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Pyramiding of multiple genes generates rapeseed introgression lines with clubroot and herbicide resistance, high oleic acid content, and early maturity

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ABSTRACT

Clubroot and herbicide resistance, high oleic acid (OA) content, and early maturity are targets of rapeseed (*Brassica napus* L.) breeding. The objective of this study was to develop new male-fertility restorer lines by pyramiding favorable genes to improve these traits simultaneously. Seven elite alleles for the four traits were introduced into the restorer line 621R by speed breeding with marker-assisted and phenotypic selection. Six introgression lines (ILs) were developed with four- to seven-gene combinations and crossed with two elite parents to develop hybrids. All ILs and their corresponding hybrids displayed high resistance to both clubroot pathotype 4 and sulfonylurea herbicides. Three ILs and their hybrids showed large increases in OA contents and four showed earlier maturity. These new ILs may be useful in rapeseed hybrid breeding for the target traits.

1. Introduction

Rapeseed (*Brassica napus* L.) is a source of edible vegetable oils. Despite efforts to breed high-yielding cultivars, few commercial cultivars combine high yield with multiple disease and herbicide resistances, high quality, and desired maturity. In the face of climate change and modern cultivation demands, cultivars possessing multiple desirable traits are a target of rapeseed breeding.

Clubroot, caused by Plasmodiophora brassicae, may cause more than 20% rapeseed yield loss in heavily infested fields in China [1]. Because clubroot is difficult to control with agricultural practices, development of resistant cultivars is considered the optimal approach for controlling the disease [2]. Among natural *B. napus* accessions, few are resistant to clubroot, whereas strong resistance has been identified in the diploid progenitor species *B. rapa*, especially in European turnips [3]. More than 27 loci have been identified as associated with clubroot resistance in *B. rapa*: *Crr2* and *PbBa1.1* on chromosome A01, *CRc* and *Rcr8* on chromosome A02, *PbBa3.1*, *PbBa3.2*, *PbBa3.3*, *CRd*, *Crr3*, *CRk*, *CRb*, *Rcr1*, *Rcr4*, *Rcr5*, *CRa*, *BraA.CR.a*, and *BraA.CR.c* on chromosome A03, *CrrA5* on chromosome A05, *Crr4* on chromosome A06, and *Crr1*, *PbBa8.1*, *Rcr3*, *Rcr9*^{wa}, *CRs*, *BraA.CR.b*, *Rcr9* and *CRA8.1* on chromosome A08 [3,4]. Of these only *Crr1a* and *CRa* have been successfully cloned, and both encode nucleotide-binding site-leucine-rich repeat proteins [4]. Recently [5], *CRA3.7.1* and *CRA8.2.4* were isolated and functionally validated: *CRA3.7.1* was identified as identical to *CRa* and *CRb*, whereas *CRA8.2.4* was identified as a candidate gene for *PbBa8.1*. Both genes conferred high resistance to race 4 of *P. brassicae*, the most prevalent pathotype in China.

It is feasible to combine multiple resistance genes in high-yielding rapeseed cultivars. By interspecific cross-breeding and marker-assisted breeding, the first clubroot-resistant conventional *B. napus* cultivar Huashuang 5R and the first clubroot-resistant hybrid *B. napus* cultivar Huayouza 62R in China have been developed by transferring respectively *PbBa8.1* and *CRb* into elite rapeseed cultivars [6,7]. Lines combining *CRb* and *PbBa8.1* showed higher resistance to most isolates in the field than those carrying only single resistance genes [8].

Development of sulfonylurea herbicide (SU)-resistant cultivars is desirable for rapeseed production. Weeds reduce rapeseed yield and quality by competing for water, sunlight, and nutrients [9].

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With the rapid expansion of rapeseed mechanized production in China, herbicides have become essential tools for effective weed control [10]. Acetolactate synthase (ALS), also known as acetohydroxyacid synthase (AHAS), is an enzyme that catalyzes the biosynthesis of branched-chain amino acids in plants, as well as a target for a variety of commercial herbicides [11]. These herbicides inhibit the activity of ALS, terminating the synthesis of branched-chain amino acids and causing plant death [12]. Among these herbicides, SUs have been extensively used owing to their broad-spectrum weed control, low residual rate, low mammalian toxicity, and wide crop selectivity [13].

The *B. napus* genome contains three functional *ALS* genes, and several herbicide-resistant *ALS* rapeseed mutants have been developed by mutation of *ALS* [14]: M45 with a P179L substitution (according to *Arabidopsis* protein sequencing) in *BnALS3* [15], M342 with a W574L substitution in *BnALS3* [9], and K5 with a P197S mutation in *BnALS1* [16], all conferring resistance to SUs. DS3, carrying two mutations in *ALS* (P197L in *BnALS1* and W574L in *BnALS3*) showed increased resistance to SUs [13]. These mutants can be used to develop herbicide-resistant rapeseed cultivars.

A high content of oleic acid (OA) increases the anti-oxidative properties and shelf life of oil, making it more suitable for human consumption [17]. Most superior rapeseed cultivars have an OA content of 55%–65%, but higher-OA oils are in increasing demand [18]. The fatty acid desaturase 2 gene (*FAD2*) influences rapeseed oil edible and processing quality [19]. Most high-OA mutants have been developed by modification of this gene. In *B. napus*, four copies of *FAD2* are located on chromosomes A01, A05, C01, and C05 [20]. *FAD2* on A01 is nonfunctional, but that on A05 shows the highest transcription and is believed to be the main target for modifying the OA level in rapeseed seeds [21]. Both a four-base-pair insertion [20] and a single-nucleotide mutation [18] in the coding sequence of *BnFAD2.A5* increased OA content. Recently [19], mutation of *BnFAD2.A5* achieved by the CRISPR/Cas9 system resulted in increased OA content. These high-OA accessions can be used to develop high-OA cultivars.

Early flowering, the major factor in early maturation, is necessary for the expansion of crop production to higher-latitude regions with shorter growing seasons, and for multiple-cropping systems in southern China [22]. Rapeseed cultivars with desired maturity times may be developed by manipulation of genes involved in early flowering. Many regulators controlling flowering time have been identified, including *FLOWERING LOCUS T* (*FT*) [23], *FLOWERING LOCUS C* (*FLC*) [24] and *FRIGIDA* (*FRI*) [25]. *FLC* controlling vernalization has been identified as one of the most important [26]. In *B. napus*, nine *FLC* copies have been identified. Of these, *BnFLC.A2* encodes a MADS-box repressor and influences the variation in flowering time of semi-winter and spring type rapeseeds [27,28]. In a recent study [24], insertion of a 2.833-kb fragment in the first intron of *BnFLC.A2* caused a loss-of-function mutation (known as *Bnflc.a2*) that promoted early flowering in diverse genetic backgrounds. Thus, development of rapeseed cultivars with early maturation by manipulating *BnFLC.A2* and *Bnflc.a2* alleles could confer adaptation to multiple cropping systems and growing regions in China.

The aim of the present study was to pyramid alleles associated with resistance to clubroot and herbicides, high OA content, and early flowering (maturity) using genotypic and phenotypic selection aided by speed breeding.

2. Materials and methods

2.1. Plant materials and breeding scheme

621R, which displays a semi-dwarf phenotype and medium tolerance to Sclerotinia stem rot, has been used as a restorers of several commercial hybrids released in China. It restores the male fertility of both the recessive genic male sterile (RGMS) line RG430A and the polima cytoplasmic male sterility (CMS) line 616A. However, 621R carries late-flowering alleles and the low-OA content allele *BnFAD2.A5*, and is susceptible to both clubroot and SUs. The inbred line ZHE226 contains the dominant clubroot-resistant gene *PbBa8.1* and has a genetic background similar to that of the conventional elite rapeseed cultivar Huashuang 5 [6]. HYZ62R is a hybrid cultivar carrying the other dominant clubroot resistance gene *CRb* [7]. Y655 carries the SU-resistance alleles *BnALS1R* (S653N) and *BnALS3R* (W574L). J-3111 carries the *Bnfad2.a5* allele (P159L in *BnFAD2.A5*) conferring high OA content. R11 was reported [24] to carry the early-flowering alleles *Bnflc.a2* and *Bnflc.c2*. All plant materials were provided by Huazhong Agricultural University.

To improve 621R (the recurrent parent, RP), a backcrossing strategy in combination with marker-assisted selection was adopted, with ZHE226, HYZ62R, R11, Y655, and J-3111 used as donor parents (DP). As shown in Fig. 1, crossing between DP and RP was performed, and then backcrossing was performed until the BC_4F_1 generation. Next, two rounds of crossing and four successive rounds of self-pollination were performed to obtain introgression lines (ILs). In every generation, the genotypes of all the selected plants were confirmed with markers tightly linked to the target genes, and then phenotypic selection was applied by selection of plants with traits similar to those of RP. The six ILs were crossed with 616A and RG430A to generate corresponding hybrids. Development of the ILs was performed in the greenhouse using the comprehensive speed breeding (CSB) system [29].

2.2. DNA marker analysis

Fresh young leaves were used for DNA extraction by the CTAB method [30]. Two gene-linked markers, A08-300 [31] and A03-50, were used for genotyping *PbBa8.1* and *CRb*, respectively. The functional marker BnALS1 designed based on the mutation (from G to A) at the 1913th position on chromosome C01 was used to detect *BnALS1R*. BnALS3, designed based on the mutation (from G to T) at the 1667th on chromosome A01 was used for detection of *BnALS3R*. PALS1 and PALS3 were used to amplify *BnALS1R* and *BnALS3R*, respectively, and the amplicons were then sent for sequencing. YQ2D26 and YQ2D27 markers were developed based on the base substitution (from C to T) at the 582th on chromosome A05 and used to confirm the *Bnfad2.a5* and *BnFAD2.A5* alleles [32]. STA2-55L/46R and STA2-55L/1R were specifically amplified alleles of *Bnflc.a2* and *BnFLC.A2*, respectively [24]. The details of the markers are presented in Table S1.

2.3. Evaluation of clubroot resistance

Clubroot galls were collected from Zhijiang, Hubei, China (30°43′N, 111°77′E), classified as pathotype 4 by the Williams classification system [33]. An inoculation test was performed in the greenhouse following Zhan et al. [34]. Rapeseed roots with severe galling were mixed with dry peat soil in a ratio of 1:20, and the mixture was stored at 25 °C for 48 h. Seeds from 621R, ILs and their hybrids were sown in the culture soil and watered regularly.

A set of 48 plants from each line and hybrid combination was tested for evaluation of clubroot resistance, with 621R and its hybrids used as susceptible controls. Four weeks after inoculation with the clubroot pathogen, disease symptoms were assessed. The roots of the plants were thoroughly washed, and clubroot severity was graded on a 0–3 scale (Fig. S1): 0, no galling; 1, fibrous root swelling, no main root swelling; 2, main root swelling with diameter $\leq$ twice that of the stem base; and 3, main root swelling

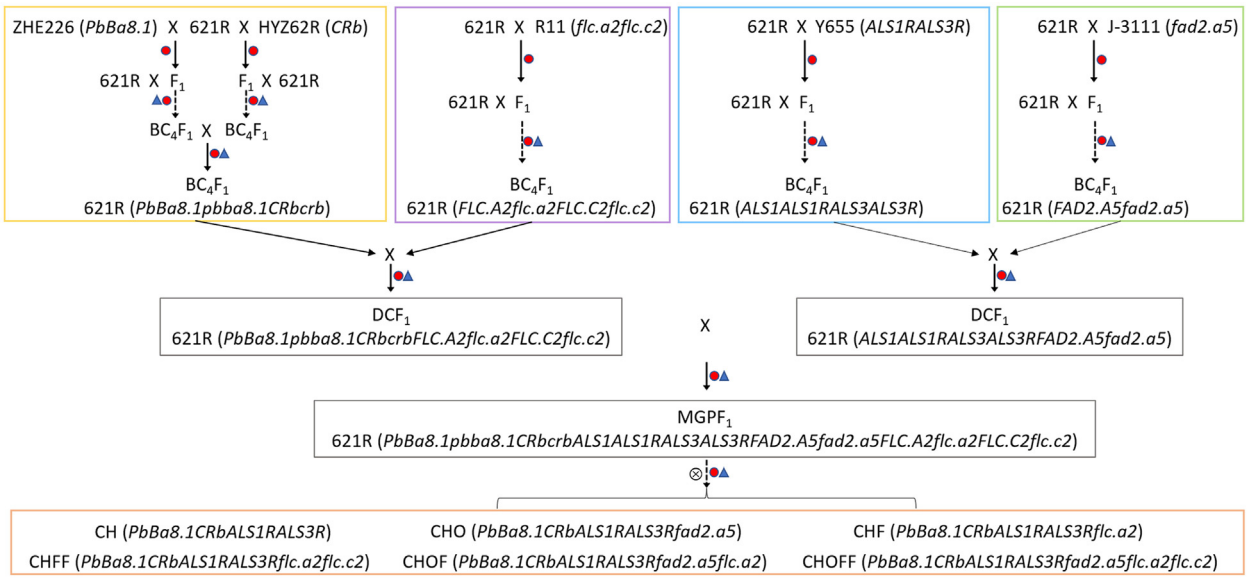

Fig. 1. Breeding scheme for introducing seven target genes into the elite restorer 621R. *BnFLC.A2*, *Bnflc.a2*, *BnFLC.C2*, *Bnflc.c2*, *BnALS1*, *BnALS1R*, *BnALS3*, *BnALS3R*, *BnFAD2.A5* and *Bnfad2.a5* are abbreviated as *FLC.A2*, *flc.a2*, *FLC.C2*, *flc.c2*, *ALS1*, *ALS1R*, *ALS3*, *ALS3R*, *FAD2.A5* and *fad2.a5*, respectively. Red circles represent marker-assisted selection, and blue triangles represent phenotypic selection.

with diameter > twice that of the stem base [35]. The disease severity index (DSI, %) was calculated as follows:

$$\mathrm{DSI} = \frac{\sum_{i=0}^{3} iN_i}{3\sum_{i=0}^{3} N_i} \times 100$$

where i is the clubroot severity grade and N_i is the number of plants in the ith grade. Resistance and susceptibility were defined as DSI $\leq$ 30% and > 30%, respectively [3].

2.4. Evaluation of herbicide resistance

Thifensulfuron, a sulfonylurea herbicide, is widely used to control broadleaf weeds in crop fields [36]. Seeds from 621R, ILs and their hybrids were sown into pots filled with nutritional soil under greenhouse conditions. The pots were placed in plastic trays and watered every other day. At the 4–6 leaf stage, 15 plants of each line were sprayed with 45 g a.i. ha^{-1} thifensulfuron (Fengle Agrochemical Ltd., Anhui, China). Each treatment had three biological replicates. Twenty-one days after herbicide treatment, survival index (SI, %) was evaluated on a five-grade scale of resistance: 1, resistant, no visible leaf damage; 2, tolerant, $\leq$ 25% leaf damage and slight heart leaf curling; 3, moderately tolerant, 25%–75% leaf damage and severe heart leaf curling; 4, sensitive, $\geq$ 75% leaf damage or dead apex; and 5, highly sensitive, death of the plant (Fig. S2) [37].

$$\mathrm{SI} = \frac{\sum_{i=1}^{5} \frac{(5-i)N_i}{4}}{\sum_{i=1}^{5} N_i} \times 100$$

where i is the grade for plant resistance and N_i is the number of plants in the ith grade [38].

2.5. Evaluation of OA content

The OA contents of the ILs and their corresponding hybrids were measured from open-pollinated seeds collected from three environments: Minle, Gansu province (38°28′N, 100°50′E) and Wuhan (30°28′N, 114°21′E) and Jiangling (30°11′N, 112°25′E), Hubei province. Total fatty acid was determined following Long et al. [17] with some modifications. Briefly, 15 seeds from each line were crushed with a glass rod and placed in a 10-mL glass centrifuge tube, followed by addition of a 1-mL mixture of ether:petroleum ether (1:1) and 1 mL KOH:methanol. The mixture was shaken well and left to stand for 45 min at room temperature after addition of 8 mL H_2O. Finally, 500 μL of the supernatant was isolated for gas chromatography (GC) analysis. Fatty acids were identified by comparison of their retention time against those of fatty acid methyl ester standards (Sigma Chemicals Co., St. Louis, MO, USA) separated on the same GC machine (HP6890 Agilent).

2.6. Determination of rapeseed flowering time

621R, ILs and their hybrids were planted in both winter-rapeseed (Wuhan and Jiangling) and spring rapeseed (Minle) areas in 2021. Flowering time was recorded three times weekly. The number of days from sowing to the day when 50% of plants in the plot opened their first flowers was recorded as the flowering time.

2.7. Evaluation of agronomic traits

621R, ILs, and their hybrids were grown in five rows with 10 seeds in each single row. The distances between rows and between plants were 0.27 m and 0.18 m, respectively. Field experiments were performed in randomized complete blocks with three replications. Eight mature plants with similar growth status were selected from each plot to investigate the agronomic traits: plant height (PH), branch number (BN), length of main inflorescence (LMI), siliques of main inflorescence (SMI), silique length (SL), number of seeds per silique (NSS), thousand-seed weight (TSW) and yield per plant (YP).

2.8. Statistical analysis

Phenotypic data for comparing traits of the ILs with those of 621R were analyzed using Microsoft Excel (Office Excel 2013, Microsoft Corporation, Redmond, WA, USA) and Graphpad Prism (Graphpad Prism 5 Software, San Diego, CA, USA).

3. Results

3.1. Construction of six ILs

621R was separately crossed with five donors and the corresponding F_1 plants were successively backcrossed to the RP until the BC_4F_1 generation. In each backcross population, three plants with the target gene were selected by marker-assisted selection combined with phenotypic selection. After two pairwise crosses between two of the four BC_4F_1, 11 double-cross F_1 (DCF_1) plants carrying the *CRb* + *PbBa8.1* + *Bnflc.a2* + *Bnflc.c2* alleles and 15 DCF_1 plants carrying the *BnALS1R* + *BnALS3R* + *Bnfad2.a5* alleles were obtained (Fig. 1). Among the 2800 plants of the multiple-gene-pyramided F_1 ($MGPF_1$) population derived from the cross between the above two DCF_1 populations, 19 positive plants carrying all the target alleles (*CRb* + *PbBa8.1* + *BnALS1R* + *BnALS3R* + *Bnfad2.a5* + *Bnflc.a2* + *Bnflc.c2*) were selected with markers linked to the corresponding genes (Fig. 1).

Based on OA content and agronomic phenotypes, three elite $MGPF_1$ plants were selected and self-pollinated to produce $MGPF_2$ seeds. After genotyping of the 4230 $MGPF_2$ plants, some plants homozygous for all the target loci or heterozygous at only one target locus were selected and continuously self-pollinated and genotyped until they were homozygous for all the target loci. The 23 introgression plants comprised six homozygous for *CRb*, *PbBa8.1*, *BnALS1R*, *BnALS3R*, *Bnfad2.a5*, *Bnflc.a2* and *Bnflc.c2*, four homozygous for *CRb*, *PbBa8.1*, *BnALS1R*, *BnALS3R*, *Bnfad2.a5* and *Bnflc.a2*, four homozygous for *CRb*, *PbBa8.1*, *BnALS1R*, *BnALS3R*, *Bnflc.a2* and *Bnflc.c2*, three homozygous for *CRb*, *PbBa8.1*, *BnALS1R*, *BnALS3R* and *Bnflc.a2*, three homozygous for *CRb*, *PbBa8.1*, *BnALS1R*, *BnALS3R* and *Bnfad2.a5*, and three homozygous for *CRb*, *PbBa8.1*, *BnALS1R* and *BnALS3R*. The 23 selected plants were self-pollinated to generate 23 ILs.

Among the 23 ILs, six with the highest similarity to the RP in agronomic phenotypes were selected for further evaluation and named CH (*PbBa8.1* + *CRb* + *BnALS1R* + *BnALS3R*), CHO (*PbBa8.1* + *CRb* + *BnALS1R* + *BnALS3R* + *Bnfad2.a5*), CHF (*PbBa8.1* + *CRb* + *BnALS1R* + *BnALS3R* + *Bnflc.a2*), CHFF (*PbBa8.1* + *CRb* + *BnALS1R* + *BnALS3R* + *Bnflc.a2* + *Bnflc.c2*), CHOF (*PbBa8.1* + *CRb* + *BnALS1R* + *BnALS3R* + *Bnfad2.a5* + *Bnflc.a2*), and CHOFF (*PbBa8.1* + *CRb* + *BnALS1R* + *BnALS3R* + *Bnfad2.a5* + *Bnflc.a2* + *Bnflc.c2*) (Fig. 1; Table S2).

3.2. Clubroot resistance identification of the six ILs and their hybrids

Seedlings from the ILs and their corresponding hybrids were scored for disease infection after four weeks (Figs. 2, S3). All six ILs showed immunity to clubroot disease with a DSI of 0%, whereas the RP showed high susceptibility with a DSI of 89.6%. Similarly, the 616A/ILs and RG430A/ILs hybrids showed complete resistance to clubroot disease with DSIs of 0%, whereas their corresponding check hybrids 616A/621R and RG430A/621R showed high susceptibility with DSIs of 91.0% and 91.7%, respectively.

3.3. Herbicide-resistance assessment of ILs and their hybrids

To assess the resistance of *BnALS1R* + *BnALS3R* to thifensulfuron herbicide, the SIs of all ILs and their hybrids were evaluated (Figs. 3, S4). All 621R plants died (with SI 0%) after 21 days of the treatment, showing high susceptibility to thifensulfuron, whereas no ILs showed symptoms of herbicide damage (SI 100%), and displayed a high-resistance phenotype. A similar phenomenon was also observed for all the hybrids. In the check hybrids of 616A/621R and RG430A/621R, the leaves suffered severe damage with SI of 0% and 1.7%, respectively. Among the 12 improved hybrids, eight had normal leaves (SI 100%), showing high resistance to herbicide, and the remaining four had slightly curled leaves and showed a tolerant phenotype (SI 63.3%–98.3%).

3.4. OA content in six ILs and their hybrids

The seed OA contents of the three *Bnfad2.a5* ILs (CHO, CHOF, and CHOFF) were 72.8%–74.7% in the three environments, significantly higher than that of the recurrent parent 621R and the three *BnFAD2.A5* ILs (CH, CHF, and CHFF) with OA content ranging from 60.9% to 63.0%, with no significant difference from that of 621R (Fig. 4a–c).

Similarly, the hybrids with the heterozygous genotype *BnFAD2.A5Bnfad2.a5* (616A/CHO, 616A/CHOF, 616A/CHOFF, RG430A/CHO, RG430A/CHOF and RG430A/CHOFF) all showed significant increases in OA content (68.6%–74.3%), whereas the hybrids with the homozygous genotype *BnFAD2.A5BnFAD2.A5* (616A/621R, 616A/CH, 616A/CHF, 616A/CHFF, RG430A/621R, RG430A/CH, RG430A/CHF, and RG430A/CHFF) had normal OA contents (60.9%–68.1%) (Fig. 4d–i).

3.5. Evaluation of flowering time of six ILs and their hybrids

CH and CHO showed the latest flowering time in all three environments, with mean times in Minle, Wuhan, and Jiangling of respectively 72.2, 134.2, and 131.7 days, showing no difference from that of 621R (Fig. 5a–c). CHFF and CHOFF showed the earliest flowering times in the three environments, on average respectively 17.5, 85.0, and 83.3 days earlier than that of 621R (Fig. 5a–c). CHF and CHOF displayed an intermediate flowering phenotype in Minle, Wuhan, and Jiangling, with flowering times respectively 12.0, 65.1, and 65.9 days earlier than that of 621R (Fig. 5a–c).

Similar results were obtained for the corresponding hybrids. The 616A/CH, 616A/CHO, RG430A/CH and RG430A/CHO hybrids showed the latest flowering times, not different from those of their corresponding check hybrids 616A/621R and RG430A/621R (Fig. 5d–i). The 616A/CHFF and 616A/CHOFF hybrids flowered earliest among the hybrids of 616A and the six ILs, on average 8.6 days earlier in Minle, 71.2 days earlier in Wuhan, and 70.8 days earlier in Jiangling than the 616A/621R hybrid (Fig. 5d–f). The flowering times of the 616A/CHF and 616A/CHOF hybrids were intermediate, respectively 4.5, 40.7, and 40.8 days earlier than that of the 616A/621R hybrid in the three environments (Fig. 5d–f). The RG430A/CHFF and RG430A/CHOFF hybrids also showed the earliest flowering time among the hybrids of RG430A and the six ILs, on average respectively 10.2, 65.7, and 68.2 days earlier than that of the check hybrid RG430A/621R in Minle, Wuhan, and Jiangling (Fig. 5g–i). The flowering times of the RG430A/CHF and RG430A/CHOF hybrids were intermediate, respectively 6.5, 10.1, and 12.4 days earlier in the three environments than the check hybrid RG430A/621R (Fig. 5g–i).

3.6. Performance of the six ILs and their hybrids for agronomic traits

The agronomic trait evaluations in the three environments are shown in Table S3. Early-flowering ILs displayed significantly reduced PH and BN in the three environments, but showed no differences in LMI, SMI, and SL from 621R. The TSWs of all six ILs were significantly higher than that of 621R. NSS was significantly increased in all six ILs in Minle and Wuhan, whereas only CH showed a significant increase in NSS in Jiangling. In comparison with 621R, the late-flowering ILs had higher YP at all three sites, whereas the early-flowering ILs showed significantly lower YP in Wuhan and Jiangling.

The hybrids showed similar results to the ILs in some agronomic traits (Tables S4, S5). Early-flowering hybrids had

significantly lower PH and BN than their corresponding check hybrids, but showed no difference in LMI, SMI, or SL. The improved hybrids generally showed higher TSW and NSS than the check hybrids, though several combinations showed no differences. Late-flowering hybrids showed no difference in YP from the check hybrids in Minle and Wuhan, but displayed significantly higher YP in Jiangling. As expected, early-flowering hybrids generally had lower YP than the check hybrids, though the reduction in several early-flowering hybrids was not significant.

Thus, the ILs and their corresponding hybrids showed clubroot and herbicide resistance, increase in OA content, and diverse flowering times. Although the earlier-flowering ILs lines and their corresponding hybrids showed lower PH and BN than 621R and their corresponding check hybrids, no difference was observed for other agronomic traits, except for the increase in TWS due to phenotypic selection. For YP, early-flowering ILs and corresponding hybrids displayed loss, whereas late-flowering ILs and their hybrids showed no difference relative to 621R and the corresponding check hybrids.

4. Discussion

PbBa8.1 with strong resistance against pathotype 4 was transferred from ZHE226 to 621R, resulting in an increase in erucic acid level in 621R due to the close link of *PbBa8.1* with *BnFAE1.A8*. A recombination rate of approximately 1.9% was observed between *PbBa8.1* and *BnFAE1.A8* [6]. Marker J64-3 was used to break the linkage drag between *PbBa8.1* and *BnFAE1.A8*, generating ILs with low seed erucic acid levels and high clubroot resistance (Fig. S5). Recently [5] it was reported that *CRA8.2.4* is a possible candidate gene for *PbBa8.1*, as a functional analysis of *CRA8.2.4* revealed that the DSI of a *CRA8.2.4* transformant was 34.9%, whereas that of the empty vector control transformant was 80.4%. In our previous study [39], Y522R carrying *PbBa8.1* resistance loci showed a DSI of 21.0%, whereas the control Y522R showed a DSI of nearly 90.0%. Thus, although clubroot severity was reduced in the improved lines with single *PbBa8.1*, some plants were still at a risk of infection. It is desirable to breed durable cultivars with a broad spectrum of resistance by pyramiding multiple clubroot resistance genes. In this study, *PbBa8.1* and *CRb* were combined in 621R, resulting in complete resistance to pathotype 4 (Fig. 2a). These ILs may be used to develop hybrids to control clubroot disease and reduce the loss of rapeseed production in clubroot-infested regions.

Recently, many mutant rapeseeds have been developed with single-point mutations of *ALS*, which conferred resistance to herbicide. However, their hybrids with heterozygous herbicide-resistant mutation sites showed low herbicide resistance, limiting their application in rapeseed breeding [40]. Synergistic effects of point mutations on both *ALS1* and *ALS3* have been observed in soybean [41] and rapeseed [13], demonstrating that stacking two herbicide-resistant genes will result in markedly higher herbicide resistance than the summed resistance from introducing single-point mutations in *ALS1* and *ALS3*. In the present study, two herbicide-resistant genes *BnALS1R* and *BnALS3R* were introduced into 621R, and all ILs showed high resistance to thifensulfuron (Figs. 3a, S4a). Different SIs were observed between the hybrids of 616A/CHO (98.3%) and RG430A/CHO (100%), and the hybrids of 616A/CHOF (100%) and RG430A/CHOF (96.7%), suggesting that the hybrids of 616A/CHO and RG430A/CHOF suffered more damage from thifensulfuron than other plants in the experiment (Fig. 3). The finding that hybrids of 616A/CHF and RG430A/CHF showed lower SI than other hybrids, despite the IL CHF showing a 100% SI to thifensulfuron, leads us to speculate that the IL CHF actually carries lower resistance to thifensulfuron than other ILs. Together, these results suggest that plants with heterozygous *BnALS1R* and *BnALS3R* may have lower thifensulfuron resistance than those with homozygous *BnALS1R* and *BnALS3R*. It is thus desirable to improve both male-sterile and restorer lines at the same time to obtain highly herbicide-resistant hybrids for rapeseed production.

High OA is preferred by the food industry because it increases shelf life and health benefits for consumers. Recently, efforts have been made to increase OA content in soybean [42], rapeseed [43], peanut [44], and cotton [45]. In rapeseed, most high-OA accessions were found to have mutations of *BnFAD2* on A05, which contributes to an increase in OA content from 64.1% to 75.0% [18,20]. In the present study, we obtained three high-OA ILs with mean OA content 74.1% by introducing the *Bnfad2.a5* allele into 621R (Fig. 4a–c). The finding that the hybrids of both 616A and RG430A with high-OA ILs showed lower OA contents than the corresponding high-OA ILs (Fig. 4d–i) indicates that both male-sterile lines and restorer lines with homozygous *Bnfad2.a5* allele are required for producing hybrids with higher OA contents. The finding that the RG430A/IL hybrids showed higher OA contents than the 616A/IL hybrids suggests that RG430A may carry other high-OA loci, given that in a recent study [46], stacking *Bnfad2.a5*, *Bnfad2.c5*, and *OLEA9* in rapeseed cultivars increased OA content by up to 88.1%.

Flowering time affects the yield and regional adaptation of rapeseed. Semi-winter rapeseed is widely grown in China, particularly in the Yangtze River basin, where farming systems include "rice–ratoon rice–rapeseed", "double-season rice–rapeseed", and "maize–rapeseed" rotations [47]. In these rotation systems, shorter growth period and earlier maturity are essential for crop rotation. In the northwest China spring rapeseed region, early flowering and early maturity are preferred for avoiding damage caused by early

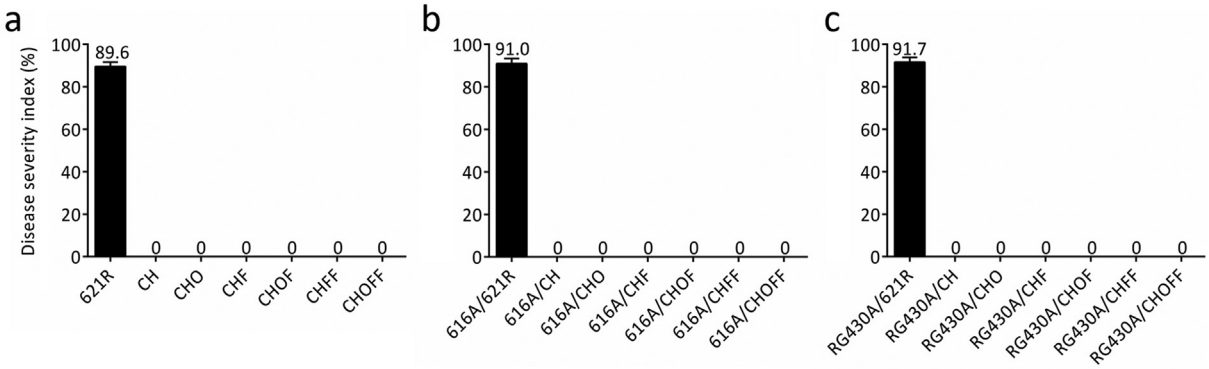

Fig. 2. Responses of 621R, six ILs, and their hybrids to clubroot disease. (a) DSIs of 621R and six ILs. (b) DSIs of the hybrids 616A/621R and 616A/ILs. (c) DSIs of the hybrids RG430A/621R and RG430A/ILs.

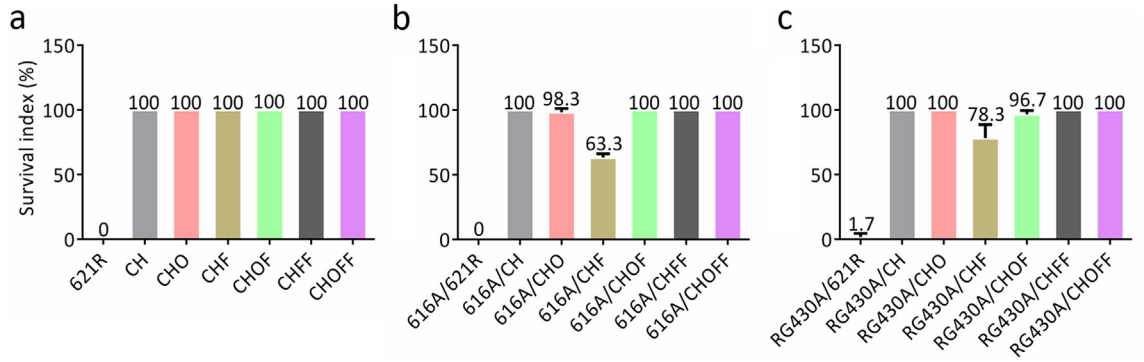

Fig. 3. Responses of 621R, six ILs, and their hybrids to thifensulfuron. (a) Survival index of 621R and six ILs at 21 days after treatment with thifensulfuron. (b) Survival index of the hybrids 616A/621R and 616A/ILs at 21 days after treatment with thifensulfuron. (c) Survival index of the hybrids RG430A/621R and RG430A/ILs at 21 days after treatment with thifensulfuron.

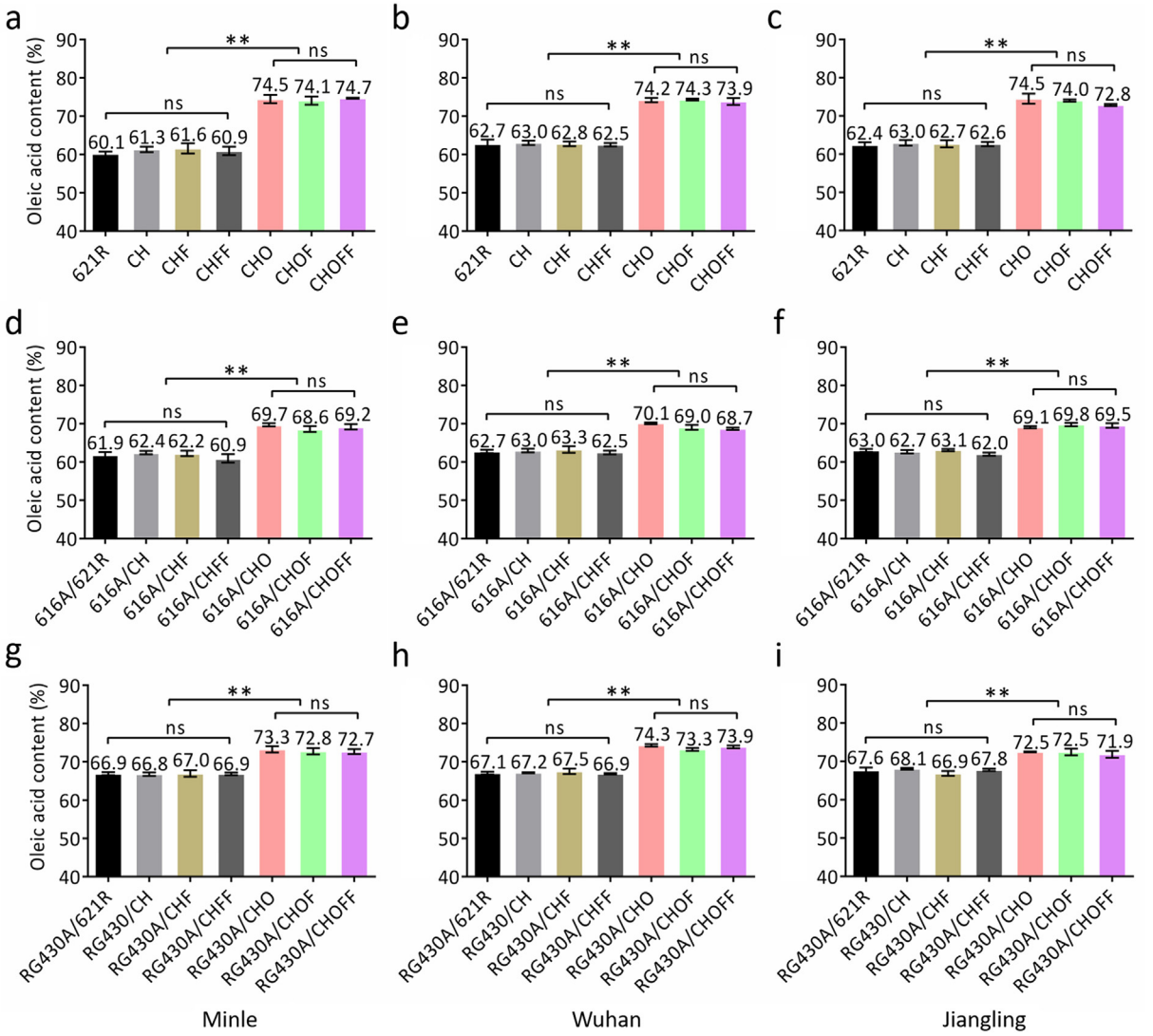

Fig. 4. Seed oleic acid (OA) contents of 621R, six ILs, and their hybrids under three conditions. (a–c) Seed OA contents of 621R and six ILs in Minle, Wuhan and Jiangling, respectively. (d–f) Seed OA contents of 616A/621R and 616A/ILs in Minle, Wuhan and Jiangling, respectively. (g–i) Seed OA contents of RG430A/621R and RG430A/ILs in Minle, Wuhan and Jiangling, respectively. Values are means ± SD. Error bars represent SDs. A two-tailed Student's *t*-test was used to generate *P*-values. ** indicates a significant difference at $P < 0.01$; ns indicates not significant at $P < 0.05$.

frost, high temperature, and drought [48]. *BnFLC.A2* and *BnFLC.C2* act as suppressors of flowering in the vernalization pathway by inhibiting expression of the central flowering regulators *BnFT* and *BnSOC1* that have been characterized in *B. napus*, and loss of their function results in early flowering [24]. In the present study, we obtained two early-maturity ILs carrying both mutant *Bnflc.a2* and *Bnflc.c2* alleles with a mean flowering time of 50.9 days, and mid-maturity ILs carrying only the mutant *Bnflc.a2* with a mean flowering time of 65.2 days, both showing earlier flowering than 621R (112.9 days) (Fig. 5a–c). Thus, manipulation of the *BnFLC* genes can help to develop hybrids suitable for diverse farming conditions by controlling flowering time [47].

We performed multiple comparisons of the phenotypic data of all six ILs in three environments to investigate the effect of target genes on agronomic traits (Fig. S6). The IL CH with clubroot and herbicide resistance showed no significant difference from 621R in PH, LMI, SMI, BN, SL, and YP, but significant increases in NSS and TSW. These results are consistent with previous findings [9,49] suggesting that improvement of clubroot or herbicide resistance has little influence on main agronomic traits. A previous study showed that high-OA germplasm has poor agronomic performance [50], whereas in our study, CHO showed no significant difference in main agronomic traits except for PH relative to CH. In a previous study [47], rapeseed with a short growth period tended to have lower PH, BN, and yield. A similar phenotype of early-flowering ILs was observed in the present study.

Molecular marker-assisted selection (MAS) has been widely used to develop pyramided lines [51–53]. However, it is still time-consuming to pyramid multiple desirable genes into elite varieties using only MAS. To cope with this problem, the speed breeding methodology [54] was introduced in some crops and accelerates generation turnover by shortening the growth cycle. In our previous study [29], a CSB system was proposed as a fast and efficient crop improvement method. It includes vernalization of germinated seeds, high-density seedling culture, and accelerated flowering and maturation with an optimized light regime. In the present study, a comprehensive breeding strategy to pyramid multiple genes was presented, which includes CSB, MAS, and

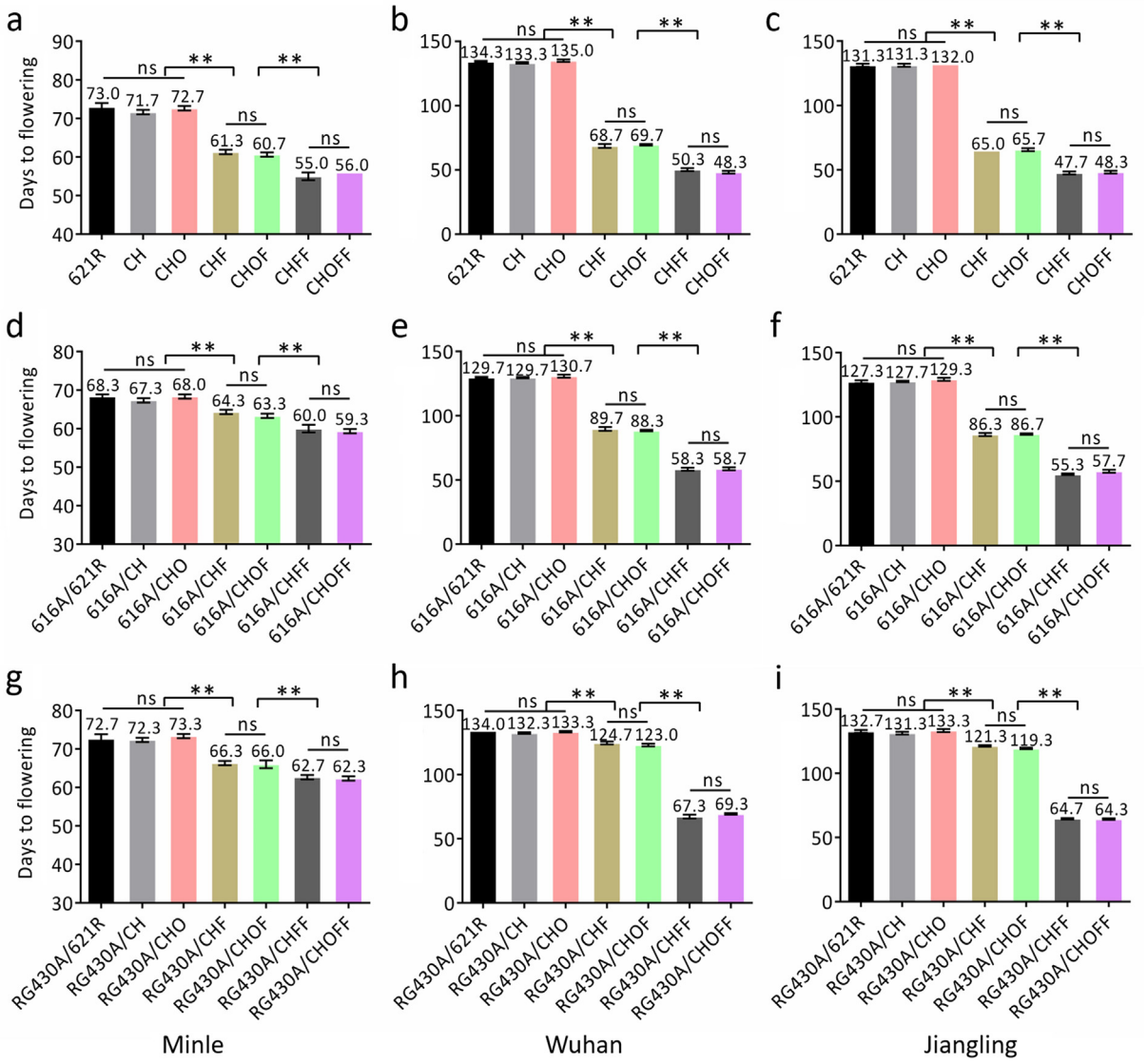

Fig. 5. Evaluation of flowering time of the six ILs and their corresponding hybrids in three environments. (a–c) Flowering times of 621R and ILs in Minle, Wuhan and Jiangling, respectively. (d–f) Flowering times of the hybrids of 616A/621R and 616A/ILs in Minle, Wuhan and Jiangling. (g–i) Flowering times of the hybrids of RG430A/621R and RG430A/ILs in Minle, Wuhan and Jiangling, respectively. Values are mean ± SD. Error bars represent SDs. A two-tailed Student's *t*-test was used to generate the *P*-values. ** indicates a significant difference at $P < 0.01$; ns indicates not significant at $P < 0.05$.

phenotypic selection. With this method we developed six ILs with genes conferring clubroot resistance, herbicide resistance, high OA content, and early maturity within a short time.

CRediT authorship contribution statement

Guangsheng Yang: Experimental design, Experimental guidelines and supervision, Writing - review & editing. **Zhaoyang Wang**: Investigation, Collated the data, Data curation, Analysis of the data, Writing - original draft. **Fucai Wang, Zihan Yu and Xiaorui Shi**: Performed the experimental work. **Xianming Zhou**: Writing - review & editing, Provided helpful suggestions. **Pengfei Wang**: Data analysis. **Dengfeng Hong and Yixian Song**: Provided helpful suggestions. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2022.10.009.

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