Cloning and Expression Analysis of $\delta$-OAT Gene from Saccharum spontaneum L

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Abstract. Full-length cDNAs of ornithine-$\delta$-aminotransferase ($Ss\delta$-OAT) were isolated from Saccharum spontaneum through homologous clone strategy. The sequence of this gene was then deposited in GenBank database with the accession number KX714115. Sequence analysis showed that the full length of the $\delta$-OAT gene was 1373bp, the open reading frame was 1365 bp, it was inferred that coding 454 amino acids, the isoelectric point and the molecular weight of coded protein were 6.16 and 49.5 kD, respectively. According to the phylogenetic tree, the evolution of $Ss\delta$-OAT corresponds with traditional biological classification. Its functional domain has a high conserved property in the process of biological evolution. Prokaryotic expression vector pEASY-E1-$Ss\delta$-OAT was established and transformed BL21 (DE3) into E. coli after IPTG induction, showing a successful gene expression. The expression pattern analysis carried out by quantitative real-time PCR indicated that the gene expression level was significantly up-regulated under drought stress. The relative expression of the gene reached to the maximum under 6d treatment. It was the first time to obtain $\delta$-OAT from Saccharum spontaneum, $Ss\delta$-OAT responded to water stress, it was inferred that $\delta$-OAT played a role in resistance to osmotic stress.

1. Introduction
Saccharum officinarum is the most imperative sugar-yielding crop. Sucrose yield takes up 80% of total yield of China’s edible sugar. As industrial structure adjustment and westward traditional sugarcane planting area, drought disaster has become the primary environmental factor in impacting China’s sugarcane production. Saccharum spontaneum L. is wild S.spontaneum, with the widest spread in Saccharum, it plays an important role in sugarcane breed improvement; most of Saccharum officinarum breeds in production have genetic relation with wild S.spontaneum. Therefore, a profound study on the mechanism of wild S.spontaneum response to drought resistance is of great significance for Saccharum officinarum drought-resistant breeding.

Proline is a type of low-molecular-weight osmolyte, and it is the most water-soluble amino acid; many plants accumulate high-level proline under non-biological stress, such as drought, salt stress and low temperature [1]. The accumulation of proline in plants is firstly relevant to the synthesis, the synthesis of proline begins from glutamic acid (Glu) or ornithine (Orn), Glu path is that Glu synthesizes $\Delta'$-pyrroline-5-carboxyate (P5C) under the condition of $\Delta'$-pyrroline-5-carboxyate synthetase (P5CS); Orn path is that Orn synthesizes PSC under the condition of $\delta$-aminotransferase ($\delta$-OAT; EC 2.6.1.13); then, Glu and Orn synthesize proline under the condition of $\Delta'$-pyrroline-5-
carboxylate reductase (P5CR) [2]. Currently, there are two perspectives on the ornithine pathway for proline accumulation under obiotic stress. Some studies suggested that δ-OAT gene realized highly expression under the condition of non-stress, cool damage, salt stress, ABA treatment and sufficient nitrogen, without being inhibited by proline feedback [3-4]. Some studies support the opposite view, that the ornithine pathway contributes to proline synthesis by increasing δ-OAT activity, when plants are treated with salinity [5].

So far, the δ-OAT gene has been cloned from a lot of plants, such as Arabidopsis, rice, tobacco, sugarcane, soybeans, maize and kidney beans [6]. However, the δ-OAT gene of Saccharum spontaneum had not been reported and the δ-OAT pathway in the Saccharum spontaneum contributes to the proline biosynthesis or not is not clear. In this study, we cloned the δ-OAT gene from the Saccharum spontaneum and analyzed the expression profile of Ssδ-OAT gene under drought stress conditions by real-time fluorescence quantitative PCR (RT-PCR). The objective of our investigation was to explore the relationship between Ssδ-OAT and drought stress tolerance of Saccharum spontaneum and provide candidate gene resources for improving the drought resistance of sugarcane.

2. Materials and methods

2.1. Plant Materials
Saccharum spontaneum stalks with two buds were planted in the bucket (35x50cm) in greenhouse. Routine management procedures were adopted, and favourable water and fertilizer conditions were maintained. Stay seedlings were grown up to the 5~6 leaf stage; consistently growing plants were taken and divided into 2 groups: a group of normal water supply and another group of natural drought stress. The sampling times were 0, 2, 4, 6, 8, 10 and 12d of treatment. All the samples collected were immediately fixed in liquid nitrogen and stored in a refrigerator at -70°C until RNA extraction.

2.2. Total RNA Extraction and cDNA Synthesis
Extract total RNA of Saccharum spontaneum leaves by EasyPure Plant RNA Kit (TransGen Biotech, Beijing), and reduce genome DNA contamination by DNase. Detect RNA integrity by 1% agarose gel electrophoresis. Synthesize the first strand cDNA by RevertAid Reverse Transcriptase Kit (TaKaRa, Dalian).

2.3. Sequence obtain of Ssδ-OAT
For amplification of the δ-OAT gene from Saccharum spontaneum, two sets of primers were designed based on the sequence of Saδ-OAT (Genbank accession number: EU113256.2) (Table 1). PCR was performed at 94°C for 5min, 30 cycles of 94°C for 30 s, 55°C for 30s, 72°C for 1.5 min, followed by a final step of 72°C for 10 min. After the amplification products were detected by 1.0% agarose gel electrophoresis, purify the PCR products according to the instruction of EasyPure PCR Purification Kit, then ligate to pEASY™-T5 cloning vector (TransGen Biotech). The aimband was sequenced in INVITROGEN Trading (Shanghai) Co., Ltd. The ProtParam (http://cn.expasy.org/tools/protparam.html) was applied to analyze the basic properties of the encoded protein. Multiple sequence alignment and phylogenetic analysis were performed with DNAMAN software (version 6.0). The phylogenetic tree was build using MEGA 6.0 software by the neighbour-joining method. The phosphorylation site was predicted with NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/).

Table 1 Description of five primer sets used for cloning.

| Use       | Primer name | Primer sequence (5’–3’)       |
|-----------|-------------|--------------------------------|
| Gene cloning | O1          | Forward: ATTCGCAGGATGTGCTCG      |
|           |             | Reverse: GCCTACGAAAATGCAGCTA     |
|           | O2          | Forward: CAGGATGTGCTCGAGCTC      |
|           |             | Reverse: GCTTCATCCGTACAGGTC      |
2.4. Real-time Quantitative PCR Analysis

According to the sequence of Ssδ-OAT, real-time qPCR primers were designed (Table 1). GAPDH rRNA was selected as the control gene in the real-time qPCR analysis [11]. In real-time qPCR amplification, the cDNA of Ssδ-OAT was used as the template and the total volume of the reaction system was 20 μL, including 10 μL GoTaq® qPCR Master Mix; 1 μL forward and reverse primers (10 μM), respectively; 1.5 μL cDNA template; and 6.5μL Nuclease-Free Water. Three replicas were set for each sample. The PCR conditions were as follows: heat denature at 95 ℃ for 120 s; denature at 95 ℃ for 15 s, anneal and extend 60 ℃ for 30 s, 40 cycles. When the reaction ended, the melting curve was analyzed. The method of $2^{-\Delta\Delta CT}$ was adopted to analyze the real-time qPCR results [7].

3. Results and Analysis

3.1. Cloning and sequence analysis of Ssδ-OAT gene

It is well known that one of the most common responses to drought stress and salt stress is the accumulation of proline which acts as an osmoprotectant [8-9]. As a key enzyme in proline biosynthesis, δ-OAT is closely related to plant stress response [10]. In this study, to study the Saccharum spontaneum ornithine-δ-aminotransferase at the molecular level, a 1373 bp full-length ornithine-δ-aminotransferase gene Ssδ-OAT (GenBank Accession No. KX714115) was cloned using RT-PCR method (Figure 1).

![PCR fragments amplified with the O2 primer set on a 1% agarose gel. M: 500bp DNA ladder (TIANGEN Biotech Co., Ltd, Beijing, China); 1: PCR result of Ssδ-OAT.](image)

The sequence contained a complete open reading frame (ORF), encoded 454 amino acids. The predicted protein had a molecular mass of 49.5 kDa with a pI of 6.16. A search at the NCBI for conserved protein domains indicated that Ssδ-OAT belonged to Orn aminotransferase family (Figure 2).

![Conserved domains analysis of Ssδ-OAT](image)

The phosphorylation sites of Ssδ-OAT protein were predicted by NetPhos 3.1Server. The results showed that the protein contained 25 phosphorylation sites, including 17 serine sites, 4 threonine sites
and 4 tyrosinethe sites (Figure 3). Therefore, It’s speculated that Ssδ-OAT protein activity may be regulated by phosphorylation.

![Phosphorylation sites prediction of Ssδ-OAT](image1)

Figure 3. Phosphorylation sites prediction of Ssδ-OAT

The phylogenetic tree was made by the neighbour-joining method with multiple alignments of amino acids from different species. Phylogenetic analysis (Figure 4) revealed that Ssδ-OAT and Soδ-OAT (Saccharum officinarum) had the closest genetic relation with the same evolution branch, and probably could be orthologous protein; the next closer genetic relation was with Sorghum bicolor and Zea mays; and the strangest genetic relation was with Araceae Anthurium amnicola and angiosperm Zostera marina.

![Phylogenetic tree of the protein encoded by Ssδ-OAT and other species](image2)

Figure 4. Phylogenetic tree of the protein encoded by Ssδ-OAT and other species

3.2. Expression Pattern of Ssδ-OAT in drought Stress

We analyzed the expression of the Ssδ-OAT gene under drought stress to obtain more information about its expression under stress. We performed real-time PCR analysis. The results showed that Ssδ-OAT obviously up-regulated expression during the drought stress period (Figure 5). At all drought stress time points, the expression of Ssδ-OAT in stressed plants leaves were significantly higher than that in the control plants (0 d). The expression of Ssδ-OAT in the leaves reached the peak after approximately 6 d of treatment. The level of the Ssδ-OAT mRNA was 4.0 times than that of in the control.
4. Conclusions and Discussion
Proline accumulation is a general adaptive response mechanism when plants are under adversity such as drought, salt stress, low temperature, many study results confirmed that the regularity of proline accumulation and plants stress adaptation presented a good correlation [11]. At present, the accumulation of proline under stress conditions which approach dominates the dispute is relatively large. There is a popular belief that Glu path plays the primary role under the adversity such as osmotic stress, salt stress, and Orn path predominated under sufficient nitrogen [12]. Yet studies show that Orn path played a vital role had great contribution to proline accumulation under water stress. The results indicated that Ssδ-OAT probably joined the osmotic adjustment process under drought stress. In this study, we isolated and characterized a complete cDNA sequence of δ-OAT from Saccharum spontaneum L. Homology comparison showed that Ssδ-OAT had high homology with other plants and the main functional domain PLP binding site was conservative in evolution. Ssδ-OAT was able to map to the OAT group in the AAT_I subfamily through phylogenetic analysis. With the increase of drought stress time, the expression of Ssδ-OAT was up-regulated. Furthermore, it was speculated that the main function of Ssδ-OAT in Saccharum spontaneum L was to catalyze proline production under drought stress to adapt to stress.

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