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Cloning and Characterization of ACC Oxidase Gene (*BnACO2*) From Ramie (*Boehmeria nivea*)

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Abstract ACC oxidase (ACO) is a key enzyme in ethylene synthesis pathway. This study by rt-pcr with RACE technology get a ACO gene cloning, named *BnACO2*, total length of the gene cDNA sequence is 1 377 bp, the open reading frame of 957 bp, coded 318 amino acid polypeptide, predict its molecular weight and isoelectric point (pl) 36.145 kD and 5.60, respectively, bioinformatics analysis showed that *BnACO2* no encoding protein signal peptide and transmembrane domain structure, subcellular localization in the cytoplasm. The similarity of ACO gene nucleotide sequence and amino acid sequence was more than 82% and 84% respectively. Phylogenetic analysis showed that the ACO gene was closely related to jute and hemp. Real-time fluorescence quantitative PCR analysis showed that *BnACO2* gene was expressed in all parts of ramie, especially in female flower buds. The prokaryotic expression vector PQe-*BnACO2* was successfully constructed by double enzyme digestion. At 37°C, 1.0mmol/L IPTG induced PQe-*BnACO2* to express the target protein, and the molecular weight of the induced protein was about 36.15kda, which was consistent with the predicted protein size. This lays the foundation for further study on the function of *BnACO2* gene.
Keywords Ramie; ACC oxidase; Clone; Expression

Boehmeria Nivea (L.) Gaud is a perennial herbaceous fiber crop, which can be harvested for three seasons every year. The third season is the period of flowering and sowing, and a large amount of nutrients will be transported to the reproductive organs, leading to the decline of fiber yield and quality. Therefore, flowering will largely determine the yield and quality of ramie. It is of great significance to study the gender differentiation of ramie and its regulation mechanism to improve the yield quality and economic benefits of ramie. Ethylene is involved in the regulation of plant growth and development (Wang et al., 2004; Lin et al., 2009; Wang et al., 2011; Shi and Zhang, 2012; Jafari et al., 2013), adversity stress (Choudhury et al., 2008) and other aspects also play an important role in the gender expression process of higher plants. In recent years, wheat (Liu Hongwei et al., 2003), rice (Li et al., 1996), rape (Liu et al., 2006), cucumber (Trebitsh et al., 1987) and rice (Li et al., 1996) have been used in various fields, including wheat (Liu et al., 2003), canola (Liu et al., 2006) and cucumber (Trebitsh et al., 1987). Louis et al. (1990), and Lin and Cao (1997) also demonstrated that ethylene release is closely related to the formation of female flowers in plants. It was also verified on ramie that ethylene was closely related to the formation of female flowers of monoecious ramie (Xing et al., 2008). ACC oxidase (ACO) is the last key enzyme in catalytic ethylene biosynthesis pathway (Bradford, 2008). ACO gene was initially cloned from tomato cDNA library by Holdsworth et al. (Holdsworth et al., 1987). Now plants have been developed from Arabidopsis et al. (Chen et al., 2005; Liu et al., 2008; Pan and Lou, 2008; Li et al., 2010; Xu et al., 2010; Tian et al., 2012; Zhu, 2018; Moon et al., 2020) was cloned and applied in studies on genetic improvement of plant quality and gender differentiation. In this study, a ACO gene (*BnACO2*) in ramie was cloned and prokaryotic expression analysis was carried out on the basis of previous research work, which provided scientific basis for elucidation molecular mechanism of ethylene regulating gender expression in ramie.

1 Results and Analysis

1.1 The cloning and verification of the full-length cDNA sequence of BnACO2 gene 5 and 3

Based on the Unigene 39 964 gene fragment obtained by solexa sequencing, BLAST comparison showed that Unigene had high homology with nucleotide and amino acid sequences of ACC oxidase gene of other species, and the 3 'and 5' end sequence of this gene was deleted. RACE technology was used to clone a 5 RACE fragment of 400 bp and a 3 'RACE fragment of 350 bp (Figure 1). After splicing, the cDNA sequence of this gene was obtained with a full length of 1 377 bp, an open read frame of 957 bp, and a code of 318 amino acids, which was named *BnACO2* (Figure 2). According to the full-length cDNA sequence of *BnACO2* gene, PCR primers were designed at both ends of its ORF and amplified to obtain an expected fragment of about 960 bp (Figure 1). The segment was connected with pmD19-T vector and transformed into Trans-T1 *E. coli*. Positive clones were screened with specific primers and M13 primers and sent to the biotechnology company for sequencing verification to obtain the correct sequence clones.

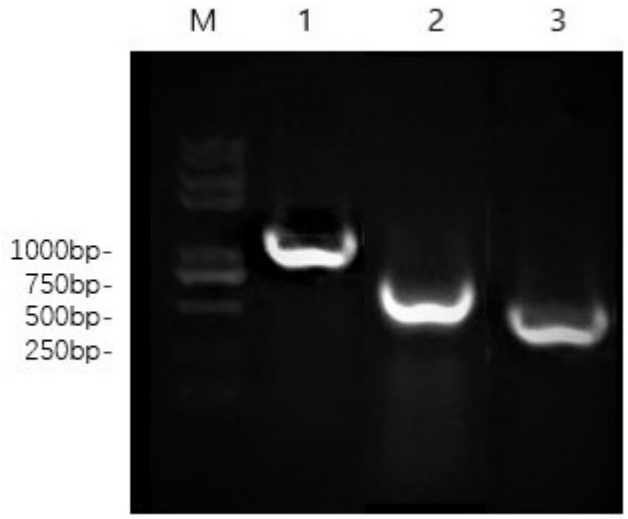

Figure 1 Electrophoresis of RACE products and RT-PCR products of ORF amplification
Note: M: Marker; 1: ORF amplified from cDNA; 2: *BnACO2* 5′RACE; 3: BnACO2 3′RACE

1.2 Protein primary prediction

The physicochemical properties of the protein encoded by *BnACO2* gene (Table 1) were analyzed by ProtParam, the protein molecular formula of *BnACO2* gene was $C_{1616}H_{2528}N_{428}O_{482}S_{15}$, the instability coefficient was 30.82, which was the stability protein, the main amino acid residues were Leu29, Glu28, Lys27, the basic amino acid residues were 39, the acid amino acid residues were 47, and the isoelectric point was 5.6.

Conservative structural analysis of the amino acid sequence of BnACO2 protein found that it has a typical 2OG-fell-oxy domain (Figure 3), indicating that *BnACO2* gene is a member of the ACC oxidase family in ramie.

1.3 Analysis of signal peptide, transmembrane region and subcellular localization

The online analysis software signalP5.0Server was used to analyze the signal peptide of the ramie BnACO2 protein sequence. The C,Y and S values were all low and the score change curve was not typical. Therefore, it was judged that the BnACO2 protein did not exist signal peptide and was a non-secreted protein.

TMHMM tool was used to analyze the transmembrane region of the protein, and the results showed that all the amino acids of BnACO2 protein were located on the surface of the cell membrane, and there was no transmembrane domain.

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   1 - ccacattaagaaactaaacttgcttaagagagaaacaaaggcaaaaagagaaagtgaaagagagattcactagag
  76 - cctaatagttcaccacccaaaaaaaaaaaATGGCCAACTTCCCTGTTATCAACTTGGAGAAGCTCAATGGTGAGG
                                    M  A  N  F  P  V  I  N  L  E  K  L  N  G  E  E
 151 - AGAGAAACACTACAATGGAGCAAATCAAAGATGCTTGTGAAAACTGGGGTTTCTTTGAGCTTGTGAATCATGGAA
          R  N  T  T  M  E  Q  I  K  D  A  C  E  N  W  G  F  F  E  L  V  N  H  G  I
 226 - TATCACATGAGCTTTTGGACACTGTTGAGAAGATGACAAAAGAGCACTACAGGAAGTGCATGGAACAAAGGTTCA
          S  H  E  L  L  D  T  V  E  K  M  T  K  E  H  Y  R  K  C  M  E  Q  R  F  K
 301 - AAGAGCATATGGCAAGCAAAGGCCTTGATGCTGTCCAAGCAGAGGTCAATGATATGGATTGGGAAAGCACTTTTT
          E  H  M  A  S  K  G  L  D  A  V  Q  A  E  V  N  D  M  D  W  E  S  T  F  F
 376 - TCTTGCGCCATCGTCCTGTCTCCAACATTTCTGAGATCCCAGATCTTGATGACCAATACAGAAGCACCATGAGAG
          L  R  H  R  P  V  S  N  I  S  E  I  P  D  L  D  D  Q  Y  R  S  T  M  R  E
 451 - AATTTGCTCAGAAGTTGGAGAAGTTGGCAGAGGAGCTACTAGACCTGTTGTGTGAGAATCTTGGCTTGGAAAAAG
          F  A  Q  K  L  E  K  L  A  E  E  L  L  D  L  L  C  E  N  L  G  L  E  K  G
 526 - GGTACCTTAAAAAGGCCTTCTATGGATCAAAGGGGTCACCAACCTTTGGCACCAAAGTCAGCAACTACCCTCCAT
          Y  L  K  K  A  F  Y  G  S  K  G  S  P  T  F  G  T  K  V  S  N  Y  P  P  C
 601 - GCCCAAAGCCTGATCTGATCAAAGGTCTCCGGGCCCACACCGATGCCGGCGGCATCATCCTCCTCTTCCAAGACG
          P  K  P  D  L  I  K  G  L  R  A  H  T  D  A  G  G  I  I  L  L  F  Q  D  D
 676 - ACAAAGTCAGCGGCCTCCAGCTCCTCAAGGACGGCCAGTGGATCGATGTCCCCCCGATGCGCCACTCCATCGTCG
          K  V  S  G  L  Q  L  L  K  D  G  Q  W  I  D  V  P  P  M  R  H  S  I  V  V
 751 - TCAACATTGGTGATCAACTTGAGGTTATCACCAATGGGAAGTACAAGAGTGTGCTTCACAGAGTGATAGCACAAA
          N  I  G  D  Q  L  E  V  I  T  N  G  K  Y  K  S  V  L  H  R  V  I  A  Q  T
 826 - CGGATGGGACACGTATGTCCATAGCCTCATTCTACAACCCTGGCAGTGATGCTGTGATCTACCCAGCACCAACGC
          D  G  T  R  M  S  I  A  S  F  Y  N  P  G  S  D  A  V  I  Y  P  A  P  T  L
 901 - TTGTGGAGAAAGAGGCTCAAGAGAAGAACCAAGTGTACCCTAAATTTGTGTTTGAGGACTACATGAAGCTCTATG
          V  E  K  E  A  Q  E  K  N  Q  V  Y  P  K  F  V  F  E  D  Y  M  K  L  Y  A
 976 - CTGGTCTCAAGTTCCAAGCCAAGGAGCCAAGATTTGAGGCCATGAAAGCTGTTGAAGCCAATGTCACCCCAATTG
          G  L  K  F  Q  A  K  E  P  R  F  E  A  M  K  A  V  E  A  N  V  T  P  I  A
1051 - CCACTGCTTAA tcaaattcaagacaagagagagacagatttattccccaaagtagtgttgatcttgagcttctgt
          T  A  *  - 318
1126 - ggtcttttgtgttacttcataattgttttctcacatgtttgagctttgtcatatggtttggtgaagtggggtttt
1201 - ctttttttcttttttttgttctttagaacaaccatagttcgttaactatatatatttgtgagaatctatttattat
1276 - ttgtagaaaaagtgaccaaagctatgtacgttcctaaataatagattattattctgagtaaaacttttttttttt
1351 - aaaaaaaaaaaaaaaaaaaaaaaaaaa - 1377
```

Figure 2 Nucleotide sequence and its deduced amino sequence of B*nACO2* gene
Note: Lowercase letters: Non-coded areas;Capital letters: Coding areas;*: The terminator

Table 1 Physical and chemical properties of the gene encoding protein *BnACO2* in ramie

Primary structural properties of proteins	Prediction
MolecuLar weight (kD)	36.145
Theoretical pI	5.60
(Asp+Glu) negatively charged residues	47
(Arg+Lys) positively charged residues	39
Instability index	30.82
half-life (h)	>10 hours (Escherichia coli, in vivo).
Aliphatic index	80.35
Grand average of hydropathicity (GRAVY)	-0.445

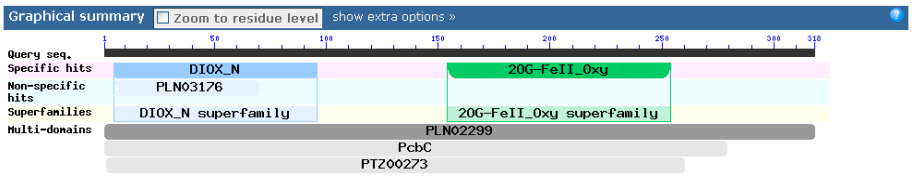

Figure 3 Conservative domain structure of *BnACO2* of protein

Subcellular localization analysis of the protein encoded by the *BnACO2* gene in ramie with PSORTB online analysis tool showed that the protein had a cytoplasmic score of 7.5 and a cell membrane score of 1.15, and its main functional site was predicted to be in the cytoplasm.

1.4 Prediction of secondary and tertiary structures of proteins

According to SOPMA online analysis, the proportion of Alpha helix is up to 40.57%. The second is Random coil, accounting for 34.59%; Extended Strand accounts for 18.55%; The proportion of Beta turn is relatively small, which is 6.29%. The BnACO2 protein was predicted to be mixed type by Garnier algorithm (Figure 4).

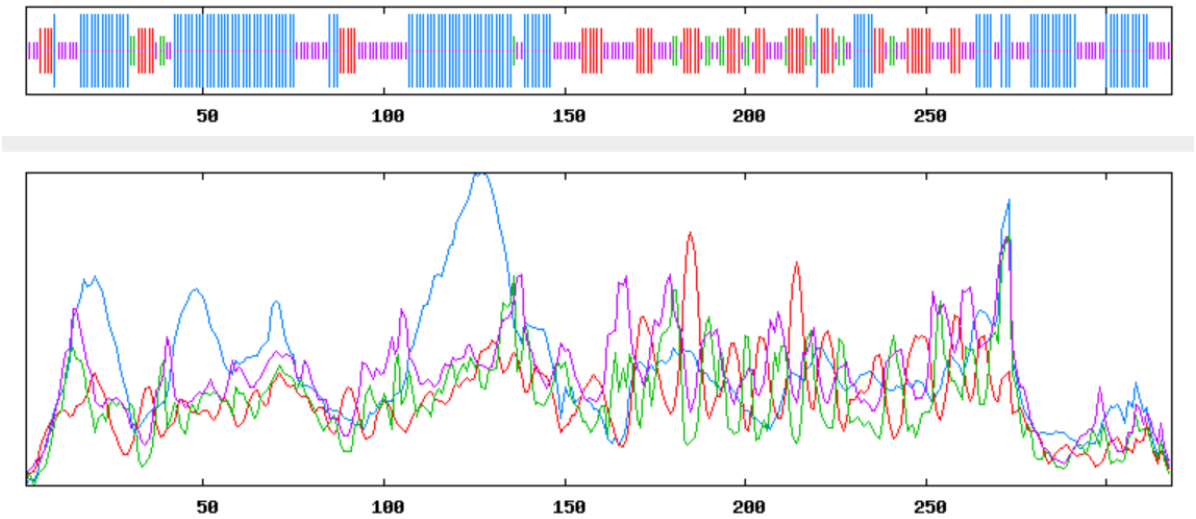

Figure 4 Secondary structure prediction of *BnACO2* protein

Using the Swiss-Model online service system to predict the three-dimensional structure of the protein encoded by *BnACO2* gene can more effectively understand the function and activity mechanism of the protein, as well as the prediction of the tertiary structure (Figure 5).

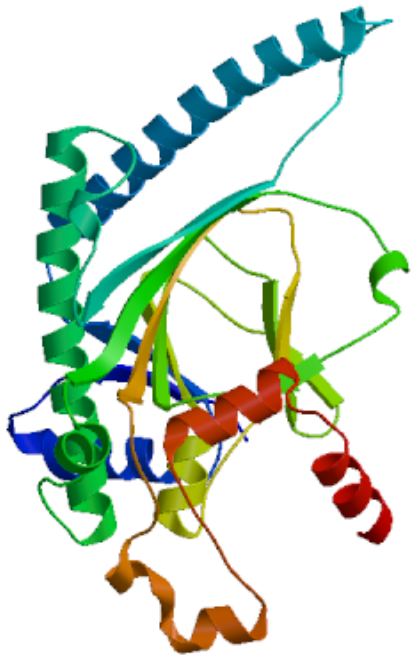

Figure 5 Prediction of tertiary structure of *BnACO2* protein

1.5 Phylogenetic analysis

By BLAST comparison of NCBI website, it was found that the homology of ACC oxidase gene sequences of BnACO2, SAN (DQ785807), sha Pear (AB042107), Apple (AB086888) loquat (GQ377219) and soybean (BT095790) were respectively 89%, 83%, 83%, 823% and 83%, respectively. The amino acid sequence similarities with that of Morus sinensis (AGN74918), PON84482, OVA20024, cayenne pepper (PHT59584) and Chinese chestnut (KAF3971868) are 92%, 92%, 87 %, 87 % and 87 % respectively. The *BnACO2* gene is similar to that of Parasponia andersonii, Cannabis, Morus notabilis, ash subspecies (Fragana vesca subsp Vesca), Capsicum Chinense, PopuLus euphratica, Macleaya Cordata and European beech (Fagus)Multiple alignment analysis of ACO amino acid sequences of sylvatica, Castanea Mollissima, Quercus Suber, and other species (Figure 6) showed that BnACO2 protein sequences were more conserved than ACC oxidase genes of other species. Phylogenetic trees based on *BnACO2* gene were constructed with MEGA software (Figure 7). The results showed that *BnACO2* gene had the closest relationship with jute and ACC oxidase genes.

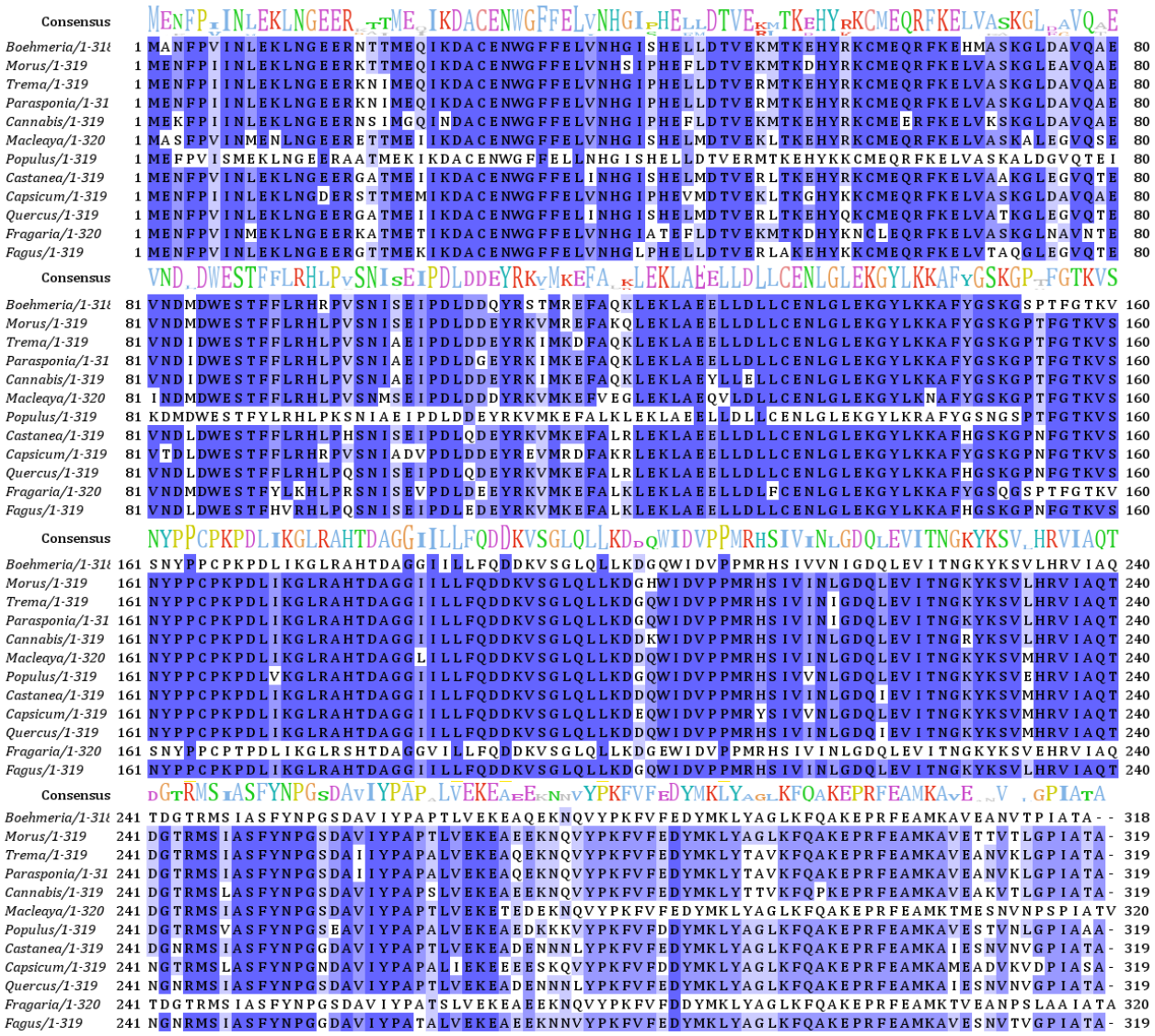

Figure 6 Comparative analysis of *BnACO2* protein sequences

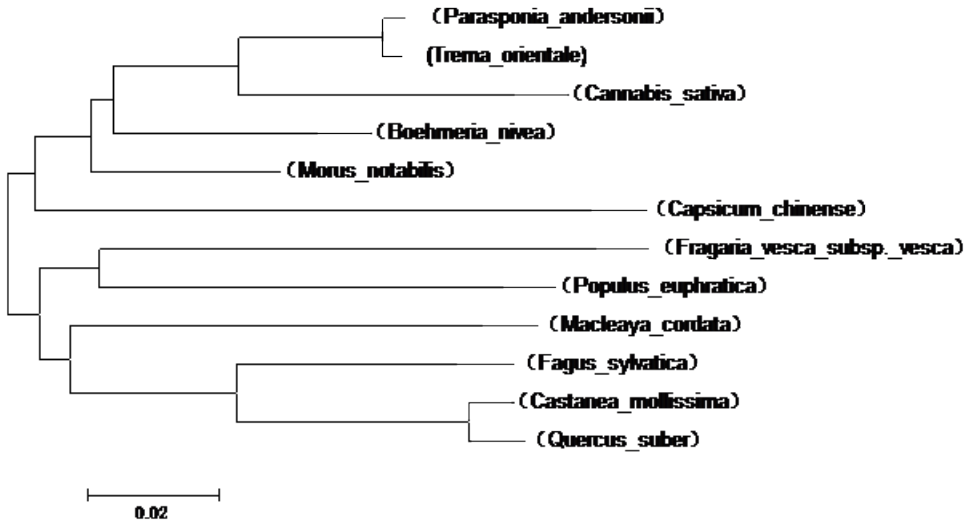

Figure 7 Phylogenetic tree based on *BnACO2* gene

1.6 Expression characteristics of *BnACO2* in different positions and lengths of flower buds

Ramie Actin (Actin) for internal genes, in ramie GBN - 08 and GBN - 09 two root of ramie material, stems, leaves, tender bud (flower bud length is less than 0.5 cm) and different lengths of flower bud, bud length 0.5 cm ~ 2.0 cm, and GBN08 for male flower bud, GBN09 female flowers bud) to extract RNA, adopt the method of real - time PCR to detect the *BnACO2* expressed in various parts of the ramie. There was no significant difference in the relative expression level of *BnACO2* gene in the roots and leaves of the two gender ramie. The expression level in the stems and flower buds of female ramie GBN-09 was significantly higher than that in the flower buds of male and female ramie GBN-08, and it was the most obvious in the flower buds (Figure 8A). *BnACO2* gene was expressed in the flower buds of ramie GBN-08 and GBN-09 of different lengths. As the flower buds of GBN-08 were male and GBN09 female, the expression level of *BnACO2* gene in the female flower buds of GBN-09 was significantly higher than that of the male flower buds of GBN-08 when the flower buds were less than 1.5cm.When the flower bud length was 2.0cm, the expression of *BnACO2* gene in male flower buds of GBN-08 increased significantly compared with the previous three stages, while the expression of *BnACO2* gene in female flower buds of GBN-09 decreased significantly (Figure 8B).

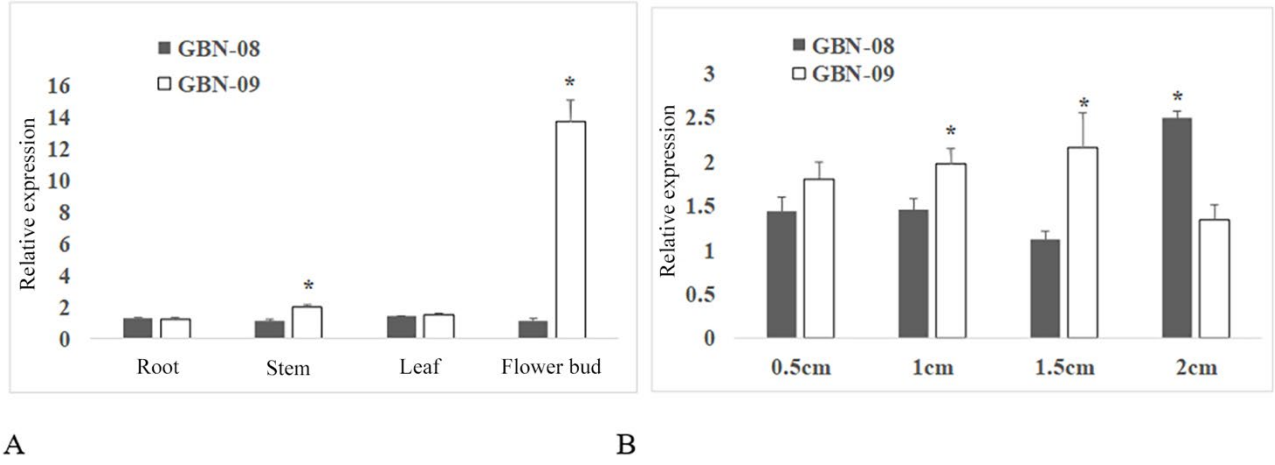

Figure 8 RNA extraction of flower buds of different lengths and expression analysis of *BnACO2* gene in various locations and flower buds of different lengths

Note: * indicates that at the level of 0.05, the two samples have significant T test; A: Expression analysis of *BnACO2* gene in different tissues of ramie (the length of flower bud is less than 0.5 cm); B: Expression analysis of *BnACO2* gene in flower buds of different lengths

1.7 Prokaryotic expression of *BnACO2* gene

The proper sequencing of plasmid pQE - BnACO2 by Sam I and BamH I restriction enzymes after enzyme digestion, for the purpose of about 4 700 bp and 957 bp fragment (Figure 9A), that recombinant plasmid pQE - BnACO2 build is successful. After the transformation of *E. coli* BL21, SDS -- PAGE electrophoresis was carried out after 1 mmol/L IPTG was used to induce expression at 37℃. It was found that the size of the target protein expressed by the recombinant plasmid 2 h and 5 h induced was similar to that expected, about 36 KD (Figure 9B), indicating that the cloned *BnACO2* gene was successfully expressed in *E. coli*.

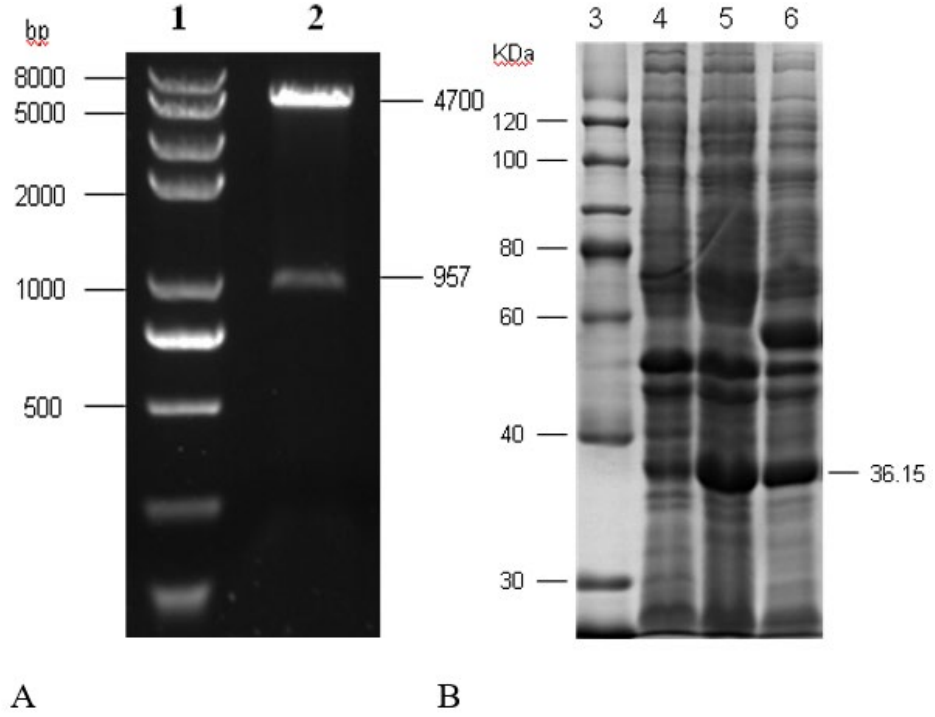

Figure 9 A: Restriction enzyme digestion of recombinant plasmid; B: Expression product of *BnACO2* gene in Escherichia coli

2 Discussion

ACO gene is a key gene in ethylene synthesis pathway. Recent studies have shown that the specific expression of ACO gene family plays an important role in the regulation of ethylene biosynthesis during the development of vegetative and reproductive organs in plants (Xia, 2009; Jafari et al., 2013). ACC oxidase gene family is expressed in many plants during flower development, but each gene has different specific expression in time and space. For example, the four ACO genes in tomato are mainly expressed in different floral apparatus (Vander et al., 1990), and PHACO1 and PHACO4 genes found in morning glory are expressed in senescence corolla and pistillate stigma respectively. This test by rt-pcr detection BnACO2 gene expression in plant monoecious and female ramie situation, found that the ACO gene in each part of the ramie root leaf bud are expressed, but in the female ramie GBN - 09 stems and flower bud of expression is significantly higher than monoecious ramie GBN - 08, and in the female flowers of both sexes ramie early developmental expression in the difference, flower bud period length is less than 1.5 cm, the gene expressed in GBN - 09 flower bud amount was significantly higher than that of GBN - 08, when flower bud length to 2 cm, The expression level of this gene in flower buds of GBN-08 was significantly higher than that of GBN-09 in the previous three stages. Whether the *BnACO2* gene may influence the gender differentiation of ramie by controlling the synthesis rate of endogenous ethylene requires further tests.

Studies have used antisense RNA technology to inhibit the expression of ACO gene, thereby inhibiting the release of ethylene to control the fruit ripening, petals falling off and other effects. The study on tomato, melon and other plants showed that after the antisense ACO gene was transferred, ethylene synthesis of fruit was

significantly reduced and fruit ripening time was significantly delayed, which was more favorable for long-term storage (Picton, 1993; Ayub, 1996). In carnation, carnation, caryophylla and other flowers, the antisense ACO gene was transferred, and ethylene production was also reduced, which delayed the decline of petals and extended the fresh-keeping period of flowers (Savin, 1995; Niu, 2012; Yu, 2004). Our previous study found that the gender differentiation of ramie was closely related to the release rate of ethylene. In the future, the antisense ACC oxidase gene could also be transferred to inhibit ethylene biosynthesis, so as to achieve the purpose of regulating the flowering of ramie and provide the breeder with ramie germplasm for vegetative growth without flowering.

This test according to ramie transcriptome sequencing information, combined the technology of RACE, from ramie *BnACO2* ACC oxidase gene cloning in the full-length cDNA sequence, through to the *BnACO2* gene bioinformatics analysis, cluster analysis, conservative structure such as domain analysis, found that belongs to 2 og *BnACO2* gene - Fe II _Oxy superfamily, is family ACC oxidase genes in ramie or members of the family, and other species ACC oxidase genes exist high conservative. Phylogenetic analysis showed that this gene was closely related to the ACO gene of jute and hemp of the same genus Nettles. The prokaryotic expression vector was successfully constructed to provide scientific basis for controlling the flowering and genetic improvement of ramie by means of genetic engineering.

3 Materials and Methods

3.1 Test materials and reagents

Experimental materials: all-female ramie "GBn-09" and monoecious ramie "GBN-08" were provided by Ramie Research Institute of Hunan Agricultural University. The roots, stems, leaves and flower buds of the plants were taken respectively at the flowering stage for subsequent analysis of the expression of target genes in different parts.

Strains and reagents: RNA extracts, reverse transcription kits, RACE kits, DNA recovery kits, Premix Taq Version 2.0 (Loading dye mix), PMD19-T, etc., purchased from Takara Biotech. DNA Maker, T4DNA ligase and Escherichia coli DH5 competent cells were purchased from TransGen Biotech. Other pure grade analytical reagents are domestic or imported; The primer and sequencing work was done by Shanghai Bioengineering Co. LTD.

3.2 Total RNA extraction and cDNA synthesis from ramie

The plant RNA extraction reagent from Bio Flux company was used to extract the total RNA of ramie flower buds according to the instructions of the kit. 2 uL of total RNA was taken for the integrity test of 0.8% common agarose gel electrophoresis, and the CONCENTRATION and purity of RNA were determined by ultraviolet spectrophotometer. The RLM-Race kit from ABI was used to reverse transcribe cDNA using total RNA as template.

3.3 Gene cloning

On the basis of Unigene 39964 gene fragment obtained from previous Solexa sequencing, 5 '-terminal and 3' -terminal PCR primers were designed for amplification using Primer 5.0 (Table 2). The correct sequences were retrieved, linked, and transformed, and then identified and sent to Shanghai Bioengineering Co., Ltd. for sequencing, and the full-length cDNA of the gene was splited.

3.4 Bioinformatics analysis

Using NCBI (https://www.ncbi.nlm.nih.gov/) to predict BnACO2 gene ORF, BLAST ratio analysis and conservative structure domain analysis; The physicochemical properties of BnACO2 protein were analyzed using Expasy online analysis website. SOPMA online software was used to predict the secondary structure of proteins. The Swiss Model was used for modeling and analysis of protein third-level structure. Online software SignalP 5.0 was used to predict protein signal peptides; TMHMM was used to predict the possible protein transmembrane regions. PSORTB was used for subcellular localization analysis. MEGA5 software was used for

multiple comparisons, Jalview was used for multiple comparison visualization drawing and DNAstar was used for the construction of evolutionary tree to analyze the evolutionary relationship.

3.5 Real-time fluorescence quantitative PCR

Ramie Actin (Actin) for internal genes, and design a fluorescence quantitative expression primers (Table 2), in the early three hemp reproductive growth take fine roots, stems, leaves, and small flower bud (less than 0.5 cm), then the flower bud length, 1, 1.5, 0.5 2 cm in flower bud, all the samples after liquid nitrogen refrigeration grinding to extract RNA, reverse transcribed into cDNA, adopt the method of Real - time PCR to detect the *BnACO2* gene expression specificity of space and time. Reaction system 20.0UL: SYBR Premix Ex Taq10.0 μL, BnACO2 BD-F and BnACO2 BD-R each 0.4 μL, ROX Reference Dye (50×) 0.4 μL, DNA template 2.0 μL, ddH_2O 6.8 μL. Amplification procedure: Pre-denaturation at 95℃ for 30 s, reaction at 95℃ for 5 s, 61℃ for 31 s for 40 cycles, dissolution at 95℃ for 15 s, 60 ℃ for 1 min, and preparation at 95℃ for 15 s. Three replicates were set for each sample, and a negative control was set. The relative expression of the target gene was calculated by $2^{-\triangle\triangle CT}$.

Table 2 Sequences of primers

Primer	Primer sequence	Purpose
F1	5′- GTTGTGTGAGAATCTTGGCTTGG-3′	Gene cloning
F2	5′- TCCATGCCCAAAGCCTGAT-3′	Gene cloning
R1	5′- TGTTGACGACGATGGAGTGG-3′	Gene cloning
R2	5′- CGTCTTGGAAGAGGAGGATGAT-3′	Gene cloning
Unigene 39964- F	5'- ATGGCCAACTTCCCTGTTATCAA -3'	ORF sequence amplification
Unigene 39964- R	5'-TAAGCAGTGGCAATTGGGGT -3'	ORF sequence amplification
Actin-F: 5'	5' GCTCCGTTGAACCCTAAG 3'	Internal reference
Actin-R: 5'	5' GCTCCGATTGTGATGATTT 3	Internal reference
BnACO2 BD -F	5' GGCATCATCCTCCTCTTCCA 3'	qRT-PCR
BnACO2 BD -R	5' CATCCGTTTGTGCTATCACTCTG 3'	qRT-PCR

3.6 Construction of prokaryotic expression vector

To sequencing the right positive of liquid expansion wave and PCR amplification, 19 - BnACO2 plasmid extraction of pMD, using restriction enzymes and Sam I BamH I confrontation and pQE - N1 carrier for double enzyme reaction after purification recycling purpose of DNA fragments, enzyme system are 20 μL: BnACO2 15 μL, 10 x Tango Buffer 2.0 μL, Sam I 0.5 μL, ddH2O 2.5 μL; PQE - N1 10 μL, 10 x Tango Buffer 2.0 μL, Sam I 1.0 μL, ddH_2O 7.0 μL. T4DNA ligase was used to connect the target fragment with the pmQe-N1 vector, transform the Escherichia coli DH5 competent cells, screen the positive colony, and take 1 mL of the culture solution shaken at 37℃ overnight to Shanghai Bioengineering Co., Ltd. for sequencing. After the plasmid was extracted and identified by double digestion, the correct recombinant plasmid was transformed into the expression strain Escherichia coli receptor cell BL21.

Authors' contributions

Luo Jin-feng and Xue Lijun were the experimental designers and executors of this research, and participated in the data sorting and the writing of the first draft of the paper. Peng Wenxian and Xiao Xu participated in part of the experiment; Xing Hucheng is the initiator and person in charge of the project, directing experimental design, data statistics, paper writing and modification. All authors read and approved the final manuscript.

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