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Research article

Transcriptome profiling reveals specific patterns of paclitaxel synthesis in a new *Taxus yunnanensis* cultivar☆

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ABSTRACT

The difference in contents of paclitaxel and 10-deacetylbaccatin III (10-DABIII) in needles between wildtype (WT) and a new cultivar (Zhongdayihao, ZD1) of *Taxus yunnanensis* was examined. Transcriptome profiling was conducted for different tissues of the ZD1 and WT to illustrate the regulation mechanism of paclitaxel biosynthesis. It was observed that average contents of paclitaxel and 10-DABIII in ZD1 were 4 folds and 32 folds higher than those in WT, respectively. More significant elevations of differential expressed genes (DEGs) from paclitaxel biosynthesis pathway were revealed in ZD1 rather than WT, which should be responsible for the higher contents of paclitaxel and 10-DABIII in the ZD1. Special tissues-dependent expression patterns of paclitaxel biosynthesis DEGs in ZD1 compared to WT were unraveled. The relative higher expressions of paclitaxel biosynthesis genes in needles than other tissues supported the higher content of paclitaxel and 10-DABIII content in needles of ZD1. Attenuation of plant hormone signal transduction pathway led to the lower expression of TFs in ZD1 rather than WT. Besides, the significant negative correlations between differential expressed TFs and DEGs from paclitaxel biosynthesis pathway displayed a possibly negative regulation pattern of these TFs on paclitaxel biosynthesis pathway genes. These results provided new insights into the molecular process of paclitaxel synthesis in *Taxus*.

1. Introduction

Paclitaxel is one of the most effective anticarcinogens for cancers such as breast and ovarian, which was approved by the United States Food and Drug Administration (FDA) (Croteau et al., 2006). The ongoing increase in cancer incidence rates has boosted market demands of the intermediates (10-deacetylbaccatin III, 10-DABIII) as well as paclitaxel (Jennewein and Croteau, 2001). More than 14 species of the *Taxus* genus have been identified so far, and the barks and roots of some *Taxus* species have been the main natural sources of paclitaxel and 10-DABIII (Croteau et al., 2006). Alternative methods such as *in vitro Taxus* cell culture have been developed to obtain paclitaxel and 10-DABIII (Arnone et al., 2006), but most of these methods are difficult to industrialize due to their high cost and low production rate (Kusari et al., 2014). A breakthrough was achieved in the *in vitro* synthesis of paclitaxel through plant cell fermentation and also partly relieved the need for 10-DABIII in the production of docetaxel. However, the need for paclitaxel and 10-DABIII is not eliminated yet due to the increasing incidences of cancer. *Taxus* yews thus still remained an ideal and reliable source for paclitaxel and other precursors. As a result, most *Taxus* species have encountered continuous destruction due to the over-exploitation (Burgarella et al., 2012), and all species of *Taxus* yew have been listed as species for first-grade protection in the National Key Protected Wild Plants List in China consequently (Yu, 1999). Therefore, exploring a method to effectively obtain 10-DABIII and paclitaxel from *Taxus* plants without destroying the population resources is an urgent task for the protection of *Taxus* from the endangerment.

For the purpose, some studies to clarify the biosynthesis mechanisms of paclitaxel have been attempted (Bonfill et al., 2003; Chau and Croteau, 2004; Koksal et al., 2011; Long et al., 2008). Several transcription factors (TFs) families such as MYC, ERF and WRKY have been found to participate in the regulation of paclitaxel biosynthesis (Hartmann et al., 2005; Lenka et al., 2015; Li et al., 2012; Zhang et al., 2015). However, TFs from the same TFs family play different roles in

☆ The nucleotide sequence reported in this paper has been submitted to the Sequence Read Archive (SRA) database of NCBI with the accession number of SRX1176252.

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Abbreviation

10-DABIII	10-deacetylbaccatin III
dbat	10-deacetylbaccatin III 10-O-acetyltransferase
tm10bh	5-α-taxadienol-10-β-hydroxylase
bapt	baccatin III 3-animo-3-phenylpropanoyl transferase
bhlh	basic helix-loop-helix
dbnbt	C13-side-chain N-benzoyltransferase
DEGs	differential expressed genes
TFs	transcription factors
ts	taxadiene synthase
tat	taxadiene-5-α-ol O-acetyltransferase
t5oh	taxadiene 5-α-hydroxylase
t13oh	taxane 13-α- hydroxylase
tbt	taxane 2-α-O-benzoyltransferase
t14oh	taxane 14-β-hydroxylase
ZD1	Zhongdayihao
WT	wild-type

regulating the biosynthesis in paclitaxel. For example, TcERF12 and TcERF15 were identified as negative and positive regulators in the expression of taxadiene synthase (*ts*) gene (Zhang et al., 2015). The remarkable divergence of TFs has led to the difficulty in understanding the regulation mechanism of paclitaxel biosynthesis. Thus, the regulation roles of TFs in paclitaxel biosynthesis in *Taxus* plants remain to be elucidated. However, the exploit on the regulation pattern of paclitaxel biosynthesis is confronted due to the lack of genomic information and effective molecular tools (Wu et al., 2011).

In 2014, a cultivar named Zhongdayihao (ZD1) with high contents of paclitaxel and 10-DABIII in not only barks and roots, but also needles was registered as a Protecting Plant Variety (20140110) as a result of a continuous investigation and selection upon a wild population of *T. yunnanensis*. However, the contents of other metabolites such as baccatin were relatively low in ZD1. Thus, ZD1 is ideal source for investigating the mechanisms of paclitaxel biosynthesis. In the present study, wildtype (WT) and ZD1 of *T. yunnanensis* were used to investigate their transcriptional differences. Candidate genes that influenced the paclitaxel and 10-DABIII contents were identified. Transcriptome profiles were employed to inspect the differential expression patterns of the paclitaxel synthesis pathway in different tissues between WT and ZD1 of *T. yunnanensis*. By comparing the differences in transcript profile between WT and ZD1, it is hopeful to find out the regulation pattern in the biosynthesis of paclitaxel in *Taxus* plants. This is the first study to illustrate the synthesis pathways of paclitaxel and 10-DABIII on the basis of the transcriptome profile of a new *Taxus* cultivar with high paclitaxel and 10-DABIII contents in needles.

2. Materials and methods

2.1. Plants of WT and ZD1

Plants of WT and ZD1 had been cultivated under the same maintenance in the fields of Songkou town of Meizhou, Guangdong, China (116°16′E, 40°01′N) since 2008. ZD1 was a cultivar selected from the population of *T. yunnanensis* in 1997 by monitoring the production of paclitaxel and 10-DABIII. For cultivating stage, the individuals of ZD1 and WT at the same age were cultivated closely and under the same management condition such as sunshine controlling method and water management. Long-term monitoring of paclitaxel and 10-DABIII contents of needles in the WT and ZD1 was conducted every year.

2.2. Samplings

Needle samples of WT and ZD1 were simultaneously collected as contrasts from 2004 to 2014. Each of the collected sample was cleaned and stocked in a plastic bag for paclitaxel and 10-DABIII determinations.

On October 2014, needles, branches and root samples of WT and ZD1 with the same age (21-year-old) grown under the same cultivating conditions were collected independently for RNA extraction and transcriptome study. Triplicate samples for each tissue (needles and branches from two year branches) were collected from different part of ZD1 while triplicate samples for each tissue were collected from three different WT plants. The collected samples were sheared into small pieces of no longer than 1 cm and stored in RNAlater stabilization solution (Life technology, Thermo Fisher Scientific, USA).

2.3. Paclitaxel and 10-DABIII determination

The measurements of paclitaxel and 10-DABIII were carried out after the samples were collected. The samples were dried at 40 °C for about 4 h to constant weight. The dried samples were further smashed in a pulverizer. Three grams of the resulting powders were weighed with high precision, added with 100 mL of ACE-H_2O (3:2), and stirred at 240 r/min for 60 min for extraction. The supernatant was collected after filtration. The extraction process was repeated four times and the solutions were combined. After elimination of Ace by rotary evaporation, the remaining solutions were extracted with 50 mL DCM for three times. The extracts were dried in vacuum-dryer and stored at 4 °C. Methanol was added to dilute the products to 100 mL in volume. One milliliter of the solutions was used for the paclitaxel and 10-DABIII determination processes after filtered with 0.22 μm filter membrane.

The Shimazu LC-20A system coupled with SIL-20A automatic sampler, CTO-20A column oven, and SPD-M20A photo-diode array detector were applied with an Agilent ZORBAX SB-C18 column (5 μm, 4.6*250 mm). The mobile phase composed of acetonitrile: water (30:70) was isocratically eluted at a flow rate of 1 mL/min under 25 °C. The volume of each injection was 10 μL. Standard solutions of paclitaxel and 10-DABIII were employed to build up the standard curves for quantification.

2.4. RNA extraction

Samples for RNA extraction were stored in the RNAlater buffer at the field for timely extraction of RNA. Total RNA was extracted from 0.5 g samples of different tissues with the RN09-EASY spin plus Plant Kit (Aidlab Biotech, Beijing, China) according to the manufacturer's instruction. DNA contamination was removed during the RNA extraction process. The RNA quality was verified by RNase free agarose gel electrophoresis and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). High quality RNA samples of the triplicates from different tissues of ZD1 and WT were mixed with equal quantity for the cDNA library construction and the subsequent RNA sequencing. Six mixed RNA samples of needles, branches, and roots of ZD1 and WT were obtained finally.

2.5. cDNA library construction and sequencing

The obtained poly (A) mRNA samples were broken into fragments of approximately 200 nt after isolation of Oligo (dT) cellulose (Qiagen, USA). After fragmentation, the first-strand cDNA was synthesized by reverse transcriptase with random primers, and second-strand cDNA was formed using DNA polymerase I and RNase H. The cDNA fragments were purified using QIAquick PCR Extraction Kit (Qiagen, USA) before end repairing, poly (A)-tailing and ligation to Illumina adapters. Agarose gel electrophoresis was performed for size fraction of the ligation products, and the fragments were excised for PCR amplification