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Identification of two tandem genes associated with primary rosette branching in flowering Chinese cabbage

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Branching is an important agronomic trait determining plant architecture and yield; however, the molecular mechanisms underlying branching in the stalk vegetable, flowering Chinese cabbage, remain unclear. The present study identified two tandem genes responsible for primary rosette branching in flowering Chinese cabbage by GradedPool-Seq (GPS) combined with Kompetitive Allele Specific PCR (KASP) genotyping. A 900 kb candidate region was mapped in the 28.0–28.9 Mb interval of chromosome A07 through whole-genome sequencing of three graded-pool samples from the F₂ population derived by crossing the branching and non-branching lines. KASP genotyping narrowed the candidate region to 24.6 kb. Two tandem genes, *BraA07g041560.3C* and *BraA07g041570.3C*, homologous to *AT1G78440* encoding GA2ox1 oxidase, were identified as the candidate genes. The *BraA07g041560.3C* sequence was identical between the branching and non-branching lines, but *BraA07g041570.3C* had a synonymous single nucleotide polymorphic (SNP) mutation in the first exon (290th bp, A to G). In addition, an ERE *cis*-regulatory element was absent in the promoter of *BraA07g041560.3C*, and an MYB *cis*-regulatory element in the promoter of *BraA07g041570.3C* in the branching line. Gibberellic acid (GA₃) treatment decreased the primary rosette branch number in the branching line, indicating the significant role of GA in regulating branching in flowering Chinese cabbage. These results provide valuable information for revealing the regulatory mechanisms of branching and contributing to the breeding programs of developing high-yielding species in flowering Chinese cabbage.

KEYWORDS

flowering Chinese cabbage, primary rosette branches, gene identification, tandem genes, GA2ox1 oxidase

Introduction

Brassica rapa is one of the most important *Brassica* species with a long cultivation history, has diverse and distinct morphological traits. Flowering Chinese cabbage [*Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *parachinensis* (L.H. Bailey)] is that bolts readily (Wang and Kole et al., 2015). It is a stalk vegetable, and the stems with flower buds and leaves are consumed after cooking, especially in southern and central China and southeastern Asian countries. Typically, only one flowering stalk (main stem, without primary rosette branches) can be harvested from the common varieties, while several stalks are produced (12 stalks, primary rosette branches) on the local flowering Chinese cabbage variety named Zengcheng (Niu et al., 2019). Thus, the primary rosette branching trait influences plant architecture and yield in flowering Chinese cabbage. Therefore, identifying genes is important to develop varieties with multiple branches and meet the growing demand for flowering Chinese cabbage.

The activities of meristems, including apical, axillary and inflorescence meristems, basically determine the branching trait (Wang et al., 2018a). In flowering Chinese cabbage, the shoot apical meristem (SAM) turns into an inflorescence meristem that produces flowers directly or flower-bearing shoots after transitioning from the vegetative to the reproductive phase. The primary rosette branches grow from the axillary buds subtended by rosette leaves. Then, the secondary inflorescence branches grow at the axils of the cauline leaves on the elongated internodes of the main inflorescence stem, like that in *Arabidopsis thaliana* (Wang et al., 2018a; Fichtner et al., 2021). Studies in rice, *Arabidopsis*, and several other species characterizing the regulatory components of tiller or branch development have improved our understanding of branching (Wang and Li, 2011; Fichtner et al., 2021).

Typically, branching is a quantitative trait controlled by multiple genes and is susceptible to the environment (Ehrenreich et al., 2007; Kebrom et al., 2013) and plant hormones (Wang and Li, 2011). The hormonal control of bud outgrowth is complex and not yet fully understood. Auxin (Morris et al., 2005), cytokinin (Xu et al., 2015), strigolactone (De Jong et al., 2014), gibberellin (Martínez-Bello et al., 2015), abscisic acid (Holalu et al., 2020), and their interactions (Chen et al., 2013; Cao et al., 2017) have been reported to affect branching. Among these, gibberellin significantly influences the growth of branches and main stems. Okada et al. (2020) found that gibberellic acid (GA₃) application increased the number of lateral branches on apple trees. Meanwhile, the exogenous spraying of GA₃ rescued the dwarf phenotype of the legume's *msd1-2* (multi-seeded1-2) mutant (Li et al., 2021). Researchers have identified a few genes controlling branching in *Brassica juncea*, *Brassica napus*, non-heading Chinese cabbage, and purple flowering Chinese cabbage (Li, 2018; Muntha et al., 2019; Li et al., 2020a; Li et al., 2020b). However, branching in flowering Chinese cabbage has not been fully clarified.

Researchers recently proposed a new quantitative trait mapping technique called GradedPool-Seq (GPS) for rapidly mapping the quantitative trait loci (QTL), it can rapidly identify QTLs for complex traits comparing conditional methods (Wang et al., 2019). GPS with high-throughput sequencing scores and assigns the F₂ populations derived from a distant cross of parental lines exhibiting contrasting phenotypes into three or more graded groups based on phenotypic values. GPS has been successfully applied to dissect heterotic genes of thousand-grain weight, plant height, heading date, flag leaf angle, and tiller angle in rice. Moreover, the candidate intervals identified by the GPS method is consistent with the mapping interval obtained by the traditional method in rice (Wang et al., 2019).

Therefore, the present study used GPS with Kompetitive Allele Specific PCR (KASP) genotyping to map and identify the candidate genes associated with primary rosette branches in flowering Chinese cabbage. We further analyzed the similarities and variations in the full length and promoters of the candidate genes between the lines with different branching phenotypes. Finally, we investigated the role of GA₃ in regulating the development of multiple primary rosette branches in flowering Chinese cabbage. The findings of our study will provide novel insights into the mechanisms of branching and lay a foundation for developing flowering Chinese cabbage cultivars with multiple primary rosette branches.

Materials and methods

Plant materials and growing conditions

'CX020' (Branching line), a doubled haploid (DH) line derived by microspore culture from Zengcheng flowering Chinese cabbage [*Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *parachinensis* (L.H. Bailey)] with multiple primary rosette branches at harvest, and 'CX010' (Non-branching line), a DH line derived from Guangdong flowering Chinese cabbage with one flower-bearing shoot and no primary rosette branch, were used as parents in this study. These DH parents were crossed to generate the F₁ and F₂ populations for the phenotypic and genetic analyses. These DH parents exhibited stable inheritance after multiple seasons of planting. All the plants ('CX010', 'CX020', and F₂ population) were grown at the Shenyang Agricultural University experimental base (Shenyang, China, 41°82'N, 123°24'E) in 2019. The plants were sown on August 1st, and the number of primary rosette branches was analyzed on September 15th. The primary rosette branches were analyzed on the axillary branches subtended by rosette leaves 5 cm away from the cotyledonary node.

GradedPool-Seq

Three types of pools (50 plants each), including 'multiple primary rosette branching (12–15 branches)', 'less primary

rosette branching (1–3 branches) and ‘moderate primary rosette branching (7–8 branches)’ plants were selected *via* phenotypic analysis from the F₂ population, consisting of 1050 individuals, were used for GPS analysis with the two parents. Young and fresh leaves of the parents and the selected F₂ individuals were harvested separately for total genomic DNA extraction using the Plant Genomic DNA Kit (Tiangen, Beijing, China), following the manufacturer’s instructions. The quantity and quality of the DNA were ensured using spectrophotometric analysis and 2% agarose gel electrophoresis. The DNA samples were quantified using a Qubit fluorometer and pooled at equimolar concentrations to generate the ‘multiple branching’, ‘less branching’ and ‘moderate branching’ pools separately. Pair-end sequencing (PEN150) on an Illumina Novaseq system (Illumina, USA) was performed following the standard protocol. The sequencing data from each hybrid pool was merged and aligned to the reference genome (http://brassicadb.org/brad/datasets/pub/Genomes/Brassica_rapa/V3.0/) to calculate the depth of each variant. After filtering the variants with low quality and depth using the default parameters, Redit analysis was performed to calculate the p-value for each variant. The sliding window size was set to 0.2 Mb, the threshold p-value to 10⁻⁸, and the candidate regions to peak intervals to reduce background noise and identify the significant variants.

KASP genotyping

KASP was performed on a high-throughput Intelliquibe genotyping platform for genotyping of the F₂ individuals. DNA was extracted from 150 F₂ individuals and the parents using the CTAB method. The DNA concentration and quality were assessed on a BioDropuLite microanalyzer (BioDrop, Britain), and the samples were diluted to a suitable concentration (5–10 ng/μL). KASP primers (Supplementary Table S1) were designed for 19 SNP loci (Supplementary Table S2) using Primer Premier 5.0 (Singh et al., 1998), and KASP assays were conducted in a 384-well plate format on a Hydrocycler (LGC, Middlesex, UK) using the following PCR protocol: 94 °C for 15 min; 94 °C for 20 s, 61 °C for 60 s (-1 °C/cycle, 10 cycles in total), and 94 °C for 20 s; 55 °C for 60 s (26 cycles). The components of the KASP reaction mixture are shown in Supplementary Table S3. The fluorescence signal generated was measured on an IntelliQube (LGC, Middlesex, UK). Finally, based on the KASP genotypic data and phenotypic data of the F₂ individuals, the QTL IciMapping software v4.2 (Liu et al., 2019) generated the linkage map to obtain the QTL and further narrow down the candidate interval.

Candidate gene analysis

Gene annotation information of the target region was obtained from the *Brassica* database (<http://brassicadb.org/brad/>

[index.php](http://brassicadb.org/brad/)) and the *Arabidopsis* database (<https://www.arabidopsis.org/>). The candidate genes’ full-length sequence and a 2000 bp long promoter sequence were amplified with the specific primers using PCR, and the amplicons were purified using a Gel Extraction Kit (CWBI, Beijing, China). The purified products were introduced into the pGEM[®]-T Easy Vector (Promega, USA) and transformed into Top10 competent cells (CWBI, Beijing, China). The colonies were sequenced at Sangon Biotech (Shanghai, China), and the sequences were aligned using DNAMAN 6.0 (Lynnon Biosoft, Canada). Meanwhile, PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to predict the *cis*-regulatory elements in the promoter regions of the candidate genes.

Expression analysis of the candidate genes

Total RNA was extracted from the whole roots, rosette stems, tender cauline leaves, flowers of top inflorescences and shoot tips of the parents. The rosette stem of CX020 at five different stages (every ten days), the first sample was taken when CX020 was in the fourth euphylla stage. The RNA was reverse transcribed using the FastKing RT Kit (Tiangen, Beijing). Real-time quantitative reverse transcription PCR (qRT-PCR) was carried out to determine the expression levels of the candidate genes using cDNA as the template with the SYBR Green PCR Master Mix on QuantStudio[™] 6 Flex (Applied Biosystems, USA), maintaining three biological replicates per sample. The *Actin* gene was used as an internal reference control. The primers used for qRT-PCR were as follows: *BraA07g041560.3C* (F: 5′-TGGAGATGATTACTGATGGGTTA-3′; R: 5′-ATT TTCGTGGATGAGAGGGC-3′); *BraA07g041570.3C* (F: 5′-TCC TGGATTTCTGTCCCTTC-3′; R: 5′-ACCCTATGCTTC ACGCTTTT-3′); *Actin* (F: 5′-ATCTACGAGGGTTATGCT-3′; R: 5′-CCACTGAGGACGATGTTT -3′). The relative gene expression levels were calculated following the 2^{-ΔΔCt} method.

Exogenous GA₃ and PAC treatment

We predicted that the genes affecting branching might be related to GA₃. An experiment was carried out by spraying GA₃ (750 mg/L) on the multiple primary rosette branching parent line CX020, and spraying Paclobutrazol (PAC, 0.3×10⁻³ mg/L on the non-primary rosette branching parent line CX010, using water as a control. The plants were sown on August 1st in green house at Shenyang Agricultural University under long-day conditions and GA₃ treatment was carried out on the fourth euphylla stage (August 15th); spraying was carried out every two days until phenotypes of fewer branches appeared. Twenty plants were maintained per treatment, using three biological

replicates. The primary rosette branches number was recorded when the branching phenotype is obvious.

Results

Phenotypic characterization of primary rosette branching in flowering Chinese cabbage

During the reproductive growth stage, ‘CX010’ (parent 1, Figure 1A) had only one main stalk but no primary rosette branches on the rosette stem, while ‘CX020’ (parent 2, Figure 1B) had 14 primary rosette branches that contributed to the yield. Moreover, significant differences were observed in the number of primary rosette branches between the non-branching line (‘CX010’) and the multiple branching line (‘CX020’). Phenotypic segregation analysis showed that F_1 progeny had a moderate number of primary rosette branches. The number of primary rosette branches in the 1050 F_2 plants ranged from 0 to 15 and showed a normal distribution (Figure 1C). These observations indicated the role of QTLs in controlling the number of primary rosette branching in flowering Chinese cabbage.

Candidate region for primary rosette branching identified by GPS

The sequences obtained from the three pools (12–15 branches, 7–8 branches, and 1–3 branches) were mapped to the reference genome (http://brassicadb.org/brad/datasets/pub/Genomes/Brassica_rapaV3.0/) to estimate the allelic frequencies. Redit analysis was implemented with allelic frequencies from three bulks to calculate p-values for each SNP. The background noise complicated the precise localization of the QTLs.

Subsequently, the statistical noise-reducing strategy narrowed the interval to about 900 kb (28.0–28.9 Mb; significant peak) on chromosome A07, which was identified as the main QTL controlling the primary rosette branching. A few minor peaks also appeared on the other chromosomes that may be minor QTLs (Figure 2A). However, we further focused on the main QTL.

Further mapping of the primary rosette branching gene

A total of 178 genes were identified in the candidate region based on the gene information in the *Brassica* database (Supplementary Table S4). Then, to locate the candidate genes controlling the primary rosette branching in this region, we developed 18 KASP markers according to SNPs in this region. The genotype of 150 F_2 individuals and two parents was detected. Only one QTL was discovered associated with the number of primary rosette branches in the F_2 based on KASP, which accounted for 20.81% of the phenotypic variance. Markers A0716 and A0717, located at the two sides of the candidate gene, were the most closely associated with the candidate gene, and the physical distance between these markers was 24.6 kb, which contained five genes (Figure 2B).

Identification of the candidate genes related to primary rosette branching

Then, all the candidate genes in the candidate region were analyzed based on the *Brassica* database to identify the candidate genes that influence primary rosette branching. To further identify the candidate genes that influence primary rosette branching, five annotated genes in the candidate region were analyzed (Table 1). Among these, two tandem genes,



FIGURE 1
Phenotype of parent lines CX010, CX020 and distribution of the number of primary rosette branches in flowering Chinese cabbage. **(A)** Non-primary rosette branching line (‘CX010’). **(B)** Multiple primary rosette branching line (‘CX020’) Bar = 10 cm. **(C)** Distribution of the number of primary rosette branches in 1050 individuals of F_2 population.

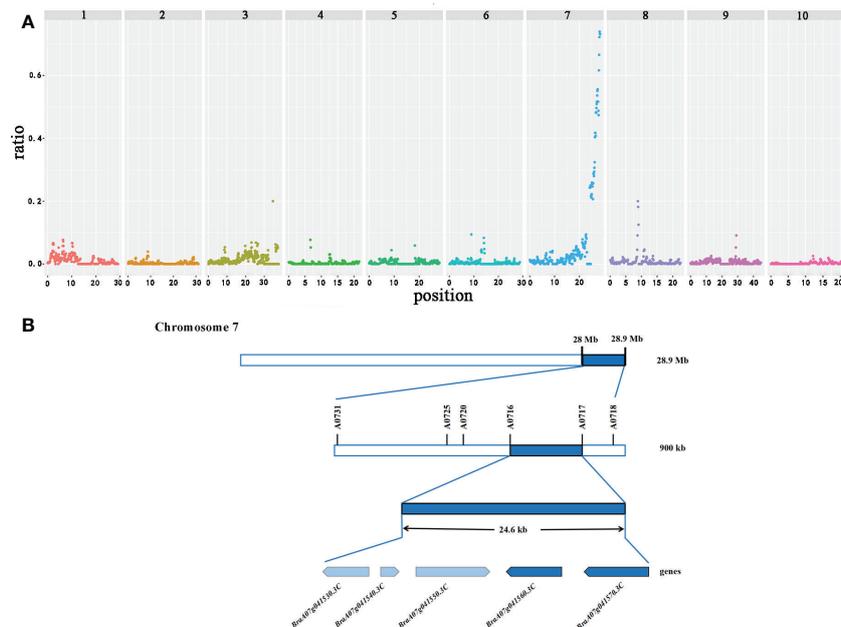


FIGURE 2

Candidate interval and genes identified by GPS on chromosome A07 in flowering Chinese cabbage. (A) The X-axis value is set at a midpoint at each defined genomic interval, and the Y-axis value corresponds to the ratio. (B) Candidate genes identified in the targeted interval.

BraA07g041560.3C and *BraA07g041570.3C*, were found homologous to the *Arabidopsis thaliana* *AT1G78440*, which encodes gibberellin 2-oxidase that acts on C19 gibberellins. In rice, *GA2oxs* influence the number of tillers (Lo et al., 2008). Therefore, we hypothesized that these two tandem genes might be associated with the number of primary rosette branches in flowering Chinese cabbage.

Candidate gene cloning and sequence analysis

These two full-length genes (*BraA07g041560.3C* and *BraA07g041570.3C*) were cloned to analyze the variances between

the parents (Supplementary Figures S1, S2). Sequence analysis revealed that the full-length sequence of *BraA07g041560.3C* was identical in the parents, whereas *BraA07g041570.3C* had a synonymous SNP mutation from A to G at the 290th position (Supplementary Figure S2). The promoter sequence of *BraA07g041560.3C* had a 31 bp deletion in CX020 (Supplementary Figure S3). There were many differences of the promoter sequence in *BraA07g041560.3C* between CX020 and CX010 that resulted in the absence of an ERE *cis*-regulatory element and the position changes of many *cis*-acting elements in CX020 (Supplementary Figure S3; Figure 3A). Compared to CX010, CX020 had an SNP in the promoter of *BraA07g041570.3C*, 103 bp upstream of the translation initiation site, resulting in the absence of an MYB *cis*-regulatory element (Supplementary Figure S4; Figure 3B).

TABLE 1 Annotation of the genes within the mapped region on chromosome A07 in flowering Chinese cabbage.

Gene	Start	End	Gene annotations (BLASTX to <i>Arabidopsis thaliana</i>)	E value
<i>BraA07g041530.3C</i>	28005077	28006447	PGX2 is a cell wall protein that codes for a polygalacturonase.	0.0
<i>BraA07g041540.3C</i>	28013792	28014106	VQ motif-containing protein; (source: Araport11)	1.72 ^{e-51}
<i>BraA07g041550.3C</i>	28018304	28020467	Activates the latent peptidases DA1, DAR1 and DAR2 by mono-ubiquitination at multiple sites. Subsequently, these activated peptidases destabilize various positive regulators of growth.	5.64 ^{e-45}
<i>BraA07g041560.3C</i>	28023878	28025430	Encodes a gibberellin 2-oxidase that acts on C19 gibberellins.	0.0
<i>BraA07g041570.3C</i>	28036581	28038308	Encodes a gibberellin 2-oxidase that acts on C19 gibberellins.	0.0

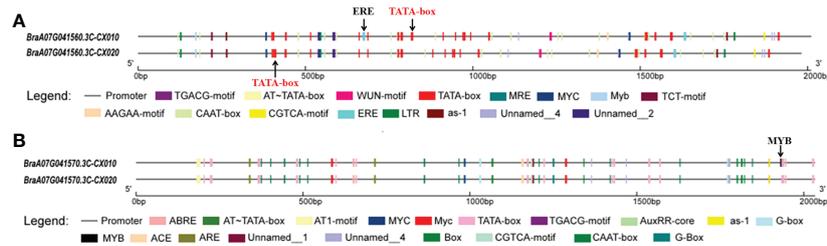


FIGURE 3

The *cis*-regulatory elements analysis of *BraA07g041560.3C* and *BraA07g041570.3C* promoters in parents CX010 and CX020. (A) Analysis of *BraA07g041560.3C* gene promoters. (B) Analysis of *BraA07g041570.3C* gene promoters.

Expression analysis of candidate genes

Further, qRT-PCR was used to detect the relative expression levels of *BraA07g041560.3C* and *BraA07g041570.3C* in CX010 and CX020. The relative expression level of *BraA07g041560.3C* in the stem and flower of CX020 was significantly higher than that in CX010, while that in the leaf and shoot tip of CX020 was markedly lower than that in CX010 (Figure 4A). The relative expression level of *BraA07g041570.3C* in CX020 was substantially higher in root, stem, flower, and shoot tip than that of CX010 but significantly lower in the leaf (Figure 4B). We further analyzed the differences in the expression levels of *BraA07g041560.3C* and *BraA07g041570.3C* in CX020 stem at different stages. The analysis revealed that the *BraA07g041560.3C* expression was the highest at the second stage (Figure 5A). Meanwhile, the *BraA07g041570.3C* expression level at the last stage was significantly different from those at the other stages (Figure 5B).

Phenotypic features after GA₃ and PAC treatment

GA₃ treatment significantly decreased the number of primary rosette branches (Figures 6A, B). On the contrary, after PAC treatment on the non-branching line CX010, the number of primary rosette branches were significantly increased (Figures 6C, D). The number of rosette branches significantly decreased from 10 to 0 after GA₃ treatment (Figure 7A), while the number of rosette branches significantly increased from 0 to 8 after PAC treatment (Figure 7B).

Discussion

Branching is an important trait that determines plant architecture, directly influences yield, and is closely related to

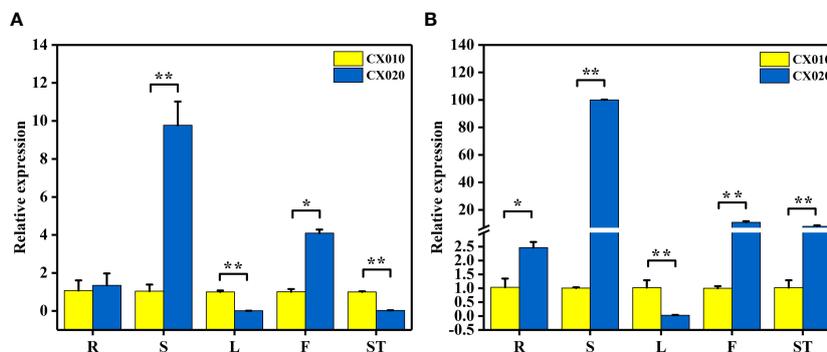


FIGURE 4

Expression levels of *BraA07g041560.3C* and *BraA07g041570.3C* in different tissues of CX010 and CX020 plants based on qRT-PCR. (A) The expression level of *BraA07g041560.3C*. (B) The expression level of *BraA07g041570.3C*. R, whole roots; S, rosette stems; L, tender cauline leaves; F, flowers of top inflorescences; ST, shoot tips. The data shown are the means of three replicates (\pm SD). * and ** indicate significant differences in expression levels at $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test).

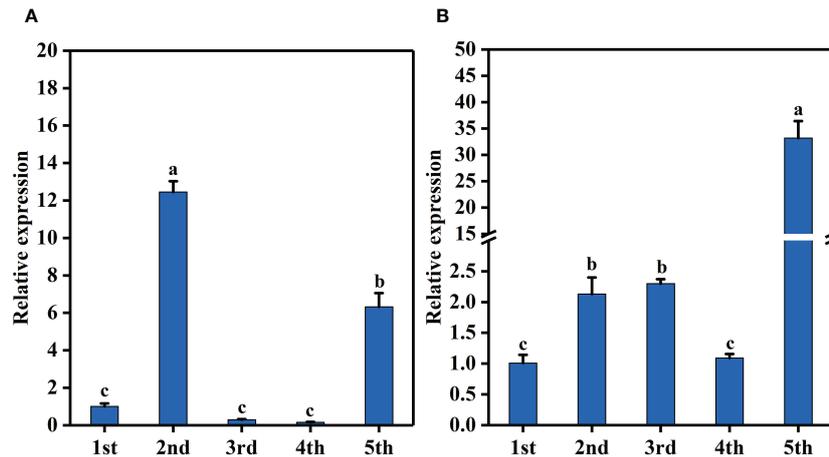


FIGURE 5

Expression levels of *BraA07g041560.3C* and *BraA07g041570.3C* in CX020 rosette stem at different stages based on qRT-PCR. (A) The expression level of *BraA07g041560.3C*. (B) The expression level of *BraA07g041570.3C*. The data shown are the means of three replicates (\pm SD). The different lowercase letters above the means are significantly different at $P=0.05$ level.

environmental adaptation (Teichmann and Muhr, 2015; Mathan et al., 2016). The primary rosette branches that contribute to yield in flowering Chinese cabbage are different from the rosette branches in non-heading Chinese cabbage and tillers in cereal crops. In non-heading Chinese cabbage, basal branches arise from axillary meristems in the leaf axils subtended by rosette leaves during the vegetative stage (Cao et al., 2016). Then the inflorescence branches grow out from the axillary meristems. In cereal crops, tillers arise from non-elongated internodes at the base of the parent shoot during the vegetative growth phase and survive even if the primary shoot dies because tillers generally produce adventitious roots (Kebrom et al., 2013). While, the branches in flowering Chinese cabbage develop only after transitioning to the reproductive phase, the stage at which tiller development ceases in cereal crops, such as wheat, barley, and rice. Studies have identified genes related to branching in *Brassica* crops. Li et al. predicted *BnaA09.ELP6* controls effective

primary cauline branching (arising from the main stem) in *Brassica napus* (Li et al., 2020a). Meanwhile, shoot branching in non-heading Chinese cabbage (*Brassica rapa* ssp. *chinensis* Makino) is controlled by *BrSB9.1* (*Bra007056*), which is homologous to *MOC1* that controls tillering in rice (Li et al., 2020b). *Bra004212*, the homolog of *TCP1*, was identified as the candidate gene for tillering in purple flowering Chinese cabbage (Li, 2018). *PAT1* (Phytochrome A signal transduction 1), which belongs to the GRAS transcription factor family, negatively regulates branching in leafy *Brassica juncea* (Muntha et al., 2019). However, the genes controlling primary rosette branching in Chinese cabbage differ from these reported species. The present study the first time identified for two tandem genes, *BraA07g041560.3C* and *BraA07g041570.3C*, which are homologous to *AT1G78440* encoding *GA2ox1* oxidase, as potential candidate genes responsible for primary rosette branching.

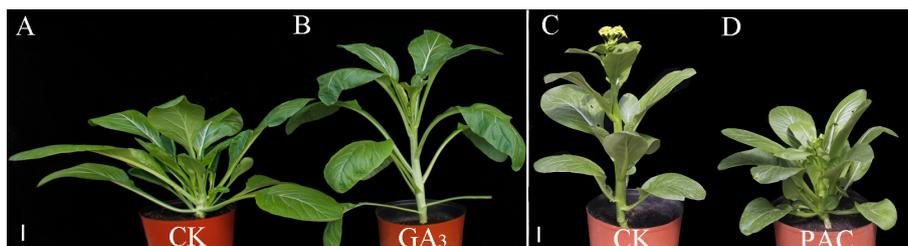


FIGURE 6

Phenotypes of CX020 and CX010 after treatments with exogenous GA_3 and PAC. (A, B) The CK and exogenous GA_3 treatment of CX020. Bar = 8 cm. (C, D) The CK and exogenous PAC treatment of CX010. Bar = 5 cm.

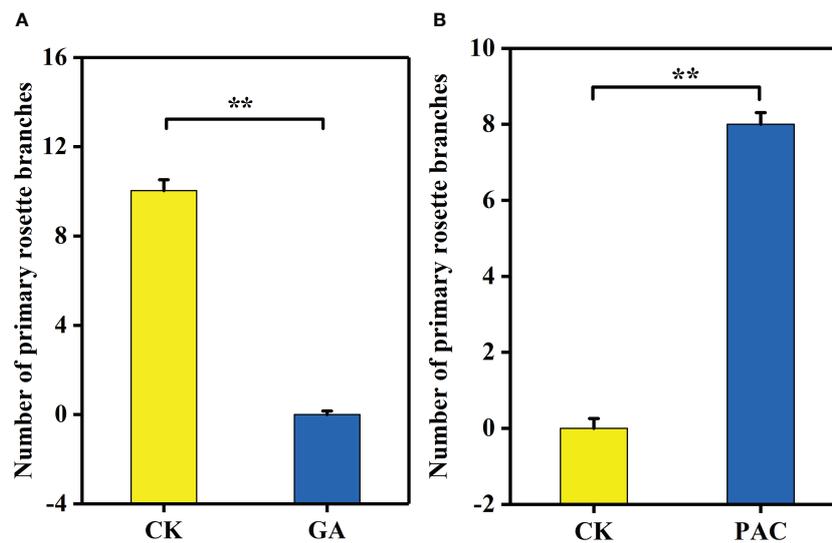


FIGURE 7

The number of primary rosette branches of CX020 and CX010 treated with exogenous GA₃ and PAC. (A) The CK and exogenous GA₃ treatment of CX020. (B) The CK and exogenous PAC treatment of CX010. The data shown are the means of three replicates (± SD). ** indicate significant differences in expression levels at P < 0.01 (Student's t-test).

Traditional QTL mapping using genetic map construction by traditional markers and phenotyping is a reliable approach to isolate genes or QTLs associated with agronomic traits (Li et al., 2020b). Hundreds of SSR (Simple Sequence Repeat), InDels, or SLAF (Specific-Locus Fragment) markers have been developed to construct a primary genetic linkage map based on the population of the F₂ generation, doubled haploid (DH) or recombinant inbred line (RIL) and locate QTL according to the phenotype (Liu et al., 2019). Usually, to narrow down the region and screen for a few candidate genes, a near-isogenic line (NIL) population is needed. However, the process of developing the NIL population is time-consuming and labor-intensive. GPS is a quick and efficient method to ascertain the genomic regions that harbor QTL for complex quantitative traits (Wang et al., 2019). It accelerates gene mapping by sequencing the graded pools; here, only the F₂ population is needed. Bulk-segregant analysis (BSA) also rapidly and effectively locates genes by constructing segregating F₂ populations from parents with significant phenotypic differences and selecting individuals with extreme traits to build a DNA pool for sequencing (Giovannoni et al., 1991; Michelmore et al., 1991; Takagi et al., 2013; Zou et al., 2016). However, the GPS has a higher resolution (~400 kb in rice) (Wang et al., 2019) than BSA (3 Mb) (Wang et al., 2018b; Liu et al., 2019; Yang et al., 2021). GPS identified a QTL controlling fruit size in melons, and traditional QTL mapping validated the results (Lian et al., 2021). Liu et al. (2020) successfully identified two candidate intervals controlling extremely late flowering in rice by GPS. In this work, one major candidate region associated with primary rosette

branching was finally located in a 900 kb region on chromosome A07 based on three graded pools according to the primary rosette branching number of the F₂ population in flowering Chinese cabbage, with 178 genes. To our knowledge, this is the first report on the genetic control of primary rosette branching in flowering Chinese cabbage using GPS.

KASP genotyping based on uniplex SNP is a novel approach to fine map genes using F₂ individuals combined with their phenotype data (Xu et al., 2018; Cheng et al., 2021). Liu et al. (2019) narrowed the candidate region from 3.29 Mb to 790 kb by QTL analysis using KASP markers with 147 F₂ individuals in tomatoes and identified the *Cf-10* gene (*Cladosporium fulvum*). Lei et al. (2020) narrowed the genome interval from 4.17 Mb to 222 kb by 26 KASP markers genotyping with 199 individuals randomly selected from F_{2,3} and identified a major QTL and candidate gene for salt tolerance in rice. In this study, we genotyped 150 F₂ individuals using 18 KASP markers and narrowed down the candidate region from 900 kb to 24.6 kb, with five genes.

GA2 oxidases are dioxygenases encoded by multiple genes and the key enzymes involved in gibberellin metabolism (Hedden and Phillips, 2000). GA2 oxidase typically transforms the bioactive GA1 and GA4 into the inactive catabolic metabolites GA8 and GA34, respectively, reducing the activity of GA and maintaining the balance between bioactive and inactive GA (Claeys et al., 2014). Studies have demonstrated the role of *GA2ox1* in regulating rosette branching in some crops. Higher expression levels of *GA2ox* genes have been correlated to low concentrations of bioactive GAs (Schomburg

et al., 2003; Dijkstra et al., 2008; Zhou et al., 2011). Meanwhile, turfgrass (*Paspalum notatum* Flugge) overexpressing *AtGA2ox1* had significantly lower levels of active GA but more tillers than wild-type plants (Agharkar et al., 2007). Overexpression of the *OsGA2ox* in rice increased tiller number (Lo et al., 2008), consistent with an increased number of tillers observed with the overexpression of *PvGA2ox5* and *PvGA2ox9* in switchgrass (*Panicum virgatum* L.) (Wuddineh et al., 2014). On the other hand, silencing of five *GA2ox* genes in tomatoes significantly increased GA4 content and inhibited lateral branches (Martínez-Bello et al., 2015). Therefore, we speculated that the two tandem genes, *BraA07g041560.3C* and *BraA07g041570.3C*, homologous to the *Arabidopsis thaliana* gene (*AT1G78440*) encoding a gibberellin 2-oxidase, found in the candidate region might be associated with primary rosette branching in flowering Chinese cabbage. While in the candidate region, the other three genes were not found to be related to branching. *BraA07g041530.3C* was the homologs gene of *AtPGX2*, which has been demonstrated regulating root hair development in response to phospho-starved (Zhang et al., 2022). *BraA07g041540.3C* was the homologs gene of *AtVQ10*, which has been shown to interact with *WRKY33* to affect plant sizes at mature stages in *Arabidopsis* (Cheng et al., 2012). *BraA07g041550.3C* was found to be homologous to *Arabidopsis At1g78420* (*DA2*), which encoded RING-type protein with E3 ubiquitin ligase activity and regulated seed size by restricting cell proliferation in the maternal integuments of developing seeds (Xia et al., 2013).

Typically, variations in the promoter regions may lead to changes in gene expression levels (Mito et al., 1996). The expression levels of these two genes were significantly higher in rosette stems of the branching line than in the non-branching line. Detailed analysis revealed differences in the promoter sequences between the parents. The promoter of the *BraA07g041560.3C* gene missed an ERE *cis*-regulatory element in branching line CX020, with a difference in the location of a TATA-box and the *cis*-regulatory element. However, there is no evidence for the role of the ERE *cis*-regulatory element in the formation of branching. Meanwhile, the promoter sequence of the *BraA07g041570.3C* gene lacked an MYB *cis*-regulatory element in CX020. Studies presented associated several genes belonging to the MYB family (*MYB2*, *MYB37*, and *MYB181*) with axillary meristem development and branching in *Arabidopsis* (Guo and Gan, 2011; Keller et al., 2006; Yang et al., 2018). *AtMYB2* protein represses the formation of axillary meristems in response to salt and drought stresses (Jia et al., 2020). Meanwhile, the overexpression of *GmMYB181* in *Arabidopsis* altered the plant architecture, increased lateral branches, and reduced plant height (Yang et al., 2018). Therefore, we hypothesized that the *MYB* gene control primary rosette branching in flowering Chinese cabbage.

GAs are a large group of diterpenoid natural products characterized by tetracyclic 6-5-6-5 ring derived from ent-

gibberellane (MacMillan and Takahashi, 1968; Peters, 2010). The biosynthesis of GA is a complex multi-step process requiring a variety of functional enzymes to catalyze the different intermediates (Wei et al., 2019). GA normally inhibits shoot branching, and plants overexpressing GA catabolic genes and GA-deficient mutants exhibit more shoot branching comparing to the wild-type (Silverstone et al., 1997; Agharkar et al., 2007; Lo et al., 2008). GA regulates internode elongation in rice, where the bioactive GA probably prevents from reaching the nodes below the shoot apex and inhibits internode elongation during the vegetative phase (Sakamoto et al., 2001). In this work, exogenous GA₃ application significantly reduced the rosette branches of flowering Chinese cabbage.

Conclusions

The present study identified two tandem genes, *BraA07g041560.3C* and *BraA07g041570.3C*, homologous to *AT1G78440* encoding GA2ox1 oxidase, as the candidates genes related to primary rosette branching in flowering Chinese cabbage. We detected differences in *cis*-regulatory elements in the promoter sequences of *BraA07g041560.3C* and *BraA07g041570.3C* between the branching and non-branching lines, which indicated the role of genes in regulating branching. These results provide valuable information for revealing the branching regulatory mechanisms in flowering Chinese cabbage. Further studies should investigate and conform the possible function and the promoter activity of *BraA07g041560.3C* and *BraA07g041570.3C* in flowering Chinese cabbage.

Data availability statement

The data presented in the study are deposited in the SRA repository of the National Center for Biotechnology Information, accession number PRJNA908111 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA908111>).

Ethics statement

The authors note that this research was performed and reported in accordance with ethical standards of scientific conduct.

Author contributions

YZ and HF designed the experiments. JG and JL conducted the experiments and wrote the manuscript. JG, JL, and QY performed the data analysis. YZ revised the manuscript. All authors reviewed and approved this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.1083528/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

The full-length of gene *BraA07g041560.3C*.

SUPPLEMENTARY FIGURE 2

The full-length of gene *BraA07g041570.3C*.

SUPPLEMENTARY FIGURE 3

The promoter sequence of *BraA07g041560.3C*.

SUPPLEMENTARY FIGURE 4

The promoter sequence of *BraA07g041570.3C*.

SUPPLEMENTARY TABLE 1

KASP primer sequence used in this study.

SUPPLEMENTARY TABLE 2

SNP site information.

SUPPLEMENTARY TABLE 3

The components of the KASP reaction mixture.

SUPPLEMENTARY TABLE 4

The 178 genes identified in the candidate region based on the gene information in the *Brassica* database.

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