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Rapid Development of Clubroot-Resistant Germplasm in Chinese Cabbage

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Abstract

Disease resistance breeding is an important direction for the genetic improvement of Chinese cabbage. The traditional elite variety 'Yutian Baojian' Chinese cabbage is highly regarded for its tall cylindrical head with a pointed tip, tightly twisted wrapper leaves, and sweet taste. However, long-term cultivation has led to a significant decline in its resistance to clubroot caused by *Plasmodiophora brassicae*. To restore clubroot resistance while maintaining its desirable horticultural traits, this study used the clubroot-susceptible 'Yutian Baojian' as the recurrent parent and the resistant donor 'Shaocai', which carries the *CRd* resistance gene, to develop backcross populations. Using marker-assisted selection (MAS), plants were comprehensively screened based on foreground selection with markers tightly linked to the *CRd* gene, background selection with 73 genome-wide polymorphic markers, and phenotypic evaluation of horticultural traits, including plant height, plant spread, head shape, and soluble solids content. In the BC_1 population, three individuals showing high genetic similarity were selected. From the BC_2 population, four elite individuals were obtained, exhibiting 99.32% genetic similarity, stable clubroot resistance, and typical horticultural characteristics. Furthermore, three homozygous resistant inbred lines (BC_2S_2) with the 'Yutian Baojian' phenotype were developed. These results enrich the clubroot-resistant germplasm resources of Chinese cabbage and provide an effective MAS-based strategy for the precise improvement and germplasm innovation of local cultivars.

Keywords: Chinese cabbage; clubroot disease; marker-assisted selection; *CRd* gene; horticultural traits

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1. Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is a major vegetable crop in Asia. It has a long history of cultivation and considerable varietal diversity [1]. Among these varieties, 'Yutian Baojian', a traditional variety originating from Hebei Province, holds particular importance. It was granted the status of a National Geographical Indication Product of China in 2010, recognizing its unique regional identity and superior horticultural traits. This variety is prized for its distinctive morphological features, including a straight, tall cylindrical head with a pointed apex that forms typical cannonball heads, tightly wrapped leaves, and a crisp, sweet taste that is well suited for storage. However, a major

constraint of its cultivation is its susceptibility to clubroot disease. Enhancing clubroot resistance in 'Yutian Baojian' Chinese cabbage is therefore essential for ensuring crop health and improving yield.

Clubroot disease, caused by the pathogen *Plasmodiophora brassicae*, is a globally widespread soil-borne disease that specifically parasitizes cruciferous crops [2]. The resting spores of *P. brassicae* can persist in soil for up to 20 years, and the disease is notorious for its wide distribution and high destructiveness. Effective clubroot control measures can reduce economic losses. Current control strategies encompass agronomic, chemical, biological, and disease-resistant breeding approaches. However, natural resistance resources are scarce in 'Yutian Baojian' Chinese cabbage, and traditional backcross breeding requires 6–8 generations to introduce resistance, making it time-consuming and inefficient. Resistance breeding is recognized as the most effective strategy for controlling this soil-borne disease [3–5]. Consequently, the identification, mapping, and cloning of resistance genes are critical for discovering new resistance resources and facilitating their rapid deployment in breeding programs.

Driven by advances in molecular biology techniques, significant progress has been made in the study of cabbage resistance genes against clubroot disease. To date, over 20 clubroot resistance genes have been identified and localized, with most resistance sources originating from European fodder turnip (*Brassica rapa* ssp. *rapifera*) [6]. More than 40 resistance gene loci have been reported to date, with *Crr1*, *CRd*, and CRa/CRb^{Kato} already cloned. Molecular marker-assisted selection (MAS) enables rapid selection of target traits by detecting molecular markers tightly linked to the target trait genes [7]. This approach efficiently screens for individuals carrying the target trait in early generations, significantly accelerating disease resistance breeding programs. This technology primarily comprises foreground selection and background selection. Foreground selection, first proposed by Tanksley et al. [8], enables marker detection at the seedling stage to select optimal individuals for backcrossing. Progeny selection utilizes markers linked to the target gene to ensure disease resistance genes are not lost during backcrossing and selfing, ultimately yielding homozygous genotypes. Background selection rapidly restores the genetic background of the recurrent parent through molecular markers distributed across the entire genome. Currently, molecular markers widely used in crop genetic breeding are primarily based on PCR detection of SSR (simple sequence repeat) and SNP (single nucleotide polymorphism) markers [9]. The objective of marker-assisted backcrossing (MABC) is to introduce a target gene from one line into another line lacking that gene, while preserving the genetic background of the recipient. This approach not only enables precise gene transfer but also maximizes the retention of desirable traits from the recipient parent. Compared to traditional backcrossing, which requires 6–8 generations to restore 99% of the parental background, MABC achieves this in just 3–4 generations, significantly enhancing breeding efficiency [10].

In this study, we selected the clubroot resistance gene *CRd*, located on chromosome A03 and conferring stable resistance to physiological race 4 of *P. brassicae*, as the target for introgression. The donor parent 'Shaocai', carrying *CRd*, was crossed with 'Yutian Baojian', which served as the recurrent parent. By establishing an MAS system, we implemented MABC to rapidly develop new breeding lines that combine robust clubroot resistance with the desirable horticultural traits of 'Yutian Baojian'. This work provides a practical strategy and technical framework for efficiently improving disease resistance and promoting germplasm innovation in traditional local varieties of Chinese cabbage.

2. Materials and Methods

2.1. Population Construction and Pathogen Isolates

To develop clubroot-resistant germplasm, the disease-resistant parent used in this study was the Chinese cabbage line 153 ('Shaocai'), which carries the *CRd* gene, is characterized by long-cylindrical heads, and demonstrates high resistance to clubroot disease. The recurrent parent was the Chinese cabbage line 188 ('Yutian Baojian'), which is susceptible to clubroot and forms typical cannonball heads.

The F_1 population was generated by crossing 153 with 188. Individual F_1 plants were then backcrossed to 188 to produce BC_1 and BC_2 populations (Figure 1). Foreground and background marker selection was applied to the BC_1 generation, while the BC_2 generation was subjected to more extensive horticultural trait evaluation. Selected BC_2 plants were self-pollinated to obtain the BC_2S_1 generation, and individuals from this population were selfed again to derive the BC_2S_2 lines. Finally, homozygous resistant lines with high genetic similarity to the recurrent parent were developed. All hybridizations and self-pollinations were performed during the flowering period. For cross-pollination, flower buds on the inflorescences of the female parent were artificially emasculated and bagged prior to anthesis to prevent uncontrolled pollination. After pollinating with fresh pollen collected from the male parent, the inflorescences were re-bagged and labeled accordingly. Self-pollination was achieved by bagging inflorescences of selected plants to exclude pollinators, allowing for natural selfing or supplemented by manual pollination.

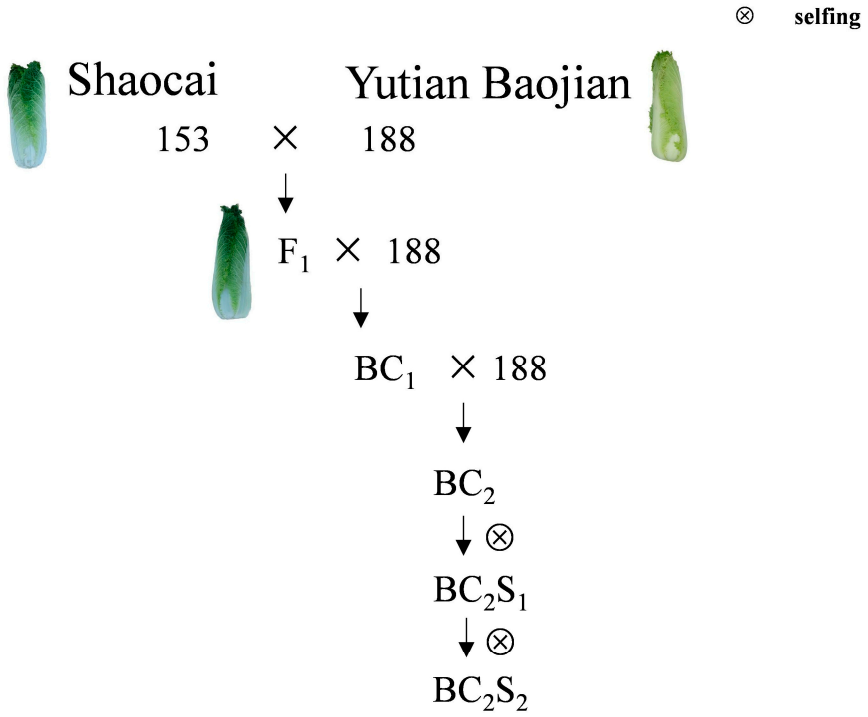

Figure 1. The schematic diagram of the complete workflow. 188: recurrent parent; 153: donor parent.

A field isolate of *Plasmodiophora brassicae* was obtained from Beijing, China. Based on the differential system established by Williams (1966) [11], this isolate was classified as pathotype 4, with a high incidence of severe clubroot disease. Infected roots were collected and stored at −20 °C until use. All plant materials and pathogen isolates were provided by the Institute of Vegetable and Flowers (IVF) of the Chinese Academy of Agricultural Sciences (CAAS).

Resting spores were adjusted to a concentration of 10^8 spores/mL. Seedlings were inoculated by using the root dip method, as described by Liu X et al. (2022) [12]. The plants were evaluated for clubroot disease severity four weeks after inoculation.

2.2. DNA Extraction and PCR Amplification Selection

Total genomic DNA was extracted from the fresh leaves of plants, according to the cetyl trimethyl ammonium bromide (CTAB) method [13], and the resulting DNA concentrations were adjusted to 50 ng μL^{-1} with TE buffer (pH 8.0) for subsequent analyses. The polymorphism of markers was validated using the PCR reaction. A 10 µL volume of PCR mixture contained 1.0 µL of template DNA (50 ng μL^{-1}), 5.0 µL of 2 × PCR Master Mix (Tiangen Biotech, Beijing, China), 0.2 µL of 10 µmol L^{-1} of each forward and reverse primer, and 3.6 µL of ddH_2O. A Thermal Cycler (BIOER, Hangzhou, China) was used to perform PCR amplification with the following procedures: first, a denaturation step at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing (temperature set was based on primers, usually 55 °C) for 30 s, 72 °C for 30 s, the final extension step at 72 °C for 5 min, and storage at 8 °C. The PCR products were resolved on a 6% polyacrylamide gel electrophoresis.

To ensure the reliability of genotyping, both biological and technical replicates were performed. For the parental lines (188 and 153) and all segregating populations, genomic DNA was extracted from each individual plant. For each PCR assay, at least three technical replicates were conducted using the same DNA template.

2.3. Foreground and Background Marker

The molecular marker yau-376 was chosen because it is tightly linked to the *CRd* gene on chromosome A03 and has been validated in multiple *Brassica rapa* populations, showing stable co-segregation with clubroot resistance [14]. The primer sequences for yau-376 are as follows: (F:5′-TGTCACCAGCGCATTATAGC-3′, R: 5′-AAGGGAGGGAAGATGGGTTG-3′).

For background selection, 364 InDel markers and 126 SSR markers distributed across the 10 chromosomes of Chinese cabbage were initially screened. These markers were obtained from Sui (2014) [15], Liu (2018) [16], Wang (2011) [17], and the BRAD database (http://brassicadb.org/brad/ accessed on 19 August 2023). A marker distribution map was constructed using Mapchart (version 2.3) [18].

The genetic similarity (G) was calculated according to the formula G(g) = [L + X(g)]/2L, where g is the specific backcross generation; X(g) is the number of molecular markers in that generation that show the same banding pattern as the recurrent parent; and L is the total number of polymorphic molecular markers.

2.4. Comprehensive Evaluation of Horticultural Traits

Field experiments were conducted at the Institute of Vegetable and Flowers of the Chinese Academy of Agricultural Sciences, using an individual plant-based evaluation strategy combined with marker-assisted selection to minimize error and maximize selection efficiency.

Horticultural traits were evaluated for individual BC_2 plants and their subsequent selfed progenies. Throughout the experiment, the recurrent parent 'Yutian Baojian' (188) was consistently used as a control for both phenotypic and genotypic comparisons. The horticultural traits included plant height, plant spread, head height, head width, head shape, blistering, leaf margin waviness, leaf margin serration, and soluble solids content. The traits were documented and evaluated according to the standards described in "Descriptors and Data Standard for Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*)." All horticultural traits were evaluated when the plants reached the heading stage (i.e., when the heads were firm

and ready for harvest), corresponding to BBCH stage 4, as redefined for Chinese cabbage in the WOFOST model by Tang et al. [19].

To quantify the overall phenotypic resemblance, a phenotypic similarity index (PSI) was calculated for each individual. All investigated horticultural traits were categorized into quantitative traits and qualitative traits. Quantitative traits include plant height, plant spread, head height, head width, and soluble solids content. These traits were obtained through measurement and recorded as numerical values. The absolute deviation from the recurrent parent's mean was standardized using the formula: $S_{quant} = 1 - (|X_i - X_{188}|)/X_{188}$, where X_i is the trait value of the individual plant, and X_{188} is the mean trait value of the recurrent parent. This approach transforms the 'distance' between the individual and the target ideotype into a 'similarity' score—a principle analogous to similarity measures based on Euclidean distance, which has been widely used in plant genetic diversity studies and cultivar recommendation systems [20,21]. This standardization allows for the combined analysis of traits measured on different scales.

Qualitative traits included head shape, blistering, leaf margin waviness, and leaf margin serration. These traits were evaluated qualitatively by comparing their morphology with that of 'Yutian Baojian' (188) according to the described specifications. Complete consistency with the recurrent parent was scored as 1 and inconsistency was scored as 0. The final PSI was the weighted average of the standardized scores for all traits, with equal weights assigned:

$$\mathrm{PSI} = \frac{\sum_{j=1}^{n} Sj}{n}$$

where S_j is the standardized score for the j-th trait, and n is the total number of traits evaluated (n = 9 in this study).

All statistical analyses were performed using Excel 2016 and SPSS (IBM SPSS Statistics version 27.0). Quantitative data are presented as the mean ± standard deviation (SD) of three technical replicates. For comparisons of quantitative traits between multiple groups, one-way analysis of variance (ANOVA) was conducted, followed by Dunnett's post hoc test for multiple comparisons against the control. The significance threshold was set at $p < 0.05$. Cluster analysis of BC_2 individuals based on genetic similarity was performed using the unweighted pair-group method with arithmetic averages (UPGMA). Euclidean distance cluster analysis was performed using IBM SPSS Statistics software with the average linkage method [22].

3. Results

3.1. Parental Characteristics and F_1 Population Development

The cross was made between 188 and 153. These two parents exhibited distinct phenotypic and genotypic differences. Parent 188 was characterized by a typical "cannonball" head shape, a plant height of 40 cm, and a high soluble solids content (3.5 Brix%), but was highly susceptible to clubroot. By contrast, parent 153 had a plant height of 46 cm, exhibited a long-cylindrical head, had a soluble solids content of 2.6 Brix%, carried the *CRd* resistance gene, and showed strong resistance to clubroot.

The F_1 population was generated through hybridization between these two parents. All F_1 plants exhibited heterozygous genotypes at the *CRd* locus, as confirmed by foreground selection using the molecular marker yau-376. The average plant height of F_1 was 45 cm, and they displayed intermediate phenotypic traits between the two parents. Following inoculation using the root dip method, disease phenotypes were evaluated four weeks post-inoculation. The roots of line 188 exhibited significant swelling, while those of line 153 and the F_1 population showed no obvious changes and remained normal. This further confirmed that line 188 was highly susceptible to clubroot disease, whereas line

153 and the F_1 population were resistant. A single F_1 plant was then backcrossed with line 188 to produce the BC_1 and BC_2 populations. The phenotypic and genotypic results for the parents and the F_1 generation are summarized in Figure 2.

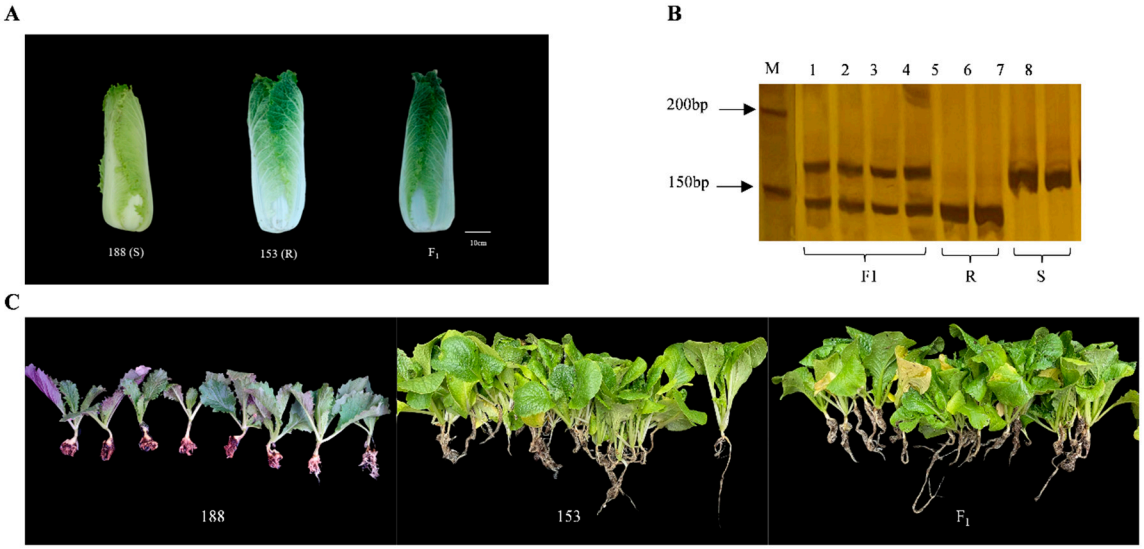

Figure 2. Differences in phenotype and genotype between parents and F_1. (**A**) Phenotypic differences in ball head between parents and F_1. (**B**) Screening different genotypes with yau-376 molecular marker. M: 500 bp DNA marker. Lane 1–4, F_1. Lane 5–6, Line 153 (168 bp). Lane 7–8, Line 188. (**C**) Phenotypes of the parental lines and F_1 progeny after inoculation.

3.2. Marker-Assisted Background Selection

A total of 364 InDel markers and 126 SSR markers were initially screened for polymorphism between the two parents. Among these, 73 markers (68 InDels and 5 SSRs) exhibited clear polymorphism, corresponding to a polymorphism rate of 14.8%. Gel electrophoresis profiles showing the polymorphism of selected markers in the parents and F_1 generation are presented in Figure 3. These 73 polymorphic markers were subsequently used for background selection, as they could effectively distinguish alleles inherited from the recurrent parent (188) and the donor parent (153), making them efficient tools for assessing the recurrent parent genome recovery rate. The average number of markers per chromosome was approximately seven, and their distribution across the 10 chromosomes is shown in Figure 4. Detailed information regarding all background markers is provided in Supplementary Table S1.

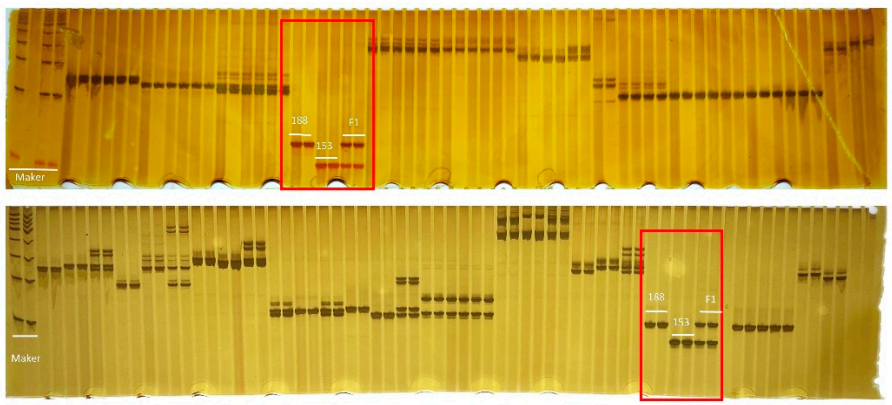

Figure 3. Polymorphism of some markers in the parents and F_1. Note: The polymorphic marker results are shown in the red box. Marker: 500 bp DNA Marker; 188: Line 188; 153: Line 153.

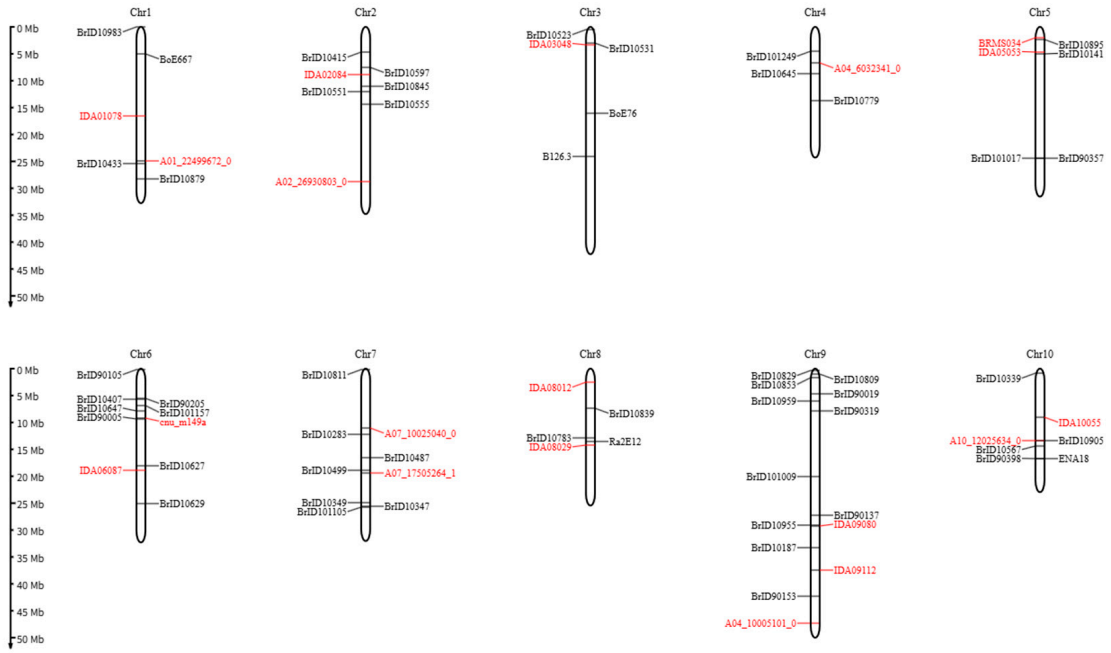

Figure 4. Distribution of 73 background-selection markers in *B. rapa* chromosomes. Note: The 19 markers for the BC_1 generation are represented in red, and the remaining 54 markers in black were used for screening the BC_2 generation.

For the screening of the BC_1 population, one or two markers were screened on each chromosome, for a total of 19 markers, which could effectively evaluate the degree of genome recovery of recurrent parents. Following the backcross of selected BC_1 individuals with parent 188 to generate the BC_2 population, the remaining 54 polymorphic markers were used for comprehensive background screening of BC_2 individuals.

3.3. Comprehensive Screening of BC_1 Plants

3.3.1. Foreground Selection

Yau-376 was used to screen and identify the *CRd* of 400 individuals in the BC_1 population. Based on the genotyping results, a total of 182 individuals were *CRd* heterozygous disease-resistant genotypes and 218 plants did not carry disease resistance genes. The Chi-square test showed that $\chi^2 = 3.24 < 3.841$ ($\chi^2_{0.05,1}$), $p > 0.05$, which was consistent with a 1:1 separation ratio ($n = 400$), confirming the single-gene inheritance of *CRd* and the accuracy of marker-assisted selection.

3.3.2. Background Selection

The 182 plants were planted, and a total of 169 plants were harvested. Owing to the need to retain the selected plants, we only calculated and screened the genetic similarity of these 169 plants. Chromosome-level analysis of recurrent parent genome recovery revealed substantial variation among the ten chromosomes of *B. rapa* (Figure 5). It was found that the consistency rate of the given marker loci on each chromosome between the individual plant and the backcross parent was between 38.5% and 60.4%. Chromosome A02 displayed the highest recovery rate (60.4%), significantly exceeding the theoretical Mendelian expectation of 50% for a BC_1 population. By contrast, chromosome A10 showed the lowest recovery rate (38.5%). The recovery rate, defined as the proportion of plants carrying the recurrent parent allele at a given marker locus. The markedly high consistency rate on A02 and low consistency rate on A10 suggested the presence of chromosome-specific selection pressures or variation in recombination efficiency during the backcross process.

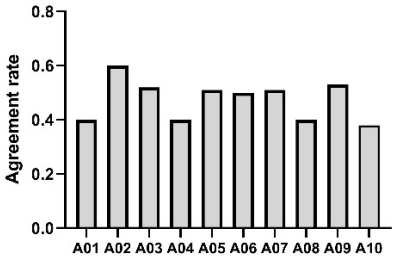

Figure 5. Consistency rate of markers on different chromosomes in single plants and parents.

The distribution of genetic similarity among these plants showed a wide range from 60% to 94.74%, with the majority clustered between 70% and 85%. Thirteen individuals exhibited a genetic similarity of more than 90%, indicating a substantial recovery of the recurrent parent genome in the first backcross generation. Among them, three individuals had the highest genetic similarity, reaching 94.74%, which were individuals 2152 and 155 (Figure 6). They were selected as for backcrossing with parent 188 to generate the BC_2 population. Based on the number of pods and seeds, the BC_2 generation created by individual 2 was finally selected for subsequent screening.

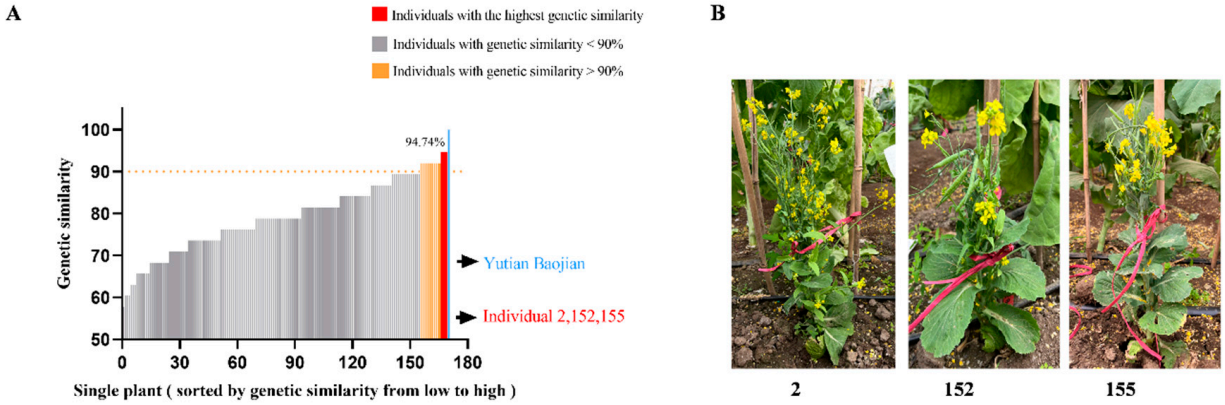

Figure 6. Screening and phenotyping of high-similarity individuals in BC_1 population. (**A**) The genetic similarity ranking distribution map of 169 single plants in BC_1 generation. (**B**) The field phenotypes of individuals 2152 and 155 (flowering stage).

3.4. Comprehensive Screening of BC_2 Plants

3.4.1. Foreground Selection

Foreground selection was performed on 300 individuals of the BC_2 population of the BC_1-2 backcross progeny. A total of 157 individuals were *CRd* heterozygous disease-resistant genotypes and 143 plants did not carry disease resistance genes. The Chi-square test showed that $\chi^2 = 0.051 < 3.841$ ($\chi^2_{0.05,1}$), which was consistent with a 1:1 separation ratio, indicating stable Mendelian inheritance of the *CRd* locus through successive backcrossing.

3.4.2. Background Marker Selection

A total of 136 plants were obtained by planting these 157 plants. To preserve selected materials, genetic similarity was assessed only for these 136 plants using the remaining 54 markers. Under the condition of a Euclidean distance of five, these 136 individual plants could be classified into four groups (BC_2-①, BC_2-②, BC_2-③, and BC_2-④), corresponding to a gradient of genetic similarity to the recurrent parent 188 (Figure 7, Table 1). Most individuals (BC_2-③, 60 plants, 44.1%) exhibited a moderate genetic similarity ranging from 90.28% to 93.84%, suggesting substantial recovery of the recurrent parent genome alongside residual introgressed donor segments. A smaller group of 31 plants (BC_2-②, 22.8%) displayed lower similarity (86.30–89.04%), indicating higher donor segment reten-

tion. Notably, only 12 plants (BC_2-④, 8.8%) reached high similarity levels (95.21–99.32%), indicating a high degree of recurrent parent genome recovery (>95% based on marker analysis.) This distribution aligned with the expected segregation pattern in a BC_2 population and supported the cluster analysis presented in Figure 6. Together, these results offer a quantitative framework for selecting individuals with preferred genetic backgrounds for subsequent breeding or functional investigations.

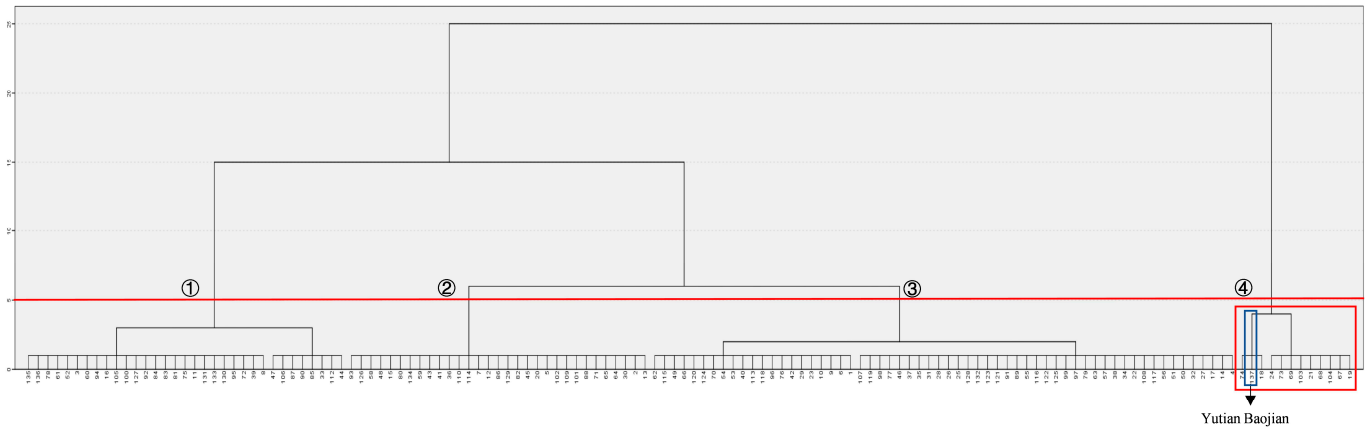

Figure 7. Cluster analysis of BC_2 individual plants. Note: The red vertical line is the Euclidean distance of five. Four clusters of 136 individual plants in the BC_2 generation were obtained: BC_2-①, BC_2-②, BC_2-③, and BC_2-④. The recurrent parent and BC_2-④ are circled in red box and blue box. 137 represents 188.

Table 1. Cluster analysis results of BC_2 population.

Cluster	Genetic Similarity	Number of Individual Plants
BC_2-④	95.21–99.32%	12
BC_2-③	90.28–93.84%	60
BC_2-②	86.30–89.04%	31
BC_2-①	<85.00%	33

3.4.3. Horticultural Traits Screening

To integrate field phenotypic screening, we systematically investigated multiple horticultural traits across these four groups of 136 plants. The traits included plant height and plant spread, leaf margin waviness, leaf margin serration, blistering, head shape, head height, head width, and soluble solids content. The phenotypic survey data of the horticultural traits of the 136 plants are listed in Supplementary Table S2.

Quantitative analysis revealed that BC_2-④ exhibited the greatest phenotypic congruence with 188 (Figure 8A). Its plant height (42.09 cm) did not differ significantly from that of 188 (41.00 cm; $p > 0.05$), whereas BC_2-① was significantly taller (45.73 cm; $p < 0.05$). Similarly, the plant spread of BC_2-④ (43.72 cm) overlapped with that of 188 (43.67 cm; $p > 0.05$), while BC_2-① showed significantly greater spread (48.96 cm; $p < 0.05$). For head-related traits, BC_2-④ did not differ significantly from 188 in either head height (33.32 vs. 32.00 cm) or head width (10.95 vs. 10.67 cm; both $p > 0.05$). By contrast, BC_2-② had greater head height (36.91 cm; $p < 0.05$), and BC_2-① had greater head width (11.44 cm; $p < 0.05$). The soluble solids content further distinguished the clusters: BC_2-④ (3.58%) did not differ significantly from 188 (3.60%; $p > 0.05$), whereas BC_2-① showed significantly lower content (2.88%; $p < 0.01$), and BC_2-② showed a trend toward lower values (3.11%; $p > 0.05$).

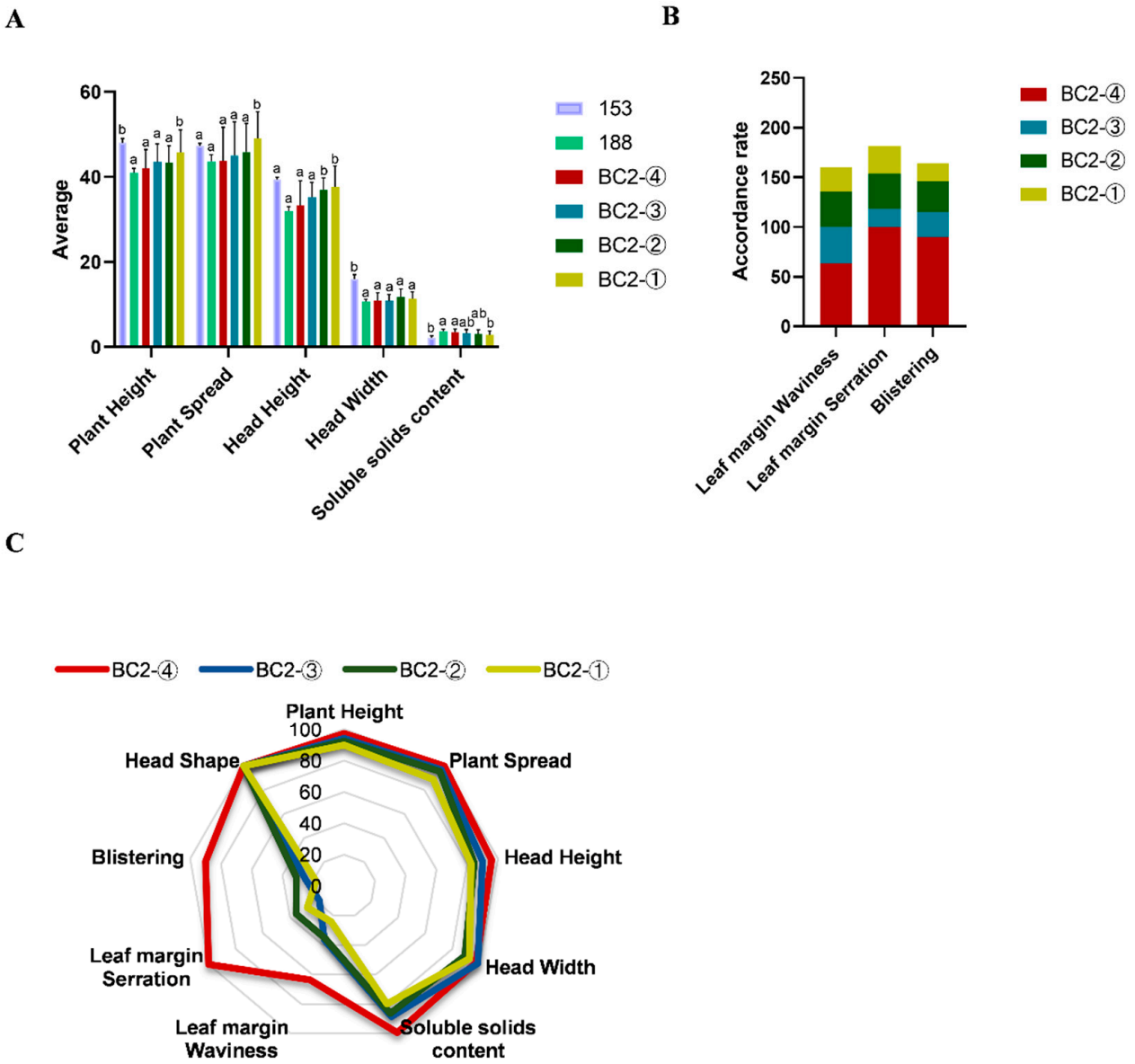

Figure 8. Horticultural traits analyze of four groups in BC_2. (**A**) Mean values and significant differences of quantitative horticultural traits (plant height, plant spread, head height, head width, and soluble solids content) among four BC_2 groups and parental lines (153 and 188). (**B**) Accordance rate of qualitative phenotypic traits (leaf margin waviness, leaf margin serration, and blistering) between four BC_2 groups and 188. (**C**) Radar chart of phenotypic similarity between four BC_2 groups and 188 across multiple horticultural traits. Note: The lowercase letters (e.g., a, b) denote significant differences.

Qualitative traits—leaf margin waviness, serration, and blistering—also supported the superiority of BC_2-④ (Figure 8B), which displayed a mean accordance rate of 95.4% with 188, exceeding that of BC_2-③ (86.7%), BC_2-② (76.6%), and BC_2-① (65.4%). Notably, BC_2-④ achieved 100% accordance with 188 in leaf margin serration, indicating stable recovery of this trait. An integrated phenotypic similarity assessment, visualized via radar chart, confirmed that BC_2-④ had the highest overall similarity to 188 (mean 98.0%), with all trait-specific similarities exceeding 95%. Other clusters showed broader divergence, particularly BC_2-①, which fell below 85% similarity for soluble solids content and head height.

These phenotypic patterns aligned closely with the previously described genetic clustering (Figure 6, Table 2). As summarized in Table 3, BC_2-④—the cluster with the highest genetic similarity to 188—displayed an average plant height of 42.09 cm, spread

of 44.00 cm, head height of 33.32 cm, and head width of 10.95 cm. Its leaves were deeply serrated, blistering was small or very small, leaf margin waviness was medium to small, and all heads were cannonball-shaped. The average soluble solids content (3.58%) closely matched that of 188 (3.60%).

Table 2. BC_2 cluster analysis and the survey results of parents.

	153	188	BC_2-④	BC_2-③	BC_2-②	BC_2-①
Plant height (cm)	48.00 ± 1.00	41.00 ± 1.00	42.09 ± 2.43	43.47 ± 4.33	43.32 ± 4.01	45.72 ± 5.35
Plant spread (cm)	47.33 ± 0.58	43.67 ± 1.53	43.72 ± 7.96	45.05 ± 7.94	45.87 ± 6.70	48.96 ± 6.38
Head height (cm)	39.33 ± 0.58	32.00 ± 1.00	33.32 ± 5.81	35.27 ± 3.44	36.91 ± 2.89	37.60 ± 4.97
Head width (cm)	16.00 ± 1.00	10.67 ± 0.58	10.95 ± 1.81	10.80 ± 1.57	11.79 ± 1.89	11.44 ± 1.52
Soluble solids content	2.10 ± 1.53	3.6 ± 0.58	3.58 ± 0.69	3.19 ± 0.93	3.11 ± 0.97	2.88 ± 0.97
Leaf margin waviness	L	M	M or S	L, M, S	L, M, S	L, M, S
Leaf margin serration	Crenate	Deeply serrated	Deeply serrated	Deeply serrated	Deeply serrated	Deeply serrated
Blistering	L	S	S or V. Small	M or S	L, M, S	S or V. Small
Head shape	Cylindrical	Cannonball	Cannonball	Cannonball	Cannonball	Cannonball

Note: L represents large, M represents medium, S represents small.

Table 3. Phenotypic similarity of BC_2-④ single plant.

Number	**74**	**67**	**19**	**104**	**18**	**69**	**103**	**24**	**73**	**21**	**68**
PSI	0.993	0.990	0.986	0.986	0.981	0.973	0.970	0.967	0.962	0.952	0.950

Collectively, these results demonstrated that BC_2-④ was phenotypically the most congruent with the recurrent parent 188, showing no significant deviation in any quantitative trait and the highest accordance rate of qualitative traits (Figure 8C). By contrast, plants in clusters with lower genetic similarity (BC_2-①, BC_2-②, and BC_2-③) showed varying degrees of phenotypic deviation. Notably, the BC_2-③ cluster also showed close resemblance to the recurrent parent, but BC_2-④ exhibited the highest level of genetic and phenotypic recovery among all clusters. Therefore, BC_2-④ was selected as the optimal group for subsequent joint screening of horticultural trait stability and genetic background homogeneity, providing a reliable basis for further breeding and genetic studies.

3.4.4. Integrated Selection of Elite Individuals

Based on the convergent evidence from genetic clustering and phenotypic characterization, a targeted selection strategy was implemented to identify individuals with optimal recovery of both the target allele and the recurrent parent's ideotype. The phenotypic survey demonstrated that plants within the BC_2-④ cluster, characterized by the highest genetic similarity to 188, collectively exhibited horticultural traits that most closely aligned with the recurrent parent. This clear association justified focusing the final selection within this genetically superior cluster. Consequently, a two-tiered selection protocol was applied. First, the plants in the BC2-④ group were considered to ensure the maximum restoration of the genetic background. Second, within this preselected subset, each individual was evaluated using a comprehensive PSI, which quantified the overall resemblance to the recurrent parent across all measured horticultural traits (Table 3). The PSI values ranged from 0.95 to 0.99, indicating a high degree of phenotypic recovery across the cluster. Four elite individuals (No.19, 67, 74, and 104) were identified using this protocol that performed best in genetic similarity and phenotypic similarity with the recurrent parent (Figure 9). These selected plants were identified as the most promising candidates from the BC_2 screening stage for further selfing to immobilize the *CRd* gene for subsequent inter-lineage development.



Figure 9. Phenotypic comparison of the selected individuals of BC_2-④.

3.5. Generation and Evaluation of BC_2S_1

3.5.1. Identification of Homozygous Resistant Plants

The BC_2S_1 population, consisting of 94 individuals from self-pollination of the selected individual 74, was genotyped using yau-376 to identify homozygous resistant plants. Statistical analysis showed that 19 individuals possessed the homozygous *CRd* genotype, 52 individuals were disease-resistant heterozygous genotypes, and 23 individuals did not carry disease resistance genes. The segregation pattern followed the expected genetic ratio of 1:2:1 ($\chi^2 = 0.936 < 5.99\ \chi^2_{0.05,2}$, $n = 94$). This final selection step validated the single-gene inheritance model of *CRd* and enabling the selection of homozygous resistant progenies.

3.5.2. Phenotypic Stability in Homozygous BC_2S_1

The phenotypic performance of the 19 homozygous BC_2S_1 plants were evaluated to assess the stability of horticultural traits following fixation of the *CRd* allele. An analysis of the qualitative traits revealed both consistent inheritance and expected segregation. All individuals uniformly exhibited the donor parent-inherited small blistering. The characteristic cannonball head shape was also fixed in all plants. However, segregation was observed for leaf margin traits: leaf serration (deeply serrated vs. serrated) and leaf margin waviness (large vs. medium) did not fully recover to the recurrent parent's state in all plants.

An evaluation of the quantitative traits (plant height, plant spread, head height, head width, and soluble solids content) showed that the population means for each trait were not significantly different from those of recurrent parent 188 (*t*-test, $p > 0.05$). Frequency distribution histograms demonstrated that, for each trait, the majority of individuals clustered around the recurrent parent's value, indicating successful recovery of the phenotypic ideotype at the population level (Figure 10A).

To quantify the overall phenotypic resemblance, a phenotypic similarity index (PSI) was calculated for each individual. The PSI values across the population followed a continuous distribution ranging from 0.73 to 0.89, with a mean of 0.82 (Figure 10B). This variation primarily stemmed from differences in the segregating qualitative traits mentioned above. Notably, a subset of individuals (individuals 2, 3, 4, 6, and 12) achieved high PSI scores (>0.87), demonstrating a combination of homozygous disease resistance and a near-complete recovery of the recurrent parent's phenotype (Figure 10C), including the desired leaf margin morphology. Based on this comprehensive evaluation, these five individuals, which best represented the ideal 'Yutian Baojian' type, were advanced to self-pollination to produce the BC_2S_2 generation, thereby initiating the development of stable inbred lines.

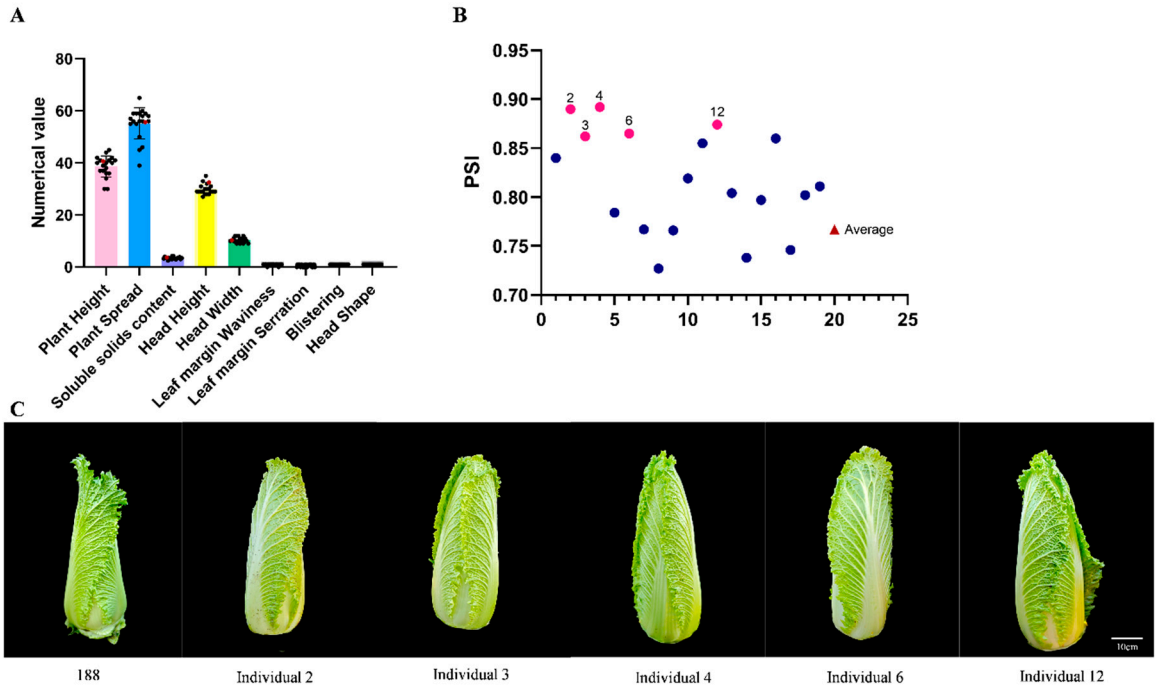

Figure 10. Phenotypic evaluation of homozygous BC_2S_1 individuals. (**A**) Frequency distribution of quantitative traits in BC_2S_1 (n = 19). Red dots indicate the value of the recurrent parent 188. (**B**) Distribution of the phenotypic similarity index (PSI) for BC_2S_1 individuals (n = 19). The PSI quantifies overall phenotypic resemblance to the recurrent parent. The blue and red dots represent individual PSI values, respectively. The average PSI value of the individual is marked with a red triangle. (**C**) Phenotypic comparison of the selected elite BC_2S_1 individuals with 188.

3.6. Development and Validation of Stable BC_2S_2 Homozygous Lines

To obtain genetically stable inbred lines, and according to the number of seeds obtained from the selfing progeny, three plants (individuals 1, 2 and 4) were selected for selfing to obtain the BC_2S_2 population. Foreground selection of these lines confirmed that all tested plants were homozygous for the *CRd* resistant allele. No heterozygous or susceptible genotypes were detected, demonstrating the complete fixation of the target gene and the attainment of genetically pure, resistant breeding lines.

Field observations showed that three lines uniformly exhibited the characteristic 'Yutian Baojian' phenotype (Figure 11). These results demonstrated that the elite BC_2S_2 lines not only carried the homozygous *CRd* gene but also fully retained the desirable horticultural traits of 'Yutian Baojian', fulfilling the breeding objective of integrating disease resistance without compromising quality.



Figure 11. Field performance of homozygous BC_2S_2 lines.

4. Discussion

4.1. Introgression of the CRd Gene and Genetic Recovery of the Recurrent Parent

This study successfully employed a marker-assisted backcrossing strategy to introgress the clubroot resistance gene *CRd* into the elite but susceptible Chinese cabbage variety 'Yutian Baojian' (188). Through the integration of foreground, background, and phenotypic selection, we developed BC_2S_2 lines that combine stable clubroot resistance with a high degree of genetic and phenotypic similarity to the recurrent parent. The foreground marker yau-376, tightly linked to *CRd* [14], proved highly effective, with segregation ratios in both BC_1 ($\chi^2 = 3.24$, $p > 0.05$) and BC_2 ($\chi^2 = 0.051$, $p > 0.05$) populations conforming to the expected 1:1 ratio for a heterozygous locus. This confirmed both the single-gene inheritance of *CRd* and the reliability of the marker for selection.

Background selection using 73 genome-wide polymorphic markers revealed substantial variation in genome recovery. In the BC_1 population, overall genetic similarity to the recurrent parent ranged from 60.0% to 94.7%, with a theoretical mean of 75.0%. Notably, chromosome-specific recovery rates varied considerably, from 60.4% on chromosome A02 to 38.5% on chromosome A10. This variation likely reflects differences in recombination frequencies among chromosomes, as well as potential selection pressures during the backcross process. For instance, the high recovery rate on A02 may have resulted from suppressed recombination in certain marker intervals, while the low recovery on A10 could be associated with linkage drag from donor segments affecting progeny viability or the inadvertent selection against specific chromosomal configurations during backcrossing. These observations underscore the power of genome-wide markers for identifying individuals that have recovered more of the recurrent parent genome than expected by chance alone—a key advantage of MABC over conventional backcrossing [10]. This chromosome-level resolution of genotypic variation provides practical guidance for optimizing background marker design in future Chinese cabbage breeding programs. Specifically, higher-density marker coverage should be allocated to chromosomes with inherently low recovery rates to improve screening efficiency and accuracy. By the BC_2 generation, we successfully selected individuals from the BC_2-④ cluster exhibiting genetic similarity as high as 99.32% based on the 73 polymorphic markers. It is important to note, however, that this figure represents an estimate derived from a finite set of markers and should be interpreted as marker-estimated similarity rather than validated whole-genome sequence identity.

4.2. Concordance Between Genotypic Background and Phenotypic Recovery

A key finding of this study is the significant positive correlation between marker-based genetic similarity and phenotypic similarity to the recurrent parent, which is the core basis for the successful improvement of 'Yutian Baojian'. Plants in the BC_2-④ cluster, which exhibited the highest genetic similarity to 188 (95.2–99.3%), demonstrated the closest phenotypic alignment with the recurrent parent. Specifically, they showed no significant differences in any quantitative trait measured (plant height, plant spread, head height, head width, and soluble solids content; all $p > 0.05$) and achieved a 95.4% accordance rate for qualitative traits, with leaf margin serration reaching 100% consistency. By contrast, clusters with lower genetic similarity (BC_2-① and BC_2-②) displayed significant deviations in quantitative traits (e.g., BC_2-① plant height = 45.7 cm, $p < 0.05$) and markedly lower accordance rates for qualitative traits (65.4–76.6%). These results confirmed that marker-based genotypic screening effectively predicted the degree of phenotypic recovery, validating the utility of background selection for concurrently tracking both the target gene and the broader genetic background.

To quantify this relationship, we employed a phenotypic similarity index (PSI), calculated as the weighted average of standardized scores for nine horticultural traits, with

equal weighting applied to all traits. Within the BC_2-④ cluster, elite individuals achieved PSI values ranging from 0.95 to 0.99. In the subsequent BC_2S_1 homozygous population, PSI exhibited a continuous distribution (0.73–0.89), with variation primarily attributable to segregating qualitative traits such as leaf margin waviness and serration. These residual phenotypic differences are common in backcross populations and reflect incomplete fixation of all background loci despite high overall genetic similarity. By selecting BC_2S_1 individuals with PSI > 0.87, we effectively eliminated these outliers and ensured the phenotypic uniformity of the final BC_2S_2 inbred lines.

Regarding trait heritability, although formal heritability estimates were not calculated in this study, the phenotypic stability observed across generations provided indirect evidence. The mean values of quantitative traits in BC_2-④ and BC_2S_1 populations did not differ significantly from those of the recurrent parent ($p > 0.05$), and trait frequency distributions clustered tightly around the recurrent parent values. These patterns suggest that the key horticultural traits of 'Yutian Baojian' are highly heritable and under polygenic control, a characteristic that is particularly favorable for MAS breeding. High heritability ensures that phenotypic recovery can be achieved concomitant with target gene introgression, minimizing the risk of losing desirable agronomic traits through genetic recombination.

All field evaluations in this study were conducted under a single environmental condition. Traits such as soluble solids content and head morphology are known to be influenced by environmental factors including temperature, soil fertility, and water availability [23]. Therefore, the stability of both resistance and horticultural traits should be validated through multi-location and multi-year trials in future studies. Such validation will be essential to confirm the broad adaptability and commercial potential of the developed germplasm.

4.3. Application Value

Marker-assisted selection for clubroot resistance has been widely applied in *Brassica* crops, and a comparison with other successful reports reveals both the consistency and the unique features of the present study. Our approach focused on single-gene introgression via MABC, which is conceptually similar to several recent studies. Wang et al. [24] successfully introduced the *CRa* gene into five elite cabbage inbred lines through five cycles of marker-assisted backcrossing, achieving BC_4 individuals with highly restored genetic backgrounds (as confirmed by whole-genome resequencing showing only 54,213 SNP differences from the recurrent parent). Similarly, Jiang et al. [25] introgressed the *CRb* gene into an elite recessive genic male sterile line of rapeseed within four backcross generations, reducing the disease index to 8.7 and significantly improving yield performance in disease-affected areas. These studies, together with our work, demonstrate the efficiency and reproducibility of MABC for single-gene resistance improvement across different *Brassica* species and genetic backgrounds.

Despite the proven efficacy of single-gene introgression, a growing body of evidence indicates that pyramiding multiple resistance genes confers broader and more durable resistance to *Plasmodiophora brassicae.* Baloch et al. [26] successfully pyramided three resistance loci (*CRA3.7*, *CRA08.1*, and *CRA3.2*) in *Brassica napus* using marker-assisted selection combined with speed breeding. Lines carrying all three homozygous resistance loci exhibited "super resistance" to six geographically distinct pathogen isolates, with clear evidence of a gene dosage effect and no negative impact on agronomic traits. Wen et al. [27] provided transcriptomic insights into this phenomenon, demonstrating that lines pyramiding two resistance genes (CRa^m and $Crr1^{rutb}$) mounted stronger defense responses with significantly higher transcript levels of defense-related genes compared to lines carrying only a single gene, suggesting synergistic interactions between resistance loci. Beyond gene pyramiding

within a single species, interspecific hybridization offers another avenue for broadening the genetic basis of resistance. Karim and Yu [28] resynthesized novel *Brassica napus* lines by hybridizing turnip (*B. rapa*, carrying species-specific resistance genes) with cabbage (*B. oleracea*, carrying non-species-specific QTLs). Through embryo rescue and marker-assisted selection, they obtained eight new lines with high resistance to all eight tested pathotypes of *Plasmodiophora brassicae*, demonstrating the immense potential of interspecific gene transfer for expanding the resistance gene pool.

In comparison with gene pyramiding and interspecific hybridization strategies, our MABC approach offers distinct advantages of speed and precision, enabling the rapid conversion of a well-adapted local variety into a clubroot-resistant version while fully preserving its unique agronomic and horticultural characteristics. This advantage is particularly valuable for geographically indicated varieties with established market identity and consumer preference, such as 'Yutian Baojian'. However, a critical limitation of the single *CRd* gene resistance in our improved germplasm is the potential for resistance breakdown due to the continuous evolution and race diversification of *P. brassicae* populations in agricultural ecosystems. Thus, future breeding efforts should prioritize pyramiding additional validated clubroot resistance genes (e.g., *CRa* and *Crr1*) into the 'Yutian Baojian' genetic background to enhance the durability and broad-spectrum of resistance. The *CRd*-homozygous, clubroot-resistant 'Yutian Baojian' germplasm developed in this study provides an ideal and genetically stable starting material for such gene pyramiding initiatives.

The clubroot-resistant 'Yutian Baojian' germplasm generated in this study holds substantial practical application value for Chinese cabbage breeding and production. First, these materials can be directly deployed as elite parental lines for the development of new clubroot-resistant 'Yutian Baojian' varieties, providing an immediate and effective solution for clubroot management in the core producing areas of this geographically indicated crop. This will reduce the reliance on chemical fungicides for disease control and contribute to the development of sustainable, environmentally friendly vegetable production systems. Second, the improved germplasm can serve as a valuable bridge parent for clubroot resistance gene pyramiding in Chinese cabbage breeding programs. By introgressing additional resistance genes through sequential crossing and MAS, it will be feasible to develop new 'Yutian Baojian' varieties with broad-spectrum and durable resistance that are capable of withstanding the challenges posed by regional variations in *P. brassicae* race composition and the continuous evolution of this soil-borne pathogen. Notably, the *CRd* gene utilized in this study confers stable resistance to *P. brassicae* physiological race 4, one of the most prevalent and virulent races in northern Chinese cabbage production areas. Nevertheless, the inherent risk of resistance loss associated with single-gene resistance necessitates the acceleration of multi-gene pyramiding to sustain the long-term utility of the improved 'Yutian Baojian' germplasm in clubroot-infested fields.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12030395/s1. Supplemental Table S1. Information of background markers. Supplemental Table S2. Phenotypic investigation of BC_2 horticultural traits.

Author Contributions: The authors have made the following declarations about their contributions: H.Z. and S.Z. (Shujiang Zhang) designed and supervised the experiments. R.H., X.X., Y.D., S.W. and Y.Z. investigated and analyzed the data. S.Z. (Shifan Zhang), F.L., G.L. and R.S. supervised the entire experiment. Y.R. and B.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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