceme.

Pigment distribution in seed coat and thickness of the palisade layer

Experimental samples were taken from the adaxial side of seeds, then 15 μm thick frozen sections were cut using a Leica CM1100 microtome at −15°C. Sections were photographed using an Olympus CX 41 light microscope without staining. The thickness of the palisade layer was measured on three seeds from each line, using the parental lines as a control.

Anatomical structure of seeds

After soaking in ddH_2O for 1 h at room temperature, seed capsules from the adaxial side and cotyledon embryos were used as experimental samples for transmission electronic microscopy. Samples were embedded in Spurr's resin, then semi-thin resin sections (1 μm) were cut on a Leica EM UC6 microtome using a glass blade, while ultra-thin sections (70 nm) were generated using a diamond blade. The semi-thin sections were counterstained with PAS-sudan black B-coomassie blue R according to Hu and Xu (1990). Observations and photographs were made via light microscopy. The number of protein bodies was counted in ten adjacent cells on each section, and the cumulative area of protein bodies along with the total area of the ten cells was measured using the morphological analysis software JD801. Subsequently, the mean area index of protein body per cell was calculated in each line. All data presented in this paper were obtained from five sections from each of the progeny and parental lines. After double-staining with uranium acetate and lead

citrate, observation and image merging of the ultra-thin sections were done with a Philips Tecnai 12 transmission electronic microscope.

Characterization of *Sclerotinia sclerotiorum* resistance

Artificial inoculation of plants with *S. sclerotiorum* was performed according to Cruickshank et al. (1983). After 72 h of mycelia inoculation with twenty detached leaves from different plants of each progeny line, the length and width of disease lesions were measured. The level of resistance to *S. sclerotiorum* was judged by the disease lesion area using the formula $S = L \times W$ (S: lesion area; L: length of lesion; W: width of lesion). All trial data were analyzed using the software DPS.

Determination of oil content

The oil content of self-pollinated seed from each of the BCF_4 progeny and parental lines was detected by Near Infrared Reflectance Spectroscopy using the NIRS Model TR-3700 from FOSS (Denmark).

Results

Variation in pod characteristics

Compared with BC_1 plants, pod length and seeds per pod among BCF_4 progeny lines were dramatically improved (Table 1). Three BCF_4 lines (D214, D224, D249) were identified with distinct variation in pod characteristics. D249 had a very high pod density of 1.8/cm, 38.5% higher than the recurrent parent 'Yangyou 6' (1.3/cm). Lines D214 and D224 had considerably longer siliques than the recurrent *B. napus* parent (Fig. 2e, f). The silique lengths of up to 9.3 and 9.1 cm in length, respectively, correspond to an increase of 69.1 and 65.5%, respectively compared to 'Yangyou 6' (5.5 cm). The number of seeds per pod in these three lines was over 26.8, a increase of 46.5, 55.2 and 60.7% respectively compared to 'Yangyou 6' (18.3 seeds per pod). Furthermore, the pods of the three selected lines had characteristically long beaks (Fig. 2), ranging from 1.4 to 1.8 cm, which resembles the pods of *S. alba* (2.0 cm beak length) rather than 'Yangyou 6' (1.1 cm beak length).

Table 1 Pod characteristics of parents and backcross progenies

Plant material	Main axis length (cm)	Compared with 'Yangyou 6' (±%)	Pods per main raceme	Compared with 'Yangyou 6' (±%)	Pod density per cm	Compared with 'Yangyou 6' (±%)	Pod length (cm)	Compared with 'Yangyou 6' (±%)	Seeds per pod	Compared with 'Yangyou 6' (±%)	Pod beak length (cm)	Compared with 'Yangyou 6' (±%)
S. alba	30.2	/	84.3	/	2.8	/	1.2	/	3.7	/	2.0	/
Yangyou6	63.8	/	79.8	/	1.3	/	5.5	/	18.3	/	1.1	/
BC_1	86.3	35.3	119.6	49.9	1.4	7.7	3.1	−43.6	4.6	−74.9	1.3	18.2
D214	73.6	15.4	110.3	38.2	1.5	15.4	9.3	69.1	28.4	55.2	1.8	63.6
D224	72.5	13.6	60.5	−24.2	0.8	−38.5	9.1	65.5	29.4	60.7	1.4	27.3
D249	68.3	7.1	120.4	50.9	1.8	38.5	7.9	43.6	26.8	46.5	1.5	36.4

Fig. 2 Comparison of silique among parents, BC_1 and BCF_4 plants. **a** *B. napus*, **b** *S. alba*, **c** BC_1, **d–f** BCF_4 lines, **d** D249, **e** D214, **f** D224

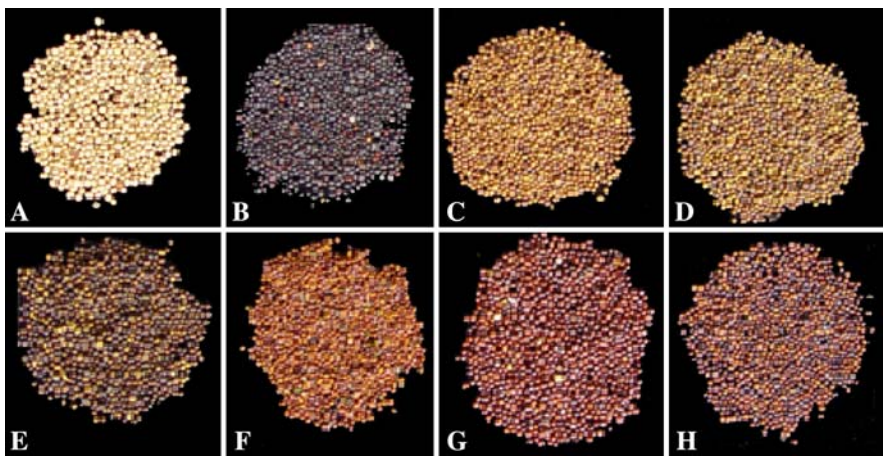

Fig. 3 Seed coat color of backcross progenies and their parents. **a** *S. alba*, **b** 'Yangyou 6', **c** D244-18, **d** D244-52, **e** D244-6, **f** D255-3, **g** D246-5, **h** D211

Oil content and anatomical structure of yellow seeded progenies

Several lines with yellow seed color derived from the *S. alba* parent were identified among the backcross progenies. The seed color of these lines ranged from brownish yellow to yellow, while the *B. napus* parents had dark brown seeds (Fig. 3a–h). The oil content of these six yellow-seeded lines was found to be increased to 43.3, 43.4, 47.6, 42.9, 42.8, 44.6%, respectively, compared to the oil content of 41.6% in 'Yangyou6' and 39.0% in the *S. alba* parental line.

The seed coat pigments were mainly distributed in the palisade layer (Fig. 4), the highest quantity of seed coat pigmentation was observed in 'Yangyou6', followed by the backcross lines D246-5, D211, D255-3 and D244-52. On the other hand, the seeds of the *S. alba* parent and the backcross progenies D244-18 and D244-6 exhibited almost no pigmentation. The thickness of palisade layer was highest in

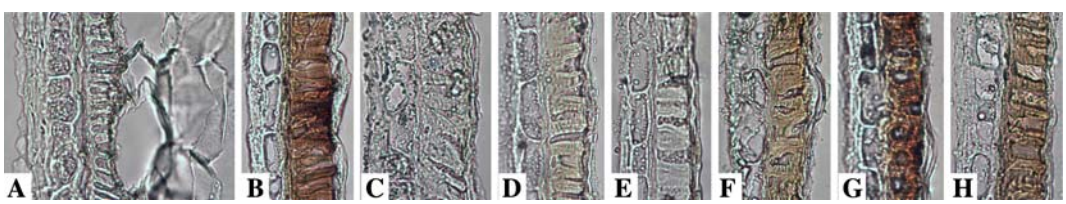

Fig. 4 Pigment distribution in seed coat of backcross progenies and their parents. **a** *S. alba*, **b** 'Yangyou 6', **c** D244-18, **d** D244-52, **e** D244-6, **f** D255-3, **g** D246-5, **h** D211. ×200

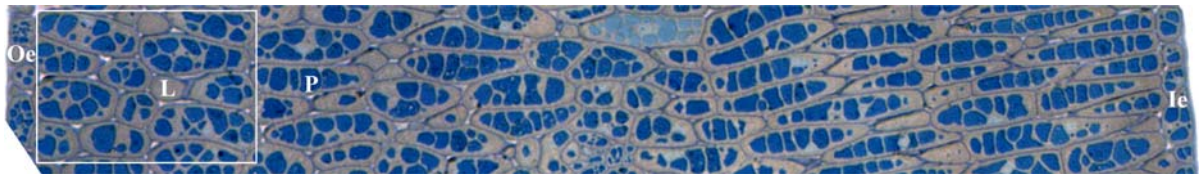

Fig. 5 The semi-thin section of cotyledon in *S. alba*. ×250. *Ie* inner epidermical cell, *L* lipid body, *Oe* outer epidermical cell, *P* protein body

B. napus, thinnest in *S. alba* and intermediate in the hybrid progenies (Fig. 4).

When treated by PAS-sudan black B-coomassie blue R, the lipid body and storage proteins on semi-thin sections of cotyledon embryos were dyed gray and blue, respectively. The first step of PAS staining stains cell walls red, however, this changes to blue–black after sudan black B and coomassie blue R counterstaining (Fig. 5). Statistical analysis of the protein body area index in the first three layers under the outer epidermical of the seed cotyledons (Fig. 5) revealed that the protein body area index in cotyledon cells was highest in *S. alba*, lowest in *B. napus* and intermediate in progenies (Fig. 6).

Characterization of resistance to *Sclerotinia sclerotiorum*

Statistical analysis of disease lesions in the twenty-four progeny lines tested showed a significant variation in disease response among lines after inoculation with mycelia ($F = 16.693$, $v1 = 494$, $v2 = 25$). Compared with line D267 and 'Zhongshuang 9', which were used as susceptible and resistant controls, respectively, different resistance reactions were observed among the tested lines. Through multi-comparison of inoculation of detached leaves with mycelia, we founded no significant difference in lines

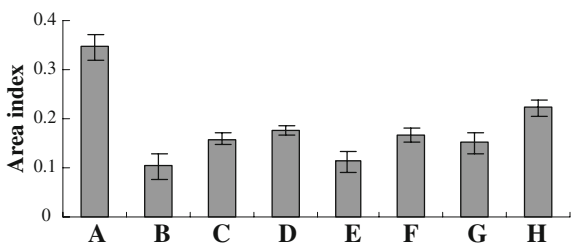

Fig. 6 Area index of protein body of backcross progenies and their parents **a** *S. alba*, **b** 'Yangyou 6', **c** D244-18, **d** D244-52, **e** D244-6, **f** D255-3, **g** D246-5, **h** D211

D216, D222, D225, D226 and D220 compared with susceptible line D267. On the other hand, lines D214, D245 and D219 were shown to have a stronger resistance than D267, but weaker than 'Zhongshuang 9'. Another eleven lines (D218, D223, D246, D212, D265, D211, D273, D241, D230, D237 and D213) were confirmed to have no obvious difference from the resistant control. Lines D244, D243, D229 and D240 exhibited the strongest resistance to *S. sclerotiorum* and showed significant differences to the resistant control 'Zhongshuang 9' (Table 2).

Discussion

Distant hybridization of sexually incompatible plant species via somatic fusion can overcome intergeneric boundaries, enlarge genetic variation, and ultimately result in the creation of new variants and species that do not occur naturally. Both sexual and somatic hybridization have been widely used to introduce traits of interest from related crucifer species to oilseed rape (Snowdon et al. 2006). Roy (1984) and Lu et al. (2006) obtained novel rapeseed with yellow seed color and resistance to blackleg through hybridization between *B. juncea* and *B. napus*, respectively. Progenies with extreme pod length variation (20 cm in length) were also created from interspecific hybrids of *B. rapa* and *B. oleracea* (Niu et al. 2005). These examples demonstrate that distant hybridization can not only introduce new characteristics from donors into phylogenetically close plants, but also create new variant types beyond the variation present in the parental species. In this study, we obtained lines with many agronomically interesting phenotypes from intergeneric somatic hybrid progenies of *B. napus* and *S. alba* through successive backcrosses. These included lines wth high pod density, yellow seed coats, resistance to *Sclerotinia sclerotiorum*, long siliques, increased numbers of seeds per pod and

Table 2 Multi-comparison of disease lesion areas after artificial inoculation of detached leaves with *Sclerotina sclerotiorum* mycelia (PLSD)

Line No.	Mean lesion (cm^2)	Significance at 1%	Significance at 1%
D216	25.12	a	A
D222	24.98	a	A
D267	24.65	ab	AB
D225	23.16	abc	ABC
D226	21.66	bcd	ABC
D220	21.39	bcd	ABC
D214	20.90	cd	ABCD
D245	20.29	cd	BCDE
D219	20.27	cd	BCDE
D215	18.74	de	CDEF
D218	16.69	ef	DEFG
D223	16.53	ef	DEFG
D246	16.22	efg	EFG
D212	15.51	efgh	FG
Zhongshuang9	15.15	fgh	FG
D265	14.53	fgh	FGH
D211	14.44	fgh	FGH
D273	14.23	fgh	FGH
D241	13.26	fghi	GH
D230	12.85	ghi	GH
D237	12.74	ghi	GHI
D213	12.08	hi	GHI
D244	10.17	ij	HIJ
D243	9.88	ij	HIJ
D229	8.07	j	IJ
D240	6.85	j	J

$LSD_{0.05} = 3.33$, $LSD_{0.01} = 4.51$

improved oil content compared to the *B. napus* parent. These variant progenies are of great interest for rapeseed breeding, offering abundant germplasm resources for the creation of new varieties with improved disease resistance, seed quality and yield.

The oil and protein content of the yellow seeds is often higher than that of black ones to the the increased contribution of the cotyledons to the overall seed volume. In addition, the thinner seed coat of yellow-seeded *B. napus* is also associated with a lower antinutritive fiber content, while the reduced pigmentation results in a clear and transparent oil and a higher palatability of the seed meal in animal feeds. The yellow-seeded progenies obtained in the present study were found to have a thinner seed coat and reduced pigmentation compared to the dark-seeded *B. napus* parent. In contrast, the oil bodies of the best progenies resembled oil bodies in *B. napus* seeds rather than the smaller oil bodies of *S. alba* which has a very low oil content. On the other hand, the protein body area was significantly reduced in seeds from yellow-seeded progenies with high oil content, indicating that the reduction in seed coat thickness resulted in an increase in oil rather than an increase in protein proportion in the seeds.

Until now, genetic variation for seed color was only introduced to *B. napus* by interspecific hybridization with the closely related *Brassica* species *B. rapa*, *B. carinata*, *B. juncea* and *B. oleracea*. The seed color of *S. alba* is however, considerably lighter than in most *Brassica* species, raising the possibility that the yellow-seed trait derived from *S. alba* could further improve the meal and oil quality traits associated with light-seeded rapeseed. The progenies identified in the present study combine the yellow seed trait from *S. alba* with other agronomic

properties that could improve the oil content, seed yield and seed quality in *B. napus*, and are therefore of considerable interest for rapeseed breeding.

Sinapis alba is phylogenetically close to *Brassica* A and C genomes (Warwick and Black 1991), and has sufficient genetic homoeology (Gaikward et al. 1996). Nelson et al. (2005) found that 95% of the *Brassica* DNA fragments could be hybridized strongly to low copy-number sequences in the *S. alba* genome. Formation of multivalents and chromosome recombination in the progenies derived from *B. napus* and *S. alba* hybrid (Wang et al. 2005c) is also indicative of intergenomic homoeology between both *S. alba* and A/C genomes. The phenotypic variation presented herein with different agronomical characteristics from BCF_4 progenies to BCF_5 exhibited almost stable. One reason is these progenies had a very good fertility and a high seedset. The other reason is these progenies had 38 chromosomes and exhibited a normal meiosis (data not shown). How to identify these introgression lines, especially with yellow seeds, using molecular markers and to use this valuable germplasm for rapeseed breeding is our present task.

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