Differentially Expression of Tua1, a Tubulin-encoding Gene, during Flowering of Tea Plant *Camellia sinensis* (L.) O. Kuntze Using cDNA Amplified Fragment Length Polymorphism Technique

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Abstract The complementary DNA (cDNA) amplified fragment length polymorphism technique was used to isolate transcript-derived fragments corresponding to genes involved in the flowering of tea plant. Comparative sequence analysis of an approximately 300 bp differential fragment amplified by primer combination E11M11 revealed 80%–84% similarity to the corresponding part of an α-tubulin gene of other species. The complete cDNA sequence of this α-tubulin was cloned by the rapid amplification of cDNA ends technique; its full length is 1537 bp and contains an open reading frame of 450 amino acid residues with two N-glycosylation sites and four protein kinase C phosphorylation sites. The deduced amino acid sequences did show significant homology to the α-tubulin from other plants that has been reported to be a pollen-specific protein and could be correlated with plant cytoplasm-nucleus-interacted male sterility. We named this complete cDNA Tua1. The nucleotide and amino acid sequence data of Tua1 have been recorded in the GenBank sequence database with the accession No. DQ340766. This Tua1 gene was cloned into the pET-32a expression system and expressed in *Escherichia coli* BL21trxB(DE3). The molecular weight of expressed protein was deduced to be approximately 49 kDa. Western blot analysis was used to identify the temporal expression of Tua1 in tea plant. Further studies of the effect of Tua1 protein on pollen tube growth indicated the Tua1 solution obviously promoted the growth of tea pollen tube.

Key words cDNA-AFLP; *Camellia sinensis*; flower bud; α-tubulin; Western blot; pollen

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Tea plant (*Camellia sinensis*) is an important economic crop in China. It is evergreen, perennial and cross-pollinated. Under cultivated conditions, a bush height of 60–100 cm is maintained for harvesting the tender leaves, a process that can continue for more than 100 years. It bears flowers and fruit 2–3 years after being planted. Under natural pollination conditions, the flowering rate of most tea plant varieties is usually very high, but there is a big difference in the fruiting rate among different tea varieties. The fruiting rate of some varieties is very low (e.g., approximately 1% in Wulong and Maoxie) or absolutely blank (e.g., 0% in Zhenghe big white tea and Foshou), but in other varieties the rate is very high (e.g., 10% in Longjing43 and Wuniuzaos) [1]. The flower bud differentiation of tea plant usually begins in early summer and lasts until late fall. It takes approximately 4 months from bud differentiation to bloom (Fig. 1). The reproductive growth of tea plant, including flower bud differentiation, flowering, blooming and fruiting, requires large amounts of energy and nutrients, which restrains vegetative growth and severely influences immunity function. As a result, reproductive growth is not only reducing the production of tea leaves, but also influencing the quality and resistance character of tea, which directly relates to the income of tea farmers. Additionally, tea is propagated either through seed or cutting. The segregation character of tea plant filial generation is often unavoidable due to the limitation of its cross-fertility.
At the beginning of the 20th century, some tea plant breeding researchers attempted to obtain a pure-breeding variety by self-fertilization, but failed. Tea vegetative propagation is an effective method for maintaining the excellent character of the maternal plant. Until now, it has been a key issue to control the reproductive growth of tea plant due to increasing product demand, the need for quality improvement, and for tea plant breeding.

Flowering of tea plant is a continuous process, meaning different developmental stages, from flower bud differentiation to full flower period, can occur in one plant simultaneously. All of these stages are asynchronous processes in nature and difficult to analyze individually. At present, research on specific characteristic products related to the quality of tea is developing quickly \[2−4\], but there is little research on the flowering period of tea plant. In 1987, Jiang and Wang published their research on the growth of pollen, the formation of pollen, the growth of ovule, the formation of embryo and the process of endosperm growth [5]. Dong classified tea plant into three types: high fruitage rate type; low fruitage rate type; and sterile type [6]. However, until now, no reports have been published on the flower developmental genes of tea plant.

In this study, we exploited the complementary DNA amplified fragment length polymorphism (cDNA-AFLP) technique to isolate transcript-derived fragments (TDFs) corresponding to genes involved in the flowering period of tea plant and aimed at finding some clues to the mechanism of the plant’s reproductive growth. Research on the flowering period of tea plant from a molecular biology angle can provide insight into the theory of the molecular mechanism of flowering of tea. Thus, it might be possible to control the reproductive growth of tea plant using biotechnology, according to product demand. It could also give us a chance to use crop hybridity, so as to lay a good theoretical foundation for the development of tea’s output, quality and resistance characters. We will attempt to provide leads for further research on the flowering mechanism, as well as the fertility mechanism, of tea plant through our study. To our knowledge, this is the first study of the flowering character of tea plant from the point of view of molecular biology.

Materials and Methods

Plant materials and RNA isolation

In this study, we selected two tea plant cultivars planted in the tea variety orchard of Anhui Agriculture University (Hefei, China), Longjing43 and Wulong. The flowering rates of both are as high as 60%, but the fruiting rate of Longjing43 (10%) is considerably higher than that of Wulong (1%). Other characters of both are basically similar. We harvested the small flower buds [Diameter (Dm) =3 mm] of both lines and the big flower buds (Dm=6 mm) of both cultivars in the same stock plant 1 month later. Flower buds were collected and immediately frozen in liquid nitrogen (Fig. 2). For each sample, total RNA was...
extracted from approximately 100 mg of flower buds using Trizol plant RNA purification reagent (Gibco BRL, Gaithersburg, USA). The total RNA quantity was measured using a spectrophotometer at a wavelength of 260 nm. The purity of the RNA was evaluated by the ratio of absorbency at 260 and 280 nm (A260/A280). Quality of the RNA was checked by formaldehyde denaturing gel electrophoresis.

cDNA-AFLP procedure

Double-stranded cDNA was synthesized using the SMART polymerase chain reaction (PCR) cDNA synthesis kit (Clontech, Palo Alto, USA). The cDNA-AFLP procedure described by Bachem et al. [7] was used with a few modifications. The EcoRI/MseI enzyme system was used. The cDNA was then digested using EcoRI and MseI. The following restriction digests were then mixed: 20 μl cDNA (approximately 100 ng), 1 μl EcoRI (10 U), 4 μl 10×PCR buffer, and 15 μl H2O. The mixture was incubated at a temperature appropriate for the EcoRI (65 ºC) for 2 h. To the first digest mixture, the following agents were added: 1 μl EcoRI adaptors (5 pM), 1 μl MseI adaptors (5 pM), 0.5 μl of 10 mM ATP, 0.5 μl 10×PCR buffer, 0.2 μl T4 DNA ligase, and 2 μl double-distilled H2O. The mixture was incubated for 3 h at 37 ºC. The ligation product was termed the primary template and its 20-fold dilution was used directly for pre-amplification.

For selective amplification, a total of 16 primer pairs were used (Table 1) with the following PCR system: 2 μl secondary template, 2 μl each of primer E11–E18 and primer M11–M18, 2 μl of 10×PCR buffer, 2.5 μl Mg2+ (25 mM), 1 μl dNTPs (25 mM), 0.3 μl Taq polymerase (10 U), and 10.5 μl double-distilled H2O. Amplification was carried out for 12 cycles with 30 s denaturation at 94 ºC, 30 s annealing at 65 ºC (the annealing temperature touching down to 1 ºC for every cycle) and 1 min extension at 72 ºC; then 23 cycles with 30 s denaturation at 94 ºC, 30 s annealing at 56 ºC, and a 1 min extension at 72 ºC. After the last cycle, the amplification was extended for 10 min at 72 ºC.

The selective amplification products were separated on a 6% polyacrylamide gel containing 8 M urea at 110 W until the bromophenol blue reached the bottom. The cDNA bands were stained with silver nitrate, following the protocol described in the DNA sequencing system kit (Promega, Madison, USA).

<table>
<thead>
<tr>
<th>Adaptor or primer</th>
<th>Nucleotide sequence (5′→3′)</th>
<th>Adaptor or primer</th>
<th>Nucleotide sequence (5′→3′)</th>
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<tr>
<td>EcoRI</td>
<td></td>
<td>MseI</td>
<td></td>
</tr>
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<td>EcoRI adaptor 1</td>
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<td>MseI adaptor 2</td>
<td>TACTCAGACTCAT</td>
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<td>M10</td>
<td>GATGAGTCCTGAGAAA</td>
</tr>
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<td>M11</td>
<td>GATGAGTCCTGAGAAA</td>
</tr>
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<td>M14</td>
<td>GATGAGTCCTGAGAAA</td>
</tr>
<tr>
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<td>E26</td>
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<td>M26</td>
<td>GATGAGTCCTGAGAAAG</td>
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† preamplification primer; ‡ selective amplification primer.
Characterization of AFLP fragments

Selected fragments were excised from the polyacrylamide gel, suspended in H2O and eluted DNA was reamplified using the same PCR conditions and the same primer combination for selective amplification. Reamplified products were checked on a 1% agarose gel [8]. The cloned DNA fragments were sequenced by Shanghai Sangon Company (Shanghai, China). The sequences obtained were compared to those in the GenBank database using BLAST sequence alignments.

Rapid amplification of cDNA ends (3′/5′ RACE) of Tua1 gene

Comparison of cDNA-AFLP patterns revealed different cDNA fragments among the four samples. These bands were excised from gels, cloned into the plasmid, and sequenced. We picked out and further analyzed a 300 bp differential fragment, ChaH-1, amplified by primer combination E11M11, and it revealed 80%-84% to α-tubulin of other species. It was particular expressed in the big flower buds of both lines. The RACE procedure was carried out using the BD SMART RACE cDNA flower buds of both lines. The RACE procedure was subjected to 30 cycles of 30 s denaturing at 94 °C, 30 s annealing at 58 °C and 1 min extension at 72 °C. Five microliters of the product was electrophoresed on an agarose gel alongside a DNA quantification ladder, and the levels of template added to the reaction were altered according to the amount of expected product.

Western blot analysis

Total soluble protein was extracted from leaves, small flower buds and big flower buds of two tea plant cultivars (approximately 250 mg per sample) by homogenizing in 3 ml extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, 100 µg/ml phenylmethylsulfonyl fluoride) and centrifuging at 15,000 g for 15 min at 4 °C. Protein concentrations were determined using the Bradford method with some modification [9]. The supernatant was mixed with 0.1 volume of Tris saline azide buffer kept on ice for 10 min and centrifuged at 3000 rpm for 5 min. The supernatant was boiled for 5 min in 5x sodium dodecyl sulfate (SDS) sample buffer and separated using 10% SDS-polyacrylamide gel electrophoresis (1 h, 120 V), then transferred to a nitrocellulose membrane using a semi-dry blotter (Mini Gel Transfer apparatus; Bio-Rad, California, USA). The membrane was incubated for 2 h in Tris-buffered saline with 5% non-fat milk power, in Tris-buffered saline/Tween-20 for 1 h at room temperature, then probed in succession with anti-α-tubulin antibody made in rabbit at a dilution of 1:1000 in 0.5% bovine gelatin in phosphate-buffered saline. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Pierce, Rockford, USA), diluted to 1:5000 to 1:10,000 in blocking buffer (both antibodies were purchased from Beijing Biosynthesis Biotechnology Company, Beijing, China). The membranes were developed with 0.33 mg/ml 1-nitroblue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl2).

Functional study of tea Tua1

To study the effect of Tua1 protein on pollen tube growth, we first optimized the isolated culture medium for tea plant pollen germination, then collected the pollen, and cultivated it for 12 h in two kinds of pollen germination medium (total volume 5 ml per medium): medium I, 10.0% (W/V) sucrose, 0.01% (W/V) boric acid, 1.0% agar; medium II, 10.0% (W/V) sucrose, 0.01% (W/V) boric acid, 1.0% agar, 0.1 µg/µl Tua1 protein expressed in E. coli

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Results

cDNA-AFLP analysis of gene differentially expressed during flower development

The gene differential expression of small flower buds and big flower buds was compared using cDNA-AFLP technology. In total, 256 primer set combinations were used per cDNA sample for selective amplification. A typical example of the obtained cDNA-AFLP gel is shown in Fig. 3(A). The cDNA-AFLP produced an average 50 amplification products per PCR and therefore a total estimated number of 12,800 fragments. Distinct transcriptional changes between the small flower buds and the big flower buds of both varieties were observed for approximately 90 cDNA fragments, of which 37 were only expressed in small flower buds of both varieties, and the other 53 appeared in big flower buds of both varieties. This indicated these fragments, which specially expressed at different times, were potentially related with the flower development of tea plant.

Characterization of AFLP fragments

We selected a part of these special expression fragments, which appeared simultaneously at different developmental stages of flower buds of both varieties, for sequencing. Twelve TDFs were successfully re-amplified by PCR using the selective primers that were used to obtain the fragments in the cDNA-AFLP analysis. Amplified fragments were sent for DNA sequencing. Those fragments were compared with those in the GenBank database using the BLAST search tool (Table 2). One sequence, named ChaH-1 [Fig. 3(B)], revealed high (80%–84%) similarity...
Table 2 Sequences of differential fragment ChaH and their homology to previously characterized genes

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer combination</th>
<th>Length (bp)</th>
<th>Homology†</th>
<th>BLAST score</th>
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<tr>
<td>ChaH-1</td>
<td>E₁₁ M₁₁</td>
<td>324</td>
<td>Pisum sativum var. Alaska α-tubulin (TubA1) gene</td>
<td>4e–25</td>
</tr>
<tr>
<td>ChaH-2</td>
<td>E₁₇ M₁₁</td>
<td>232</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>ChaH-3</td>
<td>E₁₆ M₁₁</td>
<td>210</td>
<td>No</td>
<td>NA</td>
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<tr>
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<td>E₁₁ M₁₂</td>
<td>431</td>
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<td>NA</td>
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<tr>
<td>ChaH-5</td>
<td>E₁₃ M₁₂</td>
<td>362</td>
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<td>NA</td>
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<tr>
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<td>No</td>
<td>NA</td>
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<tr>
<td>ChaH-7</td>
<td>E₁₂ M₁₅</td>
<td>440</td>
<td>Arabidopsis thaliana threonine kinase AT1G72760</td>
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<td>ChaH-8</td>
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<td>212</td>
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<tr>
<td>ChaH-12</td>
<td>E₁₂ M₁₈</td>
<td>247</td>
<td>No</td>
<td>NA</td>
</tr>
</tbody>
</table>

† GenBank accession numbers of sequences homologous to the fragment subjected to amplified fragment length polymorphism technique are in parentheses. NA, not applicable.

to the α-tubulin gene. Another fragment showed 80% homology to Arabidopsis thaliana threonine kinase AT1G72760. Other fragments showed no homology to known sequences in GenBank. They might represent previously uncharacterized genes, or the cDNA fragments might be too short to reveal significant homology.

Tua1 gene cDNA full-length sequence

The complete cDNA sequence of this α-tubulin was cloned by the RACE technique. It has 1537 bp and shares 80%–84% similarity to α-tubulin of other plants. Its complete cDNA sequence has been submitted to GenBank (accession No. DQ340766). Analysis of this cloned complete cDNA showed that it encompassed an open reading frame with 1350 bp encoding 450 amino acid residues. Homology searches with the deduced 450 amino acid residues revealed tea Tua1 shares 98% similarity with Nicotiana tabacum α-tubulin, 97% with Arabidopsis thaliana Tua6, and 84% with Oikopleura dioica putative α-tubulin. The predicted protein contains two N-glycosylation sites, four protein kinase C phosphorylation sites, seven casein kinase II phosphorylation sites, and one tubulin nucleotide-binding domain-like fragment (Fig. 4). Homology searches and domain query indicate that tea Tua1 is a member of the potential conserved tubulin family. The predicted secondary structure composition for this protein has 35.56% helix, 18.82% sheet, and 46.22% loop.

RT-PCR identification

To quantify the differential expression of the Tua1 gene at different developmental stages of tea plant, RT-PCR experiments were carried out on leaves, small flower buds and big flower buds of Longjing43. The results from these RT-PCR experiments (Fig. 5) clearly show that the expression of the Tua1 gene only exists in the big flower buds. There was no expression in leaves or small flower buds of tea plant.

Prokaryotic expression of Tua1

The recombinant plasmid pET-32a-Tua1 was transformed and expressed in E. coli BL21trxB(DE3), producing a target fusion protein (approximately 70 kDa) not produced by the control (untransformed host pET-32a). The results of protein electrophoresis are illustrated in Fig. 6(A). The size of the major protein expressed by the control was 20.67 kDa (containing TrxA, which consists of a six histidinol tag and 109 amino acids). Therefore, the molecular weight of the induced mature Tua1 was deduced to be approximately 49 kDa, consistent with the theoretical value. Fig. 6(A) also shows that the yield of the expressed fusion protein increased with time.

Western blot analysis

The pET-32a-Tua1 fusion expression proteins were subjected to SDS-PAGE and detected by Western blot analysis with anti-α-tubulin specific polyclonal antibodies. The results showed that stably accumulated proteins appeared in the anti-tubulin staining pattern on the blot. A distinct spot of tubulin protein appears in the Tua1 position, whereas the control sample failed to produce a protein spot in the corresponding location [Fig. 6(B)]. We further used this antibody to detect the expression
Fig. 4  Nucleotide sequence of the *Tua1* gene and deduced amino acid sequence
Boxed amino acids are N-glycosylation sites; the amino acids underlined are protein kinase C phosphorylation sites; the amino acids underlined with dotted lines are casein kinase II phosphorylation sites; the shading indicates tubulin subunits α, β, and γ signature. The asterisk indicates the termination codon.

Fig. 5  Electrophoresis analysis of reverse transcription-polymerase chain reaction (PCR) product of tissues from tea plant variety *Longjing43*
M, marker; B, PCR product of big flower buds; S, PCR product of small flower buds; L, PCR product of leaves. Arrow represents the aimed PCR product.

Effect of the *Tua1* protein on the pollen tube growth of tea plant

The promotive experiments of pollen germination were carried out on various mediums. The pollen germination pattern of *Tua1* protein in tea plant. The immunoblot analysis of protein obtained from different tissues is shown in Fig. 6(B). The expected band was detected in the big flower buds, but there was no protein corresponding to this band in the leaves or small flower buds. Results demonstrated that *Tua1* protein was especially expressed in big flower buds of tea plant. This result is consistent with our cDNA-AFLP and RT-PCR analyses.
Fig. 6  Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of expressed products (A) and Western blot analysis (B) of protein extracted from leaves, small flower buds and big flower buds of two tea plant cultivars

M, protein marker; 1, pET-32a induced for 4 h (as a control sample); 2–8, pET-32a-Tua1 induced for 0, 2, 4, 6, 8, 12, and 14 h, respectively. 9 and 10, the antibody could detect tea Tua1 expressed in the prokaryotic cells (Escherichia coli); the immunoblot analysis of protein obtained from different tissue showed Tua1 expression pattern, showed expected band were detected in both big flower buds. 11 and 14, the protein extracted from the leaf of tea plant; 12 and 15, the protein extracted from small flower buds of tea plant; 13 and 16, the protein extracted from big flower buds of tea plants.

Table 3  Effect of Tua1 on pollen germination and pollen tube elongation

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total pollens (n)</th>
<th>Germinational pollens (m)</th>
<th>Germination (%)</th>
<th>Pollen tube length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic medium</td>
<td>973</td>
<td>39</td>
<td>4.01</td>
<td>0.1085</td>
</tr>
<tr>
<td>Medium with Tua1</td>
<td>895</td>
<td>37</td>
<td>4.13</td>
<td>0.3024</td>
</tr>
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</table>

Basic medium: 10.0% (W/V) sucrose, 0.01% (W/V) boric acid, 1.0% agar. The concentration of Tua1 is 0.1 μg/μl.

Discussion

The combination of developmentally staged flower bud...
samples with the cDNA-AFLP technique allowed us to carry out a large screening for genes showing differential expression patterns during the flowering period. Mascarenhas distinguished two patterns of flower gene expression. “Early” genes are transcribed soon after meiosis and are reduced or undetectable in mature pollen. Transcripts of the “late” genes are first detected around the time of microspore mitosis and continue to accumulate as pollen matures [10]. In our study, we chose those TDFs that appeared simultaneously in big flower buds of both varieties. Thus, we excluded the possibility of discrepancy in the two breeds themselves and confirmed that these TDFs were exactly relevant to developmental stages of flower buds. According to the BLAST result of these fragments, we selected a “late” gene fragment that was deduced to be an α-tubulin gene for further research and named it Tua1. The remaining TDFs obtained by cDNA-AFLP will be cloned and studied in more detail in the future. The α-tubulin gene of other species has been reported to be a key protein in flower development [11–13]. The phenomenon that the Tua1 gene was expressed preferentially in the big flower buds, and no expression was found in the small flower buds or leaves of either line, indicates that the Tua1 gene might belong to the group of genes expressed at the late stage of flower development.

Microtubules are components of the filamentous cytoskeleton of eukaryotic cells and participate in many cell processes, including cell division, intracellular transport, cell motility, and cell morphogenesis [14–17]. In plants, microtubules have a number of specialized roles [18–20]. The major structural component of microtubules is tubulin, a heterodimeric protein composed of two highly conserved subunits, α and β. A less abundant form, γ-tubulin, is also found in higher plants [21,22]. Both α- and β-tubulins are encoded by multigene families in eukaryotes [23,24]. Tissue-specific preferences in accumulation of tubulin transcripts have been reported in both Arabidopsis and maize [11,25–27]. Up to the present, developmentally regulated patterns of α-tubulin transcription in pollen have been mainly studied in Arabidopsis. The Arabidopsis α-tubulin gene, Tua1, is differentially expressed in flower organ of Arabidopsis, but no expression was detected in root or leaf, and the peak transcription level of Tua1 was noted at the flowering period. It can be concluded that the Tua1 gene plays a key role in the development of pollen of Arabidopsis [11,12]. The research on maize also indicates that the α-tubulin gene has a close relationship with cytoplasm-nucleus-interacted male sterility [13,28–30].

In our present study, the transcription and characterization analysis showed that Tua1 was expressed differentially during the flowering period and might be related to fertility of tea plant. The further functional study indicated the Tua1 solution did not promote the germination of pollen, but clearly promoted the growth of the pollen tube. It will be useful to verify the gene function more deeply and elucidate the biological roles and their relationship with the flower development of tea plant. Further work will be focused on identifying the functional mechanism of Tua1 in tea plant. A long-term goal of our research is to identify expressed transcripts during flower development, and to manipulate these genes or their promoter elements to regulate flower development and fertility mechanisms in tea plant. This work constitutes the first report of genes activated during the flowering period of tea plant and has significance for tea plant breeding.

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