Genetics and Molecular Research

Regulation of bolting and identification of the α-tubulin gene family in *Brassica rapa* L. ssp *pekinensis*

Y.W. Zhang[1,2], D. Jin[1], C. Xu[1], L. Zhang[1], M.H. Guo[1] and Z.Y. Fang[3]

[1]College of Horticulture, Northeast Agricultural University, Harbin, China
[2]Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), Ministry of Agriculture, Harbin, China
[3]Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China

Corresponding author: Y.W. Zhang
E-mail: zhangyaowei@neau.edu.cn

Genet. Mol. Res. 15 (1): gmr.15017507
Received August 24, 2015
Accepted November 6, 2015
Published January 29, 2016
DOI http://dx.doi.org/10.4238/gmr.15017507

ABSTRACT. Microtubules are important components of eukaryotic cells, and they play vital roles in cell morphogenesis, carrying of signaling molecules, transport of materials, and establishing the cell polarity. During bolting of biennial plants, cell division and elongation are involved, and cell elongation inevitably involves the microtubules arrangement and expression of related genes. So we deduce that it is of great significance to figure out the mechanism of bolting and flowering in which *TUA* genes are involved. In the present study, bioinformatic methods were used to predict and identify the α-tubulin gene family (*BrTUAs*) in *Brassica rapa* L. ssp *pekinensis* (Chinese cabbage) through the alignment of *AtTUA* gene sequence from *Arabidopsis thaliana* with the *B. rapa* genome database (http://brassicadb.org/brad/) using the basic local alignment search tool. The change in the structure and functions of *BrTUAs* during the process of evolution, cis-acting elements in the promoter sequences of *BrTUAs*, and the expression of the identified genes was also analyzed. Twelve members

of the α-tubulin gene family were identified from Chinese cabbage. The gene length, intron, exon, and promoter regions were determined to have changed significantly during the genome evolution. Only five of the 12 members were encoded completely and were observed to differ in their spatial and temporal expression. The five *BrTUA* promoter sequences contained different numbers of cis-elements responsive to light and low-temperature response, cis-elements responsive among which hormonal responses were significantly different. We also report that the *BrTUAs* were involved in the regulation of the bolting in Chinese cabbage, and propose that this process could be controlled by regulating the expression of *BrTUAs*.

Key words: *Brassica rapa*; α-tubulin gene; Identification; Expression

INTRODUCTION

Microtubules, which are important components of eukaryotic cells, play vital roles in cell morphogenesis, carrying of signaling molecules, transport of materials, the establishment of polarity of cells and in other aspects (Chan et al., 2011). It is necessary for plant cells to have different morphology to adapt to the environment and functional needs during the process of growth and development. These processes are closely associated with the microtubules. Kopczak et al. (1992) reported that there were at least six *TUA* gene subtypes and nine *TUB* gene subtypes in *Arabidopsis thaliana*. They also separated some *TUA* and *TUB* gene subtypes from different plants: at least eight α-microtubules and seven β-microtubules subtypes in grapes (Parrotta et al., 2010), at least eight α-microtubules and twenty β-microtubules subtypes in poplar (Oakley et al., 2007), and nine α-microtubules subtypes in cotton (Dixon et al., 1994). The *TUA* genes of *A. thaliana* are highly conserved genetically and are very similar in structure, but they are considerably different in expression. The *TUA* genes 2, 3, 4, and 5 of *A. thaliana* express in roots, stems, and leaves, and *TUA* genes 1 and 6 express only in flowers (Kopczak et al., 1992).

During bolting of biennial plants, cell division and elongation are involved. Cell elongation inevitably involves the microtubules arrangement and expression of related genes. It is, therefore, of great significance to understand the mechanism of bolting and flowering in which the *TUA* genes might be involved. Chinese cabbage is a typical biennial plant that has undergone triploidization and double-ploidization during the evolutionary process as a new polyploidy (Cheng et al., 2013). According to the results of *Brassica rapa* genome sequencing, there are massive repeated gene fragment in *B. rapa* genome, which might contain multiple *TUA* gene subtypes. In the present study, α-tubulin gene family was identified and the effects of *B. rapa* polyploidization on the structure, function, and expression patterns of *TUA* genes were determined. The results obtained might help to understand the regulation of bolting and flowering of Chinese cabbage by the *TUA* genes.

MATERIAL AND METHODS

Plant material

An advanced generation inbred line of Chinese cabbage (A161) having uniform botanical characteristics, bolting, and early flowering, bred by the Chinese Cabbage Research Group of the Northeast Agricultural University was used in the present study.

Structure, evolution, and identification of *BrTUA* genes

Amino acid sequences of six *TUA* genes of *A. thaliana* were used as queries in the BLAST analysis against the *B. rapa* genome database (http://brassicadb.org/brad/). BLASTp was chosen as operation procedure to run Blast program online and *B. rapa* (protein) as database. For different parameters, the default values were selected. The sequences of redundant hits and those withan E value less than -10 and similarity less than 60% were removed; the remaining sequences were considered the identified sequences. The online analysis software Gene Structure Display Server2.0 (GSDS2.0) (http://gsds.cbi.pku.edu.cn) was used to determine the exon and intron regions. The Mega 5.1 software was used for sequence alignment and construction of a molecular phylogenetic tree using the mRNA and amino acid sequence of *BrTUAs* and *TUAs* of *A. thaliana* and other species. For functional analysis of the protein, tools (InterPro) available at EMBL-EBI website (http://www.ebi.ac.uk/interpro/) were used.

Analysis of cis-elements in the promoter sequences of the *BrTUA* genes

To investigate the cis-elements in the promoter sequences of *BrTUA* genes, genomic DNA sequences located 1.5 kb upstream of the initiation codon (ATG) for each gene were obtained from the *Brassica* database BRAD. The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify the cis-elements in the promoter regions.

Analysis of expression of *BrTUA* genes by reverse transcription-polymerase chain reaction (RT-PCR)

Test seeds were sown in 8 x 8 cm seedling pots. The seedlings were subjected to vernalization in an illuminated incubator after the cotyledons were laid fully-open at 8/3°C (day/night) with 16/8-h (light/dark) period for 25 days. Thereafter, the vernalized plants were transplanted on May 20, 2014 to an open field to receive optimum temperature and long-day for normal growth. Fifteen days after flowering, the new young leaves smaller than 1 cm, tender scape smaller than 2 cm, flower buds one day before flowering, and new fibrous roots and pods seven days after pollination were collected and flash-frozen in liquid nitrogen for the analysis of the difference in the spatial expression of *TUA*. We collected the shortening stems before the low-temperature vernalization, after vernalization, and during the color-change period (Figure 1) (a change in leaf-color from bright-green to gray-green was observed after the flower bud differentiation and before bolting in the Chinese cabbage) (Han et al., 2011) and scape with flower stalk up to 2 cm and then flash-frozen in liquid nitrogen for subsequent expression analysis of *TUA* during the bolting process.

For the expression analysis of *TUA*s using RT-PCR, 12 primers were designed (Table 1). The forward and reverse primers were designed, respectively, against the coding DNA sequence (CDS) and the 5'-untranslated region sequences that were determined to be highly similar among the *BrTUAs*. RNA was extracted using the EasyPure® Plant RNA Kit (TransGen Biotech Co., Ltd., Beijing, China) and was stored at -80°C, for subsequent use. The synthesis of the first strand cDNA was performed by reverse transcription using TransScript® First-Strand cDNA Synthesis SuperMixKit (TransGen Biotech Co., Ltd.) by incubating the reaction mixture at 42°C for 30 min followed by heating at 85°C for 5 min to inactivate the RNA. The RT-PCR product was analyzed by agarose gel electrophoresis. *GADPH* was used as the reference gene.

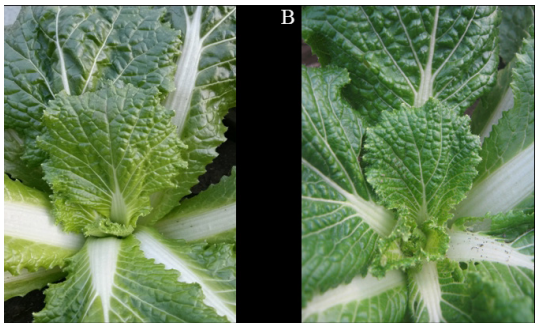

Figure 1. Change in leaf color before bolting in Chinese cabbage. Leaf color before (**A**) and after (**B**) bolting.

Table 1. Specific primer of *BrTUAs* used for RT-PCR.

Gene	Forward primer	Reverse primer
Bra039648	5'- GAGTGTTGCTGAGGTGTTC-3'	5'-AAAGAAATAAATCGGATAAGTAGTTTG-3'
Bra022914	5'-GGACAACCTTGATCGGATACTC-3'	5'-CCACCTTCAGCACCAACC-3'
Bra020572	5'-TCCTCCTACGCACCAGTG-3'	5'-TCCTCGCCTTCGTCATCC-3'
Bra018825	5'-ATCAACTACCAGCCACCAAC-3'	5'-TCAGACAGCAAGAGACAGATAG-3'
Bra020061	5'-GTATCAACTACCAACCTCCAAC-3'	5'-CGAACCAAGATAATAACAGAACG-3'
Bra020062	5'-CAAGACTAAGAGGACCGTTCAG-3'	5'-GTAATCCACAAGAATCATAAGTAGAAAC-3'
Bra018176	5'-ATGCGTCTGTTTCGGTTTTG-3'	5'-AAGAATCAGTAAGAAGAAGAGATAAGG-3'
Bra008412	5'-GCTCCTCCACCGTTCAAG-3'	5'-GGACCATAACCGCAACAG-3'
Bra006517	5'-TTAATGCTGCTGTTGGACTATC-3'	5'-AAGACGGTGGAGAATGTGC-3'
Bra002261	5'-TGGTGAAGGAATGGAGGAAG-3'	5'-GTAAGAAGATAATGGAAGGTTTGG-3'
Bra002260	5'-AAGATGTTAATGCTGCTGTTGG-3'	5'-TACCTCCTCATAGTCCTTCTCC-3'
Bra014232	5'-CAAGTGTTGCTGAGGTGTTC-3'	5'-CACAAATAGAGATGGATAGTAATAACC-3'

Gibberellic acid 3 (GA_3), methyl jasmonate (MJ), and taxol treatments

To investigate the way of regulation bolting and flowering in Chinese cabbage, the effect of tubulin specific drug, taxol, as well as GA_3 and MJ on the expression of *BrTUA* was determined by spraying their solutions on the vegetative growing point of Chinese cabbage near the time of color change. The GA_3 and MJ solutions were prepared in ethanol and diluted to 200 (Dai et al., 2010) and 10 mg/L (Lin et al., 2011) with distilled water. Taxol was dissolved in dimethyl sulfoxide and diluted to 0.3 mg/L with distilled water (Thitamadee et al., 2002). Distilled water as used as a control. The gene expression was analyzed using RT-PCR after 24 h. For each treatment, the height of floral axis was also determined at the time when it was 5 cm for the control group.

RESULTS

Identification and isolation of the *TUA* genes in *B. rapa*

To identify the members of the *TUA* gene family in *B. rapa* L. ssp *pekinensis*, sequences of all the *TUA* genes and their homologs in *A. thaliana* were retrieved from GenBank. The orthologs of

A. thaliana genes in *B. rapa* could be obtained from BRAD, a data base containing *B. rapa* genes. Each *TUA* gene loci in *A. thaliana* was used to search all the *TUA* gene sequences of *B. rapa* present in BRAD. Each predicted *B. rapa TUA* gene sequence was confirmed using FGENESH (http://www.softberry.com/berry.phtml?topic=fgenesh). The overall analysis revealed that the *BrTUA* gene family comprised of 12 members (Table 2). The different *BrTUA* genes varied in their length (from 1007 bp for Bra022914 to 3690 bp for Bra020062) and in the length of the ORFs (from 258 bp for Bra008412 to 1380 bp for Bra006517). The ORFs encoded polypeptides containing 85 to 450 aa with their predicted molecular mass ranging from 21.53 to 116.52 kDa.

The *BrTUAs* CDS and the corresponding genome sequence were used to determine the number and location of introns and exons using the GSDS webset. During the lengthy evolutionary process of *BrTUA* gene family, most of sequence was determined to have retained four introns whereas the Bra018176, Bra008412, and Bra022914 genes were observed to have lost the exons and introns (Figure2). The length of exons and introns in the 12*BrTUA* genes varied widely with the introns ranging from 65 to 2100 bp and the exons from 48 to 654 bp. This demonstrated that the exon junction components were more varied among the different copies during the evolution of *BrTUA*. Moreover, we found that intron phases were more abundant, which contained 0, 1, and 2 phases. Of these three phases, the 0 phase was most abundant with just Bra020572 containing a single 0 phase; a 128-bp regulatory sequence was inserted in front of the first codon.

The chromosomal localization and transcription directions of the 12 *BrTUA* genes (copies) were determined using the genome browse tool in BRAD. As shown in Table 2, the *BrTUA* genes were distributed on 5 chromosomes; Bra039648 was, however, not anchored on chromosomes, but located between the 212,440-214,331 bp of the scaffold 000172. Four *BrTUA* genes were located on chromosome 2, and two *BrTUA* gene copies, each, were present on chromosomes 3, 6, and 10. One *BrTUA* gene was found on chromosome 8. The *BrTUA* genes formed 2 clusters with Bra020061 in one cluster and Bra020061, Bra002260, and Bra002261 in the other, indicating that tandem duplication occurred during the evolution of *BrTUA* genes.

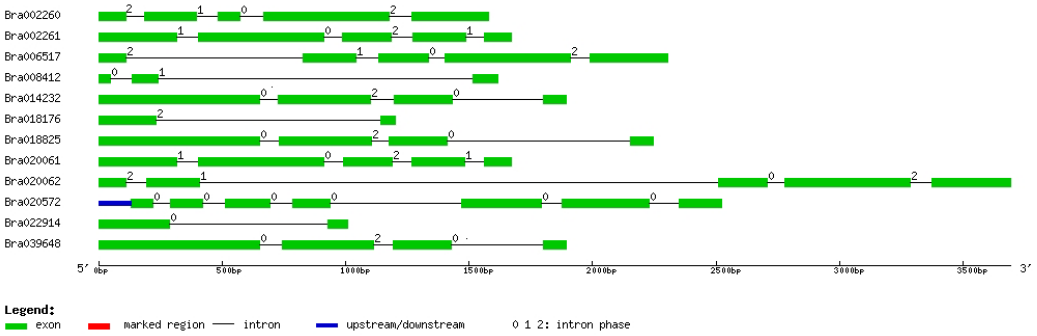

Figure 2. Intron and exon organization of the corresponding *BrTUA* genes.

Phylogenetic analysis and prediction of molecular function of *BrTUA*

The multiple alignments of full-length protein sequences and CDS of *BrTUA* were used to construct an unrooted neighbor joining phylogenetic tree with the Mega 4.1 software. The 12 *BrTUA* genes and the *TUA*s from other plants were classified into five different groups based on their CDS. Bra018825, Bra020572, Bra014232, and Bra039648 belonged to group 1 with the

genetic relationship of Bra018825, Bra014232, and Bra039648 being closer to *AtTUA*2, *AtTUA*4, and *AtTU*6. Bra002260, Bra002261, Bra006517, Bra020061, and Bra020062 belonged to group 2, with their genetic relationship being closer to *AtTUA*3 and *AtTU*5. Bra008412, Bra018176, and Bra022914 were clustered in one group. Only Bra020572 had a close genetic relationship with *Zea mays*, *Eleusine indica*, *Hordeum vulgare*, and *Alopecurus aequalis* (Figure 3A). The *TUA* phylogenetic tree reflected the genetic relationship of species, which demonstrated that the evolution of *TUA* was synchronous with evolution of the species.

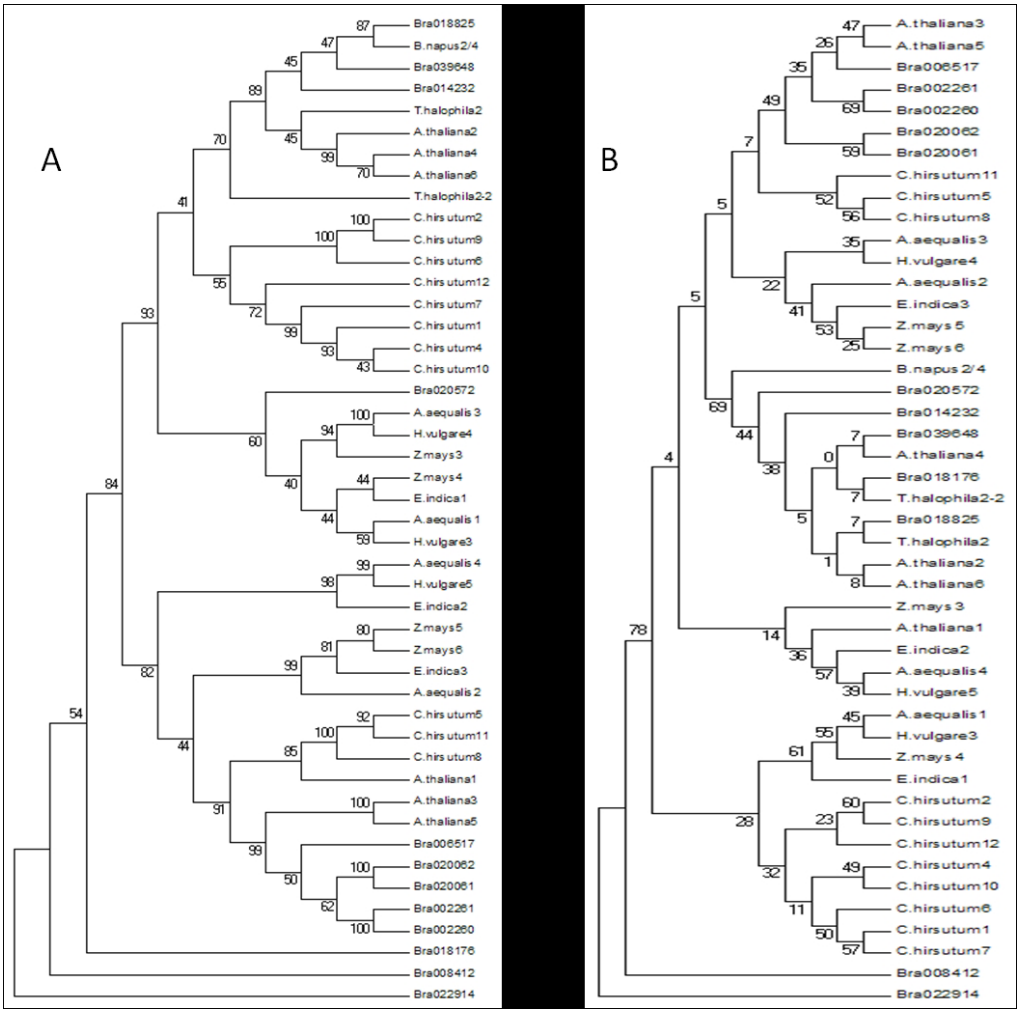

Figure 3. Analysis of genetic evolution of *BrTUA*s. Neighbor-joining phylogenetic tree based on CDS of the genes (**A**). Neighbor-joining phylogenetic tree based on the amino acid sequence (**B**).

The 12 *BrTUA* genes and the *TUAs* from other plants were classified into six different groups based on their amino acid sequences. As observed in the case of the phylogenetic tree generated on the basis of CDS, Bra002260, Bra002261, Bra006517, Bra020061, and Bra020062 belonged to

group 1, which had the closest genetic relationship with *AtTUA*3 and *AtTU*5. Bra018825, Bra014232, Bra018176, and Bra039648 belonged to group 2, which had closer genetic relationship with *AtTUA*2, *AtTUA*4, and *AtTUA*6. Among these, Bra018825, Bra039648, and Bra018176 had the closest genetic relationship with *AtTUA*, *AtTUA*4, and *ThTUA*2-2 (*Thellungiella halophila* α-tubulin2-2), respectively. Based on this phylogenetic tree, the genetic relationships of *BrTUAs* were relatively distant from the *TUAs* of *Z. mays*, *Gossypium hirsutum*, *E. indica*, *H. vulgare*, and *A. aequalis* (Figure 3B).

The molecular function of the BrTUA proteins was analyzed using the tools available at EMBL-EBI. Among the 12 BrTUA, Bra008412, Bra018176, and Bra022914 lacked a molecular function and the structural domain typical of TUA. The remaining nine BrTUAs had a molecular function and core structural domains of TUA, such as the GTPase and 2-layer sandwich domain and the C-terminal. Among the proteins of those *BrTUA* genes (copies) that had a molecular function, five (Bra002260, Bra002261, Bra006517, Bra020061, and Bra020062) had an auto-regulation site for the binding of β-tubulin (Table 2). However, Bra002260 did not have a conserved site (an invariant region rich in glycine residues).

Cis-elements in the promoter sequences of *BrTUA*

To further understand the transcriptional regulation and potential functions of *BrTUA* genes, the cis-elements of their promoter sequences were predicted. More than 50 cis-elements that could be classified into 27 types were found in the 1.5-kb upstream region of the *BrTUA* genes by Plant CARE. Five *BrTUAs*, which had the molecular function and the auto-regulation binding site for β-tubulin showed greater differentiation; their promoters had cis-regulatory elements that were involved in circadian regulation and GBF3 factor binding site (Table 3). The five *BrTUA* promoter sequences contained 12 to 14 cis-elements responsive to light. The low-temperature response cis-element was found in three *BrTUA* genes. Interestingly, cis-elements involved in hormonal response of plants were significantly different in the five *BrTUAs*; for example, the abscisic acid response element (ABRE), gibberellin-responsive element (GRE), and MJ-responsive element (TGACG-motif) were found in the different *BrTUA* promoters, but a cis-acting element involved in gibberellin-responsiveness (TATC-box) was not found in these five *BrTUAs*. Moreover, four *BrTUA* genes contained one or more cis-elements involved in tissue-specific expression, such as as-2-box, OCT, HD-Zip1, and HD-Zip2. These cis-elements in the *BrTUA* promoter might be essential in mediating the response of plants to biotic and abiotic stresses as well as during growth and development.

Analysis of gene expression by RT-PCR

The expression of *BrTUA* genes, whose proteins were predicted to have molecular function and auto-regulation binding site for β-tubulin, in various tissues and during different growth stages was analyzed by RT-PCR using gene specific primers (Figure 4A). The relative expression levels were determined after normalization for the *GADPH* gene expression. The expression of the five *BrTUA* genes was detected in the floral axis, leaf, flower, and pod whereas weak expression of Bra002260 and Bra006517 was observed in the root. The expression of the five *BrTUA* genes was not only tissue-specific but the level of expression was also different in the different tissues. The expression level of Bra006517 in leaf, flower, floral axis, and pod was much higher than that of the other four *BrTUAs*. Moreover, we found that the expression levels of Bra006517, Bra002261, and Bra020062 were relatively higher in leaf than in the other parts. The expression of Bra002260, Bra006517, and Bra002261 in the floral axis exceeded that of Bra020061 and Bra020062.

Table 2. Analysis of *Brassica rapa TUA* genes.

Arabidopsis Locus	*Arabidopsis* gene name	*Brassica* locus	Chromosome	Subgenome	Length of gene	Length of ORF	Number of introns	Predicted protein (aa)	Molecular weight (kDa)	Molecular Function	C-terminal	Conserved site	β-tubulin auto-regulation binding site
AT4G14960	TUA6	Bra039648	Scaffold000172	MF2	1892	1353	3	450	113.26	Yes	Yes	Yes	No
AT4G14960	TUA6	Bra022914	A03	MF2	1007	366	1	121	28.71	No	No	No	No
AT4G14960	TUA6	Bra020572	A02	MF1	2521	1407	6	468	116.52	Yes	Yes	Yes	No
AT4G14960	TUA6	Bra018825	A06	LF	2244	1353	3	450	112.92	Yes	Yes	Yes	No
AT5G19780	TUA5	Bra020061	A02	MF2	3690	1353	4	450	112.00	Yes	Yes	Yes	Yes
AT5G19780	TUA5	Bra020062	A02	MF2	1672	1353	4	450	111.89	Yes	Yes	Yes	Yes
AT5G19780	TUA5	Bra018176	A06	LF	1200	291	1	96	24.63	No	No	No	No
AT5G19780	TUA5	Bra008412	A02	MF1	1618	258	2	85	21.53	No	No	No	No
AT5G19780	TUA5	Bra006517	A03	MF1	2306	1380	4	450	111.56	Yes	Yes	Yes	Yes
AT5G19780	TUA5	Bra002261	A10	LF	1673	1353	4	450	111.97	Yes	Yes	Yes	Yes
AT5G19780	TUA5	Bra002260	A10	LF	1580	1233	4	410	101.93	Yes	Yes	No	Yes
AT1G50010	TUA2	Bra014232	A08	MF1	1894	1353	3	450	112.86	Yes	Yes	Yes	No

Table 3. Putative partial cis-elements in the promoters of 5 *BrTUA*s having molecular function and auto-regulation binding site for β-tubulin.

cis-regulatory elements	Bra020061	Bra020062	Bra006517	Bra002261	Bra002260
ABRE	No	Yes	No	Yes	No
ARE	Yes	No	Yes	Yes	Yes
as-2-box	No	No	No	Yes	No
ATGCAAAT motif	No	No	No	Yes	Yes
CAT-box	No	Yes	Yes	No	No
CGTCA-motif	No	Yes	No	No	No
ERE	No	No	No	Yes	Yes
GA-motif	No	No	No	Yes	Yes
GARE-motif	No	No	No	Yes	Yes
GCN4-motif	Yes	Yes	No	No	No
HD-Zip1	No	No	No	Yes	No
HD-Zip2	No	No	Yes	Yes	No
LTR	No	Yes	Yes	No	Yes
OCT	No	No	No	Yes	No
RY-element	No	No	No	No	Yes
Skn-1_motif	Yes	No	Yes	Yes	Yes
TCA-element	Yes	No	Yes	No	Yes
TGACG-motif	No	Yes	No	Yes	No

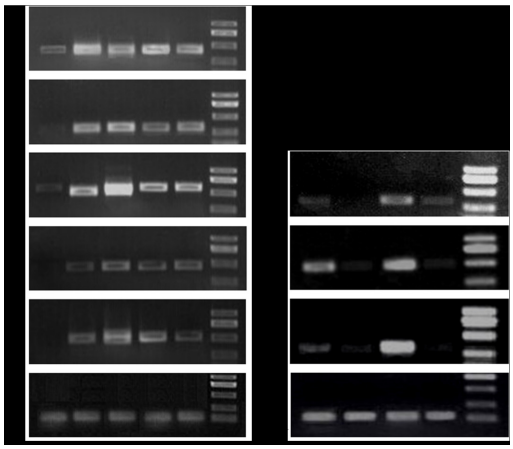

Figure 4. Gene expression of *BrTUAs* analyzed by RT-PCR. *BrTUA* expression in different organs (**A**). **a.** to **f.**, *GADPH*, Bra020062, Bra020061, Bra006517, Bra002261, Bra002260, respectively. From left to right in each figure the lanes are for root, floral axis, leaf, flower, and pod, respectively. *BrTUA* expression during the bolting process (**B**). **a.** to **d.** *GADPH*, Bra002261, Bra006517, Bra002260, respectively. From left to right in each figure the lanes are for the samples before vernalization, after vernalization, the period of leaf color change, and the period of floral axis elongation.

In a previous study, we demonstrated that the leaf color changed before bolting and after budding in the Chinese cabbage. After the leaf color changed, flower bud and floral axis of Chinese cabbage began to elongate (Hanet al., 2011). Our results, based on the proteomic analysis done before and after the color change, have revealed that tubulin levels were different during these stages (Zhang YW, Guo MH, Tang XB, Jin D, et al., unpublished data). Based on these observations, Bra002260, Bra002261, and Bra006517, whose expression was higher in the floral axis, were chosen for this experiment. The variations in their expressions were analyzed during the bolting process. The results indicated that the expression patterns of three *TUAs* were consistent, their expression reduced after vernalization, were high during the period when the leaves changed their color, and they finally reduced during the elongation of the floral axis. During the color-change of leaves, the increase in the expression of Bra002261 and Bra006517 was apparently higher than that of Bra002260, which demonstrated that Bra002261 and Bra006517 played major roles in the process of bolting (Figure 4B).

Influence of allogenic material on *BrTUA* expression

To analyze the participation of *BrTUA* in the regulation of bolting in Chinese cabbage, the effects of GA_3, MJ, and taxol on the floral axis elongation and the expression of Bra002261 and Bra006517 were studied. The floral axis elongation of Chinese cabbage apparently changed after spraying three allogenic materials approximately at the time of leaf color change. The GA_3 spray inhibited the floral axis elongation, MJ promoted its elongation, and taxol, which inhibits tubulin, had a lesser effect on the elongation; MJ in combination with taxol, however, significantly promoted the floral axis elongation (Figure 5A).

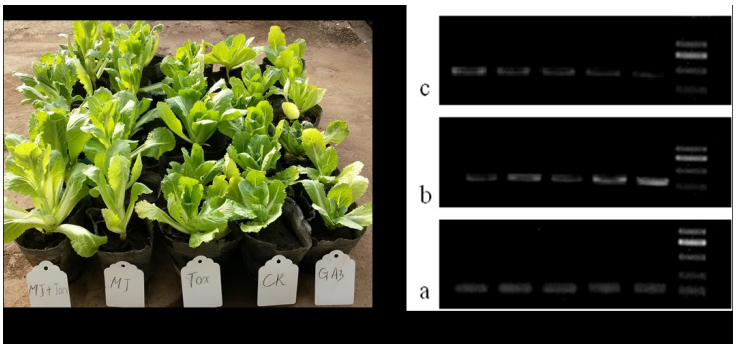

Figure 5. Effect of spraying allogenic materials on bolting and expression of *BrTUAs* in Chinese cabbage. **A.** Effect on bolting. From left to right, plants sprayed with methyl jasmonate (MJ) + taxol, MJ, taxol, control (CK), and GA_3, respectively. **B.** Effect on the expression of *BrTUAs*. **a.-c.**, G*ADPH*, Bra006517, and Bra002261. From left to right in each picture, the lanes are for samples sprayed with GA_3, CK, taxol, MJ, MJ + Taxol.

Meanwhile, we observed that after spraying GA_3, MJ, and taxol, the expression levels of Bra002261 and Bra006517 changed, and the response of the two *TUAs* to the three allogenic materials was not completely identical (Figure 5B). After spraying GA_3, the expression of Bra006517 was weakly down-regulated, whereas that of Bra002261 was weakly up-regulated. After spraying MJ, the expression patterns of the two *TUAs* were contrary to those observed for the GA_3 spray, and after the taxol spray, the expression of both the *TUAs* was weakly down-regulated. The spraying of MJ and taxol at the same time resulted in significantly up-regulation of Bra006517 expression

whereas the expression of Bra002261 was weakly down-regulated. The different response of the two *TUAs* to GA_3, MJ, and taxol might have been related to an upstream cis-acting element, which still needs to be extensively explored.

DISCUSSION

Characterization and evolution of the *BrTUA* genes in *B. rapa*

In the present study, we identified 12 members of the *TUA* gene family in Chinese cabbage. Compared to the *A. thaliana TUA* gene family that contains 6 genes, the *TUA* gene family of Chinese cabbage did not appear to have undergone obvious expansion after the evolution, including a process of triploidization and double-ploidization during the evolutionary process as a new polyploidy (Cheng et al., 2013). In terms of the structure of the *BrTUA* gene family, some *BrTUAs* lost big fragments during the process of diploidization, leading to the loss of molecular function and typical structural domains in Bra018176, Bra008412, and Bra022914, and that of auto-regulation binding site for β-tubulin in Bra039648, Bra020572, Bra014232, and Bra018825. Zhang et al. (2009) reported that after gene duplication, the exon splicing element rapidly varied in different copies and the differentiation of non-coding region was apparently greater than that of the coding region. In the present study, we also found that the promoters and introns of the *BrTUA* gene family changed a lot during the long process of evolution. Although most *BrTUA* retained four introns as in the case of *AtTUA*, the length ranged from 65 to 2100 bp and a 128-bp regulatory sequence was observed to be inserted ahead of the first codon in Bra020572. The position of introns also changed significantly; they were distributed in every region of the genes, whereas introns of plant microtubule genes were mostly distributed in the 5'-end (Dibb and Newman, 1989; Sakurai et al., 2002).

Diverse cis-elements in the promoters of *BrTUA* genes

The upstream cis-elements of *BrTUAs* revealed huge differences among the different copies, which differed in number and kinds. The differentiation of cis-acting element in the upstream regulatory regions could have followed the evolution of *B. rapa* genome. Kassahn et al. (2009) and Conant (2010) reported that the cis-acting element in the upstream regulatory regions would have rapid variation after the genome evolution and gene duplication. The evolution of *B. rapa* genome inevitably led to recombination and loss of *BrTUAs* cis-acting element.

Hormones are the basis of plant growth and development. Growth conditions of cells can be changed by regulating the organizational behavior of cortical microtubule; for example, GA_3 and ABA could regulate the cell elongation through changing the array direction of the microtubules (Mendu and Silfow, 1993; Sakiyama-Sogo and Shibaoka, 1993). In the present study, we observed that the upstream regulatory region of *BrTUAs* had numerous cis-elements involved in plant hormone response, such as ABRE, GRE, and MJ responsive element (TGACG-motif). Therefore, we sprayed GA_3 and MJ, approximately near the period of leaf color change, to investigate their effects on the expression of *BrTUAs* and on the floral axis elongation. The results showed that GA_3 restrained the floral axis elongation. In a previous study, during the period of leaf color change in Chinese cabbage, GA_3 content reached its peak (Hanet al., 2011). Thus, spraying GA_3, near the period of leaf color change, could lead to very high GA_3 content that could restrain bolting. It is reported that after MJ spray on *A. thaliana*, the expression levels of *AtGA*3 and *AtGA*20, which are the key genes of compounding, the gibberellin activity apparently reduced, resulting in late

flowering (Lin et al., 2011). However, in our experiments we found that spraying MJ could speed up the floral axis elongation and lead to early flowering of Chinese cabbage. This might be related to MJ-responsive element (TGACG-motif) in the promoter of Bra002261. Spraying MJ induced the expression of *BrTUA* (*Bra002261*), changed the arrangement of microtubules, promoted cell elongation, and accelerated the floral axis elongation.

Involvementof *BrTUAs* in regulation of bolting

Microtubule is a ubiquitous structure in the cytoskeleton, containing α-tubulin as the basic unit. Different isomers of α-tubulin protein genes have different expression patterns during a specific developmental stage or in the cells of specific tissues (Carpenter et al., 1992; Xu et al., 1999; Li et al., 2007). We had previously observed the specific expression of α-tubulin in the process of leaf color change in Chinese cabbage after the floral bud differentiation, using proteomic analysis (Zhang YW, Guo MH, Tang XB, Jin D, et al., unpublished results). Based on this observation, we could associate the variation of microtubule arrangement with the floral axis elongation and bolting after the floral bud differentiation. We analyzed the Bra002260, Bra002261, and Bra006517 expression during the process of vernalization and bolting in Chinese cabbage. The results revealed that the expression of the three *TUA*s changed with developmental process. The expression level was greatest during the period of leaf color change before bolting, which demonstrated the process of floral axis elongation and bolting needs α-tubulin protein. The *TUA*s were up-regulated after vernalization. This result was consistent with the findings of Mei et al. (2011), where they observed a differential expression of microtubular protein specificity C partner gene during winter, which is related to unfolding of the microtubular protein, and has the function of regulating the microtubular protein structure. It was found that after spraying GA_3 and MJ, the speed of floral axis elongation in the Chinese cabbage changed along with the expression of *TUA*s. The results presented in this report, establish the involvement of *BrTUA* genes in the regulation of bolting in Chinese cabbage. However, spraying taxol had little effect on *BrTUA* expression and on the speed of floral axis elongation during the period of color change in leaves, which might be due to the change in the arrangement of microtubules during this period.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Foundation for Key Technologies R&D Program (#2012BAD02B01-13), the National Natural Science Foundation of China (#31401895), and the Natural Science Foundation of Heilongjiang Province of China (#C201221).

REFERENCES

Carpenter JL, Ploense SE, Snustad DP and Silflow CD (1992). Preferential expression of an α-tubulin gene of *Arabidopsis* in pollen. *Plant Cell* 4: 557-571.

Chan J, Eder M, Crowell EF, Hampson J, et al. (2011). Microtubules and CESA tracks at the inner epidermal wall align independently of those on the outer wall of light-grown *Arabidopsis* hypocotyls. *J. Cell Sci.* 124: 1088-1094. http://dx.doi.org/10.1242/jcs.086702

Cheng F, Mandáková T, Wu J, Xie Q, et al. (2013). Deciphering the diploid ancestral genome of the Mesohexaploid *Brassica rapa. Plant Cell* 25: 1541-1554.http://dx.doi.org/10.1105/tpc.113.110486

Conant GC (2010). Rapid reorganization of the transcriptional regulatory network after genome duplication in yeast. *Proc. Biol. Sci.* 277: 869-876.http://dx.doi.org/10.1098/rspb.2009.1592

Dai ZL, Pan YP, Xiao Y, Qin WB, et al. (2010). Effects of gibberellin treatments at different concentrations on bolting and flowering of common head cabbage. *Acta Agric. Shanghai* 26: 69-71.

Dibb NJ and Newman AJ (1989). Evidence that introns arose at proto-splice sites. *EMBO J.* 8: 2015-2021.

Dixon DC, Seagull RW and Triplett BA (1994). Changes in the accumulation of α- and β-tubulin isotypes during cotton fiber development. *Plant Physiol.* 105: 1347-1353.

Han DP, Li CG and Zhang YW (2011). Physiological changes before and after leaf color transition in the bolting process of Chinese cabbage. *China Veget.* 1: 34-38.

Kassahn KS, Dang VT, Wilkins SJ, Perkins AC, et al. (2009). Evolution of gene function and regulatory control after whole-genome duplication: comparative analyses in vertebrates. *Genome Res.* 19: 1404-1418. http://dx.doi.org/10.1101/gr.086827.108

Kopczak SD, Haas NA, Hussey PJ, Silflow CD, et al. (1992). The small genome of *Arabidopsis* contains at least six expressed α-tubulin genes. *Plant Cell* 4: 539-547.

Li L, Wang XL, Huang GQ and Li XB (2007). Molecular characterization of cotton *GhTUA*9 gene specifically expressed in fibre and involved in cell elongation. *J. Exp. Bot.* 58: 3227-3238. http://dx.doi.org/10.1093/jxb/erm167

Lin CC, Chu CF, Liu PH, Lin HH, et al. (2011). Expression of an *Oncidium* gene encoding a patatin-like protein delays flowering in *Arabidopsis* by reducing gibberellin synthesis. *Plant Cell Physiol.* 52: 421-435. http://dx.doi.org/10.1093/pcp/pcq206

Mei JF, Tang CQ, Xu DL, et al. (2011). Study on diggerential gene expression of tea plant (*Camellia sinensis*) during cold acclimation in winter. *Chin. J. Tropical Crops.* 32: 648-652.

Mendu N and Silfow CD (1993). Elevated levels of tubulin transcripts accompany the GA_3-induced elongation of oat internode segments. *Plant Cell Physiol.* 34: 973-983.

Oakley RV, Wang YS, Ramakrishna W, Harding SA, et al. (2007). Differential expansion and expression of α- and β-tubulin gene families in *Populus. Plant Physiol.* 145: 961-973. http://dx.doi.org/10.1104/pp.107.107086

Parrotta L, Cai G and Cresti M (2010). Changes in the accumulation of α- and β-tubulin during bud development in *Vitis vinifera* L. *Planta* 231: 277-291. http://dx.doi.org/10.1007/s00425-009-1053-9

Sakiyama-Sogo M and Shibaoka H (1993). Gibberellin A_3 and abscisic acid cause the reorientation of cortical microtubules in epicotyl cells of the decapitated dwarf pea. *Plant Cell Physiol.* 34: 431-437.

Sakurai A, Fujimori S, Kochiwa H, Kitamura-Abe S, et al. (2002). On biased distribution of introns in various eukaryotes. *Gene* 300: 89-95. http://dx.doi.org/10.1016/S0378-1119(02)01035-1

Thitamadee S, Tuchihara K and Hashimoto T (2002). Microtubule basis for left-handed helical growth in *Arabidopsis. Nature* 417: 193-196. http://dx.doi.org/10.1038/417193a

Xu L, Zheng WZ, Zuo ZH, et al. (1999). Progress in molecular biological studies of α-tubulin in maize. *Chin. B. Botany* 16: 488-494.

Zhang Z, Zhou L, Wang P, Liu Y, et al. (2009). Divergence of exonic splicing elements after gene duplication and the impact on gene structures. *Genome Biol.* 10: R120. http://dx.doi.org/10.1186/gb-2009-10-11-r120