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Cloning, Expression and Vector Construction of *BrSOT16* Related to Glucosinolate Synthesis in *Brassica rapa* ssp. *chinensis* L.

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Abstract Sulfotransferase is an enzyme that catalyzes the transfer of sulfate groups from 3'-phosphoadenosine 5'-phosphosulfate to various receptor molecules, and plays an important role in plant growth and development as well as resistance to diseases and insect pests, etc. In this study, the 1 020 bp gene sequence of *BrSOT16* was obtained by homologous cloning from *Brassica rapa*. Bioinformatics analysis showed that *BrSOT16* encoded a stable hydrophilic protein containing 339 amino acids, whose molecular mass and theoretical isoelectric point were 39.25 kD and 5.50, respectively. It was a member of the plant sulfotransferase family containing four conserved regions and having no signal peptide and transmembrane domain. Evolutionary analysis showed that *BrSOT16* had the closest relationship with *BcSOT16* and *BnSOT16*. QRT-PCR analysis showed that *BrSOT16* showed an expression pattern that increased first and then decreased as the plants continued to develop, and it had the highest expression level in the leaves of pakchoi at the twelve-leaf stage. The overexpression vector of *BrSOT16* was constructed by the method of homologous substitution for the biological function identification, which could provide experimental materials and technical assistance for further study on the genetic engineering of pakchoi.

Keywords *Brassica rapa*; Sulfotransferase; Glucosinolates; Gene clone; Gene expression; Vector construction

Glucosinolates are important secondary metabolites rich in sulfur in plants. The system of glucosinolate-myrosinase plays an important role in plant defense against herbivores and pathogens (Redovnikovic et al., 2008). When the tissues of cruciferous plants are damaged, glucosinolates are hydrolyzed by myrosinase to produce isothiocyanates, nitriles and thiocyanates, which have antibacterial, anti-inflammatory and anti-cancer effects on human body (Bones and Rossiter, 2006; Brunelli et al., 2010; Azeem et al., 2015; Wu et al., 2015; Wu et al., 2017). For example, indole-3-carbinol, a degradation product of indole-3-methylthioglycoside, has been proved to be a good anticancer agent. Indole-3-carbinol induces cell cycle arrest at G0/G1 through the activation of p53 at Ser 15, increase the formation of fragmented DNA and apoptotic bodies, and thus inhibit the growth of cancer cells (Choi et al., 2010). Indole-3-acetonitrile production from indole-3-methylthioglycoside can stimulate the intestines and stomach of *Pieris rapa* and deters oviposition (Devos et al., 2008). Therefore, indole glucosinolates play an important role in human health and plant defense.

Sulfotransferase (SOT) can transfer sulfuryl from the 3'-phosphoadenosine 5'-phosphosulfate to the appropriate hydroxyl group of the specific substrate, making the substrate sulfuric acid. Reports have shown that sulfate compounds play an important role in plants. It not only as hormones and secondary metabolites in stress defense, but also as sulfur storage (Klein and Papenbrock, 2004). SOT16 is a member of the plant sulfotransferase family. It can transform non glucosinolates into desulfoglucosinolate. It is one of the key enzymes in the formation of indole glucosinolates (Klein et al., 2006). In the early 1990s, the full-length cDNA of flavonol 3-and 4'-SOTs was first cloned from *Flaveria chloraefolia* (Klein and Papenbrock, 2004), followed by the cloning of SOT genes from

Brassica, *Limonium*, *Mimosa* and other species (Varin et al., 1992). At present, 18 AtSOTs have been found in *Arabidopsis thaliana*. Among them, AtSOT16, AtSOT17, and AtSOT18, are involved in the sulfurization of desulfoglucosinolate (Marion et al., 2006). AtSOT16 clearly prefers tryptophan- and phenylalanine-derived desulfoglucosinolate, whereas derived from methionine are the preferred substrates of AtSOT17 and AtSOT18. A total of 56 *SOT* genes were identified in *Brassica rapa*., which can be divided into 9 groups. Among them, 14 *SOT* genes in group VII may be related to glucosinolate synthesis (Jin et al., 2019). A total of 71 *SOT* genes were identified in *Brassica napus*, of which 11 were homologous genes of Arabidopsis glucosinolate synthesis related *SOT* genes, and BnSOT16 and BnSOT17 were found to be substrate specific (Hirschmann and Papenbrock, 2015).

Chinese cabbage (*Brassica rapa* ssp. *chinensis* L.) is an important vegetable crop of Brassicaceae, *Brassica*, which is popular among consumers. The results showed that 8 glucosinolates were detected in Chinese cabbage, including 3 aliphatic glucosinolates, 4 indole glucosinolates and 1 aromatic glucosinolates. Among them, the content of aliphatic glucosinolates was higher, and the variation of each glucosinolate content was highly dependent on genotype (Kang et al., 2006; Liao et al., 2011). The components and content of glucosinolates in Chinese cabbage are closely related to quality traits and disease resistance. The changes of 3-butenyl glucosinolates and 4-Pentenyl glucosinolates can affect the flavor of Chinese cabbage. And the increase of indole glucosinolates can inhibit the infection of plant pathogens on Chinese cabbage (Guillermo et al., 2007; Klein and sattely, 2017). In this study, a homologous gene of AtSOT16 in *Arabidopsis thaliana* was cloned from Chinese cabbage by homologous cloning, and its sequence, structure, evolution and expression pattern were analyzed. Finally, the overexpression vector of the gene was constructed, which provided experimental materials and technical support for further study on the relationship between glucosinolate metabolism and plant stress resistance in Chinese cabbage.

1 Results and Analysis

1.1 Cloning and identification of *BrSOT16* gene in *Brassica rapa*

The results of total RNA quality detection showed that the RNA band was clear (Figure 1A), which could be used for the next experiment. The total RNA of *Brassica rapa* was reverted into cDNA by TaKaRa's reverse transcription kit, and a pair of specific primers were designed as the template for PCR amplification. The resulting amplified fragment was about 1 000 bp (Figure 1B). Then, send the amplified product to the sequencing company for sequencing. The sequencing results are consistent with the predicted target sequence (BraA02g022100.3C) in *Brassica* Database, which encodes a protein containing 339 amino acids (Figure 2).

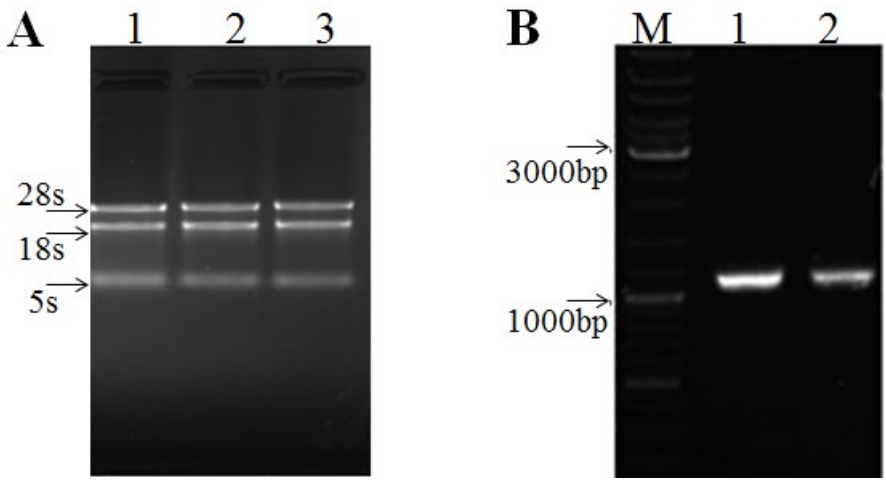

Figure 1 Total RNA extraction and PCR amplification of *BrSOT16* in *B. rapa*

Note: A: Detection of total RNA in Chinese cabbage; 1,2,3: RNA bands; B: Amplification of *BrSOT16*; M: DL5000 DNA Marker; 1, 2: PCR amplification products

```
1   ATGGAACCAACCACGACCCAGAACGGATCCGAACTCGAACTCGAACTCTCGGAGTTCGAGAAGACCCAGAAG
1    M  E  P  T  T  T  Q  N  G  S  E  L  E  L  E  L  S  E  F  E  K  T  Q  K
73  AAGTACCAAGACTTCATCGCTTCCCTCCCAAAGAGCAAAGGCTGGAGACCCAAAGAGATCCTCATCCAACAC
25  K  Y  Q  D  F  I  A  S  L  P  K  S  K  G  W  R  P  K  E  I  L  I  Q  H
145 GGCGGACACTGGTGGCAAGAATGTCTCCTCGAAGGCCTCCTCCACGCCAAAGACCACTTCCAAGCCCGACCC
49  G  G  H  W  W  Q  E  C  L  L  E  G  L  L  H  A  K  D  H  F  Q  A  R  P
217 ACCGACTTCCTCGTCTGCAGCTACCCGAAAACCGGAACCACGTGGCTCAAAGCCCTGACGTACGCCATCGTC
73   T  D  F  L  V  C  S  Y  P  K  T  G  T  T  W  L  K  A  L  T  Y  A  I  V
289 AACCGCTCTCGCTTCGACGACGCCACGAACCCGCTCCTCAAACGGAACCCTCACGAGTTCGTGCCTTACGTG
97   N  R  S  R  F  D  D  A  T  N  P  L  L  K  R  N  P  H  E  F  V  P  Y  V
361 GAGATAGACTTCGCGTTTTACCCGACCGTCGACGTCCTTCAAGATCAAAAGAACCCACTATTCTCTACCCAT
121  E  I  D  F  A  F  Y  P  T  V  D  V  L  Q  D  Q  K  N  P  L  F  S  T  H
433 ATCCCAAACGGGTCGTTACCGGACTCGATAGTGAACTCCGGTTGCAAGATGGTTTACATCTGGAGAGACCCC
145  I  P  N  G  S  L  P  D  S  I  V  N  S  G  C  K  M  V  Y  I  W  R  D  P
505 AAGGACACGTTCATCTCCATGTGGACTTTCTTGCACAAGGAGAAGTCTCAAGAAGGTCAACTCGCGAGTCTT
169  K  D  T  F  I  S  M  W  T  F  L  H  K  E  K  S  Q  E  G  Q  L  A  S  L
577 GAAGAGTCTTTTGATATGTTCTGCAAAGGCTTGTCGGTGTACGGTCCTTATCTTGATCATGTGTTGGGTTAC
193  E  E  S  F  D  M  F  C  K  G  L  S  V  Y  G  P  Y  L  D  H  V  L  G  Y
649 TGGAAGGCTTACCAAGATAACCCGGAGAGGATTCTGTTCCTTAGGTACGAGACGATGAGAGCTAATCCTTTG
216  W  K  A  Y  Q  D  N  P  E  R  I  L  F  L  R  Y  E  T  M  R  A  N  P  L
721 CCGTTTGTGAAGAGGTTGGCTGAGTTCATGGGTTATGGGTTTAGTGCTGAGGAAGAGGAGAAAGGGGTTGCT
241  P  F  V  K  R  L  A  E  F  M  G  Y  G  F  S  A  E  E  E  E  K  G  V  A
792 GAGAATGTTGTGAAGCTTTGTAGTTTCGAGACGTTGAAGAATCTTGAAGCTAATAAAGGAGATAAGGAGAGG
265  E  N  V  V  K  L  C  S  F  E  T  L  K  N  L  E  A  N  K  G  D  K  E  R
865 GAGGACCGTCCTGCGGTTTATGCGAATAGTGCTTATTTTAGGAAAGGGAAGGTTGGAGATTGGGCGAATTAT
289  E  D  R  P  A  V  Y  A  N  S  A  Y  F  R  K  G  K  V  G  D  W  A  N  Y
937 CTGACTCCGGAGATGGCTGCTCGTATCGATGGGTTAGTGGAAGAGAAGTTTAGAGATACTGGCTTGCTTGAA
313  L  T  P  E  M  A  A  R  I  D  G  L  V  E  E  K  F  R  D  T  G  L  L  E
1009CATGATCAGTGA
337  H  D  Q  *
```

Figure 2 cDNA and amino acid sequence of *BrSOT16*

1.2 Physical-chemical properties of *BrSOT16* in *Brassica rapa*

The results of compute pl /Mw software showed that the relative molecular weight of *BrSOT16* gene was 39.25 kD and the theoretical isoelectric point was 5.50. With the help of Protparam, we found that in the amino acid sequence of *BrSOT16*, the total number of negatively charged amino acid residues was 51, the total number of positively charged amino acid residues was 41, the fat coefficient was 72.21, and the instability coefficient was 32.91, indicating it is a stable protein. ProtScale was used to analyze the hydrophobicity of *BrSOT16* protein. The stronger the hydrophobicity, the greater the positive value. The results showed that *BrSOT16* protein was a hydrophilic protein (Figure 3).

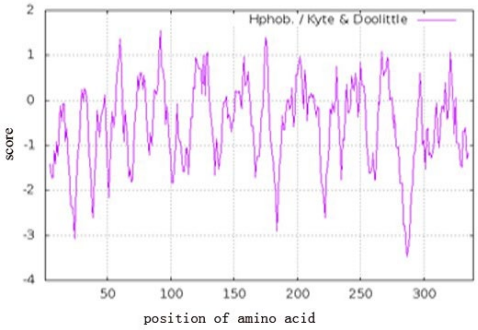

Figure 3 Hydrophobicity analysis of *BrSOT16*

1.3 Functional prediction of BrSOT16 protein in *Brassica rapa*

TMHMM was used to predict the transmembrane structure of BrSOT16 protein. The results showed that there was no transmembrane helix and no transmembrane structure in the amino acid sequence of this protein. Analysis by SignalP-5.0 software showed that the amino acid sequence of BrSOT16 protein did not contain signal peptide. Using SMART to predict the key functional sites of BrSOT16 protein, the results showed that the protein contained a typical sulfotransferase domain (Figure 4).

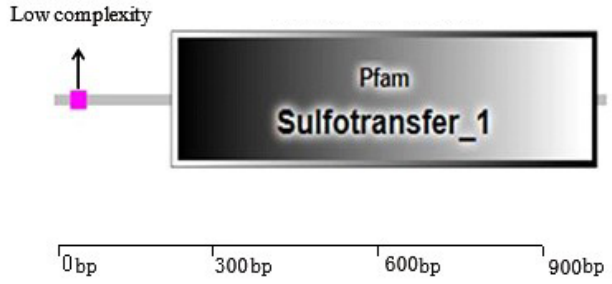

Figure 4 Functional domain analysis of *BrSOT16*

1.4 Multiple sequence alignment and evolutionary analysis of *BrSOT16* in *Brassica rapa*

The amino acid sequences encoded by 12 SOT genes in Genebank (Table 1) and *BrSOT16* genes were used for multiple sequence alignment. The analysis results showed that the amino acid sequences of the 13 conserved domains of sulfotransferase were highly similar, and BrSOT16 sequences had four conserved domains of sulfotransferase (Figure 5). Domain I is located in the N-terminal part of *BrSOT16*, and domain IV is located in the C-terminal part of *BrSOT16*. They are the most conserved domains in the amino acid sequence of SOT. They mainly recognize and bind to the co substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Using MEGA4.0 software and adjacency method to construct rootless evolutionary tree, the results showed that *BrSOT16* was the closest to BcSOT16 and BnSOT16 (Figure 6), indicating that they had the closest evolutionary relationship and had some functional similarity.

Table 1 Information of the *SOTs* from 12 species

Gene name	Species	GenBank No.
BnSOT16	*Brassica napus*	XP_013676725.1
AnSOT16	*Arabis nemorensis*	VVA95228.1
AtSOT16	*Arabidopsis thaliana*	NP_177550.1
EsSOT16	*Eutrema salsugineum*	XP_006390466.1
BoSOT16	*Brassica oleracea*	ADW54463.1
BcSOT16	*Brassica cretica*	RQM01601.1
CrSOT18	*Capsella rubella*	XP_006302485.1
CrSOT16	*Capsella rubella*	XP_006302516.1
EsSOT18	*Eutrema salsugineum*	XP_006390467.1
CsSOT16	*Camelina sativa*	XP_010428378.1
RsSOT16	*Raphanus sativus*	XP_018448684.1
ThSOT16	*Tarenaya hassleriana*	XP_010537407.1

1.5 Expression pattern analysis of *BrSOT16* in *Brassica rapa*

Fluorescence quantitative PCR was used to analyze the expression of *BrSOT16* in Chinese cabbage leaves of different development periods (Cotyledon stage, two leaves stage, four leaves stage, eight leaves stage, twelve leaves stage and sixteen leaves stage) and in roots, stems and leaves of sixteen leaves stage. The results showed that with the continuous growth of plants, the expression of *BrSOT16* in Chinese cabbage leaves increased at first and then decreased, and the expression of *BrSOT16* reached the highest level at the twelve leaves stage (Figure 7A). Quantitative results in different tissues and organs of Chinese cabbage at the sixteen leaves stage showed that *BrSOT16* was specifically highly expressed in the roots (Figure 7B).

1.6 Construction of *BrSOT16* gene over expression vector in *Brassica rapa*

The PCR product of *BrSOT16* gene was extracted, purified and recovered. With the help of homologous substitution, the target fragment was connected to linearized pBI121 vector. The recombinant plasmid was transformed into competent cells of *E.coli* by heat shock transformation. The positive strain was identified by colony PCR (Figure 8A) and sent to sequencing company for sequencing. The recombinant plasmids with correct sequencing were selected and verified by Sal I fast digestion. There is a Sal I site in *BrSOT16* gene sequence, but there is no in pBI121 vector, so the recombinant plasmid can be digested by Sal I. Lane 1 is a linear recombinant plasmid, lane 2 is a recombinant

plasmid, its structure is complex, and the running bands are more complex, which further indicates that *BrSOT16* gene is successfully connected to pBI121 vector (Figure 8B).

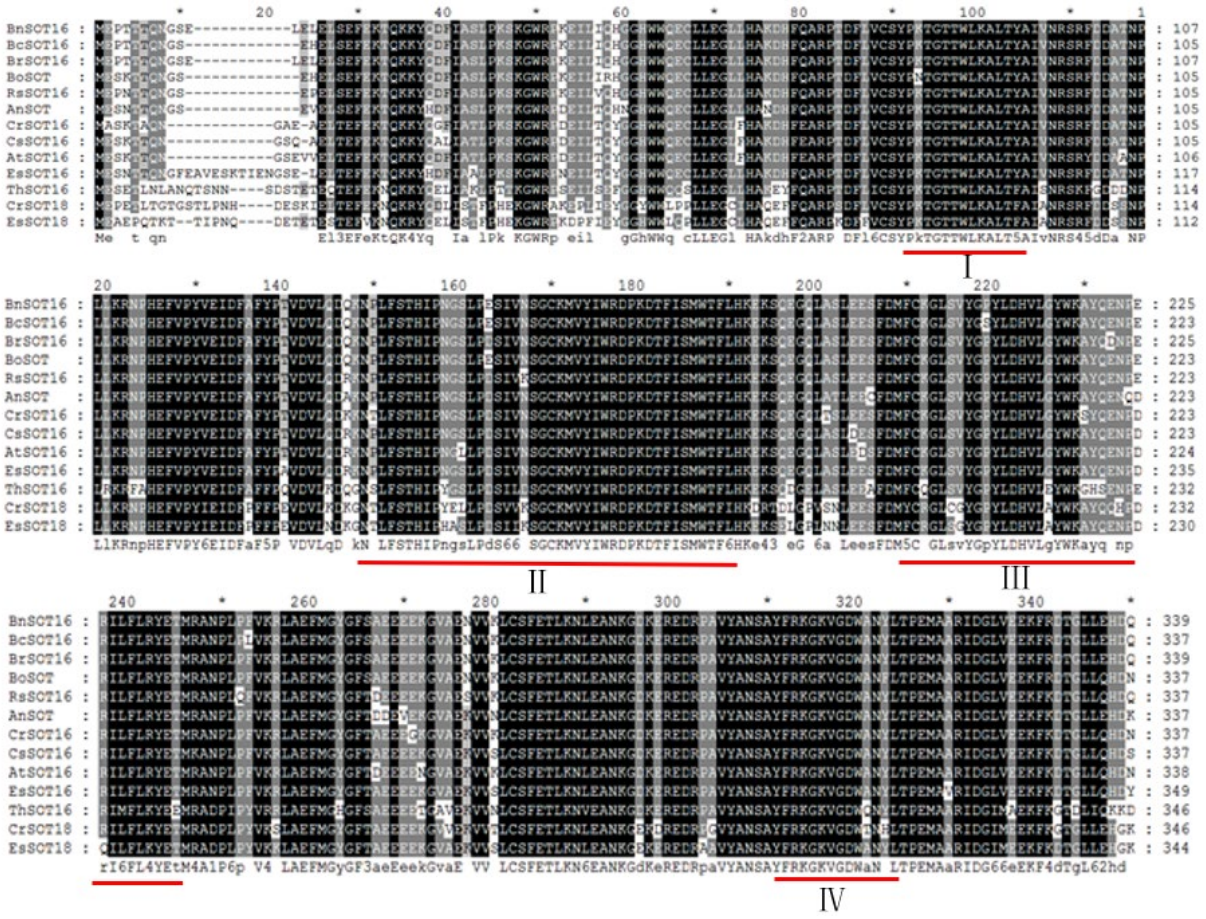

Figure 5 Multiple sequence alignment analysis of *BrSOT16* and the other SOTs

Note: I, II, III, and IV represent the four conserved domains of SOTs

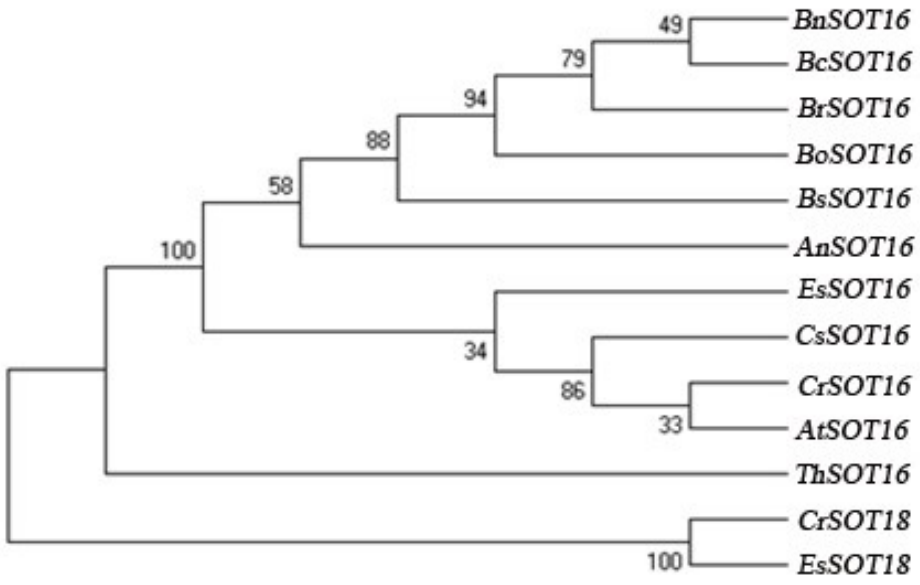

Figure 6 Evolutionary analysis of *BrSOT16*

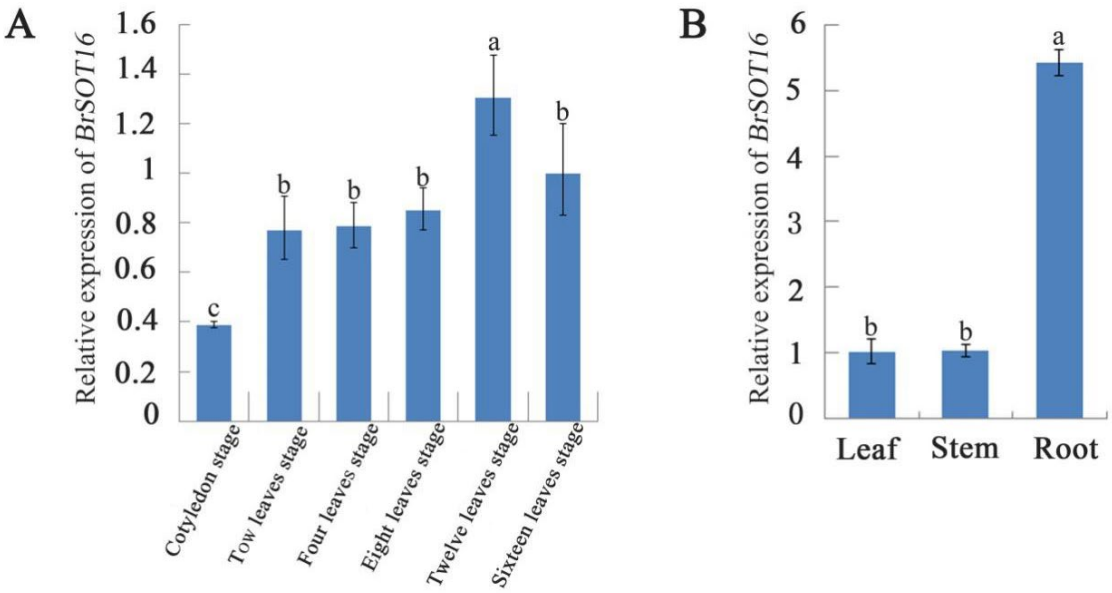

Figure 7 Expression pattern analysis of *BrSOT16* in Chinese cabbage leaves of different development periods (A) and different organs of the stage having 16 leaves (B)

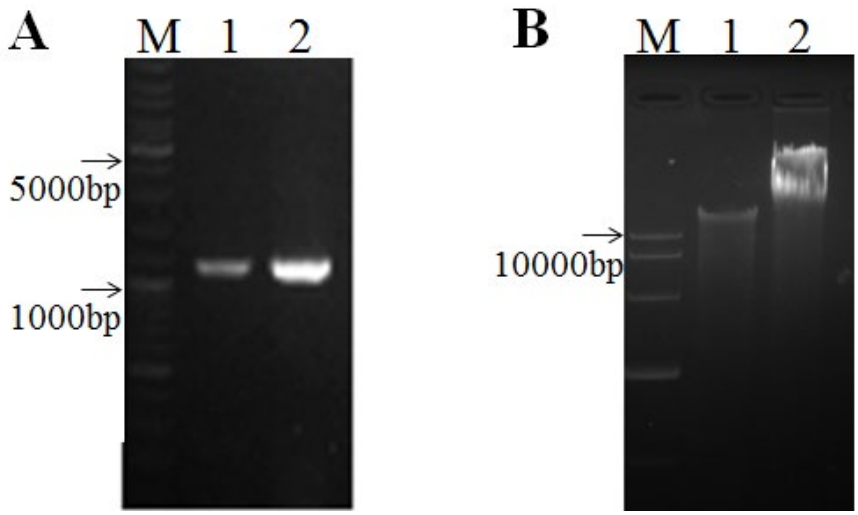

Figure 8 PCR and digestion verification of the *BrSOT16* overexpression vector

Note: A: PCR verification of recombinant plasmid; M means DL5000 DNA Marker; 1, 2: PCR amplification products; B: Digestion verification of recombinant plasmid; M means DL10000 DNA Marker; 1: digestion products of pBI121-*BrSOT16* recombinant plasmid; 2: pBI121-*BrSOT16* recombinant plasmid

2 Discussion

In order to adapt to the external environmental factors and various biotic and abiotic stresses, plants produce a wide range of metabolites in response to the environmental changes or stresses (Björkman et al., 2011; Kliebenstein and Osbour, 2012). Glucosinolates are a class of secondary metabolites of sulfur and nitrogen. Its biosynthesis and degradation pathways in *Arabidopsis thaliana* have been studied deeply and thoroughly (Sønderby et al., 2010b). Meanwhile, *Brassica rapa* and *Arabidopsis thaliana* belong to Cruciferae. However, after the triploid event, the genome of *Brassica rapa* becomes complicated, and affecting the function of specific genes (Franzke et al., 2011). In this study, *BrSOT16* gene of *Brassica rapa* was obtained by homologous cloning. The full length of this sequence is 1 020 bp, encoding a stable hydrophilic protein with 339 amino acids. Its molecular weight is 39.25 kD, theoretical isoelectric point is 5.50, and there is no signal peptide and transmembrane domain. It has been reported that the

binding site of PAPS in the functional domain of sulfotransferase is highly conserved, which can be used to identify whether the protein encoded by a gene has the function of sulfotransferase (Chapman Dr et al., 2004). In this study, the cloning and sequence analysis of *BrSOT16* showed that it contained four conserved domains of sulfotransferase, including one binding to PAPS, indicating that *BrSOT16* could encode a protein with sulfotransferase function.

With the growth of *Arabidopsis thaliana*, the expression of AtSOT16 first increased and then decreased, and the highest expression was found in roots (Klein et al., 2006). We analyzed the expression pattern of *BrSOT16* in *Brassica rapa* and found it was different in different growth stages and organs in *Brassica rapa*. With the continuous growth of the plant, the expression level of *BrSOT16* in the leaves of *Brassica rapa* increased first and then decreased and reached the highest level at the twelve leaves stage. And it was highly expressed in roots at sixteen leaves stage. Therefore, the functions of *BrSOT16* in *Brassica rapa* and AtSOT16 in *Arabidopsis thaliana* may have great similarity. Analysis of the expression pattern of *OsCST5* gene in rice showed that the highest expression level was in the embryo, followed by the root (Zhai et al., 2019). In addition, a large number of studies have shown that under biotic or abiotic stress, the expression pattern of SOT gene in plants will change more significantly in response to adverse environmental conditions. Sclerotinia sclerotiorum infection of *Brassica oleracea* can induce the expression of 4 sulfotransferase genes ST5b-Bol026202, ST5c-Bol030757, ST5a-Bol026200, and ST5a-Bol039395 related to glucosinolate biosynthesis, thus changing the content of 3-methylthiopropylglucosinolate and indole-3-methylthioglycoside (Robin et al., 2016). CaCl2 treatment can increase the expression of sulfotransferase in broccoli, which can increase the content of aliphatic glucosinolates and improve the nutritional quality of broccoli (Yang et al., 2016). Sulfotransferase CST5V11 in rice can catalyze the conversion of salicylic acid to sulfonated salicylic acid, enhance the resistance of rice to stripe virus, and inhibit the replication and transmission of virus in rice (Wang, 2013). The sulfotransferase AtSOT12 in *Arabidopsis thaliana* can sulfonate the toxin cycloheximide produced by bacteria to resist the invasion of toxic molecules (Chen et al., 2015).

In general, sulfotransferase plays an important role in plant growth and development, virus resistance and disease resistance. Therefore, it is of great significance to construct *BrSOT16* overexpression vector and study its function in plants for obtaining metabolic engineering with improved pest resistance.

3 Materials and Methods

3.1 Experimental materials

The material of this experiment is "Meidu Heiyoutong", and the seeds are sown in a small flowerpot. The proportion of peat, vermiculite and perlite was 3:1:1, and the watering standard was dry and wet. The restriction endonucleases (Xba I, Sma I, Sal I) and PCR amplification system (*rTaq* enzyme) were purchased from TaKaRa. Seamless ligase (Clone express II) was purchased from Vazyme, San Prep column DNA gel recovery kit was purchased from Sangon Biotech (Shanghai) Co., Ltd, and *E. coli* DH5α and pBI121 plasmids were preserved in our laboratory.

3.2 Total RNA extraction and cDNA synthesis in *Brassica rapa*

The total RNA in *Brassica rapa* leaves was extracted by Trizol and detected by agarose gel with a concentration of 1.2%. The reverse transcription kit of TaKaRa was used for cDNA synthesis, and it was carried out according to the instructions. 2 μL total RNA was taken as the template, and the double stranded cDNA was synthesized by reverse transcription and stored at -20℃.

3.3 Cloning of *BrSOT16* gene in *Brassica rapa*

According to the SOT16 sequence of *Arabidopsis thaliana*, BLAST was performed in *Brassica* Database (http://brassicadb.org/brad/) to find the predicted gene of *BrSOT16*. And PCR primers (SOT16-F: ATGGAACCAACCACGACCCAGAA; SOT16-R: GTCACTGATCATGTTCAAGCA) were designed according to the sequences. The above reverse transcriptional cDNA was used as template and the sequence of *BrSOT16* was amplified by KOD enzyme from TOYOBO.

The PCR reaction system was as follows: 10 μL 2×PCR Buffer, 4 μL dNTPs (2 mmol/L), upstream and downstream primer F/R 0.6 μL each, 0.4 μL KOD, 1 μL DNA, and complement ddH2O to 20 μL system. The PCR procedure was as follows: pre denaturation at 94℃ for 2 min, denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s,

extension at 68℃ for 1.5 min, and finally extension at 68℃ for 10 min. A total of 40 cycles were performed. PCR products were recovered and purified by gel recovery kit after 1.2% agarose gel electrophoresis, and the operation procedures were strictly carried out according to the instructions. It was sent to TsingKe Biotechnology Co., Ltd. for sequencing identification.

3.4 Nucleotide and amino acid sequence analysis

ProtParam was used to predict the physicochemical properties of SOT16 protein. TMHMM was used to predict the transmembrane structure of SOT16. Analyzed hydrophilicity and hydrophobicity of SOT16 protein by ProtScale. Analyzed the signal peptide of SOT16 protein by SignalP-5.0. SMART software was used to predict the functional domain of SOT16 protein. Sequence similarity analysis was performed by BLAST program of NCBI. Gene Doc software was used for multiple sequence alignment. Constructed evolutionary tree with ClustalW and NJ of MEGA4.0.

Chinese cabbage leaves of different development periods (cotyledon stage, two leaves stage, four leaves stage, eight leaves stage, twelve leaves stage and sixteen leaves stage) and in roots, stems and leaves of sixteen leaves stage were selected as materials, RNA was extracted and reverse transcribed into cDNA. Primers were designed by Primer Premier5, and *BrUBC10* gene was used as internal reference (BrUBC10-F: GGGTCCTACAGACAGTCCTTAC and BrUBC10-R: ATGGAACACCTTCGTCCTAAA), *BrSOT16* gene was used as quantitative primers (SOT16-RT-F: GGGTTATGGGTTTAGTGCTG; SOT16-RT-R: CTCCAACCTTCCCTTTCCTA). The reaction system was constructed according to the instructions of SYBR fluorescent quantitative kit of TaKaRa company. The relative expression level of target gene was calculated by $2^{-\triangle\triangle CT}$.

3.6 Construction of over expression vector

The plasmid pBI121 was digested with XbaI and SmaI and cloned with homologous primers. The linearized pBI121 vector was connected with the gel recovery product by clone express II. The system was as follows: 4 μL 5×CEII Buffer, 2 μL Exnase II, 240 ng PBI121, 80 ng target fragment, and add ddH_2O to 20 μL. The samples were placed in a PCR machine with a constant temperature of 37℃ for 30 min. After the end of the reaction, it was immediately cooled in ice water bath for 5 min. The ligation products were transformed into competent cells DH5α, and the positive clones were selected and sent to TsingKe Biotechnology Co., Ltd. for sequencing. The correct sequencing bacterial solution was added to 30 mL LB and shaken overnight at 37℃. The plasmid was extracted according to the instructions of plasmid extraction kit from TaKaRa and verified by Sal I digestion.

Authors' contributions

GEB designed and carried out the study, drafted the manuscript. MYH and SQY participated in the partial data analysis and test result analysis. LML and ZZJ conceived of the project. YYK directed the design of the study and manuscript revision. All authors read and approved the final manuscript.

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