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Cloning and Characterization of a Tyrosine Aminotransferase (TAT) Gene in *Gerbera hybrida*

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Abstract In our previous study, a gene predicted to encode a Tyrosine aminotransferase (TAT) was found to be significantly up-regulated in root rot diseased *Gerbera* by transcriptome sequencing. To confirm the genes and investigate the function, we cloned the gene by RT-PCR and then conduct bioinformatic analyses. In this study, a 1 537 bp long cDNA sequence of this gene (named as *GhTAT*) was firstly cloned, which contained a coding region of 1 233 bp, which was predicted to encode a protein of 410 amino acids. Bioinformatic analysis showed that the *GhTAT* was a stable hydrophobic protein without signal peptide. Subcellular location prediction result indicated that this protein located in chloroplast, which is the biosynthesis position of tyrosine and the derived products of tyrosine biosynthesis pathway. Moreover, typical Tyrosine aminotransferase domain was found in this protein, indicating that it is a TAT. According to the TAT-based phylogenetic analysis and similarity analysis, the closest relationship and highest similarity was found between *GhTAT* and *Halianthus annuus* TAT, which again verified the TAT property of *GhTAT*. Tyrosine aminotransferase (TAT) is the first enzyme in tyrosine biosynthesis pathway, whose products include many antioxidant substances such as tocopherols and tocotrienols. The up-regulation of *GhTAT* in root rot diseased gerbera suggests that it may play an important role in response to the root rot pathogen infection. In addition, 60 phosphorylation sites (accounting for 14.6%) were found in this protein, suggesting that the expression of this protein and its encoding gene were greatly influenced by the phosphorylation reactions.

Key words Cloning, Characterization, Tyrosine aminotransferase, *Gerbera hybrida*

1 Introduction

Plants are exposed to various biotic and abiotic environmental stresses such as light, wounding, pest attack and so on during their life cycle. The biotic or abiotic stresses will lead to the production of reactive oxygen species (ROS) in plants as a defense response. However, the ROS accumulation will cause some changes like lipid and chlorophyll oxidation and cell damage to plants^[1–3]. Fortunately, plants possess efficient enzymatic and non-enzymatic antioxidant defense system which helps to protect the plants by scavenging ROS. Tocopherols are part of the antioxidant mechanism. Many experimental evidences have proved that tocopherols can defend the plant photosynthetic mechanism through scavenging singlet oxygen^[4], ROS and lipid radicals^[5–6] by increasing the level of α -tocopherol^[6–7]. The precursor of tocopherol is homogentisic acid that is synthesized from 4-hydroxyphenylpyruvate (4-HPP)^[8]. And 4-HPP is produced from amino acid tyrosine. Tyrosine is the originator of several plant secondary metabolites like prenylquinones, alkaloids, and cyanogenic glycosides^[9–14] and some other amino acids such as methionine^[15]. The reaction from Tyr to 4-HPP and from 4-HPP to Tyr is catalyzed by TAT^[16–17], which is the first enzyme in tyrosine biosynthesis path-

way. And the TAT catalyzed reactions could provide the substrate for plastoquinone, rosmarinic acid, benzyloquinoline alkaloids and tocopherols^[18–22]. Therefore, TAT is considered as an essential enzyme in carbohydrate metabolism, biosynthesis, and degradation of amino acids and many other metabolic pathways^[14].

In mammals and fungi, structural and biochemical characterization of Tyrosine aminotransferase is widely studied^[14,23–24]. But this enzyme is less studied in plants. In model plant *Arabidopsis thaliana*, 7 TATs were identified, accounting for 16% of all aminotransferases^[21]. Up to now, however, there is no report about *Gerbera* TAT. In our previous study, we identified several TAT genes by using RNA-Seq, and one of them was found to be significantly up-regulated in root rot diseased *Gerbera*. *Gerbera* is one of the most important cut flower and pot plant in national and international market. It ranks fourth after rose, carnation and chrysanthemum, in the global cut flower trade^[25]. Root rot disease is threatening the *Gerbera* industry worldwide and the losses caused by it is still increasing^[26]. So, measures should be taken for the healthy development of *Gerbera* industry. Among all the methods tried by scientists and growers, genetic breeding was considered as the most rapid and effective one. For genetic breeding, candidate resistance genes are very necessary. Transcriptome profiling of *Gerbera hybrida* ray florets by using RNA-seq technology encodes putative genes that were associated with signal transduction and other phytohormones that involved in gibberellin metabolism^[27]. According to the identified functions of TAT in other plants and its

disease induced significant up-regulation, *TAT* was predicted to be a potential candidate resistance related gene. Therefore, it is very necessary to know its sequence, protein structure and functional domain of its encoded protein. In this study, the gene was firstly cloned and was then subjected to series of bioinformatics analysis. The result generated in this study may provide basis for the understanding of its functions, especially its function in the *Gerbera*-root rot pathogen interaction.

2 Materials and methods

2.1 Materials The *G. hybrida* cv. Daxueju plants used in this study were provided by Sanming Modern Agriculture sci-tech demonstration garden.

2.2 Methods

2.2.1 Isolation of DNA and RNA. Total RNA was extracted by using Trizol reagent. Experiments were done according to protocol suggested by the manufacturer. The quantity and quality of the total RNA were measured by agarose gel electrophoresis and spectrophotometer analysis. Complementary DNA (cDNA) was obtained by using SMARTer® PCR cDNA Synthesis Kit according to the manual.

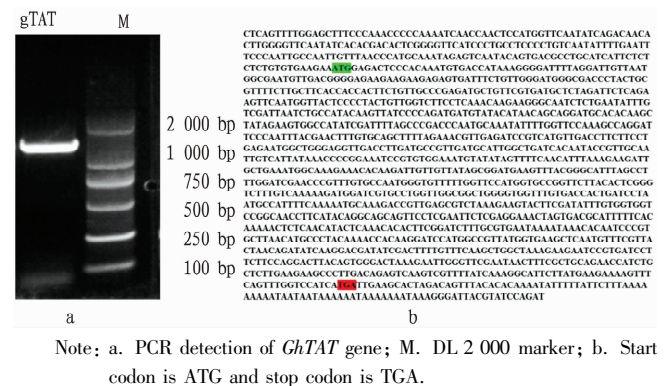
2.2.2 Primer design and gene cloning. Primers (Forward primer: GCA TCA TTC TCT CTC TGT GTG AAG AA; Reverse primer: TAT TTT GTG TGT AAA CTG TCT AGT GCT TC) were designed according to the gene sequence of our transcriptome data by using DNAMAN 6.0. Gene is cloned from cDNA of *G. hybrida* by using Pfu DNA polymerase. PCR was performed under following conditions: 94°C for 4 min; 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 30°C cycles; and 72°C for 7 min. The PCR product was purified by using gel extraction kit (GeneJET Gel Extraction kit) and was then cloned into B-zero vector and were transformed into *E. coli* TH5α. And the positive clones proved by PCR were sent to Huada biotechnology Co. Ltd for sequencing. The obtained cDNA sequence was submitted to the GenBank database (<http://www.ncbi.nlm.nih.gov>).

2.2.3 Bioinformatics analysis. Bioinformatics analysis of these obtained genes and their deduced protein sequences were performed as follows: the similarity between *GhTAT* and *TATs* from other plants were compared by using blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>); ORF of *GhTAT*, molecular mass, physical and chemical properties of *GhTAT* were predicted by using ExPASy (<http://www.expasy.org>); the secondary structure of *GhTAT* was predicted by using Predictprotein (<https://www.predictprotein.org/>); subcellular localization was predicted on <http://www.scbio.sjtu.edu.cn/bioinf/Cell-PLoc/>; InterProScan (<http://www.ebi.ac.uk/Tools/InterProScan/>) was used for the domain prediction. SignalP server (<http://www.cbs.dtu.dk/services/SignalP>) was used to predict the signal peptide; transmembrane helix structure of *GhTAT* protein was analyzed by using TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html); phylogenetic analysis of *Gerbera TAT* and other *TAT* from other plant species was performed by using MEGA 6.06 software using neighbor-join-

ing (NJ) method; Phosphorylation sites of *GhTAT* was predicted on NetPhos2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>).

3 Results

3.1 Gene cloning results of *GhTAT* For the *Gerbera TAT* gene amplification, primers were designed according to the sequence obtained in our transcriptome data. After PCR, a 1 537 bp band was obtained. ORF prediction result showed that this gene contains a 1 233 bp ORF, which was predicted to encode a protein containing 410 aa. The BLAST results showed that *GhTAT* has high similarity with the *TATs* of other plants and also with some animals, indicating that the *TAT* genes were very conserved. In plants, the highest similarity was found between *GhTAT* and *Halianthus annuus TAT* (XP_021999922.1) (89%), followed by *Populus euphratica* (XP_011029528.1) (75%), *Arabidopsis thaliana* (BAB10727.1) (72%), *Cucumis sativus* (XP_004146045.1) (72%), *Glycine max* (NP_001238408.1) (70%) and *Petunia × hybrid* (AHA62827.1) (70%). Due to its high similarity to *TATs* of other plant species, the gene was named as *GhTAT*. The sequence of it was submitted to NCBI and its accession number at GenBank is KY990406.



Note: a. PCR detection of *GhTAT* gene; M. DL 2 000 marker; b. Start codon is ATG and stop codon is TGA.

Fig. 1 Gene cloning results of *GhTAT*

3.2 Physical and chemical properties analysis result By using expasy (<http://www.expasy.org>), the *GhTAT* was found to contain 410 amino acids, among which Leu (37, 9%), Val (36, 8.8%) and Ala (33, 8%) take very large part. The carbon, hydrogen, nitrogen, oxygen and sulfur percentage of *GhTAT* was respectively 31.99%, 50%, 8.2%, 9% and 0.2%. The total number of negatively charged residues (Asp + Glu) was 42, and the total number of positively charged residues (Arg + Lys) was 43. Theoretical PI of this protein was predicted to be 7.54. Estimated half-life of *GhTAT* was > 20 hours in yeast (*in vivo*) and > 10 hours in *Escherichia coli* (*in vivo*) and its instability index is computed to be 32.84, which suggest that *GhTAT* is a stable protein. The aliphatic index of this protein was 97.24 and grand average of hydropathicity (GRAVY) was 0.079, which indicate that *GhTAT* is a hydrophobic protein.

3.3 Phosphorylation site prediction results NetPhos2.0 server was used to predict the phosphorylation sites in *GhTAT*. Results showed that it has 60 phosphorylation sites, including 23 on serine, 27 on threonine and ten on tyrosine (Fig. 2).

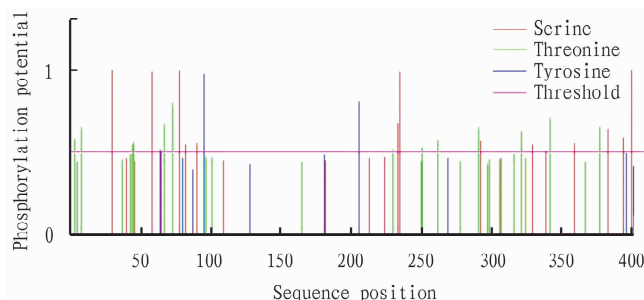
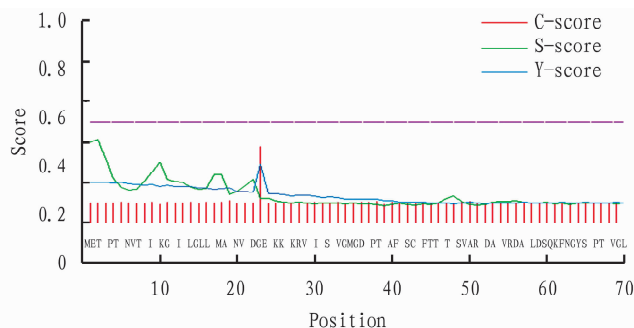


Fig. 2 Prediction of phosphorylation sites in GhTAT sequence

3.4 Signal peptide analysis results SignalP-4 server software was used to predict the signal peptide of protein GhTAT. The results showed that *D* value is 0.25, which means that GhTAT has no signal peptide (Fig. 3).



Note: C- and Y- scores represent the plotted probabilities for raw cleavage sites and S-score represent signal peptide.

Fig. 3 Signal peptide cleavage site prediction

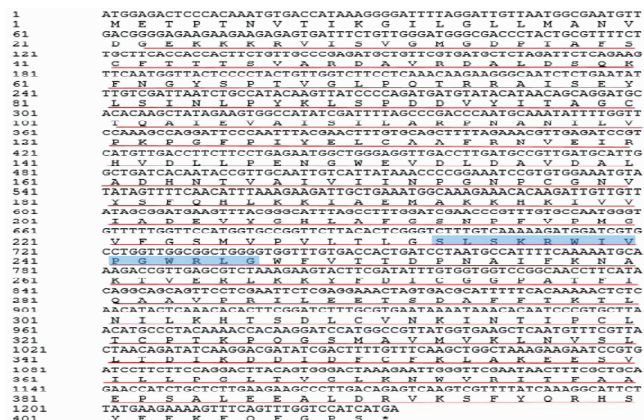
3.5 Transmembrane helices analysis result Transmembrane helix structure of GhTAT protein was analyzed by using TMpred. Transmembrane α -helices were found on both orientations of the protein. And the significant transmembrane helices were predicted to be located in from 29 to 52 (i-o) and from 215th aa to 234th aa. The TM-Helix is from the 17th aa to the 33th aa, and preferred model is N-terminus inside.

3.6 Interpro analysis results Interpro software was used to classify the protein sequences into domain, sites, and family. Results showed that a Tyrosine aminotransferase domain ranging from 22nd aa to 398th aa (Fig. 4). And the conserved typical sequence of TAT was also found in this domain (Fig. 4).

3.7 Secondary structure prediction results Secondary structure analysis result showed that GhTAT consists of different components such as transmembrane helices, protein binding sites, polynucleotide binding sites. And helix, strand and loop accounts for 46.3%, 13.73% and 40% respectively.

3.8 Subcellular localization analysis results Subcellular localization prediction was important for protein function and annotation. Our results predicted the protein in the chloroplast.

3.9 Multiple alignment of GhTAT protein BLASTp was firstly performed by using the protein sequence of GhTAT. TAT proteins share high similarity with GhTAT were downloaded and then aligned by using ClustalW embedded in MEGA 6.0. Multiple alignment of GhTAT protein and TAT protein of other plant species are shown in Fig. 5. The blast results showed that GhTAT protein



Note: The characters underlined represent the Tyrosine aminotransferase domain, and the characters in blue represent the conserved sequences.

Fig. 4 Coding sequence and deduced amino acid of GhTAT

shared high similarity with the TAT of *Halianthus annuus*. Many essential sites for TAT activities were conserved in different plants also found in GhTAT. Moreover, the conserved aminotransferase family-I pyridoxal-phosphate attachment site (SKRWIVPG-WRLG) was also identified in GhTAT (Fig. 5).

3.10 Homology and phylogenetic analysis of GhTAT The encoding protein of *GhTAT* gene and some TATs of some plant were subjected to phylogenetic analysis. The closest relationship was found between GhTAT and the TAT of *Helianthus annuus* by Phylogenetic analysis (Fig. 6).

4 Discussions

4.1 TATs and their encoded proteins are very conserved

The TAT genes have been cloned and characterized for few plants such as *Trypanosoma cruzi*^[28], *Arabidopsis thaliana*^[29], *Medicago truncatula*, *Coleus blumei* and *Glycine max*^[20,30-31]. In this study, the full-length cDNA sequence of *GhTAT* was cloned and characterized. According to the results of series of bioinformatic analysis, very high conservation was found in plant TATs especially in the aminotransferase domain, which suggest that plant TATs might play similar role in different plants and the highly conserved domain are very necessary for the function of the enzyme.

4.2 GhTAT was predicted to locate in chloroplast

The subcellular localization of TATs differed in different plants. In *petunia* phenylpyruvate aminotransferase localize in the cytosol^[32]. In *Arabidopsis*, very high TAT activity was found in cytosolic and plastidic fractions of leaf tissue^[33]. TAT plays an important role in the catabolism of aromatic amino acids in *Trypanosoma cruzi* and catalyzes the transformation of the amine group^[34]. The TAT activity and its presence have been detected in the cytosol of *T. cruzi*, and in mitochondria at lower levels^[35]. Previous studies showed that tyrosine is synthesized in plastids, and further steps of HPPD in plastoquinone and tocopherol biosynthesis occurs in plastid^[12,36-40]. The enzymes take part in tyrosine biosynthesis are mostly localized in plastid^[37,41-42]. According to our results, the GhTAT was predicted to be present in the chloroplast, which is greatly consistent with previous reports.



Note; The TATs of *Helianthus annuus* (XP_021999922.1), *Solanum lycopersicum* (XP_004248255.1), *Ipomoea nil* (XP_019194524.1), *Populus euphratica* (XP_011029528.1), *Malus domestica* (XP_008386209.1), *Gossypium hirsutum* (XP_016705900.1), *Nicotiana tabacum* (XP_016452180.1), *Capsicum annuum* (XP_016545101.1), *Prunus persica* (XP_007211793.1), *Eucalyptus grandis* (XP_010060624.1), *Ziziphus jujuba* (XP_015899240.1), *Cucumis sativus* (XP_004146045.1), *Petunia x hybrida* (AHA62827.1), *Cucumis melo* (NP_001284465.1), *Vitis vinifera* (XP_002282664.1), *Sesamum indicum* (XP_020549896.1), *Arabidopsis thaliana* (NP_200208.1), *Raphanus sativus* (XP_018449279.1), *Ricinus communis* (XP_002523289.1), *Glycine max* (NP_001238408.1), *Citrus clementina* (XP_006451776.1) and *G. hybrida* (KY990406) are shown. The characters in black box represent the conserved typical of TATs.

Fig. 5 Multiple sequence alignment of GhTAT protein and its homologs from other plant species

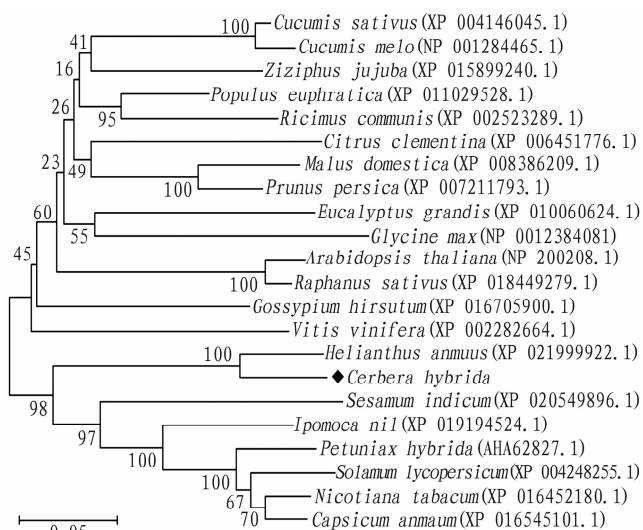


Fig. 6 Neighbor-joining Phylogenetic relationship of GhTAT and other plant species

4.3 The significant up-regulation of GhTAT in root rot diseased gerbera suggested that it may function a lot in gerbera in response to root rot pathogen infection The TAT enzyme

has different functions in Tyr-derived pathways and that are studied in several plant species. For example, in *Arabidopsis thaliana*, level of α -tocopherol and γ -tocopherol increase significantly in aging leaves under stress condition and the activity of Tyrosine aminotransferase also increased as well^[17]. The TAT activity and level of antioxidant tocopherol were observed when subjected to oxidative stress^[17]. Full length cDNAs microarray analysis of *Arabidopsis* genes showed that Tyrosine aminotransferase responsive genes were upregulated under high salinity, cold and drought stress^[43]. Tyrosine aminotransferase (TAT) activity was found to be induced by coronatine, octadecanoids methyl jasmonate (MeJA), 12-oxophytodienoic acid (MeOPDA), herbicide oxyfluorfen, wounding, UV light and high light^[29,44]. The significant expression changes of TAT under different stresses suggested that this enzyme might play important role in plant defense. Interestingly, in our previous study, the gene encoding GhTAT was found to be significantly up-regulated in root rot diseased gerbera, which suggest that the gene might function a lot during the gerbera-root rot pathogen interaction.

4.4 Phosphorylation may be important for the regulation of the TAT expression In addition, 60 phosphorylation sites (accounting for 14.6%) were found in this protein, suggesting that

the expression of this protein and its encoding gene were greatly influenced by the phosphorylation reactions.

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