CLONING AND BIOINFORMATIC ANALYSIS OF A NOVEL THIOLASE II GENE (BPLTHI2) FROM BETULA PLATYPHYLLA

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ABSTRACT
A full-length cDNA sequence encoding a thiolase II of Betula platyphylla was identified from a constructed cDNA-AFLP library and obtained by the rapid amplification of cDNA ends (RACE) method. The cDNA gene, designated as BplTHI2, is 1,203 bp in length and encodes an enzyme of 400 amino acid residues with a calculated MW of 40.9 kDa. The BplTHI2 displays high similarity to the thiolase II of Camellia oleifera (90 % sequence identity) and Populus trichocarpa (89 %). It was predicted to possess two main domains, an N-terminal domain (10–268) and a C-terminal domain (278–398), which contain three predicted active sites (Cys96, His356 and Cys386). Its three-dimensional structure was predicted based on the crystal structure of human mitochondria thiolase (2ib8B). Expression analysis of the BplTHI2 gene showed that it was highly expressed in female inflorescences and not expressed in male ones. We suggest that BplTHI2 is a female inflorescence-specific gene and may be responsible for the development of female inflorescence.

Keywords: birch; thiolase, female inflorescence, development, gene expression

Introduction
Thiolases II (E.C. 2.3.1.9, acetoacetyl-CoA synthases) catalyze the condensation of two units of acetyl-CoA to form acetoacetyl-CoA, a key step in the biosynthetic pathways by which beta-hydroxybutyric acid and cholesterol are made (5). This thioester-dependent Claisen condensation reaction, by which a C–C bond is formed, is an important reaction in the living world (11). Another type of thiolases, designated as Thiolases I (E.C. 2.3.1.16), exhibit the reverse enzyme activity when compared to Thiolases II and are also called degradative thiolases (16). These two types of thiolases, involving both the metabolic synthesis and degradation of carbon chains, are found both in eukaryotes and prokaryotes (1, 13, 22).

Betula platyphylla, as the most prevalent of the birch hardwoods, is widely applied in architecture, furniture, and paper production (19). At present, in order to shorten the breeding period and accelerate the breeding process, a breakthrough has been made in early flower induction. However, little was known about the background of gene expression in the flowering phase of B. platyphylla, until several genes were successfully cloned and functionally analyzed, such as BplAGL (18), BplMADS (20), and BplHEN (21). For the sake of providing some important information relevant to the study of floral development, gene differential expression in male and female inflorescences was analyzed using cDNA-AFLP technology. A large number of potential floral development EST candidates have been determined and are available in the NCBI database.

In our previous studies, some cDNA-AFLP libraries were constructed using birch female- and male-inflorescences as materials (14). According to the expression profile, we isolated a myriad of female and male inflorescence-specific ESTs, which play potential roles in floral development. In this study, the gene encoding B. platyphylla thiolase II, designated BplTHI2, was cloned from the female inflorescence library and submitted to GenBank under accession number JQ406680. The physicochemical properties and structure of the protein were predicted by bioinformatics analyzing tools, for the first time. Our present results provide a basis for further studies of the function and structure of BplTHI2, as well as its female inflorescence-specific expression manner.

Materials and Methods
cDNA-AFLP library construction and isolation of BplTHI2
The cDNA-AFLP library of birch female inflorescence used in this study was constructed in our previous work (14). Parts of expressed female-inflorescence-specific ESTs, which play potential roles in floral development. In this study, the gene encoding B. platyphylla thiolase II, designated BplTHI2, was cloned from the female inflorescence library and submitted to GenBank under accession number JQ406680. The physicochemical properties and structure of the protein were predicted by bioinformatics analyzing tools, for the first time. Our present results provide a basis for further studies of the function and structure of BplTHI2, as well as its female inflorescence-specific expression manner.

Cloning of the full-length cDNA encoding BplTHI2
The total RNA was extracted from female inflorescence of birch, using trizol reagent (Sangon, China). The extracted total RNA had a ratio of A260 to A280 of 1.89. Then, 3' RACE and 5' RACE was performed using the 3' RACE kit and 5' RACE
kit, respectively. According to the EST sequence, two primers were designed using Primer Premier 5 software, P1: 5’-TAA CGA GGG AGG AGC AGG-3’, and P2: 5’-TCA GAG CTT TGA GCG TGG TA-3’, for 3’ RACE. P2 was used for nested amplification. In the same way, P3: 5’-CCA AGC TTC AGT GCT GTC T-3’; and P4: 5’-GAG CAG CAT CAG CAT ACC C-3’ were designed for 5’ RACE. The RACE procedure was performed using the BD SMART™ RACE cDNA Amplification Kit (ClonTech, PaloAlto, CA, USA). Other manipulations followed the manufacturer’s instructions. Fragments amplified by 3’ RACE and 5’ RACE were purified by gel extraction, then cloned into pMD18-T Vector (TAKARA, Dalian, China) and sequenced on a Perkin Elmer Applied Biosystems apparatus 3700.

Bioinformatics

Sequence information was obtained by comparing with the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) database, using BLAST sequence alignment with default parameters, and significant similarity was scored with the ESTs with an E-value lower than $10^{-10}$. The physicochemical properties were analyzed using the ProtParam tool (http://web.expasy.org/protparam/). The multiple protein sequence homological alignment was carried out using the software package DNAMAN 6.0. Phylogenetic analysis was analyzed using the software MEGA 4.0. Signal peptides and subcellular location were predicted by the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP-3.0/) and TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Conserved domains and motifs were predicted by the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and Pfam 25.0 (http://pfam.sanger.ac.uk/) together. Secondary structure and topology was predicted by the SOPMA SECONDARY STRUCTURE PREDICTION MEHTOD (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). Tertiary structure was established using SWISS-MODEL (http://swissmodel.expasy.org/) based on homology modeling.

RT-PCR analysis

For semi-quantitative RT-PCR, two primers P5: 5’- ACG AGG GAG GAG CAG GAT-3’ and P6: 5’-TGC CAG CAG TGA CAG AAC C-3’) were designed according to the cDNA sequence of the BplTHI2 gene. The actin gene was chosen as an endogenous control (P7: 5’-CAT CTC TGA TCG GAA TGG AAG-3’ and P8: 5’-AGA TCC TTT CTG ATA TCC ACG-3’). The ca. 30-year-old birches in Pangquangou Tourist Scenic Area, Shanxi Province, were selected as RT-PCR experimental materials. The same size and developmental phase of male and female inflorescences were chosen for this experiment. Male (from 20 May 2009 to 30 May 2009) and female (from 20 Apr. 2009 to 30 Apr. 2009) inflorescences were a mixture of samples with an interval of every 2 d, respectively. Fresh shoots and leaves were also a mixture of samples from the same phase. All samples were immediately frozen into liquid nitrogen after collection, and stored at -80 °C for RNA isolation. RT-PCR was carried out using 50 ng cDNA of fresh shoots, leaves, male inflorescences and female inflorescences as a template, respectively. The PCR mixture included 2 µL of 10×PCR buffer, 0.5 µL of dNTP (10 µmol/L), 0.5 µL of primer (20 µmol/L), 0.5 µL of cDNA, 0.2 µL of Taq polymerase (TAKARA, Dalian, China), and sterile H2O to a total of 20 µL. The PCR procedure was initially started with 95 °C denaturation for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and an additional extension for 5 min at 72 °C.

Results and Discussion

Cloning of the BplTHI2 gene

The cloning of this gene depended mainly on the combination of cDNA-AFLP library construction and the screening of candidate genes (17). To clone the full-length of thiolase II cDNA from female inflorescence of birch, 5’ and 3’ RACE protocols were used. DNA fragments ca. 600 bp and 550 bp was cloned by 3’ RACE and 5’ RACE, respectively. According to the selected EST of BplTHI2, these three fragments were assembled by DNAMAN and an entire ORF of 1203 bp was confirmed finally by sequence analysis (Fig 1). This entire ORF has been deposited in the GenBank database (accession number JQ406680).

Fig. 1. Nucleotide and deduced amino acid sequences of thiolase II from birch (Betula platyphylla) female inflorescence. This nucleotide sequence has been deposited in the GenBank database (accession number JQ406680).
Bioinformatics analysis of the BplTHI2 gene

Bioinformatics represents a new field between molecular biology and computers, which focus to use of computer databases and computer algorithms to analyze proteins and genes (10). Tools of bioinformatics including computer programs help to reveal fundamental mechanisms underlying biological problems related to the structure and function of macromolecules and biochemical pathways. In our study, we used online tools to isolate and analyze BplTHI2 in order to deduce some fundamental physicochemical properties.

According to the obtained data, we can predict possible BplTHI2 functions and design novel experimental approaches to verify whether they are correct or not.

The result from the GenBank BLAST showed that the cloned cDNA belongs to thiolase II of the thiolase family. Its deduced protein is composed of 400 amino acids with a calculated MW of 40.9 kDa and a pI value of 6.54. The extinction coefficients of this enzyme were found to be in the range from 19940 (assuming all pairs of Cys residues form cystines) to 20565 (assuming all Cys residues are reduced) M⁻¹·cm⁻¹, measured in water at 280 nm. Its estimated half-life is 30 h when it is expressed in mammalian reticulocytes in vitro, whereas in yeast in vivo or in Escherichia coli in vivo, the estimated half-life of this enzyme is ca. 20 h or 10 h, respectively. The instability index of BplTHI2 is computed to be 29.34, which means this enzyme is stable. Its aliphatic index is 95.42, while the grand average of hydropathicity is 0.208, which indicates this enzyme is soluble in water.

Multiple alignments of the deduced amino acid sequence with other thiolases were performed by the software package DNAMAN 6.0. As shown in Fig. 2, B. platyphylla thiolase II showed a high degree of sequence homology to Camellia oleifera thiolase II (90 % sequence identity), Populus trichocarpa thiolase II (89 %), Nicotiana tabacum thiolase II (86 %), Ricinus communis thiolase II (84 %), Hevea brasiliensis thiolase II (88 %), Vitis vinifera thiolase II (88 %), and Arabidopsis thaliana thiolase II (86 %). The sequence comparison revealed high similarity between the deduced amino acid of BplTHI2 and the other seven thiolases, indicating their relatively conserved evolution relationship at the protein level (12).

Fig. 2. Alignment of the amino acid sequence of the B. platyphylla thiolase II with Camellia oleifera thiolase II (GenBank Ac. No. ADD10719.1), Populus trichocarpa thiolase II (GenBank Ac. No. EEE98843.1), Nicotiana tabacum thiolase II (GenBank Ac. No. AAU95618.1), Ricinus communis thiolase II (GenBank Ac. No. EEE939476.1), Hevea brasiliensis thiolase II (GenBank Ac. No. BAF98276.1), Vitis vinifera thiolase II (GenBank Ac. No. CBI28461.3) and Arabidopsis thaliana thiolase II (GenBank Ac. No. NP_568694.2). The identical amino acids are shown as white letters on a black background.
A phylogenetic tree of these thiolases, including BplTHI2, was constructed by the software MEGA 4.0 as shown in Fig. 3A. The phylogenetic tree showed that the eight proteins can be divided into two main groups, each group containing several subgroups. R. communis thiolase II belongs to the mitochondrial thiolases, while the others belong to cytosolic thiolases. Based on the topology of the phylogenetic tree, BplTHI2 is closest to P. trichocarpa thiolase II, whereas farthest from Arabidopsis thiolase II among the cytosolic thiolases. This result may be explained with the fact that B. platyphylla and P. trichocarpa are both perennial hardwood, whereas Arabidopsis is a herbaceous plant (6).

Thiolases are best known as the final enzyme in the β-oxidation cycle in both prokaryotes and eukaryotes (7). In eukaryotes, there are two forms of 3-ketoacyl-CoA thiolase II: one located in the mitochondria and the other one, in the peroxisomes. In mammalian cells, thiolase II is located both in mitochondria and peroxisomes. For example, mammalian nonspecific lipid-transfer protein (nSL-T) is a protein which seems to exist in two different forms: a 14 kDa protein (SCP-2) and a larger 58 kDa protein (SCP-x). The former is found in the cytoplasm or the mitochondria and is involved in lipid transport; the latter is found in peroxisomes. The C-terminal part of SCP-x is identical to SCP-2, while the N-terminal portion is evolutionarily related to thiolases (7). In plants, controversy remains regarding the subcellular location of thiolase: whether it is peroxisomal activity, cytosolic activity or mitochondrial activity (3). For example, AtTHI2 targeted to peroxisomes not to mitochondria (2, 15). In our study, BplTHI2 was predicted to possess cytosolic activity.
In our future studies, we are planning to construct transgenic lines for better understanding the female inflorescences development. Isolation and characterization of this gene will be helpful for studies of biochemical functions related to mevalonate pathway (3), while the mitochondrial thiolase II is involved in the degradation of 2-methylacetoacetyl-CoA (4). Therefore, expression analysis of BplTHI2 showed that it was highly expressed in female inflorescences and we suggest that BplTHI2 is a female inflorescence-specific gene and may be responsible for the development of the female inflorescence.

Expression profile analysis of the BplTHI2 gene

Expression analysis was performed by means of RT-PCR using primer 5 and primer 6 primer pairs with an equal amount of cDNA templates prepared from the total RNA of different organs. Female inflorescences in different developmental stages, male inflorescences, stems, and leaves were selected as PCR templates. Previous studies showed that the floral primordium of female inflorescences forms early in June and grows to a mega bract primordium in the next 10 days. In July, the ovary primordium forms from the mega bract primordium and the primordium of bracteole develops during the middle ten days of September. Female inflorescences appear in mid-April the following year (9).

The expression results indicated that the BplTHI2 gene was female inflorescence-specific and was not detected in male inflorescence (Fig. 5). This result indicated that the cDNA-AFLP library of female inflorescence-specific genes was constructed successfully and also showed that BplTHI2 may play a role in female floral development. However, there were some transcripts expressed at a lower level in stems and leaves, suggesting that BplTHI2 may play other roles in other organs and in different cellular compartments. Take human thiolase II for example, the cytosolic thiolase II appears to have an important role in the mevalonate pathway (8), while the mitochondrial thiolase II is involved in the synthesis and degradation of ketone bodies as well as in the degradation of 2-methylacetoacetyl-CoA (4). Therefore, isolation and characterization of this gene will be helpful for better understanding the female inflorescences development. In our future studies, we plan to construct transgenic Arabidopsis and birch harboring BplTHI2 to testify its function.

Conclusions

In this work, the gene (BplTHI2) encoding a thiolase II from birch (B. platyphylla) was synthesized via a cDNA-AFLP library and the RACE method. It is 1 203 bp in length and encodes an enzyme of 400 amino acid residues with a calculated MW of 40.9 kDa. It was also predicted to possess two main domains, an N-terminal domain (10–268) and a C-terminal domain (278–398), and to contain three predicted active sites (Cys96, His356 and Cys386). Expression analysis of BplTHI2 showed that it was highly expressed in female inflorescences and we suggest that BplTHI2 is a female inflorescence-specific gene and may be responsible for the development of the female inflorescence.

Acknowledgements

The research was supported by programs for Science and Technology Development of Shanxi province (No. 201103110114-2; 201103110153; 201103110254; 201103121233). We would like to thank Dr Hu Xu for help in the experimental design and proofreading the manuscript.

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