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Development of Novel Markers and Creation of Non-Anthocyanin and Anthocyanin-Rich Broccoli (*Brassica oleracea* var. *italica*) Cultivars

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Abstract: In broccoli, anthocyanin pigments can be accumulated in the flower bud epidermis, resulting in a purple-green head. This study aimed to create non-anthocyanin green broccoli varieties and anthocyanin-rich purple broccoli varieties using new *F3′H* and *Pur7.1*-K1 molecular markers, respectively. The breeding program started with crosses of the recipient (superior variety and line) LF02 line with the donor line SN60 carrying the recessive allele *f3′h* and the donor line BT126 carrying the dominant allele *Pur7.1*. The F1 hybrids were confirmed with molecular markers and backcrossed with the recurrent parent LF02, followed by cycles of foreground and background selection at each stage. A total of 161 green plants with the *f3′hf3′h* genotype and 152 purple plants with the *Pur7.1Pur7.1* genotype were selected from the BC3F2 line. Among these, 34 green plants and 28 purple plants demonstrated >85% background recovery. The identified plants were selfed to obtain 301 green and 416 purple BC3F3 plants for assessment of major agronomic traits. After these investigations, two green broccoli lines without anthocyanin and three anthocyanin-rich purple lines with the best yield/quality characteristics were obtained. The development of these lines might help provide basic materials and the theoretical basis for breeding commercial broccoli varieties.

Keywords: broccoli; marker-assisted selection; anthocyanin; quantitative trait loci (QTL); agronomic traits

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1. Introduction

Broccoli and other Brassica vegetables, with a global production of 71.7 million tons, have attracted increasing attention in the context of healthy diets in recent years [1]. Most cruciferous vegetables are green because of their association with photosynthesis. However, in certain other species, red pigments can persist throughout the life span of the leaves, or they may be induced and retained only after the plant has experienced stress [2,3]. Broccoli (*Brassica oleracea* var. *italica*) has a traditional, dark bluish-green head, some of which can develop a slight purple pigmentation at low temperatures. Green broccoli is rich in dietary fiber, vitamins, and minerals and contains valuable amounts of bioactive compounds such as sulforaphane, glucosinolates, glycosylated flavonoids, and vitamin C [4,5]. Purple broccoli was less well-known in marketplaces until Alan Gray and P. Crisp introduced the seeds of a cold-tolerant and better-tasting cultivar for commercialization in the early 1980s [6]. Purple broccoli is gaining popularity as a gourmet produce item and has similar morphology to regular broccoli, except that its florets have a deep purple tinge and the heads are typically smaller. It has been demonstrated that purple broccoli contains higher nutrition levels of antioxidant compounds, vitamin C, phenolic compounds, and

thioglucosides but has lower productivity [7–9]. The purple color of broccoli is due to the accumulation of anthocyanins [10,11].

Anthocyanins are water-soluble pigments. They not only provide plants with a range of colors, such as pink, red, purple, and blue, for attracting animal pollinators [12,13] but also play important roles in protecting plants against biotic and abiotic stresses, such as UV light, pests, and diseases [14]. For humans, the antioxidant function of anthocyanins allows them to serve as strong free radical scavengers against many chronic health issues related to aging, mutagenesis, and cardiovascular diseases [11,15]. Purple vegetables, including purple-leaved sweet potato, heartleaf houttuynia, purple-leaved perilla line 1, and bicolored-leaved perilla line 2, can protect lymphocyte DNA against oxidative damage due to their strong antioxidant activities [16]. A study showed that red kale extracts at a concentration of 2500 μg/mL were effective in inhibiting cancer cell growth and increasing the death rate of cancer cells [17]. More than 20 naturally occurring anthocyanins have been identified, and only six are commonly found in higher plants: cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin [18]. Studies have reported that the main component of anthocyanin in purple *Brassica* vegetables is cyanidin, with small amounts of delphinidin and pelargonidin [11].

The biosynthesis and regulatory mechanisms of anthocyanins have been reported in a variety of model plants, such as *Arabidopsis* [19], maize [20], petunia [21], and grapes [22], as well as many other cruciferous plants, including oilseed rape [23], radish [24], kale [25], cabbage [26], and cauliflower [27]. The genes involved in anthocyanin accumulation are divided into two categories: regulatory genes and structural genes [28]. The regulatory genes mainly consist of three major classes of transcription factors (R2R3-MYB, bHLH, and WD40), which directly affect the transcription and expression of structural genes through the formation of the MYB–bHLH complex or MYB–bHLH–WD40 complex (MBW complex). In *Brassica oleracea*, the anthocyanin biosynthetic pathway is controlled by the R2R3MYB transcription factor *BoMYB2*. Studies on transgenic plants have also shown that the *BoMYB2* gene serves as a key regulator of anthocyanin accumulation in flower coloration through interaction with BobHLH and BoWD40 proteins and binding to the promoter of late anthocyanin biosynthesis genes [25,29–31]. Structural genes encode enzymes required by the anthocyanin synthesis pathway. They are divided into early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs). EBGs include chalcone synthase, chalcone isomerase, flavonoid 3-hydroxylase, flavonoid 3′-hydroxylase (F3′H), and flavonol synthase. LBGs include dihydroflavonol 4-reductase (DFR), leucine anthocyanin oxygenase (LDOX), anthocyanin reductase, and UDP-glucose: flavonoid 3-O-glucosyltransferase. Among these, *F3′H* belongs to the CYP75B subfamily of the cytochrome P450-dependent monooxygenase (P450) superfamily. It converts dihydroflavanol into colorless cyanidin and is the key enzyme for the synthesis of cyanidin [32,33]. Tao et al. recently crossed the white radish inbred line YAAS-WR1 with the red radish inbred line YAAS-RR1. The genetic analyses highlighted *RsF3′H* and *RsMYB1.3* as key candidates for controlling anthocyanin pigmentation in radish [34].

Green broccoli can sometimes produce light purple buds due to various environmental factors, significantly reducing its market value. The plant receptors detect the changes in environmental factors and activate a series of downstream transcription factors, which directly or indirectly regulate the expression of anthocyanin biosynthetic genes [35,36]. In purple kale, the expression of *DFR* and *LDOX* genes in the anthocyanin synthesis pathway was induced under UV-A and UV-B treatments, increasing anthocyanin accumulation [37]. He et al. found that *BrMYB2* and *BrTT8* might co-activate anthocyanin structural genes in purple-head Chinese cabbage after low-temperature induction, while the *BrMYB2* gene was downregulated and some negative regulators were upregulated in white-head cabbage [38]. Studies have shown that green leaves accumulated anthocyanins under low temperatures with excessive light exposure in maize, thereby shading chloroplasts and preventing the risk of photo-inhibition [39,40]. The *HY5* transcription factor may be a central integrator

of light and temperature signaling pathways in regulating anthocyanin biosynthesis in *Arabidopsis* [36].

Several previous studies have been performed on anthocyanins biosynthesis in broccoli. In purple broccoli, cyanidin was identified as a predominant anthocyanin isomer using HPLC–UV photo-diode array detection (PAD)–electrospray ionization (ESI)-MS/MS (HPLC–PAD–ESI-MS/MS) analysis [10,41]. Yu et al. used 127 doubled-haploid segregation populations of broccoli to construct a genetic map and localized a major locus and two minor loci on chromosome C1 controlling the purple sepal trait of flower heads at low temperatures [42]. Rahim et al. analyzed the dynamic transcript levels of structural and regulatory genes related to anthocyanin synthesis in purple broccoli with real-time quantitative analysis. They showed that the transcription factor *BoTT8* was expressed in coordination with anthocyanin biosynthesis genes and activated their expression at the same time [43]. Fine localization and the candidate gene identification in purple cauliflower mutants also clearly revealed that the insertion of transposons upstream of the *BoMYB2* promoter resulted in the activation of *BoMYB2*, which led to the ectopic accumulation of pigments in the mutants [27].

Molecular marker-assisted selection (MAS) plays an important role in modern plant breeding thanks to the aid of molecular markers closely linked to target genes. The genome-wide selection of single plants can be performed using MAS, which quickly recovers the genotype of the recurrent parent. As a general rule, two to four markers per 100 cM can be efficiently used to accelerate the recovery of the recipient parent in the early generations, such as BC1 or BC2 [44]. Kompetitive allele specific PCR (KASP), a single-step genotyping technology, has the major advantage of improved cost-effectiveness and has been widely used in linkage maps accompanied by simple sequence repeats (SSRs), cleaved amplified polymorphic sequences (CAPSs), sequence characterized amplified regions (SCARs), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD) [45]. Molecular markers are also required in purple plant breeding because of the plants' environmentally sensitive and semi-dominant traits during the introgression process. In purple cauliflower, 17 primers were designed using the homologous sequences of anthocyanin-related genes in the *B. oleracea* genome and *Arabidopsis*. Three polymerase chain reaction (PCR)-based markers, namely *BoMYB2*, *BoMYB3*, and *BoMYB4* (<0.2 cM), were detected to provide a link to the high-resolution map of the *Pr* gene in "Graffiti" purple cauliflower [27]. However, among the three molecular markers, only the *BoMYB3* marker could distinguish the Sicilian purple "PC-1" and white curding Indian cauliflower parents, but it did not show co-segregation during the investigation in the F2 population [46]. Cauliflower (*B. oleracea* var. *botrytis*) and broccoli (*B. oleracea* var. *italica*) are different botanical varieties of *B. oleracea* and, hence, the evolutionary levels may be different. Identifying molecular markers closely linked to the quantitative trait locus (QTL) can make selection more efficient, effective, rapid, and cost-effective. Therefore, the construction of detailed molecular and genetic maps of the genome of purple broccoli is a prerequisite for MAS.

In a previous study, a purple broccoli inbred line "BT126" was crossed with a green broccoli inbred line "SN60" without anthocyanin accumulation. The genetic analysis of the segregating F2 population revealed two QTLs located on chromosomes C7 and C9 named *Pur7.1* and *Pur9.1*, respectively. During QTL-seq analysis, the target genes were localized in the intervals of 6.92 MB (36.78 MB–43.70 MB) on chromosome C7 and 73 K (C9:51.71 MB–51.79 MB) on chromosome C9. The blast search of the reference genome revealed that the C9 candidate interval (C9:73 Kb) contained 14 candidate genes, and one candidate gene *Bo9g174880* was homologous to AT5G07990 in *Arabidopsis*, encoding a flavonoid 3′-hydroxylase (F3′H) involved in anthocyanin synthesis [47]. Since the color distinction between different green broccoli varieties was not obvious at low temperatures and the character of the purple head was investigated only in the mature plant stage, this study aimed to develop new molecular markers using the molecular genetic mechanism of the purple broccoli phenotype and accurately perform early screening for non-anthocyanin and anthocyanin-rich broccoli plants with superior market value.

2. Materials and Methods

2.1. Materials and Breeding Procedures

The study material consisted of three inbred broccoli lines: BT126, SN60, and LF02 (Table 1). BT126 (with the *F3′H* gene and the *Pur7.1* gene for the anthocyanin content) belonged to the purple broccoli variety and contained a high anthocyanin content. This variety produces a flat, compact, and small head. The average weight of the heads was 400 g. Transplantation to first harvest took 90–100 days. SN60 (without the *F3′H* gene for the anthocyanin content) belonged to the light green broccoli variety, which do not produce anthocyanin. The plant had a spreading growth habit and smooth and green leaves. This variety produces a round, light green, and compact head. The average weight of the heads was 450.2 g. This variety matured 70–75 days after transplanting. LF02 (with the *F3′H* gene and without the *Pur7.1* gene for the anthocyanin content) was a green broccoli variety. It had a semi-spreading growth habit and dark green leaves, and the variety produces heads with green-purple color at low temperatures. The plants showed good stress and disease resistance. The average weight of the heads was 620 g. Transplantation to first harvest took 85–90 days.

Table 1. Recurrent and donor parental lines of broccoli.

Line	Color	Anthocyanin Content	Characterization
BT126	Red	High anthocyanin	Late ripening
SN60	Green	No anthocyanin	Early ripening
LF02	Green	Low anthocyanin at low temperatures	Medium ripening and good head quality

Two crosses were made. BT126 was used as the donor parent crossed with the LF02 recurrent parent to select purple broccoli with high yield and high anthocyanin contents. SN60 was used as the donor parent crossed with the LF02 recurrent parent to select green broccoli without anthocyanin in cold weather (Figure 1). After three-round backcrossing, the BC1F1, BC2F1, and BC3F1 plants were selected for the presence of molecular markers for *f3′h* and *Pur7.1* and as a result of the background selection, respectively. The selected BC3F1 plants were selfed and advanced to the BC3F2 generation stage in the fields. BC3F2 seeds from BC3F1 plants were bulk-harvested for each cross to keep all possible genotypes. Plants with good agronomic traits were visually selected from the BC3F2 generation to produce individual BC2F3 lines. The BC2F2 and BC2F3 lines were tested with markers for *f3′h* and *Pur7.1* to select homozygous lines. All materials were grown at Zhuanghang Experimental Base at the Shanghai Academy of Agricultural Sciences, Shanghai.

2.2. DNA Extraction

Young leaves from the test materials were harvested and total plant genomic DNA was extracted, referring to the modified CTAB method proposed by Kidwell et al. [48]. DNA purity, quality, and concentration were determined using 0.7–2% agarose gel electrophoresis and UV spectrophotometry. The DNA concentration was diluted to 20–30 ng/μL as a template for PCR analysis.

2.3. Primer Synthesis and PCR Detection

Three molecular markers, namely F3′H-F/R, Pur7.1-K1, and Pur7.2-K2, were present in the linkage groups Chr09 and Chr07, respectively. After screening, the F3′H marker was found to be polymorphic between parents LF02 and SN60, and Pur7.1-K1 was found to be polymorphic between parents LF02 and BT126. The two markers were used to transfer the green and purple traits in the background of the LF02. The sequences of these primers are listed in Table 2.

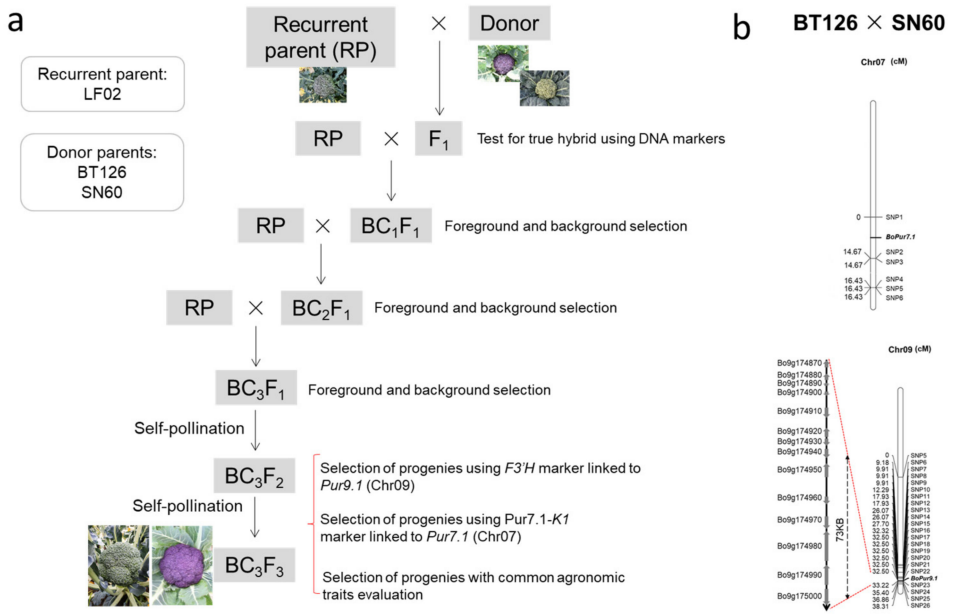

Figure 1. Breeding strategy for the development of broccoli cultivars (**a**) and *BoPur7.1* and. *BoPur9.1* linkage map (**b**).

Table 2. Molecular markers used for foreground selection in the marker-assisted breeding program.

Marker Name	Primer Sequence	Linked to *Pur* Gene
F3′H-F/R	CACGGACATGCTTAGCACTTTAAT GCCGTAAACATGTTCTGTAATGGA	*Pur9.1*
Pur7.1-K1	GAAGGTGACCAAGTTCATGCTCTCGTGGCTTCTCTACAATTATCG GAAGGTCGGAGTCAACGGATTACTCGTCGCTTCTCTACAATTATCA TGGTTAAGACAAAGTCACAAAACCAT	*Pur7.1*
Pur7.2-K2	GAAGGTGACCAAGTTCATGCTATCGAGTCCGAAGACGAAACGG GAAGGTCGGAGTCAACGGATTCATCGAGTCAGAAGACGAAACGC CATCACTCCCCAGTCCATAGCA	*Pur7.1*

A total of 76 pairs of primers (Tables S1 and S2) with clear amplified bands and good polymorphism were used for data analysis by selecting 133 pairs of SSR markers uniformly distributed on the C genome, as reported by Li et al. [49,50]. The primers were synthesized by Shanghai Sangon Biotechnology (Shanghai, China). The reaction system had a volume of 10 µL and included the following: 10× PCR buffer, 1 µL; 10 mmol/L dNTP, 0.2 µL; 5 U/uL Taq enzyme, 0.1 µL; DNA template, 1 µL; upstream and downstream primers, 0.5 µL each; and ddH_2O, 6.7 µL. All these reagents were purchased from Bao Biological Company (Dalian). The PCR amplification products were detected using 8% non-denaturing gel electrophoresis, and the electrophoretic profiles were obtained for subsequent analysis.

2.4. Anthocyanin Content Identification

The total anthocyanin content was calculated using the pH differential method [51]. Further, 1 g of fresh broccoli buds was weighed, and methanol (containing 1% formic acid) was added to the extraction solution at a material-to-liquid ratio of 1:5 (*w*/*v*). The extract was obtained by using ultrasound for 30 min and centrifugation at 4000 rpm for 5 min. The extraction was repeated twice. After extraction three times, the supernatants were combined and stored in a refrigerator at −20° until usage. Three replicates of each species were extracted. For the determination of the total anthocyanin content, the pH differential method was used. First, 800 µL of KCl buffer (pH = 1) or 800 µL of NaAc buffer (pH = 4.5) was mixed with 200 µL of sample extract and then placed in a cuvette with a light range of

1 cm for 20 min at room temperature. The absorbance values at 510 nm and 700 nm were measured using a spectrophotometer with ddH_2O as blank.

$$\text{Total anthocyanin content (mg/100 g FW)} = \frac{A \times MW \times DF \times V}{\varepsilon \times 1 \times m} \quad (1)$$

where $A = (A_{510\ nm} - A_{700\ nm})_{PH1.0} - (A_{510\ nm} - A_{700\ nm})_{PH4.5}$, the molecular weight (MW) = 449.2 g mol^{-1} for cyanidin-3-glucoside (C3G), DF is the dilution factor of 5, ε is the molar extinction coefficient of C3G (26900 L cm^{-1} mol^{-1}), V is the total volume of the extraction solution, and m is the sample mass.

2.5. Field Planting and Agronomic Trait Examination

The cultivars were planted at Zhuanghang Experimental Base at the Shanghai Academy of Agricultural Sciences. The sowing period was from late July to the end of August, and the seedlings were transplanted at the age of about 30 days. Generally, the seedlings were transplanted at the age when they had four to six true leaves, and attention was paid to controlling light and water in the seedling stage. The cultivation density was 50 × 50 cm^2, and the general density was about 2200–2400 plants per 667 m^2. The advanced backcross lines, along with the recipient parents, were evaluated with three replications. The agronomic traits—plant height, plant spread, head length, head diameter, head shape, stalk diameter, anthocyanin content, glucoraphanin content, head color, and head weight—were examined in five plants. The data obtained were statistically analyzed using Statistical Product and Service Solutions (SPSS) 16.0.

3. Results

3.1. Analysis of F3′H Homologous Sequences and Development of Specific Markers

The development of molecular markers associated with a specific agronomic trait is important when breeding new broccoli varieties from different crosses. Previous genetic analysis showed that two QTLs, *Pur7.1* and *Pur9.1*, located on chromosomes C7 and C9 controlled anthocyanin accumulation. *F3′H*, which is related to anthocyanin biosynthesis, may be a candidate gene on chromosome C9 [47]. The differences in the *F3′H* gene between the two parents were investigated to confirm the linkage between *F3′H* and anthocyanin-related trait segregation. The sequence analysis showed that a deletion of the 43-bp fragment existed at the second exon of *F3′H* in SN60 green broccoli, which formed a premature termination codon (Figure 2). The insertion/deletion site of *F3′H* was used to design the InDel marker (F3′H-F/R); ~187 and 144 bp amplified products were obtained for BT126 and SN60, respectively. PCR-amplified bands indicated that the deleted fragment was heterozygous in the F1 plants, indicating that it could be used as a marker to distinguish *F3′H/F3′H*, *F3′H/f3′h*, and *f3′h/f3′h* genotypes. It was subsequently validated in 720 F2-generation populations with phenotypic data. The results indicated that all of the f3′h/f3′h genotypes showed a green head consistent with that of SN60, whereas *F3′H/F3′H* and *F3′H/f3′h* genotypes could be obtained in purple individuals (Figure 3a). In addition, the marker was detected in 63 inbred lines. The *f3′h/f3′h* genotype was detected in green broccoli inbred lines without anthocyanin at low temperatures. In contrast, the *F3′H/F3′H* genotype was obtained in both purple broccoli inbred lines and green broccoli inbred lines with low anthocyanin at low temperatures (Figure 3b). Therefore, the introduction of a premature termination codon led to loss of function in *F3′H*, which is the key gene for determining broccoli coloration. However, both the deep purple and light purple populations had *F3′H/F3′H* and *F3′H/f3′h* genotypes, which indicated that *F3′H* genotypes were not related to pigmentation grades.

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BoF3'H-SN60       GGAATCAAGCTCTTCTTGGGCTTTTCTCATTATTTCCGGGTGACGGATTAATTCAGCTAT  600
NC_027756.1       GGAATCAAGCTCTTCTTGGGCTTTTCTCATTATTTCCGGGTGACGGATTAATTCAGCTAT  600
BoF3'H-BT126      GGAATCAAGCTCTTCTTGGGCTTTTCTCATTATTTCCGGGTGACGGATTAATTCAGCTAT  600
                  ************************************************************

BoF3'H-SN60       GGCCCAGTCCACCGTACTTGCTGACGTGTCAGTTCCGGCCGTAAACATGTTCTGTAATGG  660
NC_027756.1       GGCCCAGTCCACCGTACTTGCTGACGTGTCAGTTCCGGCCGTAAACATGTTCTGTAATGG  660
BoF3'H-BT126      GGCCCAGTCCACCGTACTTGCTGACGTGTCAGTTCCGGCCGTAAACATGTTCTGTAATGG  660
                  ************************************************************

BoF3'H-SN60       AAAGAAAATAAATCAATAATCATCGTGTATCCGGTATTAAAAATTAATGATTATACGAAG  720
NC_027756.1       AAAGAAAATAAATCAATAATCATCGTGTATCCGGTATTAAAAATTAATGATTATACGAAG  720
BoF3'H-BT126      AAAGAAAATAAATCAATAATCATCGTGTATCCGGTATTAAAAATTAATGATTATACGAAG  720
                  ************************************************************

BoF3'H-SN60       TACGAACCAATAGCAAGGCTTTGATCTCAGT----------------------------  751
NC_027756.1       TACGAACCAATAGCAAGGCTTTGATCTCAGTATCCGTTAGAGTTCCACCCTCACCGTCAA  780
BoF3'H-BT126      TACGAACCAATAGCAAGGCTTTGATCTCAGTATCCGTTAGAGTTCCACCCTCACCGTCAA  780
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BoF3'H-SN60       --------------AGCGAGATTAAAGTGCTAAGCATGTCCGTGTGCTTTTGATCCTGGC  797
NC_027756.1       AATCAGTTCCCTTAAGCGAGATTAAAGTGCTAAGCATGTCCGTGTGCTTTTGATCCTGGC  840
BoF3'H-BT126      AATCAGTTCCCTTAAGCGAGATTAAAGTGCTAAGCATGTCCGTGTGCTTTTGATCCTGGC  840
                                ********************************************

BoF3'H-SN60       CGTTCTTCATCGTCTCGTGCTCTTCCAAGATCGACGATAGAAAAGCGTCGAACCTCTTGT  857
NC_027756.1       CGTTCTTCATCGTCTCGTGCTCTTCCAAGATCGACGATAGAAAAGCGTCGAACCTCTTGT  900
BoF3'H-BT126      CGTTCTTCATCGTCTCGTGCTCTTCCAAGATCGACGATAGAAAAGCGTCGAACCTCTTGT  900
                  ************************************************************

BoF3'H-SN60       GTAGACGTTTCATTTTACCAGCGACGCCTTGTAAATCTAAACAATCAAGTGCGGGCACGA  917
NC_027756.1       GTAGACGTTTCATTTTACCAGCGACGCCTTGTAAATCTAAACAATCAAGTGCGGGCACGA  960
BoF3'H-BT126      GTAGACGTTTCATTTTACCAGCGACGCCTTGTAAATCTAAACAATCAAGTGCGGGCACGA  960
                  ************************************************************

BoF3'H-SN60       AATCTCCGATGTTGAATACTCCGGCGAGAGCCATCATTTCTGTGACCATTGATCGAAACT  977
NC_027756.1       AATCTCCGATGTTGAATACTCCGGCGAGAGCCATCATTTCTGTGACCATTGATCGAAACT 1020
BoF3'H-BT126      AATCTCCGATGTTGAATACTCCGGCGAGAGCCATCATTTCTGTGACCATTGATCGAAACT 1020
                  ************************************************************

BoF3'H-SN60       CCTCCGCTTTGTGATCGGCATCGGCGCCGAACAGTCGCCGTCCGATCATCTCTCGTCCAA 1037
NC_027756.1       CCTCCGCTTTGTGATCGGCATCGGCGCCGAACAGTCGCCGTCCGATCATCTCTCGTCCAA 1080
BoF3'H-BT126      CCTCCGCTTTGTGATCGGCATCGGCGCCGAACAGTCGCCGTCCGATCATCTCTCGTCCAA 1080
                  ************************************************************

BoF3'H-SN60       GGGCGTTGAGTACGC       1052
NC_027756.1       GGGCGTTGAGTACGC       1095
BoF3'H-BT126      GGGCGTTGAGTACGC       1095
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Figure 2. Sequence alignment of *F3′H* candidate genes.

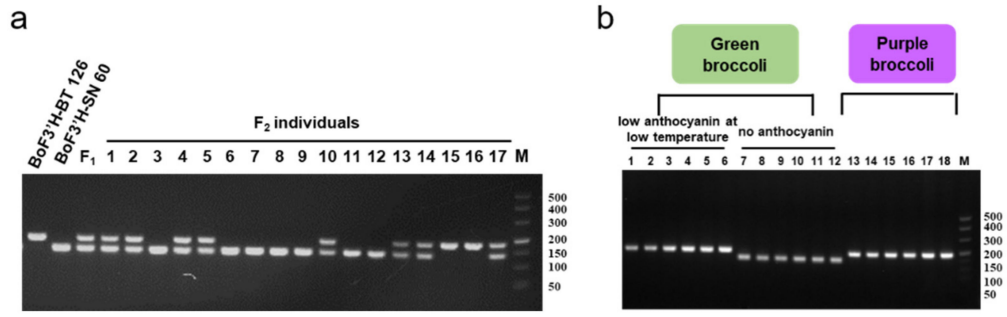

Figure 3. Results from the development of the primer PCR to label the F1, F2, and inbred lines. (**a**) Numbers 3, 6, 7, 8, 9, 11, and 12 represent individual F2 plants with green flower bulbs; numbers 1, 2, 4, 5, 10, 13, 14, and 17 represent individual F2 plants with purple flower bulbs; (**b**) numbers 1–5 represent inbred green broccoli lines with low anthocyanin accumulation at low temperatures; numbers 6–12 represent inbred green broccoli lines without anthocyanin accumulation; numbers 13–18 represent inbred purple broccoli lines. M, marker.

Examination of the *Pur7.1* candidates in the two parental lines resulted in the detection of six single-nucleotide polymorphism (SNP) differences. Two KASP primers were designed by selecting SNP sites in the *Pur7.1* candidate interval (C7:3 6.78 MB–43.70 MB) and used to genotype the 720 F2 individuals. The results showed that one KASP marker (Pur7.1-K1) at the 42,567,442 bp position on chromosome C07 had a C/T mutation in this SNP base (C for BT126 and T for SN60). The purple F2 individuals could be classified into three categories: deep purple, purple, and light purple. The results proved that green individuals had CC, TT, and CT genotype, whereas the deep purple, purple, and light purple individuals had CC, CT, and TT genotypes, which fit a 1:2:1 Mendelian ratio in the F2 generation (Figure 4). In addition, the *Pur7.1*-linked *Pur7.1*-K1 marker was detected in 63 inbred lines. The allele with mutation "C" was present in 91.1% of the purple lines and the allele with mutation "T" was present in 93.2% of the light purple lines.

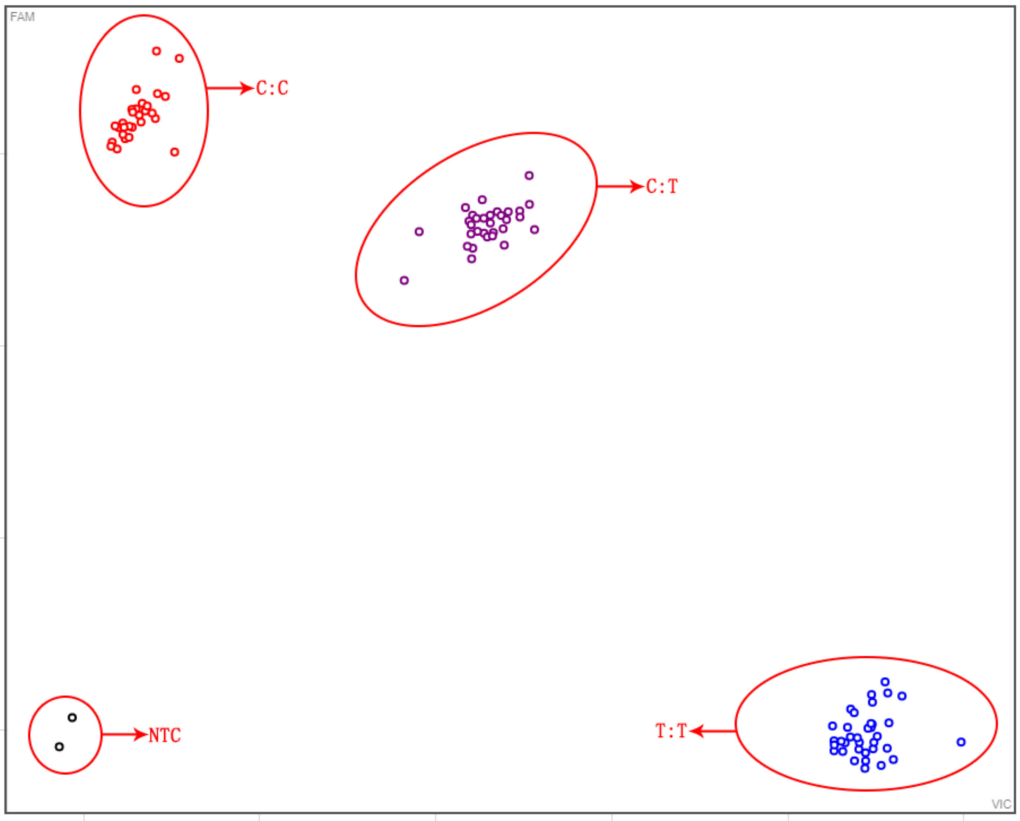

Figure 4. KASP genotyping of the F2 population with primers *Pur7.1*-K1. NTC, no template control.

3.2. Crossbreeding and Backcross Transfer

In the green broccoli selection, SN60 was used as the donor parent to transfer the *f3′h* gene in the background of the LF02. In the purple broccoli selection, BT126 was used as the donor parent to introgress the *Pur7.1* gene to the recurrent parent LF02. SN60 was crossed with LF02 to obtain 15 F1 plants, and BT126 was crossed with LF02 to obtain 12 F1 plants, which were confirmed to obtain 14 and 12 true hybrids, respectively, using DNA markers. The F1 hybrids were backcrossed with LF02 to generate 132 and 146 BC1F1 plants, respectively. All BC1F1 plants were screened with foreground and background selection. The foreground selection using the marker *F3′H*-F/R revealed 61 plants to be heterozygotes, while the foreground selection using the marker *Pur7.1*-K1 revealed 65 plants to be heterozygotes. These 61 and 65 BC1F1 plants were further subjected to background selection with SSR markers, and seven and six BC1F1 plants with 60–70% genome recovery were used for the second round of backcrossing to obtain 83 and 96 BC2F1 plants, respectively. As a result of the foreground selection, 37 and 44 plants were selected

as heterozygotes, respectively. All these plants were subjected to background selection, and the background recovery estimation ranged from 73% to 86% in all the plants. Among these, the ten plants with the highest genome recovery rates were used for the third round of backcrossing, obtaining 93 and 146 BC3F1 plants, respectively. Molecular markers were used to identify the *F3′H* gene and *Pur7.1* gene in the BC3F1 isolate population singletons, and 45 and 63 plants were heterozygotes, respectively. Background selection was performed using SSR markers, and the background recovery rate ranged from 81% to 93%. The top 15 plants with >70% background recovery were screened, and 161 and 152 BC3F2 plants were obtained by self-crossing, respectively. Using foreground genome selection at the seedling stage, 34 homozygotes with the *f3′h/f3′h* genotype and 28 homozygotes with the *Pur7.1/Pur7.1* genotype were selected for background selection, with a background recovery rate of more than 85%. Self-crosses were performed to obtain 301 and 416 BC3F3 plants, respectively. Finally, three purple broccoli lines and two green strains with recovery rates of >90% were selected.

3.3. Screening of Foreground and Background Selection Primers

One hundred thirty-three previously published single-locus SSR markers covering the whole genome [49,50] were selected for the genetic background analysis of high-anthocyanin strains, first in the SN60 and LF02 lines and then in the BT126 and "LF02 lines, comparing the two groups for parental polymorphism screening. Finally, 34 and 42 SSR markers showing polymorphisms between the two groups were selected for genetic background assessment.

In the SN60 backcross population, the F3′H marker in the foreground selection of the 71 green individuals without anthocyanin accumulation (Figure 5) resulted in the highest recovery rates at >90% for 20PBL23, 20PBL62, and 20PBL103 (Figure 6a). For the BT126 backcross population, the *Pur7.1*-K marker in 89 purple individuals with large amounts of anthocyanin accumulation, among which were 20GBL47 and 20GBL128, demonstrated the highest recovery rates of 91.3 and 92.1%, respectively (Figure 6b).

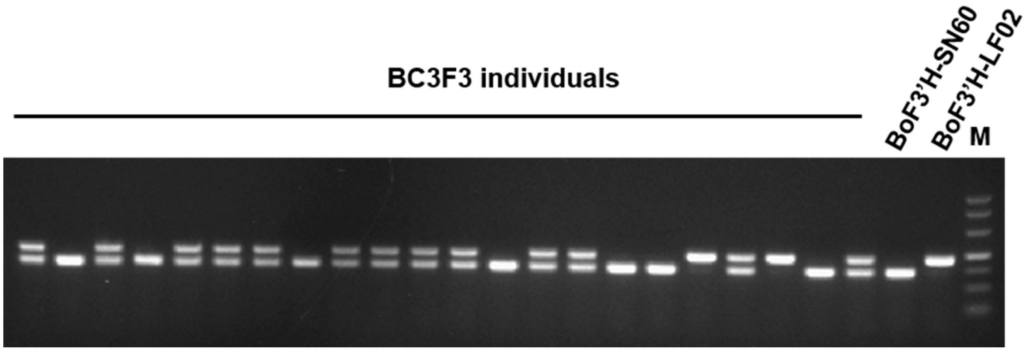

Figure 5. Foreground selection of primer *F3′H* for isolation in LF02, SN60, and BC3F3 populations.

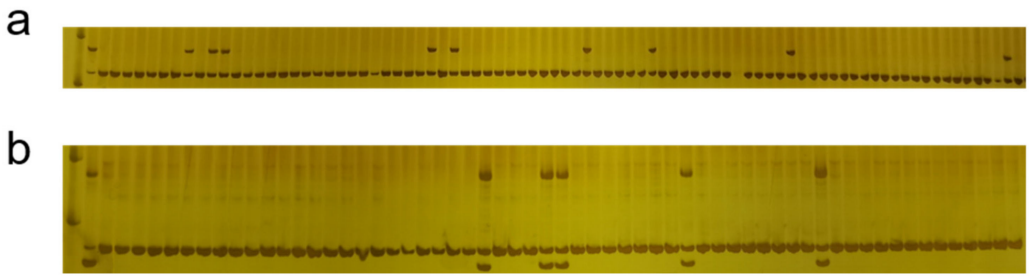

Figure 6. Background selection of primer BoGMS0771 for isolation in LF02, SN60, and BC3F3 populations (**a**) and of primer BoGMS0576 for isolation in LF02, BT126, and BC3F2 populations (**b**).

3.4. Agronomic Traits and Yield Performance in the Field

Purple broccoli pure lines and green broccoli lines with excellent agronomic traits were selected and planted in plots for *F3′H* and *Pur7.1* gene identification and determination of major agronomic traits, such as plant height, plant spread, head height, head diameter, head shape, head color, anthocyanin content, stalk diameter, glucoraphanin content, and head weight. The mean anthocyanin contents of the three parents, BT126, SN60, and LF02, were 63, 1.5, and 12 mg/100 g FW, and the selected purple progeny lines had 46–71 mg/100 g FW, with one exceeding that of their donor parent BT126. In contrast, the mean anthocyanin contents of the two selected green progeny lines without purple-green heads at low temperature were 1.3 and 1.7 mg/100 g FW. The mean head weights were 400, 450.2, and 620 g, respectively, whereas the selected progeny lines had mean head weights of 523.3–617.3 g, which is a desirable range for breeding traits. The mean head diameters for the three parents were 13.85, 12.25, and 15.9 cm, respectively, and the head diameters in the selected progeny lines ranged from 14.37 to 15.45. The mean head lengths for each parent were 6.36, 7.03, and 10.72 cm. Their selected plants had head lengths mostly between 8.48 and 10.2 cm. The mean glucoraphanin contents for the three parents were 8.75, 3.24, and 7.32, respectively, while the mean glucoraphanin content for their selected progeny lines mainly ranged from 4.65 to 7.11 μmol/g DW. The head shapes for the three parents were flat, round, and round, respectively, while the selected progeny lines had round or semi-round head shapes, which were more attractive than their donor parents (Table 3).

The results showed that the anthocyanin contents of the three new purple broccoli lines, 20PBL23, 20PBL62, and 20PBL103, and the two new green broccoli lines, 20GBL47 and 20GBL128, were significantly different from those of the LF02, while the main agronomic traits were not significantly different from those of the LF02 (Figure S1). Their agronomic traits were similar or superior to their elite parents, providing more options for broccoli breeding.

Table 3. Characterization of the best BC2F3 lines.

Parents and Lines	Growth Period (d)	Plant Height (cm)	Plant Spread (cm)	Head Length (cm)	Head Diameter (cm)	Head Shape	Stalk Diameter (cm)	Anthocyanin Content (mg/100 g FW)	Head Color	Glucoraphanin Content (μmol/g DW)	Head Weight (g)
Purple broccoli											
BT126	90–100	63.2	82.03	6.36	13.85	Flat	4.13	63	Purple	8.75	400.0
20PBL23	90–100	62.4 *	90 *	8.48 *	14.62 *	Round	4.63 *	46 **	Purple	7.11	523.3
20PBL62	90–100	68.5	78 *	10.2	15.45	Round	5.1	57 **	Purple	5.26	617.3
20PBL103	85–95	66.35	84.55 *	9.11	14.37 *	Semi-round	4.62 *	71 **	Purple	6.39	592.2
Green broccoli											
SN60	70–75	79.45	79.3	7.03	12.25	Round	4.34	1.5	Light green	3.24	450.2
LF02	85–90	70.7	67.01	10.72	15.9	Round	5.1	12	Green-purple	7.32	620.0
20GBL47	70–80	70.67	68	9.56	14.5 *	Round	5.14	1.3 **	Green	6.02	600.4
20GBL128	75–85	76.3	73.32 *	9.74	15.35	Round	4.9	1.7 **	Dark green	4.65 *	571.0

* indicates BC2F3 lines with LF02 showing a significant difference ($p < 0.05$), ** indicates BC2F3 lines with LF02 showing a highly significant difference ($p < 0.01$).

4. Discussion

Anthocyanin-rich vegetables and fruits appear as red, blue, black, or purple and provide humans not only with essential nutrients but also with antioxidants that are beneficial to health [52,53]. Many in vitro experimental studies have confirmed that purified anthocyanins or anthocyanin-rich extracts are effective in preventing liver injuries, significantly lowering blood pressure, improving eyesight, inhibiting mutations caused by cooked-food mutagens, and suppressing the proliferation of human cancer cells and that they possess strong anti-inflammatory and antimicrobial activities [54]. Some types of traditional green broccoli produce low amounts of anthocyanins in flower buds under low winter temperatures, which reduces their market value and planted area [43].

In our multi-year field tests, LF02, an elite inbred line of green broccoli with green-purple color in cold weather, was found to be excellent broccoli breeding parent material due to its superior seed quality and high yield potential. SN60 is an inbred green broccoli line without anthocyanin accumulation and lower productivity. As is well-known, head yield and head color are important traits in green broccoli breeding. In the present study, SN60 was crossed with LF02 and unfavorable traits were significantly improved. Richness in anthocyanins has attracted more and more attention as an important trait in purple broccoli breeding. BT126 is an anthocyanin-rich, inbred line with a flat, small head shape. In the present study, BT126 was crossed with LF02, and the anthocyanin content and head appearance were significantly improved.

Purple plant breeding can be accelerated through the use of gene functional markers, which can be used to quickly eliminate heterozygous genotypes, select individuals at the seedling stage, and promote early generation stabilization [55]. They are especially efficient in cases when the character of the light purple pigmentation in green broccoli depends on cold-stress environments, as the conventional methods used for selection in breeding programs are always slow and unreliable [42,56]. A previous study indicated that the purple head trait in broccoli is controlled by two complementary dominant genes. In this study, we developed InDel molecular markers based on the insertion/deletion (InDel) variant loci of the *F3′H* gene, which was the key gene controlling anthocyanin biosynthesis, and a tightly linked KASP molecular marker based on the candidate genes of *Pur7.1* on chromosome C7, which was the key gene controlling the purple grades. In radish, the insertion of a Gypsy/Ty3 retrotransposon in the first exon of the *F3′H* gene or a 507 bp fragment inserted into the second exon of *RsF3′H* introduced a premature termination codon, which resulted in a lack of functional *F3′H* [34,57]. This suggests that a lack of *F3′H* may be responsible for the red or purple coloration.

The breeding strategy for the development of the non-anthocyanin and anthocyanin-rich cultivars was based on the transfer of the structural genes *f3′h* and *Pur7.1* controlling anthocyanin biosynthesis on chromosomes 9 and 7, respectively, into the background of elite LF02 breeding lines. The critical point of the strategy was the selection of the homozygous plants for the *f3′h* or *Pur7.1* genes during the BC3F2 generation after crossing the donors with the recurrent cultivars/lines, and the remaining progeny were reduced by 75% according to the analyses of the *F3′H* and *Pur7.1* genes, respectively. In this study, foreground selection, using the closely linked molecular markers *F3′H*-F/R and *Pur7.1*-K1 from the donor parents SN60 and BT126, respectively, and background selection, using microsatellite markers in the genetic background of the recurrent parental genome [58], provided practical tools to ensure accurate transfer of non-anthocyanin and anthocyanin-rich traits in early generations while recovering the majority of the genome of the recurrent parent LF02 in the advanced backcross generations.

The genetic background recovery percentage is an important issue considered in backcross breeding, and it is difficult to accurately reflect the genetic similarity between improved selected lines and recurrent parents in traditional agronomic character investigation [59,60]. In this study, in order to retain the elite traits of the recurrent parent, genotype comparison between improved selected lines and recurrent parents was conducted using SSR markers to evaluate the background recovery rate in backcross breeding. Individuals

with high background recovery percentages were selected to participate in the next round of backcrossing, and the background recovery rate was more than 85% in BC3F2 individuals. Using MAS combined with backcross breeding, the green or purple trait improvements in the existing excellent broccoli varieties was completed within 3–4 years. Many downy mildew-resistant, heat-stress-resistant, and clubroot-resistant broccoli varieties have been successfully developed using MAS [61,62]. Many cultivars producing obvious purple heads have been selected for their rich anthocyanin contents, such as "Purple Sprouting Early" [63]. As a functional and nutrient-rich food, one of the most outstanding characteristics of purple sprouting broccoli is its winter hardiness. The Organic Seed Alliance evaluated "Red Head", "Red Fire", and "OSA 206" as the most winter-hardy purple sprouting broccoli varieties, with survival rates ranging from 44% to 78% at temperatures down to −10 °C in 2014 and 2015.

Green broccoli lines developed using SN60 as the donor parent appeared more attractive with completely green heads. This color trait could be associated with a loss of function of the *F3′H* gene affecting the anthocyanin biosynthesis. The purple lines derived from BT126 had significantly higher productivity, but the anthocyanin contents were not significantly different between the 20PBLs and BT126 lines. The 20PBL23, 20PBL62, 20PBL103, 20GBL47, and 20GBL128 lines produced higher head lengths, head diameters, and head weights, which are the key components of productivity. The materials obtained from the backcross breeding in this study also provided the parent materials for the breeding of next-generation hybrids.

5. Conclusions

In this study, two novel markers were developed to create green and purple broccoli cultivars, respectively, using QTL-seq analysis. Through MAS, three purple lines and two green lines were ultimately selected based on their desirable agronomic traits. The five lines showed higher head yield-related traits and quality, which makes them more attractive to consumers. These lines can be used as genetic resources with superior market value in future broccoli breeding efforts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12126267/s1, Figure S1: Phenotype screening of marker-assisted backcrossed lines along with parents. (a) represents the donor parent BT126; (b) represents the donor parent SN60; (c) represents the recurrent parent LF02; (d–f) represent the anthocyanin-rich broccoli cultivars/lines 20PBL23, 20PBL62, and 20PBL103; (g,h) represent the non-anthocyanin broccoli cultivars/lines 20GBL47 and 20GBL128. Table S1: SSR markers used for background selection in the marker-assisted breeding programs to breed for green broccoli lines, Table S2: SSR markers used for background selection in the marker-assisted breeding programs to breed for purple broccoli lines.

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