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ORIGINAL PAPER

Transfer of sclerotinia resistance from wild relative of *Brassica oleracea* into *Brassica napus* using a hexaploidy step

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Abstract
Key message **Sclerotinia resistance was transferred into rapeseed from a wild relative of *Brassica oleracea* (*B. incana*) using hexaploids derived from crosses between *B. incana* and rapeseed as a bridge.**
Abstract A high level of resistance against *Sclerotinia sclerotiorum* has been documented in wild *Brassica oleracea*, but not in cultivated rapeseed (*Brassica napus*). To transfer sclerotinia resistance from a wild relative into rapeseed, a strategy was proposed using hexaploids (AACCCC) derived from crosses between the wild *B. oleracea*-related *B. incana* genotype 'C01' and the Chinese rapeseed variety 'Zhongshuang 9' as a bridge. Progenies (BC1F1) generated by backcrossing the hexaploid to 'Zhongshuang 9' could be generated with a high crossability (average 18.3 seeds per pod). Seventy-three individuals in BC1F1 were firstly screened for resistance with five molecular markers linked to the major resistance QTL on chromosome C09 in 'C01', and 11 individuals harboring resistance loci were selected to develop vegetative clones. Of these, five exhibited significantly higher resistance than 'Zhongshuang 9' and the most resistant individual was chosen to develop the BC1F2 progeny. Finally, five individual genotypes with nearly twofold higher resistance than 'Zhongshuang 9' were found among 100 BC1F2 individuals by using marker-assisted selection and resistance evaluation. Hereof, one rapeseed-type individual with 38 chromosomes and good self-fertility (15.0 ± 3.56 seeds/pod) was identified. Our results indicate that the proposed strategy is effective for transferring sclerotinia resistance from a wild relative of *B. oleracea* into rapeseed.

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Introduction

Rapeseed (*Brassica napus* L; genome AACC, $2n = 38$) originated through spontaneous interspecific hybridization between the diploid progenitors, turnip rape (*B. rapa* L., syn. *B. campestris*; genome AA, $2n = 20$) and kale (*B. oleracea* L.; genome CC, $2n = 18$) (Nagaharu 1935). Today, rapeseed is the world's third-leading source of both edible oil and extraction meal or cake after soybean and oil palm (Friedt and Snowdon 2010). Unfortunately, the crop frequently suffers from attack by the fungal pathogen *Sclerotinia sclerotiorum*, which can cause serious yield losses (del Río et al. 2007; Koch et al. 2007).

While partial resistance has been reported in *B. napus* (Falak et al. 2011; Li et al. 1999; Wang et al. 2004), complete or highly resistant lines are obviously not available in the *B. napus* gene pool including oilseed rape (OSR).

Therefore, resistance breeding in OSR has been difficult and rather unsuccessful (Bradley and Hamey 2005; Yu et al. 2010). Recently, highly effective sources of sclerotinia resistance were found in wild relatives of *B. oleracea* (Mei et al. 2011), and resistance QTL from a wild *B. oleracea*-related *B. incana* genotype 'C01' were identified (Mei et al. 2013). These resistance sources are considered a promising basis to improve sclerotinia resistance of current OSR breeding material and to develop better cultivars in the future.

Resynthesizing rapeseed is a routine strategy to introduce genetic components of the parental species *B. oleracea* and *B. rapa* into rapeseed. However, sclerotinia resistance appeared to be suppressed in resynthesized rapeseed lines due to the high susceptibility of *B. rapa* (Ding et al. 2013). Therefore, we propose here a new strategy to transfer resistance against *S. sclerotiorum* using hexaploid progeny derived from a cross between *B. oleracea* and *B. napus* as a bridge.

Materials and methods

Development of plant materials

Interspecific hexaploid hybrids (AACCCC) were generated according to the method described by (Li et al. 2013). Briefly, a wild *B. oleracea*-related genotype 'C01' (*B. incana*) showing a high level of resistance (Mei et al. 2011) was used as a male parent to pollinate 40 individual florets of a single plant of cv. 'Zhongshuang 9', a registered Chinese semi-winter rapeseed variety with partial resistance against *S. sclerotiorum* (Wang et al. 2004). The developing hybrid siliques were excised 10 days after pollination and the ovules were cultured in vitro according to Wen et al. (2008), followed by chromosome doubling in vitro before transplantation into the field. The hexaploid progeny was backcrossed with 'Zhongshuang 9' and the BC1F1 progeny was self-pollinated to develop BC1F2 which was characterized for its fertility and cytological status according to Li et al. (2013). A schematic diagram of the whole process was presented in Fig. 1.

Molecular marker-assisted selection

In a previous study, two major QTL for stem resistance to *S. sclerotiorum* in 'C01' were detected on linkage group C09, jointly explaining 26–32 % of phenotypic variance for stem resistance tested in 2 years (Mei et al. 2013). Among eight simple sequence repeat markers (SSR) within the confidence interval of the QTL, five markers exhibiting polymorphism between 'C01' and 'Zhongshuang 9' were used to track these resistance loci in the subsequent breeding progenies.

Vegetative propagation of plants

Individuals carrying the resistance loci, together with a negative control not harboring the resistance loci, were vegetatively propagated. A number of clones were generated for multiple resistance tests according to the following procedure: (1) cut the stem into several pieces at the eight- to ten-leaf stage, with one or two leaf nodes on each cutting; (2) place the cutting into a water-filled tube after touching with rooting powder (Neudofix® WurzelAktivator in Germany) on the bottom of the cutting and keep it in the tube for 1 week; (3) place each stem cutting into moist soil (around 60 % of humidity) and grow in a greenhouse for 1 month; (4) vernalize the plants at 4 °C for 12 weeks; and (5) transplant the vernalized plants in the greenhouse until resistance evaluation. The plants were grown in the greenhouse for 16 h photoperiod under 450 μmol m^{-2} s^{-1} light at 25 °C and 8 h dark at 20 °C with normal management.

Sclerotinia resistance screening

Two *S. sclerotiorum* isolates, one collected from the field in Southwest University at Beibei, Chongqing, China (isolate *C*), and the other collected from a field at Hohenlieth in Kiel, Northern Germany (isolate *G*), were used in this study. The detached stem inoculation technique was applied at the end of flowering according to Mei et al. (2012). A 30-cm-length stem segment of each individual was collected and inoculated with two mycelium-covered agar plugs in 10-cm interval. At least three individuals were evaluated for each genotype. The temperature during infection was 22 °C and humidity was 85 %. As a criterion of resistance reaction, lesion length was measured 3 days after inoculation (dai).

Data analysis

Statistical analyses such as Pearson's simple correlation, analysis of variance (ANOVA), and *F* and *t* tests were performed using the SAS software, version 6.07 (SAS Institute 1992).

Results

Characterization of hexaploid progenies

A total of 40 individual florets of a single plant of *B. napus* cv. Zhongshuang 9 were pollinated by *B. incana*. Via in vitro ovule culture in 2011, three hybrid embryos were obtained and treated with colchicine to develop hexaploid progenies by chromosome doubling. Hexaploidy (AACCCC, $2n = 56$) of the hybrids was confirmed by fluorescent

in situ hybridization (FISH), where 36 chromosomes from the C genome and 20 chromosomes from the A genome were identified (Fig. 2). The hexaploid plants were morphologically similar to 'Zhongshuang 9', but characterized by a greater plant height. The hexaploids showed good fertility (ranging 90.6–92.7 % for pollen fertility and 3–7 seeds/pod after self-pollination) and stronger sclerotinia resistance (average lesion length 4.6 cm at 3 dai) than 'Zhongshuang 9' (lesion length 9.6 cm) (Fig. 3a). It was interesting that a high crossability (17–19 seeds/pod) was detected in the backcross progenies between the hexaploid (female) and 'Zhongshuang 9' (male).

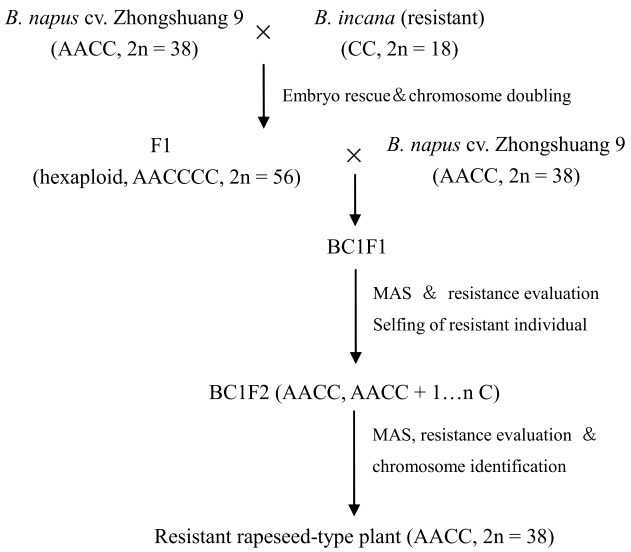

Fig. 1 Breeding process of introducing resistance to *S. sclerotiorum* from a wild relative of *B. oleracea* (*B. incana*) into *B. napus*

Identification and selection of BC1F1

A total of 73 BC1F1 individuals from the cross between the hexaploid and 'Zhongshuang 9' were planted in the greenhouse and screened for resistance loci using five SSR markers located within the resistance QTL regions on C09. Of these, 11 (15.1 %) individuals with the same banding patterns as 'C01' were chosen for vegetative propagation; the clones were subsequently evaluated for resistance with *S. sclerotiorum* isolates *G* and *C*. The isolate *G* caused longer lesions (8.0 cm on average) than isolate *C* (5.0 cm on average) ($P = 0.0002$), but the lesion length caused by the two isolates was significantly correlated ($r = 0.599$, $P < 0.01$). Significant differences for sclerotinia reaction were detected among the 11 BC1F1 genotypes, of which 5 showed significantly higher sclerotinia resistance than 'Zhongshuang 9' ($P = 0.01$, Fig. 3b). An individual with good fertility (averages 16.3 and 17.4 seeds/pod after open- and self-pollination, respectively) and twofold higher resistance (averages 3.4 and 5.5 cm for lesion length caused by isolates *C* and *G*, respectively) than 'Zhongshuang 9' (9.7- and 10.5-cm lesion length due to isolates *C*

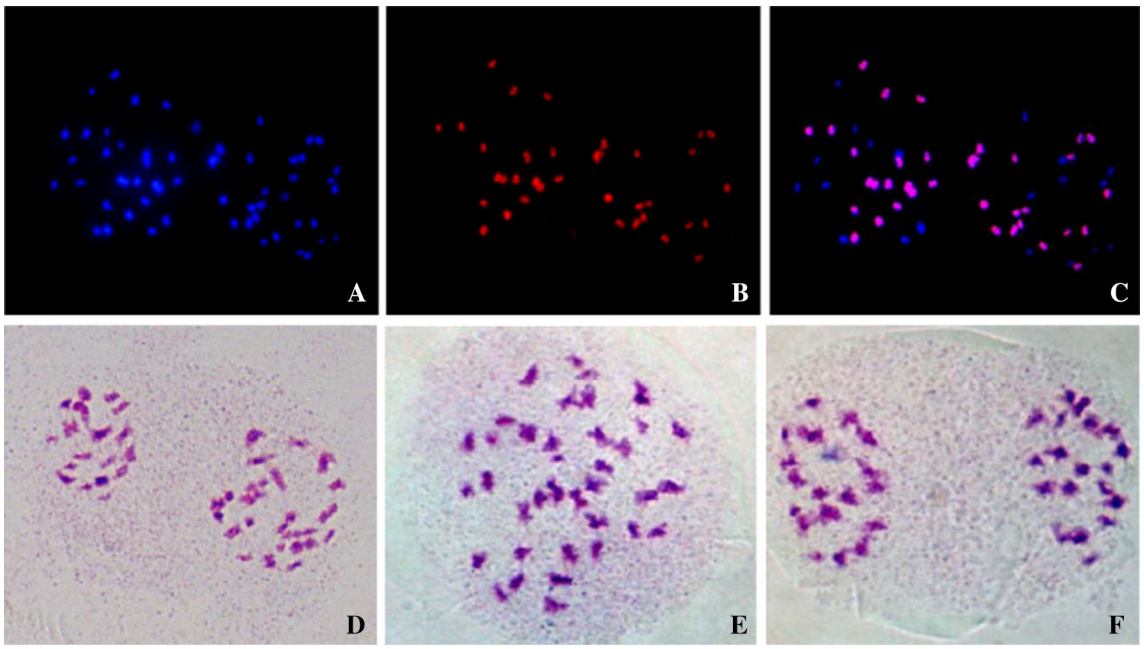

Fig. 2 Cytological analysis of a hexaploid and its offspring. **a–c** Genome composition in meiotic anaphase I of a pollen mother cell of a hexaploid plant shown by FISH. Chromosomes were counterstained with 4′-6-diamidino-2-phenylindole solution (*blue*), while the C-subgenome chromosomes were labeled *red* by C genome-specific probe; **d** chromosome distribution pattern of 19:28 in BC1F1; **e, f** PMCs of rapeseed-type plants in BC1F2 carrying 38 chromosomes; **f** 19:19 distribution pattern of rapeseed-type plants in BC1F2

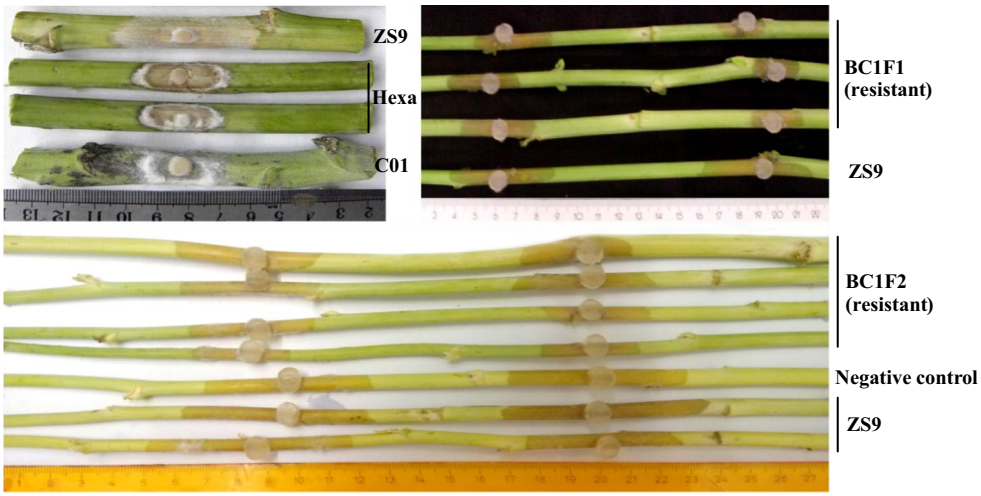

Fig. 3 Stem responses to *S. sclerotiorum* at 3 dai. The abbreviations 'ZS9' and 'Hexa' represent 'Zhongshuang 9' (a rapeseed cultivar with partial resistance) and hexaploid (F1), respectively; 'C01' represents the resistance donor *B. incana*; the 'negative control' means the BC1F2 individual without resistance QTL on chromosome C09

Table 1 Sclerotinia reaction (lesion length), pollen and seed fertility of marker-selected BC1F2 individuals derived from a cross between 'Zhongshuang 9' and 'C01' (mean values ± standard deviation)

Individual	No. of clones	Lesion length (cm)		Pollen	Seed set (seeds/pod)	
		Isolate *C*	Isolate *G*	Fertility	Open pollinated	Self-pollinated
BC1F2-1	4	1.6 ± 1.02**	2.3 ± 1.02**	88.4 ± 2.92	16.2 ± 3.27	17.3 ± 3.23
BC1F2-2	3	3.1 ± 1.50**	4.0 ± 0.90**	92.1 ± 1.80	10.4 ± 2.40	5.7 ± 1.00
BC1F2-3	3	3.1 ± 0.31**	4.1 ± 1.42**	85.7 ± 6.61	6.7 ± 4.40	15.0 ± 3.56
BC1F2-4	3	3.1 ± 0.74**	4.2 ± 1.15**	91.1 ± 1.30	8.3 ± 2.85	1.5 ± 0.71
BC1F2-5	3	3.2 ± 0.25**	3.3 ± 0.82**	60.7 ± 8.00	9.7 ± 2.42	0.4 ± 0.70
BC1F2-6	8	4.8 ± 1.09*	5.8 ± 0.94	80.8 ± 8.02	12.2 ± 3.06	3.0 ± 1.25
BC1F2-7	5	5.0 ± 0.37*	5.6 ± 0.77*	88.1 ± 4.94	10.3 ± 2.16	15.4 ± 2.87
BC1F2-8[a]	3	5.2 ± 0.12*	6.1 ± 0.40	85.9 ± 6.18	5.3 ± 2.45	5.4 ± 1.35
'Zhongshuang 9'	10	6.1 ± 1.05	6.8 ± 0.76	96.2 ± 1.76	15.3 ± 4.47	15.3 ± 1.57

*, ** Significant differences to 'Zhongshuang 9' at the 0.05 and 0.01 level, respectively

[a] BC1F2-8 represents the negative control without resistance QTL on C09

and *G*, respectively) was chosen to develop the BC1F2 generation by self-pollination. The cytological analysis in three randomly selected BC1F1 plants at anaphase I revealed a major chromosome distribution pattern of 19:28 (Fig. 2d), indicating the possibility of producing rapeseed-like progenies with 38 chromosomes in the BC1F2 generation.

Selecting resistant rapeseed-like plants in BC1F2

A total of 100 BC1F2 plants were screened with the markers linked to the resistance QTL. Of these, seven were recognized to possess the C09-resistance QTL from 'C01' and were chosen to develop clones for stem resistance evaluations together with a negative control not carrying resistance loci from 'C01'. Stem resistance test was conducted with four replications in BC1F2 and 'Zhongshuang 9'. A significantly high correlation was detected between the stem resistance results for the two sclerotinia isolates (average lesion length caused by isolates G and C were 5.0 and 4.4 cm, respectively, $r = 0.881$, $P < 0.01$), in accordance with the observation in BC1F1. ANOVA revealed insignificant genotype × isolate interaction ($P = 0.6346$) and significant differences for *Sclerotinia* resistance between genotypes ($P < 0.001$). Five of the seven BC1F2 carrying resistance QTL exhibited significantly higher sclerotinia resistance than the negative control and the OSR parent 'Zhongshuang 9' ($P = 0.01$) (Fig. 3c; Table 1). Of these, one rapeseed-type individual with 38 chromosomes and good self-fertility (15.0 ± 3.56 seeds/pod) was identified. This progeny will be further used in future oilseed rape breeding.

Discussion

The genetic basis of current rapeseed germplasm is narrow in comparison to its wild relatives and the parental species,

B. oleracea and *B. rapa*, owing to its relatively short history of domestication. Today, a major route of pre-breeding to broaden genetic diversity in rapeseed is using the 'resynthesis' approach to combine different types of A and C genomes from wild or alien *B. rapa* and *B. oleracea*. However, the aim of transferring a trait may be challenging if the ideal phenotype is determined by one of the parental species only, possibly resulting in interference caused by the other parental species in resynthesized (RS) rapeseed. Accordingly, in a previous study we found that the resistance against *S. sclerotiorum* in RS rapeseed was lower than that of parental *B. oleracea*, possibly owing to the suppression of resistance by the susceptible *B. rapa* parent (Ding et al. 2013). A similar observation was reported for the rapeseed–clubroot interaction in the study of Diederichsen and Sacristan (1996), where the resistance from *B. oleracea* was strongly reduced in RS rapeseed due to the presence of the A genome. Although it is theoretically possible to transfer genomic components between A and C subgenomes in rapeseed by homeologous chromosome pairing, it requires extensive and long-term selection due to the low frequency of homeologous recombination in rapeseed (Cui et al. 2012).

In the present study, a hexaploidy strategy was applied to transfer sclerotinia resistance from *B. oleracea* into rapeseed without implementing genomic components from the A genome of *B. rapa*. The first advantage of this approach is that it is easy to accomplish hybridizations between hexaploids and natural rapeseed. Second, the selection of rapeseed-like individuals in the backcross progenies is straightforward. In this study, a high frequency of disomic meiotic behavior was found in the hexaploid (data not shown), yielding predominantly gametes with the ACC genome constitution. This was in accordance with the previous study by Li et al. (2013) in which 70 % of the pollen mother cells (PMCs) from one hexaploid individual showed a chromosome distribution pattern of (10A + 18C) : (10A + 18C) at meiotic anaphase I. In the subsequent BC1F1 generation, a chromosome distribution pattern of 19:28 was found in meiotic anaphase I, indicating the formation of euploid gametes with AC genome constitution which is a prerequisite for the appearance of rapeseed-like individuals in BC1F2. A similar observation of producing euploid gametes was reported by Li et al. (2004) on a pentaploid hybrid (AACCB) derived from crossing natural rapeseed and hexaploids (AABBCC) originating from crosses between *B. carinata* (BBCC) and *B. rapa* (AA).

Finally, we have succeeded in generating a highly resistant rapeseed-like individual from BC1F2, suggesting that it is an effective approach to transfer elite traits from *B. oleracea* into rapeseed bridged by a hexaploidy step. In fact, all of the BC1F2 genotypes carrying resistance loci on chromosome C09 as shown by molecular markers are more resistant than the negative control, and the majority of BC1F2 individuals exhibited a significantly better resistance to *S. sclerotiorum* than the rapeseed parent 'Zhongshuang 9'. These findings demonstrate the feasibility of using the identified markers for selection of resistant individuals and variety candidates. However, it should be kept in mind that the QTL on C09 only explains around 30 % of the phenotypic variance in an F2 population of *B. oleracea* itself (Mei et al. 2013), and that the resistance was only evaluated in laboratory. Therefore, it would need to be combined with other resistance QTL and field resistance evaluation to achieve a very high level of sclerotinia resistance in oilseed rape. Finally, the combination of molecular marker-assisted selection and resistance evaluation is still required and important for the successful establishment of resistant and stable elite individuals from the pre-breeding progenies for subsequent variety development.

Author contribution statement J. M. conducted the whole experiment and wrote the manuscript; Y. L., D. W., B. W, Y. D. and H. W. participated in the field experiment and resistance evaluation; Y. L., Q. L., Z. L. and X. G. performed the cytological analysis; W. Q. designed the experiment; J. L., M. F., R. S., W. Q. and W. F. directed the project and contributed to the writing.

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Conflict of interest The authors declare that they have no conflict of interest.

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