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BcBRC1a is a negative regulator for tillering in non-heading Chinese cabbage

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Abstract

Shoot branching is a decisive factor for crop yield. Molecular mechanism for regulating shoot branching (tillering) needs to be determined. Plenty of previous studies have illustrated that *BRANCHED1* (*BRC1*) is a key integrator of shoot branching regulating signals. However, *BcBRC1* function in non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis*) (NHCC) remains unknown. Here, we defined two *BRC1* orthologs, *BcBRC1a* and *BcBRC1b*, from NHCC and focused on the *BcBRC1a* gene to describe its alternative splicing characteristic and structure. *BcBRC1a* was expressed rhythmically and mainly in leaf axils at the 'Maertou' cultivar tillering stage. *BcBRC1a*^{L} encoded a nuclear location protein. Its ectopic expression caused *Arabidopsis* growth inhibition and silencing *BcBRC1a* led to increased tiller numbers in 'Maertou'. Removing the shoot tips of NHCC caused axillary buds to be released from apical dominance and *BcBRC1a* expression down-regulation. Our research determined that *BcBRC1a* acts as a negative regulator for tillering in non-heading Chinese cabbage and sets the foundation for further studies.

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INTRODUCTION

Shoot branching determines the architecture and yield of crops. In non-heading Chinese cabbage (NHCC), it is known as tillering, a special mode of shoot branching.

Lateral shoot development consists of two stages: the formation and outgrowth of axillary buds. Axillary buds are derived from axillary meristems (AMs) which are located in leaf axils. Leaf axil meristematic cells undergo three phases to form axillary buds: the maintenance and activation phase are controlled by *SHOOTMERISTEMLESS* (*STM*) and the emergence phase is characterized by the establishment of the *WUSCHEL* (*WUS*) - *CLAVATA3* (*CLV3*) feedback loop[1]. *LATERAL SUPPRESSOR* (*LAS*) and *CUP-SHAPED COTYLEDON 2* (*CUC2*) are putative regulatory hubs based on the abundance of transcription factors that bind to their promoters[1]. *Arabidopsis las* mutants are not able to form lateral shoots during vegetative development[2]. *Lateral suppressor* (*Ls*) and *MONOCULM 1* (*MOC1*) are orthologs of *LAS* in tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*). They encode GRAS family transcription factors and have a similar effect on shoot regulation[3,4].

Axillary buds remain dormant or develop into branches after forming. Axillary buds dormancy or activation are regulated by multiple factors including genes, environment and phytohormones. In rice, *TEOSINTE BRANCHED1* (*OsTB1*)/*FINE CULM 1* (*FC1*) represses axillary buds outgrowth but does not affect axillary buds formation[5]. In other words, *OsTB1*/*FC1* works at the second stage of lateral shoot development. *BRANCHED1* (*BRC1*), the ortholog of *OsTB1*, engages in multiple regulatory pathways and acts as an integrator of branching signals within axillary buds[6]. *BRC1* encodes a TCP (teosinte branched1, cycloidea, proliferating cell factors) transcription factor which contains a basic helix-loop-helix (bHLH)-type DNA binding motif named TCP domain and a R domain rich in polar residues (arginine, lysine and glutamic acid)[7]. *BRC1* prevents axillary bud outgrowth and its mutation leads to rosette branches increasing in *Arabidopsis*[6]. The function of *TB1*/*BRC1* seems conserved to regulate lateral branches outgrowth in monocot[5,8,9] and dicot[6,10–12] plants.

In non-heading Chinese cabbage, *BcBRC1* (named *BcBRC1b* here) can respond to the treatment of GR24 and 6-Benzylaminopurine (6-BA) which are synthetic analogs of strigolactone (SL) and cytokinin respectively[13]. It suggests *BcBRC1b* might be also located in the downstream of SL and cytokinin and be involved in tillering regulation. However, the function of its paralog, *BcBRC1a*, remains unknown. In this study, we cloned *BcBRC1a* from non-heading Chinese cabbage and preliminarily analyzed its function.

RESULTS

Identification of *BcBRC1* genes in non-heading Chinese cabbage

In NHCC, TCP transcription factor family contained 37 members with a TCP domain (Supplemental Table S1). To identify genes from NHCC most similar to *BRC1*, a neighbor-joining (NJ) phylogenetic tree based on the full-length protein sequences of *B. campestris* and *A. thaliana* TCP family was constructed. The TCP family could be divided into two subfamilies, class I (PCF) and class II. The basic domain of class I has a four-amino-acid deletion compared with Class II[14]. Class II contained two

clades, CYC/tb1 and CIN. *BcBRC1a* (*BraC03g038980/BcTCP17*) and *BcBRC1b* (*BraC01g038590/BcTCP2*) in CYC/tb1 clade were closest to *BRC1* (*AtTCP18*) (Fig. 1a).

To investigate the structure and function of *BcBRC1a*, we cloned it and found that *BcBRC1a* had two transcripts from alternative splice conforming to the GT-AG rule[15], a long one named *BcBRC1a*^{L} and a short one named *BcBRC1a*^{S} which had an alternate acceptor site (AA) in Intron III (Fig. 1b, c).

Interestingly, the extra segment of *BcBRC1a*^{L} exon Ⅳ had a termination codon TGA which led to early termination of *BcBRC1a*^{L} translation. *BcBRC1a*^{L} had a 1227-bp open reading frame (ORF), while *BcBRC1a*^{S} had a 1278-bp ORF. They could encode two proteins with identical TCP domain that is the main domain and R domain but different C-terminal domains (Fig. 1b). BcBRC1a^{S} was the ancestral isoform (Supplemental Fig. S1).

Expression pattern of *BcBRC1a*

Non-heading Chinese cabbage cultivar 'Maertou' initiated tillering at the early vegetative stage, while 'Suzhouqing' began tillering at the late vegetative stage close to the reproductive stage[13]. To reveal expression pattern of *BcBRC1a* clearly, total RNA of non-heading Chinese cabbage cultivars 'Suzhouqing' and 'Maertou' from four tissues at 'Maertou' tillering stage (Supplemental Fig. S2) was extracted for qRT-PCR. The results showed that transcript level of *BcBRC1a* in 'Maertou' was higher than 'Suzhouqing' generally during the tillering period of 'Maertou' (Fig. 2a). Both in 'Maertou' and 'Suzhouqing', *BcBRC1a* was expressed mainly in leaf axil (LA) and secondly in shoot tip (ST), which suggested that *BcBRC1a* worked in the leaf axil primarily (Fig. 2a).

In terms of two transcripts of *BcBRC1a*, *BcBRC1a*^{L} played a major role in shoot tip (ST) and was expressed at a higher level than *BcBRC1a*^{S} in almost all tissues except for leaf (L) of 'Suzhouqing' (Fig. 2b). So *BcBRC1a*^{L} was chosen as a candidate for further study.

Previous studies showed that *BRC1/TB1* expression had a daily oscillation like other TCP transcription factors[16,17]. So we detected *BcBRC1a* expression levels every four hours under

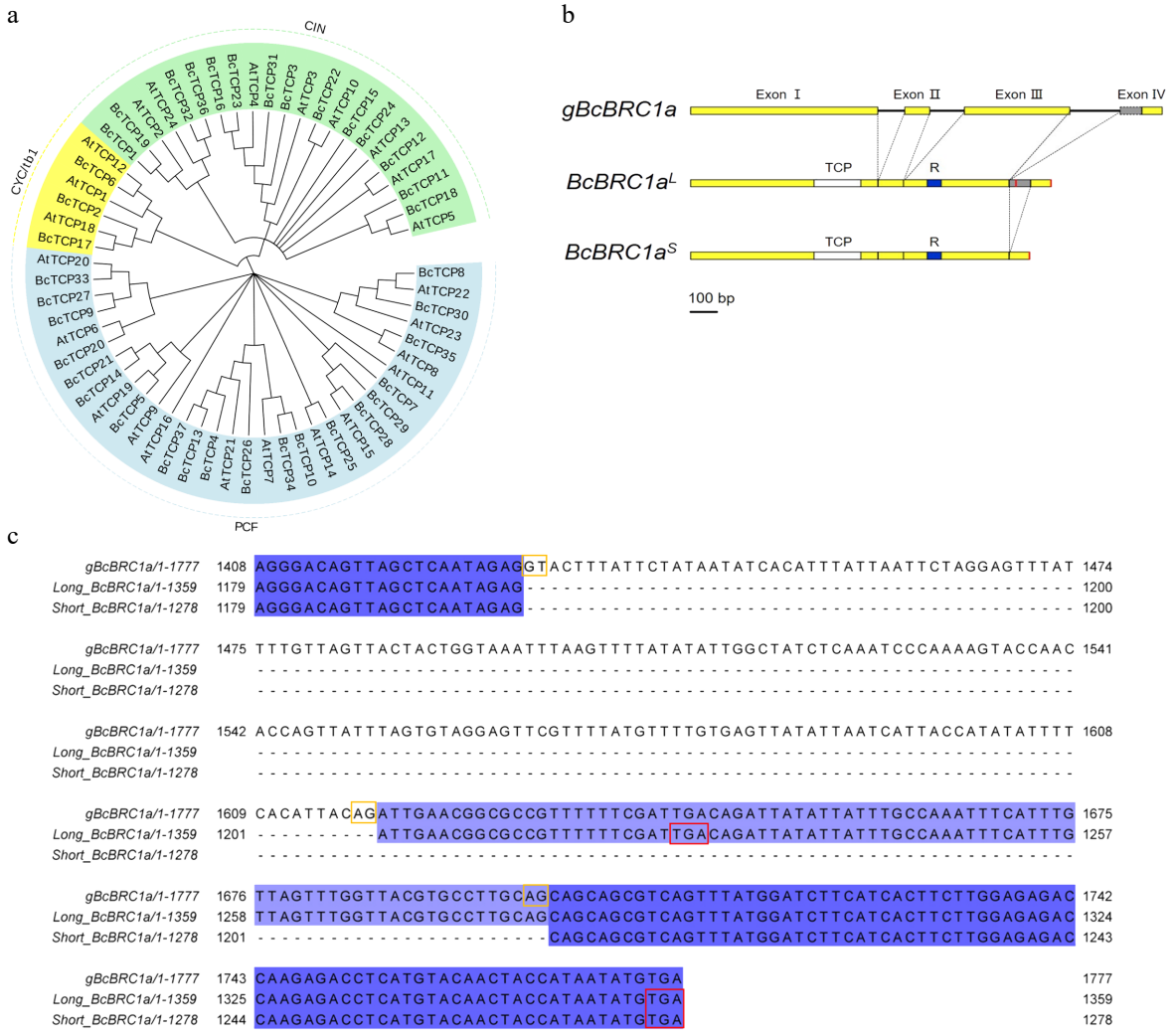

Fig. 1 TCP family analysis and *BcBRC1a* gene structure and partial sequence. (a) Phylogenetic tree showing relationships between TCP family transcription factors in *B. campestris* and *A. thaliana*. Blue, yellow and green backgrounds represent PCF, CYC/tb1 and CIN clades respectively. *AtTCP18* is *BRC1*, *BcTCP17* is *BcBRC1a* and *BcTCP2* is *BcBRC1b*. (b) *BcBRC1a* gene structure. Yellow boxes represent exons and black lines represent introns. Grey box is alternative splicing region. TCP and R domain are filled with white and blue, respectively. The red color indicates termination codon. Scale bar = 100 bp. (c) *BcBRC1a* 3′ end sequence. Termination codons are bounded by red squares and alternative splicing sites are bounded by yellow squares.

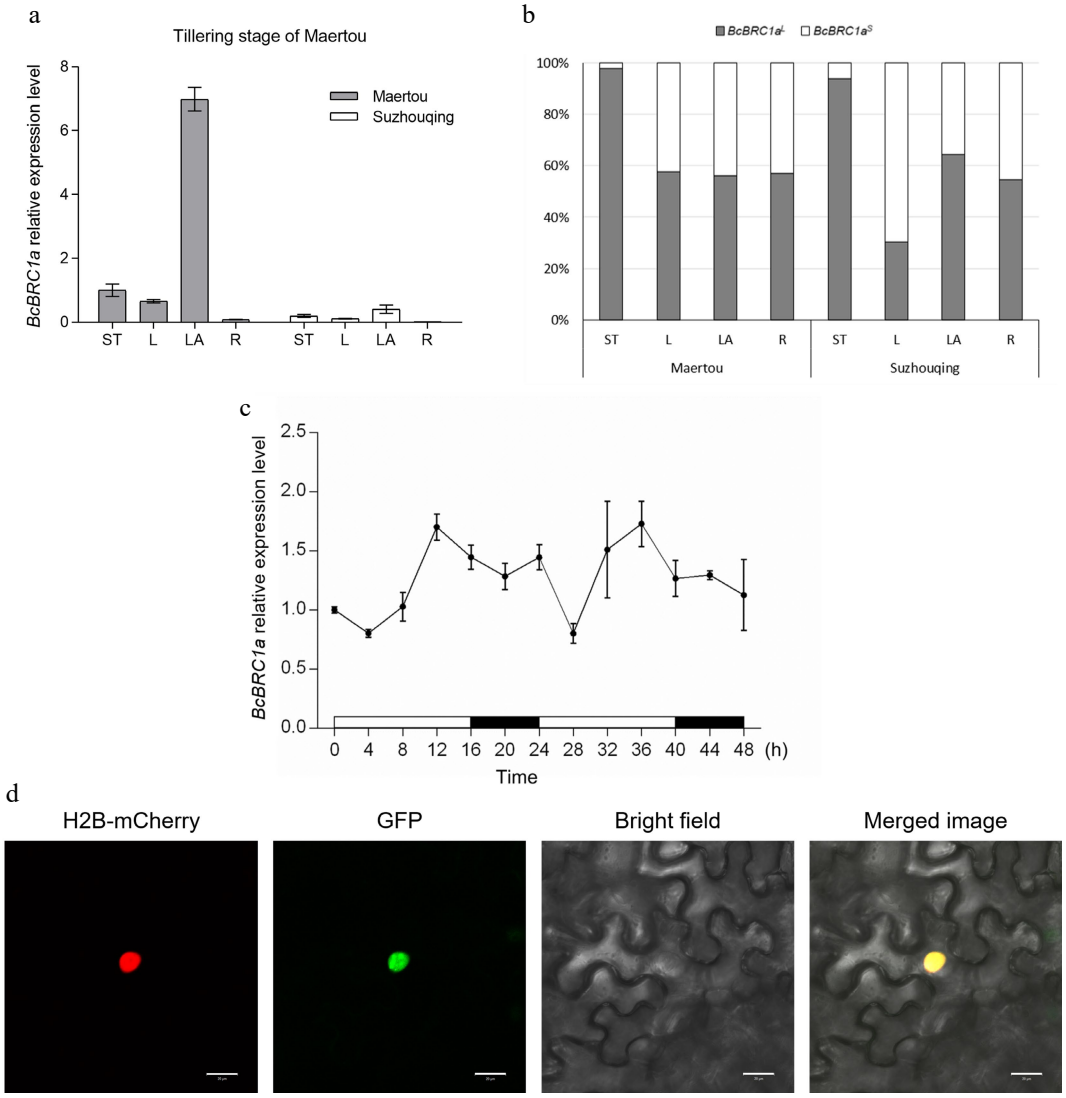

Fig. 2 Expression pattern of *BcBRC1a* in 'Maertou' and 'Suzhouqing' at the 'Maertou' tillering stage. (a) *BcBRC1a* relative expression level within four tissues. ST, shoot tip; L, leaf; LA, leaf axil; R, root. Error bars represent SE. (b) Proportion of *BcBRC1a*^{L} and *BcBRC1a*^{S} expression within four tissues. (c) Circadian expression of *BcBRC1a*. White bars represent daylight and black bars represent darkness. Error bars represent SE. (d) Subcellular location of BcBRC1a^{L}. Scale bar = 20 μm.

long-day conditions (16 h light and 8 h dark) during two continuous photoperiods. We found that *BcBRC1a* was expressed rhythmically. *BcBRC1a* expression peaked at 12 h and 36 h and was lowest at 4 h and 28 h (Fig. 2c). Evening elements (EE) motif is one of the binding targets of *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) which is a key clock regulator and can prevent tillering in rice by positively regulating SL pathway gene expression including *D14* and *OsTB1*[17]. Interestingly, EE motif of which sequence is (AA)AATATCT was also found in the promoter region of *BcBRC1a* (Supplemental Fig. S3).

BcBRC1a^{L} is located in the nucleus

To analyze BcBRC1a^{L} subcellular location, a 35S: BcBRC1^{L} (CDS)-GFP recombinant plasmid was constructed and *BcBRC1a*^{L} was transiently expressed in tobacco epidermal cell through the *Agrobacterium*-mediated method. Signals of GFP and mCherry (fused with a nuclear marker) merged in the nucleus (Fig. 2d), which indicated that BcBRC1a^{L} was a nuclear location protein.

Ectopic expression of *BcBRC1a*^{L} inhibits the growth of *Arabidopsis*

To analyse the function of *BcBRC1a*, 35S: BcBRC1a^{L} (cDNA)-GFP was transformed into *Arabidopsis* (Supplemental Fig. S4). As shown in Fig. 3a, the crown diameter of *BcBRC1a*^{L} overexpression (OE) lines were much smaller than WT during the vegetative period. OE14 growth was almost stagnant and *BcBRC1a* mRNA level of OE14 was significantly higher than other lines (Supplemental Fig. S5). During the reproductive period, primary rosette-leaf (R1) branch numbers of OE14 and OE17 decreased significantly (Fig. 3b, c). The phenotypes of *BcBRC1a*^{L} transgenic lines illustrated that ectopic expression of *BcBRC1a*^{L} at least inhibited the growth of *Arabidopsis* and probably inhibited its shoot branching.

Silence of *BcBRC1a* enhances tillering of 'Maertou'

To reveal the function of *BcBRC1a* more reliably, VIGS experiment was conducted in 'Maertou' (Fig. 4a). In pTY-BRC1-1/2 plants, BcBRC1a mRNA level reduced by 88.6% and 79.7%, respectively, which meant that the experiment was effective

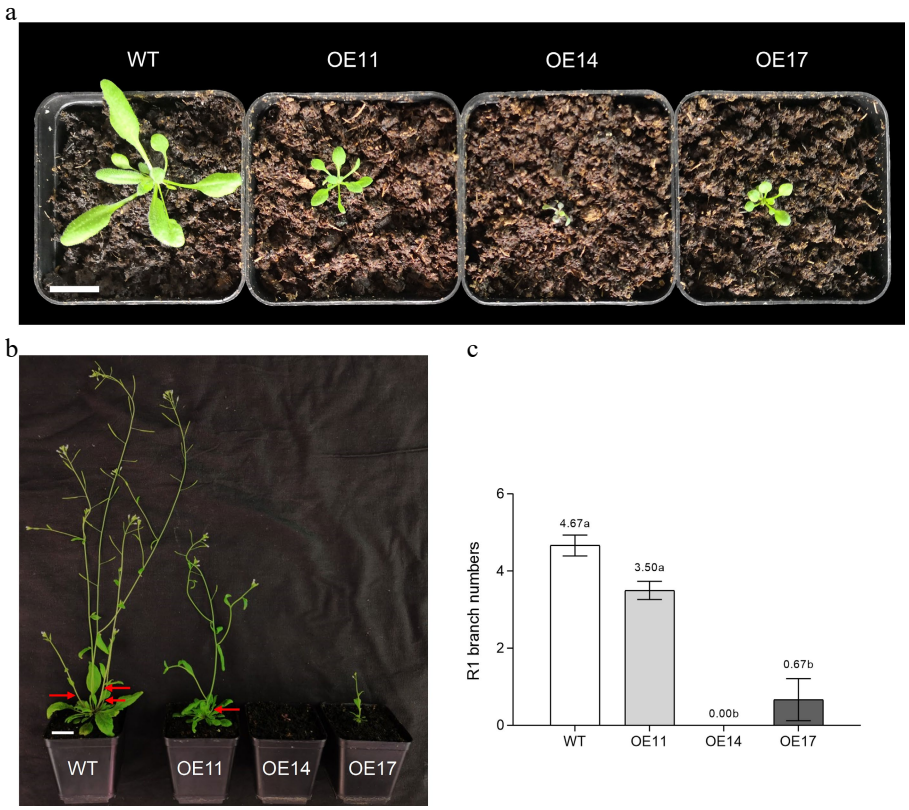

Fig. 3 Overexpression of *BcBRC1a*^{L} in *Arabidopsis* (T_2 lines). (a) Phenotype during vegetative period. Scale bar = 2 cm. (b) Phenotype during reproductive period. Red arrows indicate primary rosette-leaf (R1) branches. Scale bar = 2 cm. (c) R1 branch numbers of *Arabidopsis* transgenic lines. Three biological replicates, $\alpha = 0.05$.

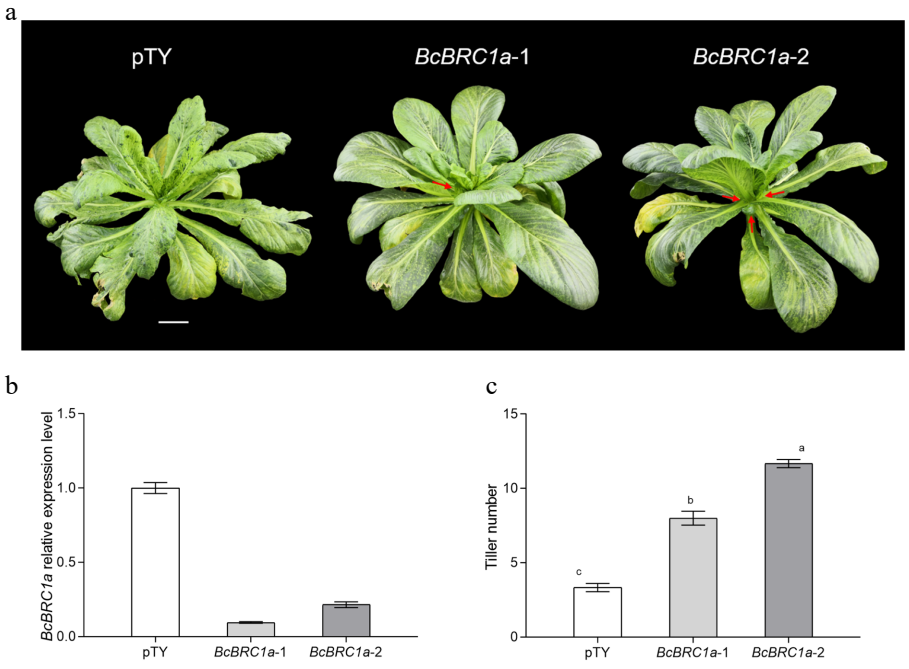

Fig. 4 *BcBRC1a* silencing in 'Maertou'. (a) Phenotype of 'Maertou' after silencing *BcBRC1a*. Scale bar = 2 cm. Red arrows indicate tillers. *BcBRC1a* relative expression level (b) and tiller numers (c) of *BcBRC1a* silencing plants. Three biological replicates, $\alpha = 0.05$.

(Fig. 4b). In addition, tiller numbers of pTY-BRC1-1/2 plants significantly increased compared with pTY plants (Fig. 4c). The result showed that *BcBRC1a* could prevent the outgrowth of tillering.

BcBRC1a responds to decapitation

Dormant axillary buds can be activated by decapitation due to the breakdown of apical dominance.

Although tillering buds of 'Maertou' would elongate constantly under suitable conditions, their elongation was accelerated after removing the stem tips (Fig. 5a), while axillary buds of 'Suzhouqing' were stimulated from dormancy to activation (Fig. 5b). Tiller numbers and length of decapitated 'Maertou' and 'Suzhouqing' were significantly larger and longer than intact ones (Fig. 5c, d). However, decapitation could not stimulate the nodes which could not have had axillary buds to generate tillers. In other words, decapitation only had an effect on axillary buds outgrowth but not on formation.

To investigate whether decapitation would influence *BcBRC1a* expression, we conducted qRT-PCR to detect *BcBRC1a* relative expression level. In the control (intact) group, *BcBRC1a* transcript abundance decreased within 3 h and then increased presenting a similar trend in two cultivars (Fig. 5e, f). *BcBRC1a* was immediately down-regulated within 1 h after decapitation compared with the control in both 'Maertou' and 'Suzhouqing' and the down-regulation was stronger in 'Suzhouqing' than in 'Maertou' (Fig. 5e, f). After 1 h, *BcBRC1a* expression had a rebound in decapitated groups but the overall level was lower than intact groups (Fig. 5e, f). Therefore, decapitation could result in down-regulation of *BcBRC1a*.

Together, these results suggested that *BcBRC1a* responds to decapitation and the increase of tillers caused by decapitation in NHCC might be associated with the reduction of *BcBRC1a* expression.

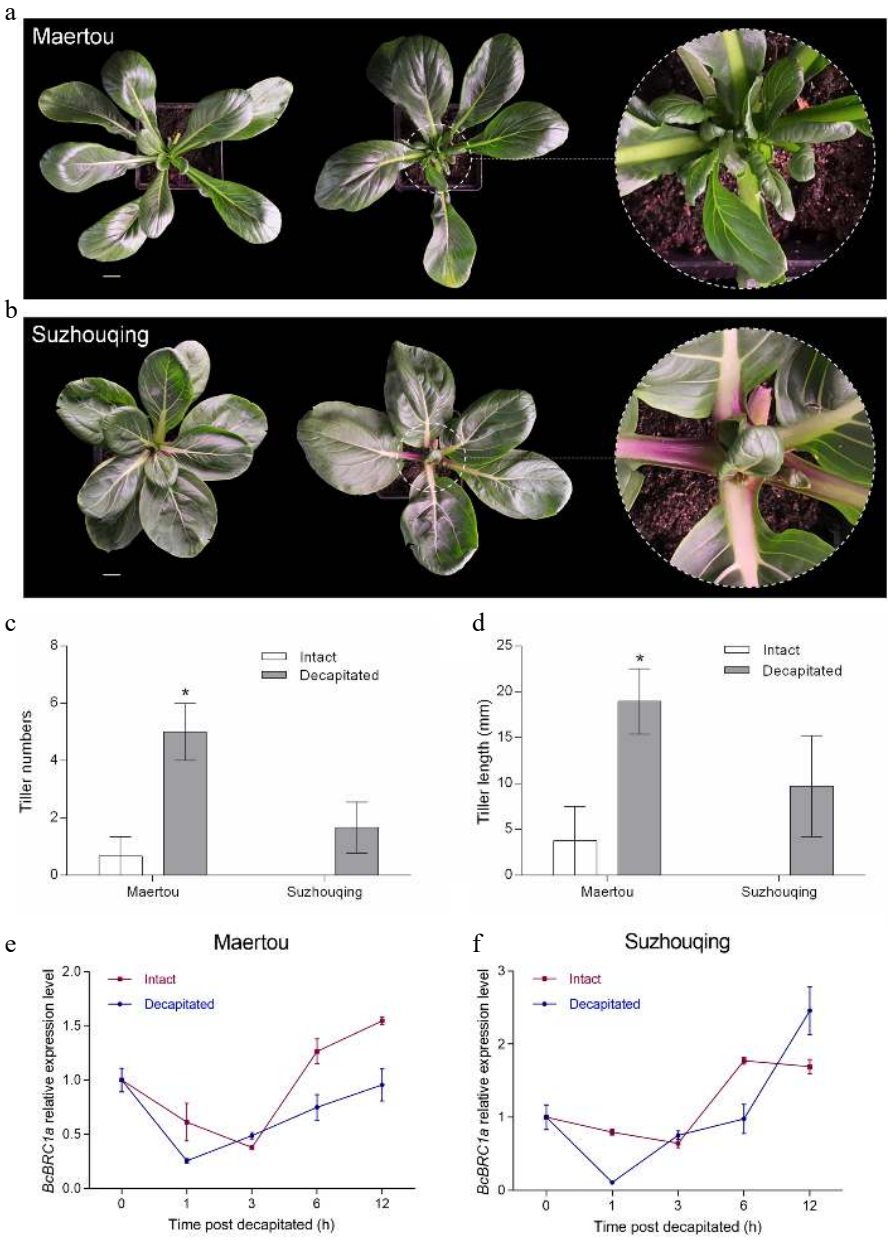

Fig. 5 Decapitated treatment for NHCC. 'Maertou' (a) and 'Suzhouqing' (b) tillering phenotype after 10 d of decapitation. Scale bars = 2 cm. Left, intact plant; right, decapitated plant. Tiller numbers (c) and length (d) of 'Maertou' and 'Suzhouqing' after 7 d of decapitation. Error bars represent SE (the same below). *BcBRC1a* expression in 'Maertou' (e) and 'Suzhouqing' (f) responds to decapitation. * represents $P < 0.05$.

DISCUSSION

Branching/tillering is an important agronomic trait. In many crops, multiple branches/tillers are usually regarded as an adverse trait for crop yields. However, in NHCC which is harvested mainly for leaves, multiple branches/tillers contribute to the increase in yield. The function of *TB1/BRC1* has been studied in many species, which is the hub regulator of shoot branching and related to the dormancy of axillary buds.

In this study, we identified two orthologs of *BRC1* in NHCC and cloned *BcBRC1a*. *BcBRC1a* was active in leaf axils at vegetative stage of 'Maertou' (Fig. 2a), which is consistent with previous studies[5,6,10]. The circadian clock plays an important role in regulating the growth and development of plants. CCA1 is a key clock regulator and the overexpression and repression of *OsCCA1* reduce and enhance tiller numbers in rice[18]. *BcBRC1a* was expressed rhythmically (Fig. 2c) probably due to *OsTB1/BRC1* acting as the target of CCA1[17,19,20]. Therefore, in NHCC, *BcBRC1* expression may also be regulated by CCA1 in a circadian-dependent manner like in rice. It is worth investigating how circadian clock is related to tillering in NHCC and verifying whether CCA1 binding to the promoter of *BcBRC1a* in the following experiments.

Alternative splicing gives proteins much more diversity than genes[21]. There was an alternate acceptor site in Intron III of B*cBRC1a*, which made *BcBRC1a* generate two transcripts and proteins (Fig. 1b, c). The alternative splicing phenomenon of *BRC1* was also detected in tomato (*S. lycopersicum*) and potato (*Solanum tuberosum*)[10,11]. Splicing of intron I causes a frameshift, which leads to short SlBRC1a and StBRC1a have a divergent C-termination (H domain) from long SlBRC1a and StBRC1a. StBRC1a^{S} can affect StBRC1a^{L} subcellular localization and reduce the transcriptional activity of StBRC1a^{L}[11]. As for *BcBRC1a*, whether alternative splicing makes difference in its function still needs further study.

The ectopic expression of *BcBRC1a*^{L} promoted by 35S in *Arabidopsis* made the growth of transgenic lines seriously inhibited (Fig. 3a), which was similar with what was found in *Arabidopsis* and potato[11,16]. During the reproductive period, branch numbers of OE14 and OE17 were significantly smaller than WT (Fig. 3b, c). Besides, silencing *BcBRC1a* increased tiller numbers of 'Maertou' (Fig. 4a, c). The results indicated that *BcBRC1a* could inhibit tillering in NHCC.

It is worth noting that *BcBRC1a* transcript abundance was higher in 'Maertou' (with tillers) compared with 'Suzhouqing' (without tillers) (Fig. 2a), which seems inconsistent with its inhibiting effect. This may be interpreted as there being a *BRC1*-independent way to regulate tillering in NHCC like pea and *Arabidopsis*[16,22–24]. On the other hand, *BRC1/TB1* only controls the outgrowth of axillary buds[5,6]. As for axillary buds formation, it is regulated by many other genes. It is likely that another tillering regulatory factor plays a decisive role in 'Suzhouqing'.

Sugars play a central role in early outgrowth of axillary buds and act prior to phytohormones[25]. In this study, it was found that *BcBRC1a* expression was down-regulated within 1 h after decapitation, which suggests that *BcBRC1a* may prevent sink strength of axillary buds. Previous research shows that *BcBRC1b* responds to exogenous GR24 and 6-BA[13]. It may have a synergistic effect with *BcBRC1a* because the dimerization of TCP transcription factors can affect their DNA binding activity[26–28]. But this assumption still needs experimental proof.

CONCLUSIONS

In non-heading Chinese cabbage, *BcBRC1a* has two transcripts, *BcBRC1a*^{L} and *BcBRC1a*^{S}. The proteins they encode have identical TCP domains and R domains but different C-termination domains. *BcBRC1a* expression is more active in leaf axil than shoot tip, leaf and root and is controlled by circadian rhythms. *BcBRC1a* is a negative regulatory factor for tillering in NHCC. Besides, *BcBRC1a* can respond to decapitation treatment. Our findings are a supplement for *BRC1*-like gene function in diversified species and lay a foundation for revealing internal tillering regulatory networks in non-heading Chinese cabbage.

MATERIALS AND METHODS

Plant materials

The non-heading Chinese cabbage 'Maertou' and 'Suzhouqing' cultivars were used. The seeds were grown in plastic pots containing soil mixture in a plant growth chamber (16 h light and 8 h dark at 27 °C). Tobacco (*Nicotiana benthamiana*) and *Arabidopsis* wild type (ecotype Col-0) plants were grown in the plant growth chamber (16 h light at 24 °C and 8 h dark at 18 °C).

Sequence alignment and phylogenetic analysis

The sequences of *B. campestris* were acquired from NHCC001 genome[29]. The Hidden Markov Model profile (HMM) and BLASTP were used to search for *B. campestris* TCP family proteins taking *O. sativa* (http://rice.plantbiology.msu.edu/index.shtml) and *A. thaliana* (www.arabidopsis.org/index.jsp) as references[14,30,31].

The protein sequences of *B. campestris* and *A. thaliana* TCP family were aligned with MUSCLE in MEGA 7.0 using the default options[32,33]. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method[34]. We set 1000 bootstrap replications, p-distance model and complete gaps deletion and other options retained the default settings. The tree was polished with Evolview v2[35].

Cloning of *BcBRC1*

Total RNA of 'Maertou' was extracted with an RNA easy Mini Kit (Tiangen, Beijing, China) and the first strand of cDNA were synthesized via reverse transcription using a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The full-length *BcBRC1a* was amplified with PrimeSTAR® Max DNA Polymerase (Takara, Dalian, China) and *BcBRC1a*-F/R primers (Supplemental Table S2). The PCR product was inserted into the pEASY-Blunt Cloning vector (TransGen Biotech, Beijing, China) and sequenced by TSINGKE Biological Technology (Nanjing, China).

Vector construction for subcellular localization and *Arabidopsis* transformation

The pRI101-GFP vector was cut with Nde Ⅰ and BamH Ⅰ restriction enzymes and the linearized pRI101-GFP vector was obtained. The coding sequence (CDS) region and full-length cDNA of *BcBRC1a*^{L} was connected with linearized pRI101-GFP vector using In-Fusion® HD Cloning Kit (Takara, Dalian, China) to construct a complete 35S:BcBRC1a^{L} (CDS/cDNA)-GFP vector.

Subcellular localization

35S:BcBRC1a^{L} (CDS)-GFP recombination plasmid was introduced into *Agrobacterium* GV3101 strain (Tolo Biotech,

Shanghai, China). *Agrobacteria* were cultured overnight until OD_{600} reached 0.8 and injected into tobacco leaves. After 24 h dark and 24-36 h light culture, the leaves were cut into pieces to detect fluorescence under a confocal laser scanning microscope (Zeiss, LSM780, Jena, Germany).

Arabidopsis transgenic plants

35S:BcBRC1a^{L} (cDNA)-GFP recombination plasmid was introduced into *Agrobacterium* GV3101 strain (Tolo Biotech, Shanghai, China). *A. thaliana* plants were transformed with *Agrobacterium* using the floral dip method[36]. Seeds of transgenic lines were screened on 1/2 Murashige and Skoog (MS) medium with 50 mg/L kanamycin and 16 mg/L timentin in the illuminatuin incubator (16 h 75 μmol·m^{-2}·s^{-1} light and 8h dark at 25 °C) and the green seedlings were transferred into pots containing soil mixture in the plant growth chamber after 14 d. The phenotype of WT and T_2 lines were analyzed.

Silencing of *BcBRC1* via the virus-induced gene silencing (VIGS) technique

The pTY vector we used for VIGS is derived from Turnip yellow mosaic virus (TYMV). Two 40-bp specific DNA fragments were designed in the conserved coding region of *BcBRC1a*^{S} and *BcBRC1a*^{L}, and then they were reversely complemented to obtain two 80-bp palindrome sequences (5'-CTAGATGTCGCCA AAGAGTTGTTCGGCTTACAAGACATGCGCATGTCTTGTAAGCCGA ACAACTCTTTGGCGACATCTAG-3' and 5'-CTAAAGGGACAAGAG ATCGTAGGATGAGGCTCTCGCTAGATCTAGCGAGAGCCTCATCCT ACGATCTCTTGTCCCTTTAG-3', *BcBRC1a*-1 and *BcBRC1a*-2) and form hairpin structures. The synthesis of hairpin structures and construction of pTY-*BcBRC1a*-1 and *pTY-BcBRC1a*-2 vectors were done by the GeneScript company (Nanjing, China).

The pTY and pTY-*BcBRC1a*-1/2 plasmids were imported into 'Maertou' using the method of Yu et al.[37].

Decapitation for NHCC

One month after germination, half of 'Maertou' and 'Suzhouqing' seedlings had their stem tips removed for phenotyping and quantitative real-time PCR and the other half were kept intact as a comparison. The tiller (length > 5 mm) numbers and length were counted and measured on the 7th day after decapitation. Three biological replicates were set. The t-test method was adopted to analyze the data.

Quantitative real-time PCR

Total RNA was extracted from NHCC and *Arabidopsis* with an RNA easy Mini Kit (Tiangen, Beijing, China) and reverse transcription was conducted with Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (YEASEN, Shanghai, China). SYBR Green method was used for qRT-PCR with Hieff® qPCR SYBR Green Master Mix (High Rox Plus) (YEASEN, Shanghai, China). The following pairs of primers were used (Supplemental Table S2): RT-*BcBRC1a*-F/R, RT-*BcBRC1a*^{L}-F/R, RT-*BcActin*-F/R, RT-*AtActin*-F/R. Three biological and technical replicates were applied. The results were calculated as the $2^{-\Delta\Delta CT}$ method[38]. Ratio(*BcBRC1a*^{L}) was the expression level of *BcBRC1a*^{L} relative to *BcBRC1a*. Ratio(*BcBRC1a*^{S}) was calculated by substracting Ratio(*BcBRC1a*^{L}) from one.

$$\mathrm{Ratio}(BcBRC1a^{L}) = \frac{\mathrm{E}(BcBRC1a^{L})}{\mathrm{E}(BcBRC1a)} \times 100\%$$

$$\mathrm{Ratio}(BcBRC1a^{S}) = 1 - \mathrm{Ratio}(BcBRC1a^{L})$$

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Conflict of interest

The authors declare that they have no conflict of interest.

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