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Genetic mapping of a lobed-leaf gene associated with salt tolerance in *Brassica napus* L.

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ABSTRACT

Lobed leaf is a common trait, which is related with photosynthesis and plant stress resistance in crops. In order to fine map and isolate the lobed-leaf gene in *Brassica napus*, an $F_{2:3}$ population derived from 2205 (salt tolerance) and 1423 (salt sensitive) was constructed, and the quantitative trait locus (QTL) technology was adopted to identify the QTLs related to lobed leaf formation. As a result, one major QTL was identified on LG10, and two intron polymorphic (IP) markers and one sequence characterized amplified region (SCAR) marker were successfully developed in QTL region. The lobed-leaf gene was mapped to a region from 15.701 to 15.817 M on A10. In light of annotations of the genes in candidate region, a leaf morphological development related gene, *Bra009510*, was primary identified as the candidate gene. The full length of the candidate gene was 1390 bp containing three exons and two introns in the two parents. The open reading frame (ORF) was 693 bp and encoded a protein of 229 amino acids. Eight amino acid differences between the two parents in CDS (coding sequences) region were identified. qRT-PCR analysis showed that the expression of the candidate gene was significantly different between the two parents under salt stress. These results showed that the candidate gene might be related to leaf morphological development and abiotic stresses. Our study will lay a solid foundation for studying lobed leaf mechanism in *B. napus* L.

1. Introduction

Leaf shape in *Brassica napus* is classified into round leaf, semi-lobed leaf and lobed leaf [1], which is easily identified by visual inspection. Thus, it can be used as an ideal morphological marker in rapeseed breeding, especially in the identification of hybrid authenticity, purity identification, as ornamental plants and other aspects [2,3]. Moreover, rapeseed leaves are the vegetative organs for photosynthesis, which provide energy for the activities of plants [4]. Studies have shown that appropriate dense planting is an important measure to increase crop yield, and reasonable plant types can effectively increase plant photosynthesis [5–7]. In general, for the plants with larger leaves, when they are densely planted, only the upper leaves can effectively carry out photosynthesis, and the photosynthetic efficiency of the lower leaves is poor, resulting in low yield of the plants [6–8]. On the contrary, because of the small leaf area and no accumulation of leaves for lobed leaves, they can make full use of light, and have higher photosynthetic efficiency and higher plant yield [6,8]. Specific leaf area (SLA) reflects the ability of plant leaves to obtain light resources, and thus, it is positively related to net photosynthetic rate (NPR) and dark respiration [9]. The leaf index influences the exchange of material and energy on the leaf surface by influencing the size of the leaf area [10,11]. In addition, some studies also showed that leaf shape is related to plant stress resistance, such as drought resistance and cold resistance [12–14]. Similar studies have been reported in maize, leaf shape and leaf direction were positively correlated with drought resistance [15]; Leaf area is relatively easy to be measured, which has a significant correlation with drought resistance [16].

Lobed leaf is related to the heterosis utilization, stress resistance and photosynthetic utilization. Studies on lobed leaves have been carried out. In *Brassica*, the studies are mainly focused on the inheritance and gene mapping of lobed leaf. Several studies showed that lobed leaf formation was controlled by one pair of incompletely dominant genes in *B. rapa* and *B. napus*, and the lobed leaf is dominant over the round

Abbreviations: QTL, quantitative trait locus; IP, intron polymorphic; SCAR, sequence characterized amplified region; CDS, coding sequences; ORF, open reading frame; SLA, specific leaf area; NPR, net photosynthetic rate; LS, leaf shape; LA, leaf area; RL, root length; LFW, leaf fresh weight; LDW, leaf dry weight; RDW, root dry weight; EC, electrical conductivity; SOD, superoxide dismutase; SP, soluble protein; SPAD, chlorophyll content; STR, salt tolerance rating; CIM, composite interval mapping; LOD, log likelihood of the odds

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leaf [1,17,18]. Some molecular markers linked to lobed-leaf genes were identified. For example, a SCAR marker linked to the lobed-leaf gene was developed in Chinese cabbage [19]; two QTLs related to the lobed leaf were identified in *B. rapa* [20]; and a lobed-leaf gene was mapped to A10 in *B. napus* [18]. However, a few studies on the molecular mechanism of lobed leaf formation in *B. napus*, especially on the relationship between the lobed-leaf and photosynthesis and stress resistance were reported [18,21]. With the publication of the genome sequence of *B. napus* and *B. rapa*, it is possible to finely map and clone the candidate genes, which brought great convenience for studying the mechanism of lobed leaves.

In a previous study, a lobed-leaf *B. napus* inbred line 1423 was identified, which is a restorer line (530C) of two *B. napus* varieties 'Shanyou 16′ and 'Shanyou 107′. These two varieties have some advantages, such as lobed leaf, high yield, stress resistance, and short stem. A genetic map derived from lines 2205 and 1423 has been constructed, and some QTLs related to salt tolerance have been identified [22]. These results provided a good basis for studying the correlation between lobed leaves and salt tolerance. However, the studies on lobed-leaf gene mapping and cloning, and relationship of lobed leaf and salt tolerance have not been reported. Thus, the purpose of our study is that: 1) analyzing the relationship between lobed leaf characteristics and plant photosynthetic efficiency; 2) studying the correlation between lobed leaf and salt tolerance; 3) fine mapping the lobed-leaf gene; 4) cloning the candidate gene. This research will lay a foundation for studying the mechanism of lobed leaf formation and the utilization of lobed leaf in *B. napus*.

2. Materials and methods

2.1. Materials

An F_2 population including 196 individuals derived from two *B. napus* lines, 2205 (round leaf, salt-tolerant) and 1423 (lobed leaf, salt-sensitive) was constructed for lobed-leaf gene mapping (Fig. 1). Each individual of F_2 was selfed to generate 196 $F_{2:3}$ lines, which were used to determine the leaf shape (LS), net photosynthetic rate (NPR) and leaf area (LA). Ten plants of each $F_{2:3}$ line were measured per replicate. In order to study inheritance of lobed leaves and finely map the lobed-leaf gene, line 2205 crossed with line 1423 to produce F_1, and then, F_1 was selfed to produce an F_2. F_1 backcrossed to lines 2205 and 1423, respectively, to produce two BC_1 populations. These four populations were used to study the inheritance of lobed leaves. And the BC_1 population derived from F_1 and line 2205 was developed to finely map the lobed-leaf gene.

2.2. Correlation between lobed leaf and photosynthesis

The LS of $F_{2:3}$ constructed by Lang et al. (2017) was determined by a visual method. The plants of $F_{2:3}$ were assessed individually for leaf shape using a 1–3 scale, where 1 = round leaf; 2 = semi-lobed leaf; 3 = lobed leaf. The third leaf of seedlings in the 6–7 leaf stage was selected, the NPR of leaves was measured by the LI-6400XT portable photosynthesis system, and the LA of leaves was determined by the Yaxin-1242 portable leaf area meter. Each leaf was measured for three times. And the r-test [23] was conducted to analyze the correlation among LS, NPR and LA by the SPSS 20.0 software.

2.3. Correlation between lobed leaf and salt tolerance

Since the morphological and physiological indexes have been measured in the previous study using the $F_{2:3}$ population [22], including root length (RL), leaf fresh weight (LFW), leaf dry weight (LDW), root dry weight (RDW), electrical conductivity (EC), superoxide dismutase(SOD), soluble protein (SP), chlorophyll content (SPAD), and salt tolerance rating (STR). Therefore, in this study, this $F_{2:3}$ population was used to study the relationship between the leaf shape and these salt tolerance related indexes by the SPSS 20.0 software.

2.4. QTL mapping of lobed leaf

The genetic linkage map of *B. napus* was constructed by Lang et al. (2017). The QTLs related to lobed leaves were mapped using the composite interval mapping (CIM) function of the Win QTL Cartographer v.2.5 [24]. The LOD thresholds of QTL were determined by a 1000 permutation test at a 95% confidence level. The QTL mapping was performed followed the method of Fan et al. [25].

Fig. 1. Performance of the two parents 2205 and 1423 at seedling stage.

2.5. Inheritance of lobed leaf

The segregation of the lobed leaf was studied in the F_1, F_2, and two BC_1 populations. All the segregated populations were grouped into three classes: round leaf, semi-lobed leaf and lobed leaf. A $\chi 2$ test was also performed on the grouped data to assess the goodness-of-fit of the segregating populations to the expected ratio for mendelian phenotypic segregation.

2.6. Fine mapping of the lobed-leaf gene

To identify a putative syntenic region around the lobed-leaf gene in the A genome of *B. rapa* or *B. napus*, the collinearity between the sequences of the markers around the QTLs and the A genome was compared using the BLAST tool (http://brassicadb.org/brad; http://www.genoscope.cns.fr/brassicanapus/). After determining the physical position of the QTL on the A genome, the genes within the QTL region were randomly selected for designing intron polymorphism (IP) primers. The detailed method can be referred to Huang et al. [26]. All IP primers and the SCAR markers located in the QTL region (http://brassicadb.org/brad) were used to amplify the two parents. The primers that showed polymorphism were used to screen the BC_1 population derived from F_1 and line 2205 including 874 individuals. The results were analyzed using MAPMAKER/EXP 3.0 program [27,28]. A minimum log likelihood of the odds (LOD) score of 3.0 was used for map construction. Map distances were calculated using Kosambi's mapping function [29].

2.7. Analysis of the candidate gene

Mapdraw 2.9 [30] was used to construct a high resolution genetic map around the target gene. All of the gene annotations within the mapping region were retrieved from the publicly accessible *Brassica* genetic database (http://brassicadb.org/brad). The genes related to leaf morphological development were selected as the candidate genes responsible for lobed leaves in *B. napus*.

2.8. Total RNA extraction and cDNA synthesis

A RNA prep Pure Plant Kit (TIAN GEN, Beijing, China) was used for total RNA extraction from the leaf and roots tissues of the two parental seedlings that were ground in liquid nitrogen after being processed for 24 h with 0, 100 and 200 mM NaCl, respectively. After DNA removal by DNaseI digestion, agarose gel (mass-to-volume ratio was 1.2%) electrophoresis was performed to measure the RNA concentration and integrity. cDNA synthesis was performed following the instructions of the TIAN GEN FastQuant RT Kit (Beijing, China). These cDNAs were used for analyses of the candidate gene cloning and expression.

2.9. Amplification of the candidate genes

After determining the candidate genes, the full-length DNA sequence of the candidate genes was amplified using the genomic DNA and cDNA as templates. The primer sequences were: Forward 5-'ATGGAATGGACAACGACAAGAAACT −3′; Reverse: 5'-TTACGGGAAAGGGGGCCAGCAAGAGGAA-3′. And the amplification reaction was as follows: denaturation at 95 °C for 10s, followed by renaturation at 55 °C for 5s, and elongation at 72 °C for 1 min for a total of 35 cycles. The Prime STAR Taq DNA polymerase (Takara) was added once the PCR program was suspended. PCR products were purified by a TIAN gel Midi Purification Kit (TIAN GEN, Beijing, China). The purified products were connected to the carrier of the pMD19-T vector, and transformed to *Escherichia coli* DH5α cells. A total of 10 positive clones were sent to Shanghai Sangon Biological Engineering Technology& Service Co., Ltd for sequencing. Full-length cDNA sequences were analyzed by the DNAMAN5.0 software. Multiple sequence alignment of the amino acid sequences was performed using the Clustal W. Phylogenetic tree was constructed by the ML (maximum likelihood) method with bootstrap analysis (1000 replicates) from alignment of protein sequences of the candidate genes in *Arabidopsis, B. rapa, B. napus*, and *B. oleracea* using the MEGA5.0 program. The conserved domains, homologues and physicochemical properties of amino acids were analyzed by SMART (http://smart.embl-heidelberg.de/), Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and PROTPARAM (http://web.expasy. org/cgi-bin/protparam), respectively.

2.10. Expression of the candidate genes

RT-qPCR was carried out to analyze the expression of the candidate genes in the leaf and roots tissues from the two parental seedlings. The SYBR Green Supermix kit purchased from Takara was used for PCR reactions, and the reaction conditions were as follows: pre-denaturation at 95 °C for 10 min, followed by denaturation at 95 °C for 15s, and renaturation at 60 °C for 1 min for a total of 40 cycles; at the end of the reaction, the system was kept at 95 °C for 15s, followed by 60 °C for 1min, then at 95 °C for 15s, and at last at 60 °C for 15s. The replicates were carried out for each experiment following the manufacturer's instructions. For the aimed gene, the forward primer sequence was 5'-TGCACGAGGAGGTGAAGAAG-3′ and the reverse primer was 5'-CCAGCAAGAGGAAGCGATGA-3′. β-actin was selected as the reference gene with a forward primer sequence of 5'-TCAAGAAGGCTATCAAGGAG-3′ and the reverse primer sequence of 5'-GTAACCCCATTCGTTGTCAT-3′. All of primers were designed according to the principle of real-time fluorescence quantitative PCR using Primer Premier 5.0 software.

3. Results

3.1. Correlation between lobed leaf and photosynthesis

The result showed that the three indexes, LS, NPR and LA, had a significantly correlation among them. LS was significantly negatively correlated with NPR and LA, with a correlation coefficient of −0.457 and −0.427, respectively. NPR was found to be significantly positively correlated with LA, with a correlation coefficient of 0.381 (Table 1).

3.2. Correlation between lobed leaf and salt tolerance

LS was significantly correlated with the nine salt tolerance related indexes. Among them, LS was significantly positively correlated with EC and STR with a correlation coefficient of 0.538 and 0.550, respectively. LS was significantly negatively correlated with SPAD, SP, SOD, RL, SH, LDW, and LFW with a correlation coefficient of −0.476, −0.530, −0.560, −0.501, −0.529, −0.400, and −0.398, respectively (Table 2).

3.3. Inheritance of lobed leaf

The inheritance of lobed leaf was investigated using the F_1, F_2 and two BC_1 populations. For F_1 populations, all individuals had semi-lobed leaves, indicating that the lobed-leaf gene was incompletely dominant over the round leaf gene. However, the F_2 populations segregated into lobed leaves, semi-lobed leaves and round leaves. As shown in Table 3, among the 472 F_2 plants derived from the cross 2205 × 1423, a total of

Table 1
Correlation coefficient between LS, NPR and LA under salt stress.

	LS	NPR	LA
LS	–	−0.457**	−0.427*
NPR		–	0.381*
LA			–

** and * indicates significance at the level of 1% and 5%, respectively.

Table 2
Correlation coefficient between LS and indexes related to salt tolerance.

	SPAD	EC	SP	SOD	RL	SH	LDW	LFW	STR
LS	−0.476[a]	0.538[a]	−0.530[a]	−0.560[a]	−0.501[a]	−0.529[a]	−0.400[a]	−0.398[a]	0.550[a]

[a] indicates significance at the level of 1%.

Table 3
Segregation of the crosses between lobed leaf and semi-lobed leaf parents.

Generations	Lobed leaf number	Intermediate lobed leaf number	Round leaf number	Total number	Expectant ratio	χ2	*P*
F_1	0	45	0	45	0:1:0		
F_2	121	221	130	472	1:2:1	2.25	0.30–0.50
BC_1 ($F_1 \times 2205$)	0	178	163	341	0:1:1	1.17	0.20–0.30
BC_1 ($F_1 \times 1423$)	115	97	0	212	1:1:0	1.53	0.20–0.30

342 F_2 plants showed either lobed leaves or semi-lobed leaves, while the remaining 130 plants had round leaves. The chi-squared test showed that the lobed leaf were segregated at a ratio of 3:1 ($\chi 2 = 2.25$, $P = 0.30$-0.50), suggesting a monogenic inheritance of the trait. The BC_1 populations derived from the cross F_1 and 2205 were segregated into the semi-lobed leaves and round leaves groups. Of the 874 BC_1 plants, 421 had semi-lobed leaves and the remaining 453 had round leaves. Chi-squared tests showed that the progenies fitted a monogenic segregation ratio of 1:1 ($\chi 2 = 1.17$, $P = 0.20$-0.30). For the BC_1 populations derived from the cross F_1 and 1423, the individuals were segregated into the semi-lobed leaves and lobed leaves groups. Therefore, it was determined that the lobed leaf of *B. napus* was controlled by a major gene, and the lobed leaf was incompletely dominant over the round leaf.

3.4. QTL mapping of lobed leaf

A major QTL related to lobed leaf was identified on the LG10. The QTL was located between SSR markers BnGMS385 and BnGMS114, which accounted for 36.0% of the total phenotypic variance (Fig. 2). In order to identify more molecular markers linked to the lobed-leaf gene, the SSR markers located on the LG10 were used to screen a small population including 10 lobed leaf and 10 round leaf plants. As a result, only three markers, BnGMS385, BnGMS114 and CB10079, showed polymorphism in this small population. And these three markers were used to screen the BC_1 derived from F_1 and 2205 including 874 individuals, and a primary genetic map around the lobed-leaf gene was constructed. These three markers were located on one side of the lobed-leaf gene (Fig. 3).

3.5. Fine mapping of the lobed-leaf gene

The three SSR markers linked to the lobed-leaf gene were sequenced. Through comparing the marker sequences with the *B. rapa* and *B. napus* genome, it was found that these three marker sequences showed good collinearity with the genome of *B. rapa* (Fig. 3). The homologues of BnGMS385, CB10079 and BnGMS114 were located on 12.887, 14.563 and 15.701 M on the A10 chromosome, respectively (Table 4). Therefore, we primarily justified that the homologue of the lobed-leaf gene might exist on the downstream of 15.701 M on the A10 chromosome. In order to narrow down the region of the lobed-leaf gene, 12 gene sequences from 15.701 to 18.230 M on the A10 of *B. rapa* were selected to design IP primers. As a result, two IP primers and one SCAR marker amplified polymorphic bands between the two parents and the small population (Table 4). Thus, these three primers were used to screen the BC_1 population. Through linkage analysis, a genetic map around the lobed-leaf gene was constructed, and the two markers, BnGMS114 and IP2, were the either two closest markers linked to the target gene with a distance of 0.6 and 0.3 cM, respectively. And one IP marker, IP1, derived from *Bra009511* gene was co-segregated with the target gene. Preliminary analysis showed that the homologue of the lobed-leaf gene existed between 15.701 and 15.817 M, and 25 genes were resisted in this region in *B. rapa*. The function of these 25 genes in this region was analyzed according to the annotations published on the Brassica database (http://brassicadb.org/brad). *Bra009510* is highly homologous with *At5G03790* (*LATE MERISTEM- IDENTITY1*, *LMI1*) of *Arabidopsis thaliana*, and a recessive mutant of this gene can make the *Arabidopsis* leaf margin into a notch (http://www.arabidopsis.org/). In addition, a molecular marker, IP1, closely related to *Bra009510,* is co-segregated with the target gene. Therefore, we considered *Bra009510* was the candidate lobed-leaf gene.

3.6. Candidate gene cloning

The candidate gene was amplified in the two parents 2205 and 1423 by homologous cloning according to the sequence of *Bra009510*. These two genes were named as *BnHB2205* and *BnHB1423*, respectively. The full-length of the genes in both parents, 2205 and 1423, were 1390 bp, which contained three exons and two introns, and the gene sequences (GM637033 and GM637032) were deposited to NCBI database. The ORFs of them were 693 bp encoding a protein of 229 amino acids, with the isoelectric points of 6.85, and the molecular weights of 26.55 kD and 26.61 kDa, respectively. Ten single nucleotide differences between them were detected, resulting in eight amino acid changes (13, 14, 17, 211, 212, 219, 220 and 221) (Table 5, Fig. 4). Two typical domains, homeobox domain and HALZ super family domain, were found between residues 77–130 and 132–166 (Fig. 4). The two domains were conservative among *BnHB2205*, *BnHB1423*, *Bra009510* and *GSBRNA2T00136430001*. No amino acid difference for these two domains was identified. Only one amino acid change was detected in the Homeobox domain for *Bol010030*, but many amino acid changes were identified in *Arabidopsis*.

The BLAST tool developed by NCBI was used to search for the homeobox-leucine zipper proteins in *Brassica* species reported in the GenBank, and the phylogenetic analysis was conducted. The results showed that *Bra009510* from *B.rapa*, *Bol010030* from *B. oleracea*, *GSBRNA2T00136430001*, *GSBRNA2T00032256001* and *GSBRNA2T00010767001* from *B. napus* clustered with *BnHB1423* and *BnHB2205* (Fig. 5). It was found that the above genes were homeobox-leucinezipper protein (HD-Zip proteins) ATHB-51 by the swissprot annotation, which is the palisade mesophyll cell differentiation regulation element. A sequence of 1500 bp upstream of the ATG of *BnHB2205* and *BnHB1423* was analyzed for cis-acting elements using Plant CARE. The results showed that a number of cis-acting elements HD-Zip 1, HD-Zip 2, LTR and HSE were related to leaf morphological development and plant stress resistance (Table 6), suggesting that the *Bra009510* gene may be related to leaf morphological development and abiotic stresses.

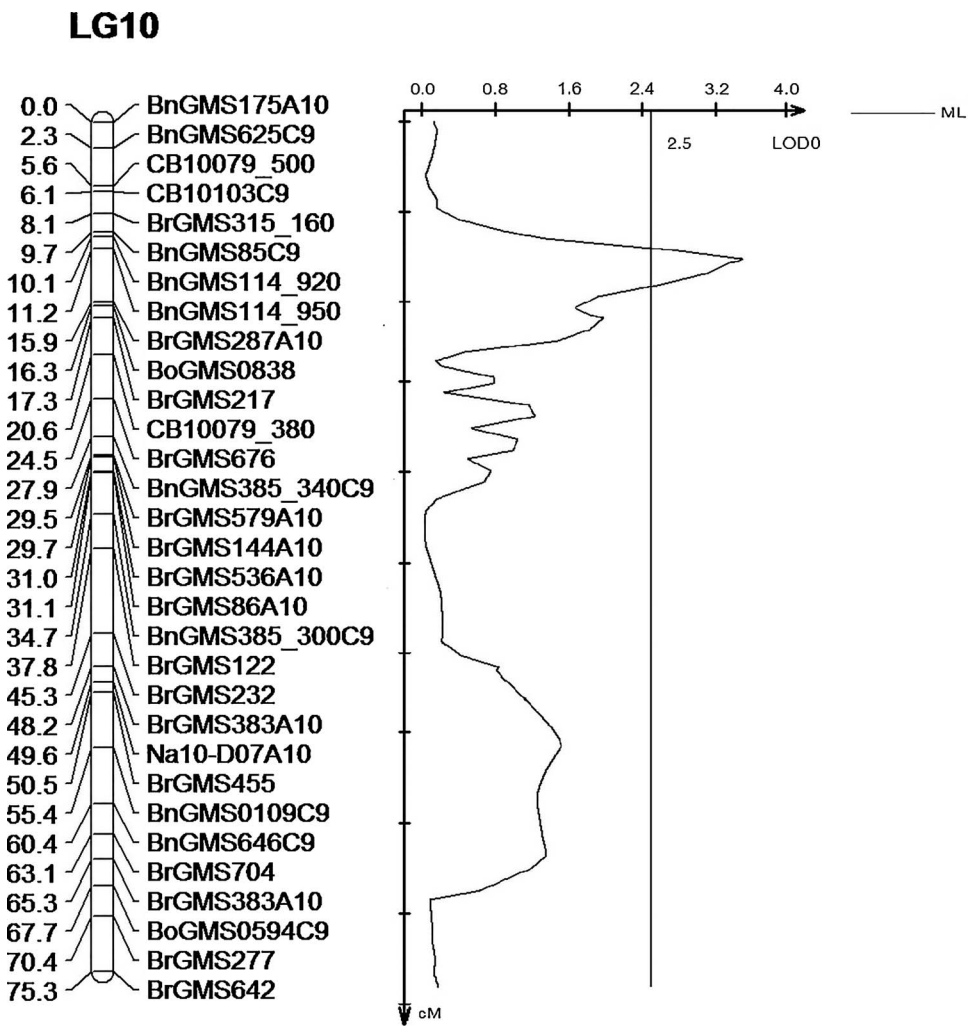

Fig. 2. Mapping of QTLs related to lobed leaf gene on LG10. The markers names are shown to the right of the linkage group (LG10), and genetic distance (cM) is on the left. The vertical line is LOD threshold for QTL.

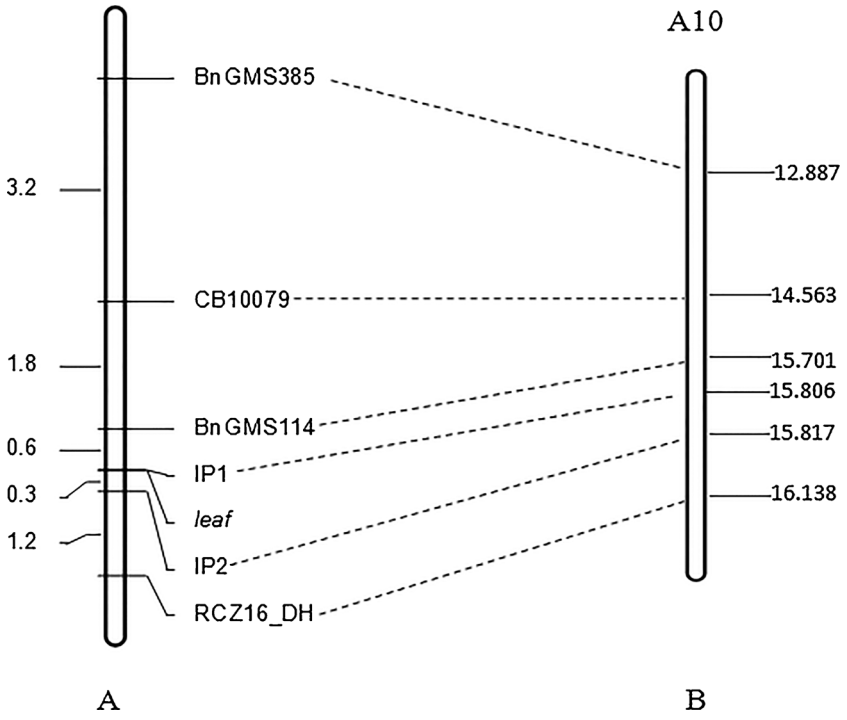

Fig. 3. The left map is a genetic map surrounding the lobed-leaf gene from the BC_1 population; the right map is a partial physical map of the A10 chromosome of *B. rapa* showing the homologues of mapped markers sequences. Dotted lines indicate the relationship of the two maps.

Table 4
Information of molecular markers linked to lobed leaf gene.

Markers	Sequences (5′–3′)	Location on A10(Mb)
BnGMS385	TTTCATGACTTAGCCACCTT/ CCAAGTATTCAATTTCTGGC	12.887
CB10079	TACAGGAAGATTAAATGCC/ ACCGTTTGTCAGTGTCATAG	14.563
BnGMS114	GTGATTGAGCTGATGGAAAT/ AGAAGGACGTAGAAGCCTTT	15.701
IP1	GAACGGTTCGGTCATCCCTC/ AATGACAAAGGGTGGTGCCT	15.806
IP2	CGACATCGAGCTCGGAAACT/ CGTTGAAACCGAAGAATGCGT	15.817
RCZ16_DH	ACATTGTGGACACACTCAAA/ GCTTAGCGAAAGAGAGAGAA	16.138

Table 5
Structure analysis of *BnHB1423* and *BnHB2205*.

	Loci							
Gene	13	14	17	211	212	219	220	221
BnHB1423 (Aminoacids)	K	I	M	G	I	I	P	L
BnHB2205 (Amino acids)	R	V	L	V	V	M	L	I

3.7. Expression of the candidate gene

As shown in Fig. 6A and Fig. 6B, the expression of the candidate gene was low in the leaves and roots without salt stress; however, after salt treatment, the expression of the gene increased significantly. Moreover, the expression profiles of the genes in leaves and roots were also different (Fig. 6C). In leaves, the expression of this gene in line 1423 was significantly higher than that in line 2205 in 100 mM salt stress. In roots, under 100 mM and 200 mM salt stress, the expression of this gene in line 1423 was also significantly higher than that in line 2205. With the increase of the salt concentration, the expression in the leaves first increased and then decreased, but increased continuously in roots. Probability, the leaves are more sensitive to salt stress, and easy to be damaged, when the plants were subjected to high salt stress, the expression of the gene on the leaves decreased.

4. Discussion

Lobed leaf is an ideal morphological marker in rapeseed breeding. By comparing the leaves of the parents and F_1, it is easy to distinguish true and false hybrids in F_1. Previous studies have showed that the lobed leaf formation in *B. napus* is controlled by one or two dominant genes [18,31]. Our result is similar with those of previous studies. Because the inheritance of the lobed-leaf characteristics is simple and stable, it has been used in the identification of true hybrids, purity identification and breeding ornamental plants. In breeding, a method for effective utilization of lobed-leaf characteristics is to generate a new line with lobed leaves by transferring it to a sterile line. The F_1 plants will have semi-lobed leaves, although only one parent has lobed leaves; furthermore, an improved purity of the hybrid seeds can be achieved by the removal of false individuals.

In addition to be a morphological index, lobed leaf is thought to be related to photosynthesis. The leaf area of a single lobed leaf is smaller, and its photosynthetic efficiency is weaker. However, the photosynthesis of the population is stronger because the accumulation of leaves is smaller than that of the round leaves [6,8]. In this study, it was found that LS was significantly negatively correlated with NPR and LA; therefore, increasing the plant density of lobed-leaf plants can improve the photosynthetic efficiency of population. Line 1423 is a lobed-leaf line, which is also the restorer line (530C) of two Chinese *B. napus* varieties, Shanyou 16 and Shanyou 107. These two varieties have the advantages of lobed leaf, short stature and high yield, which have been planted in the north of China.

In the previous study, an $F_{2:3}$ population derived from lines 2205 and 1423 has been used to identify the QTLs related to salt tolerance [22]. Line 1423 is salt sensitive and has lobed leaves. Therefore, one of our ideas is to study whether lobed leaf is related to salt damage. It was found that lobed leaf was significantly positively correlated with EC and STR, and negative correlated with SPAD, SP, SOD, RL, SH, LDW, and LFW, indicating that the lobed leaf is possibility negatively correlated with salt tolerance. Thus, we proposed that certain leaf shape formation related genes affected the salt tolerance. Under salt stress, the physiological indicators related to salt tolerance of plants will be changed, meanwhile the morphological indexes such as leaf shape will also be change to resist the stress. In subsequent candidate gene screening, we identified a candidate gene *Bra009510* which is related to leaf morphological development and abiotic stresses. Through gene structure and expression analysis, it was found that the gene sequence and expression between the two parents was different, particularly the presence of eight different amino acids within CDS region. This new finding also confirms the correlation analysis in this study. To confirm whether the variation of eight amino acids is the key reason causing the leaf-shape change, it is better to clone the full-length sequence of the gene, including the promoter region, and analyzed whether there are structural differences in the promoter region. Meanwhile, the transgenic technology is used to verify the function on leaf shape formation and salt stress, however, this work will last a long time.

Based on the current research, the conserved domains of genes can also be used to initially illustrate their functions, the amino acid sequence analysis showed that *Bra009510* had a homeobox domain and a conserved homeobox binding leucine zipper domain. According to the structure of the predicted conserved domain and the HD-Zip family, this gene belongs to the HD-Zip I family [32]. Some studies have showed that the genes in the HD-Zip I family were involved in plant abiotic stress responses, and some members of the HD-Zip I were mainly through the regulation of the carbohydrate metabolism pathway [33], and leaf traits [13], reducing the content of endogenous ABA [34] to improve the antioxidant capacity in response to a series of stress. The lobed leaf of rapeseed is controlled by *BnLL1* gene [35]. *LMI1* is considered to correspond to *BnLL1*, and *LMI1* regulates floral meristem determinacy, bract formation, and leaf morphology [36]. These genes are expressed in various tissues [37]. One study showed that a *LMI1-like2* gene, *BnaA10g26320D*, has high expression in lobed leaves, but low expression in toothed-leaf plants [21]. Under drought and salt stresses, the expression of HD-Zip I transcription factor, *Oshox22*, increases significantly in rice [34]. Similar results were found in Alfalfa; when induced by NaCl and ABA, the expression of a HD-Zip I transcription factor, *MsHB2*, increases significantly [38]. The results in our study showed that the expression of the candidate gene in the lobed-leaf parent 1423 was consistently higher than that in the round leaf parent 2205 under salt stress, which is consistent with the studies reported by others [34,38]. It was proposed that when the plants were subjected to salt stress, the self-defense mechanism was activated, through changing their leaf shape to resist salt damage. Lobed leaf is more sensitive to salt damage compared to round leaf, when it was subjected to salt stress, the leaf-related genes of it are highly expressed, such as *Bra009510*. Therefore, this gene may be related to salt stress regulation and leaf development. Meanwhile, it was found that the leaves are more sensitive to salt stress compared to the roots, and a severer damage on the leaves than the roots was observed. Under the high concentration of salt stress (200 mM), the expression of some genes on the leaves was decreased, but the expression in roots was still raised, which is also observed in other plants [39–41].

In order to study the mechanism of lobed leaf formation, it is

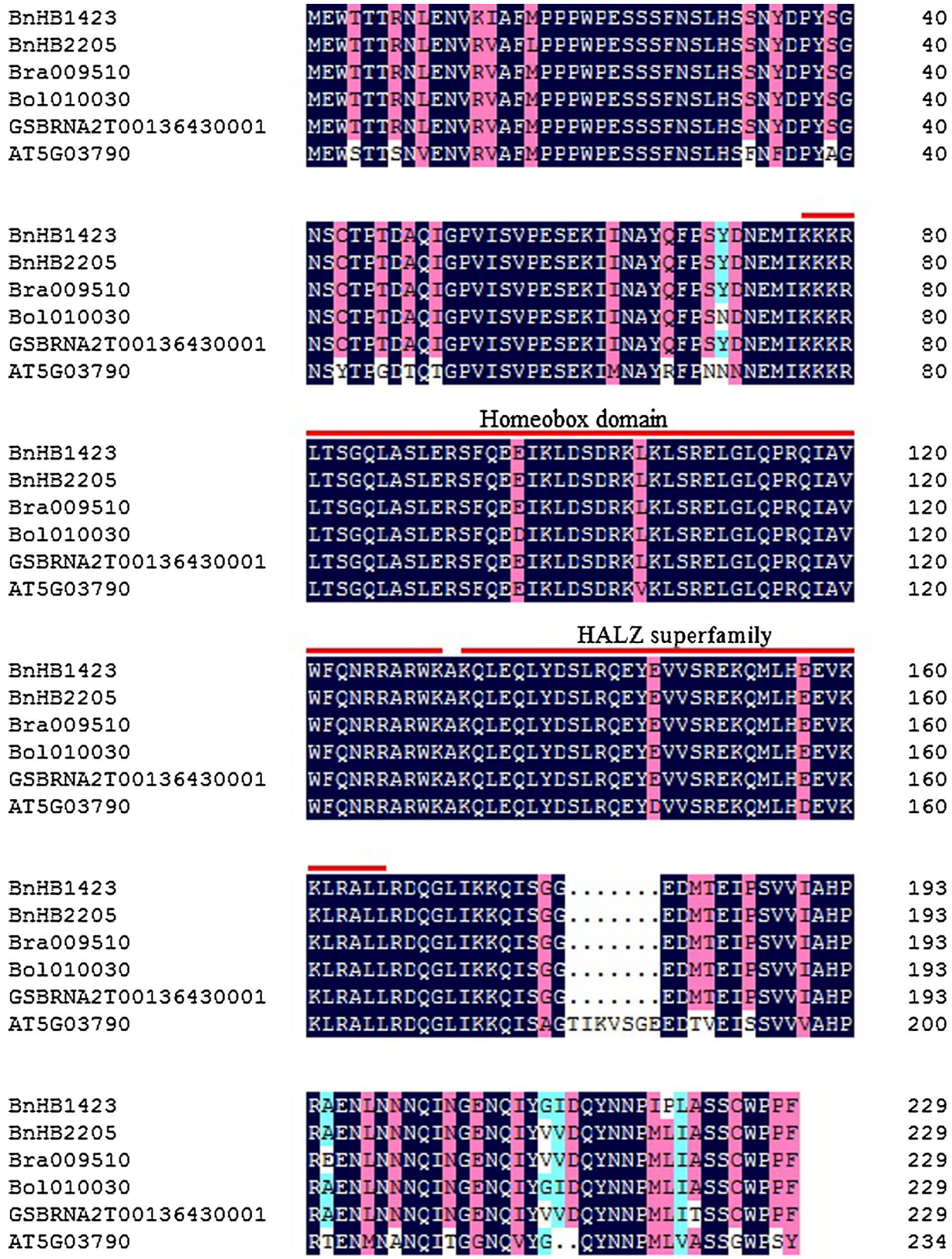

Fig. 4. Sequences comparison of *Bra009510* between *Arabidopsis* and *Brassica* species. The red lines indicate the homeobox domain and HALZ super family domain. The mutant amino acids are indicated in red, blue and white.

necessary to understand the function of the lobed-leaf related genes. Map-based cloning is considered as an effective method to map and clone the target genes. In this study, QTL technology was used to map the lobed-leaf genes, and the gene was mapped to a large space. The key step is to narrow down the DNA regions for the candidate genes. Development of IP markers is a very effective method to finely map the candidate genes, through selecting the genes located in the candidate region. The IP primers were designed based on the conserved sequences

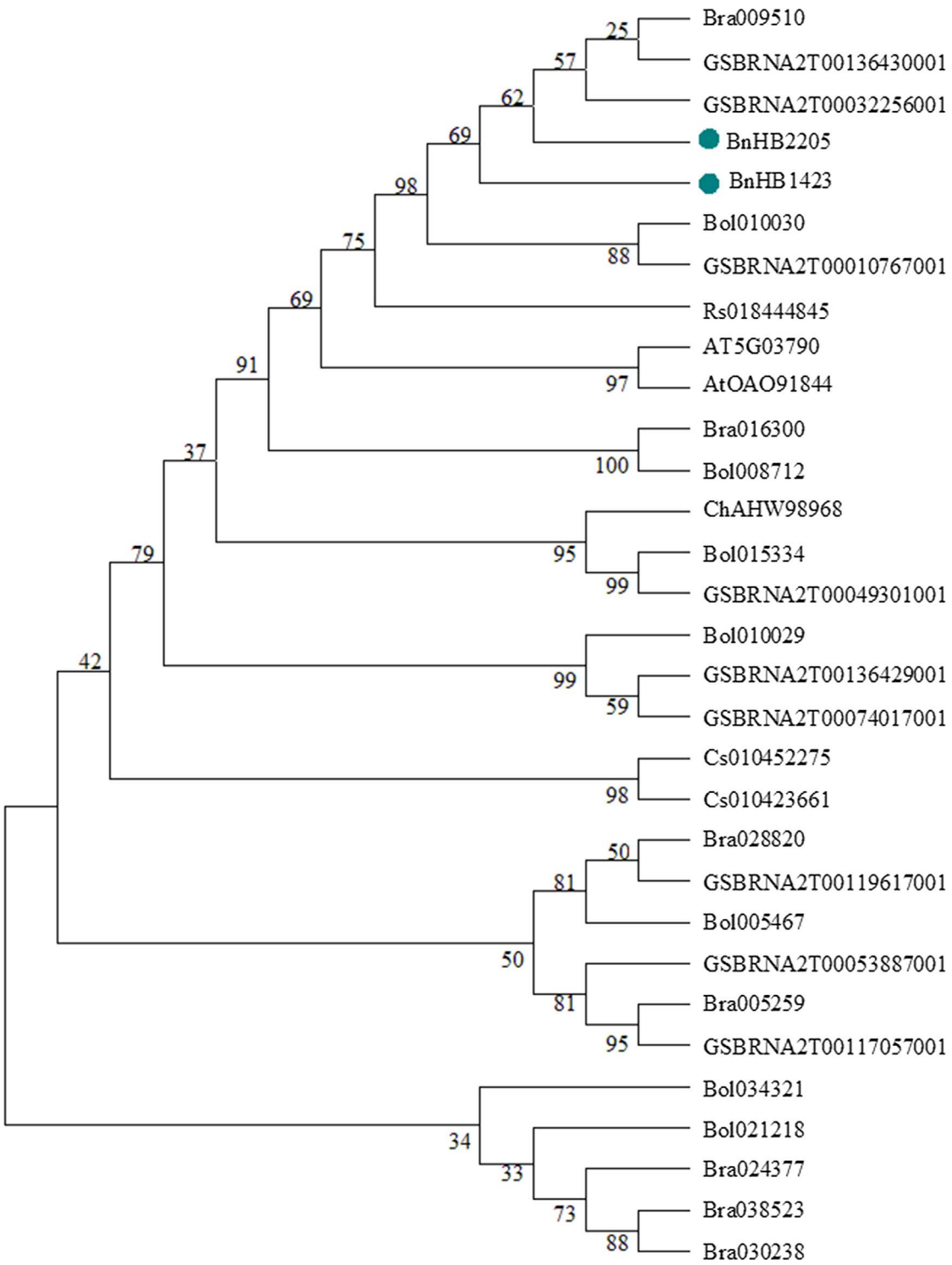

Fig. 5. Phylogenetic tree analysis of the candidate genes. Numbers are bootstrap values indicating frequencies of respective furcations found in 1000 replications of subset tree calculations.

of the exons, which were used to amplify the introns between two exons. Molecular markers were selected from the intron sequences which were linked to lobed-leaf genes. As a result, two markers were identified using 12 pairs of primers; the gene region was narrowed to a region of 0.117 M. According to the genes annotations of this region, a candidate gene *Bra009510* was selected. The functional studies are currently under investigation in our laboratory. The use of the IP markers greatly reduced the range of the candidate genes.

Authors and contributors

ZH was responsible for designing this study and drafting the manuscript; YZ and AX carried out gene cloning and expression. LL carried out the QTL mapping of lobed-leaf gene; YZ, ML and YS carried out analysis of correlation; YW, XL, FL, BZ, MQ and JD collected important background information and provided assistance for data acquisition, data analysis and statistical analysis. All authors have read and approved the content of the manuscript.

Table 6
Analysis of cis-acting elements in the upstream of the candidate gene.

Cis-acting elements	Core sequence	Annotation
CAT-box	GCCACT	cis-acting regulatory element related to meristem expression
G-box	CACGTGG	cis-acting regulatory element involved in light responsiveness
LTR	CCGAAA	cis-acting element involved in low-temperature responsiveness
HSE	AAAAAATTTC	cis-acting element involved in heat stress responsiveness
HD-Zip 1	CAAT(A/T)ATTG	element involved in differentiation of the palisade mesophyll cells
HD-Zip 2	CAAT(G/C)ATTG	element involved in the control of leaf morphology development
ABRE	ACGTGGC	cis-acting element involved in the abscisic acid responsiveness

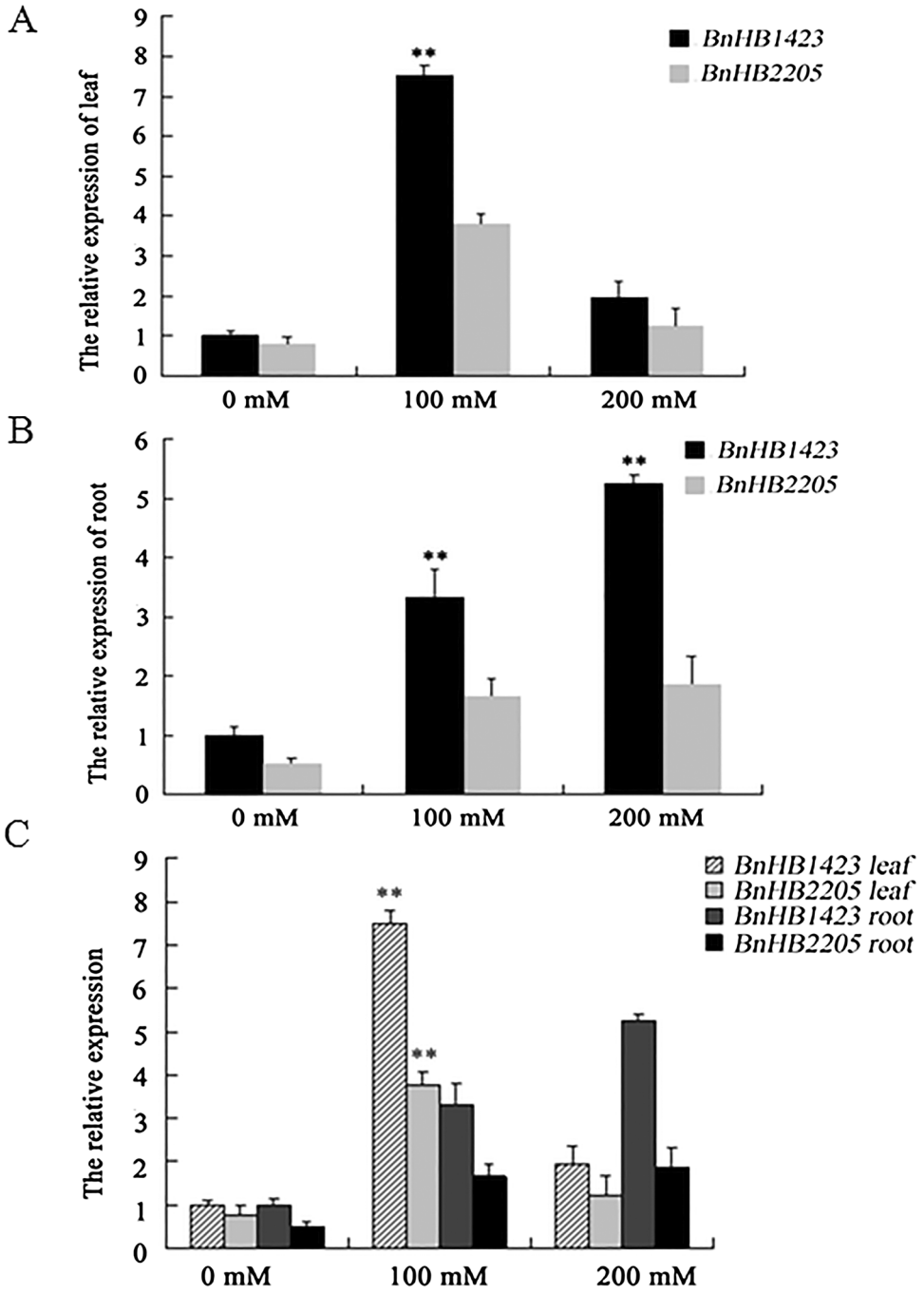

Fig. 6. A. Expression of the candidate gene in leaves of the two parents. B. Expression of the candidate gene in roots of the two parents. C. Expression of the candidate gene in leaves and root of the two parents. ** stands for significance level at $P < 0.01$.

Competing interests

The authors declare that they have no competing interests.

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