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THE ACTIVITY AND GENE EXPRESSION LEVELS OF *P5CS* AND *δ-OAT* IN *MEDICAGO SATIVA* CV. PIANGUAN UNDER DROUGHT STRESS

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Abstract

Medicago sativa cv. Pianguan, an excellent local germplasm in Shanxi province, China, with the resistance to cold and drought is broadly planted. Many studies have showed that drought resistance of plants is positively correlated with the accumulation of proline, *P5CS* and *δ-OAT* play important roles in synthesis of proline. In this study, the proline content, activities and expression of *P5CS* and *δ-OAT* mRNA were detected in *Medicago sativa* cv. Pianguan under drought stress. The results showed that the proline content in *Medicago sativa* cv. Pianguan increased in the process of drought stress. The expression levels of *P5CS* and *δ-OAT* mRNA changed correspondingly at the different stress time point. In conclusion, *P5CS* and *δ-OAT* could participate in the drought resistance process by regulating proline content in *Medicago sativa* cv. Pianguan.

Key words: *Medicago sativa* cv. Pianguan; *P5CS*; *δ-OAT*; Drought resistance.

Introduction

Medicago sativa cv. Pianguan, as a kind of high-quality forage in Shanxi, possesses the characteristics of strong adaptability, cold resistance, high productivity and nutritional value, etc. However, just like other forages, the distribution, growth and production of *Medicago sativa* cv. Pianguan could be affected continuously by the drought (Reddy *et al.*, 2004). In 1954, it was reported for the first time that proline, as a kind of osmotic substances, should be accumulated when plants suffered abiotic stress(Kemble & MacPherson, 1954). There is a significant positive correlation between the accumulation of proline and drought resistant of plants (Anjum *et al.*, 2012). Meanwhile, high-concentration accumulation of proline in cytoplasm has no adverse effect on the normal metabolism of plants (Chen & Murata, 2002). It has been reported that $\triangle^1$-pyrroline-5-carboxylate synthetase (*P5CS*) and δ-aminotransferase (*δ-OAT*) have critical regulating effects in the process of proline synthesis(Roosens *et al.*, 2002; Sekhar *et al.*, 2007).

Under catalysis of *P5CS*, glutamate-γ-semialdehyde (GSA) is produced by glutamate, then GSA spontaneously cyclized into pyrroline -5-carboxylic acid (P5C). Proline is produced by P5C with the reduction of $\triangle^1$-dihydropyrrolre-5-carboxylate reductase (P5CR). The proline synthetic quantity is 10-18 times in tobacco and rice genetically modified with *P5CS* than that in the wild types. In addition, they keep strong tolerance to the drought, other plants are reported similarly (Zheng *et al.*, 2014; Dobrá *et al.*, 2011; Ramadan & Hassanein, 2014). There is another proline synthetic pathway. Ornithine was turned into P5C by the catalysis reaction of *δ-OAT*, then P5C was reduced into proline(Nada *et al.*, 2010). *δ-OAT* plays an important role in salt-tolerance improvement in various kind of plants(Sekhar *et al.*, 2007; Da Rocha *et al.*, 2012). It is reported that the proline content, the activities and gene expression levels of *P5CS* and *δ-OAT* increased significantly under salt stress in *Arabidopsis thaliana* seedling (Roosens *et al.*, 1998). Both the biomass and budding percentage of genetically modified tobacco are higher than that in the wild type (Roosens *et al.*, 2002). These results showed that the *P5CS* and *δ-OAT* pathway plays an important role in the process of proline synthesis.

In this study, proline content, the activities and gene expression levels of *P5CS* and *δ-OAT* were detected in *Medicago sativa* cv. Pianguan under drought stress in order to explore the function of *P5CS* and *δ-OAT* in drought resistance in *Medicago sativa* cv. Pianguan.

Materials and Methods

Test materials: *Medicago sativa* cv. Pianguan seeds were derived from the territory of Pianguan in Shanxi, China (Altitude1375 m，E111º22′48″，N39º12′37″). One hundred of full and even grained seeds were selected and sowed in the cultivating basin with 25 cm upper diameter and 20 cm height, then filled with sand and perlite (volume ratio: 3:1) and cultivated in solar greenhouse with 20°C-25°C temperature and 65%-75% relative humidity. After the emergence of seeds, poured with nutrient solution at 1/2 Hoagland every two days and supplied water with distilled water in the free time. When the plants appearing four leaves, selected those plants with similar growth vigor and moved in nutrient solution at 1/2 Hoagland to have a renewal cultivation in three days, and then induced drought stress into nutrient solution at 1/2 Hoagland which contains 20% of PEG. Samples were collected at 0h. 8 h, 16 h, 24 h, 32 h, 40 h, 48 h, 72 h respectively. The sample collected at each time point was divided into 2 duplicated, one was stored at liquid nitrogen to be used for extraction of the whole RNA and the other was used for detecting enzyme activity.

Determination of proline contents: Proline content was detected by sulfosalicylic acid method(Bates *et al.*, 1973). 0.3-0.5 g sample, 5 ml 3% sulfosalicylic acid solution was ground into homogenate, centrifuged at 3000 rpm, 10 min, put 2 ml supernatant into the tube, added water and acetic acid about 2 ml respectively, and then added 4 ml

acidic-ninhydrin coloured solution (mass ratio: 2.5%), after 1 h coloring in boil water bath, took out and cooled, added 4ml toluene and extracted by oscillating fully. Standing, when it occurred stratified, absorbed the toluene layer as a comparison with 520 nm wavelength. The standard curve was made in the proline concentration range of 1-15 μg ml^{-1} using the specific method. Extracted 2 ml standard solution, added 3% of 2ml sulfosalicylic acid, 2 ml acetic acid and 4 ml acidic-ninhydrin, put into the boiling water, colored 1 h. After cooled, added 4 ml toluene and extracted red material, standing, and took toluene to determine the absorption value when the wavelength at 520 nm. According to proline amount and corresponding absorption value, drew the standard curve.

***P5CS* activity determination:** Extraction of P5CS according to the method Kishor (Kishor *et al*., 1995) has reported. First, took 0.5 g sample, added 5 ml extract solution (pH 7.2, 100 mM Tris-HCl buffer, 20 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF), after the ice ground, centrifuged at 7000 rpm for 20 min, got supernatant also named crude enzyme and stored at 4°C. Protein content was determined by micro-protein analyzer. *P5CS* activity was detected according to the method reported by García Rios(García-Ríos *et al*., 1997), added 1.6 ml reaction buffer (pH 7.2, 100 mM Tris-HCl buffer, 20 mM $MgCl_{2,}$ 75 mM sodium glutamate, 5 mM ATP) into 0.2 ml 0.4mM NADPH, 30°C for 20 min, record the absorption value at 340 nm, A_{340} take reducing 0.001 per minute as enzyme activity unit (U).

***δ-OAT* activity determination:** With reference to the method of Roosens (Roosens *et al*., 2002), made the extraction solution of crude enzyme. Took 0.5 g sample, added 1.5 ml 50 mM phosphoric buffer (pH8.0, contains 1mM DTT) and ground on ice, centrifuged at 9000 rpm for 20 min, collected supernatant, also named crude enzyme, and stored at 4°C. Determination of δ-OAT activity consulting Kim's method (Kim *et al*., 1994). 0.1 ml enzyme solution was added in 0.9 ml reaction mixture (50 mM phosphoric buffer, 35 mM L-ornithine, 5 mM α-ketoglutarate and 0.05 mM pyridoxa) at 25°C for 20 min, then added 0.3 ml 3 M perchloric acid to the termination reaction, then added 0.2 ml 2% ninhydrin, heat boiling water for 20 min and colored, after cooling centrifuged at 10000 rpm, discarded the supernatant. 1.5 ml absolute ethyl alcohol was used to dissolve red precipitate and centrifuged, took the supernatant 510 nm to determinate absorption value. The amount of 0.001 μmol P5C was considered as *δ-OAT* activity unit (U).

Extraction of RNA and PCR: To confirm the expression of *P5CS* and *δ-OAT* in *Medicago sativa* cv. Pianguan using PCR method, the total RNA were extracted from *Medicago sativa* cv. Pianguan using Trirol kit (Invitrogen, Carlsbad, CA USA) following the manufacturer's instructions (n = 3 in each group). The reverse transcriptase reaction was performed using 1μg total RNA for 15 min at 37°C and for 5 s at 85°C according to the manufacturer's instructions of the RT-PCR kit (Takara, Dalian, China). RNA concentration and integrity was determined by ND-1000 and electrophoresis, respectively.

Based on *P5CS* and *δ-OAT* gene sequences of other plants in NCBI, specific primers of *P5CS* and *δ-OAT* of *Medicago sativa* cv. Pianguan were designed by Primer 5.0 plus software. *P5CS*: F: 5'-AGGAGGCAAGGAGGCTAATC-3', R: 5'-CTCTGCGCCTAATCCAAATC 3'; δ-OAT: F: 5'-TTTCATGTTGTGGCTGCTTC -3', R: 5'-CATGGTCCGGGTATTGTTTC -3', the sizes of expected products were 694 bp and 606 bp, respectively. The primers were synthesized in Da Lian TaKaRa Company.

The 25 μL PCR reaction mixture (2 μL cDNA, 2 μL dNTP, 2.5 μL 10×buffer, 0.5 μL forward primer, 0.5 μL reverse primer, 0.25 μL TaqE, 17.25 μL water) was incubated for 10 min at 95°C, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s, 72°C for 5 min. The PCR product was examined by electrophoresis using 1% agarose, and the amplified product was sequenced in Da Lian TaKaRa Company.

Real-time fluorescence quantitative PCR: The mRNA expression levels of *P5CS* and *δ-OAT* were measured by the quantitative real-time PCR method using SYBR®Premix Ex Taq™ (Takara, Dalian, China) and the BioRad QPCR System according to the manufacturer's instructions. Based on the PCR results, *P5CS*, *δ-OAT* and β-actin primers were designed by Primer 5.0 plus software. *P5CS*: F: 5'-AGAAAGGCTGGGTCAATA-3', R: 5'- AACGAGCAAGTGGAACAT-3'; *δ-OAT*: F: 5'-TCATGTTGTGGCTGCTTC-3, R: 5'-CTGGAAGGTGCTGAGGGA -3'; β-actin: F: 5'-AACATCGTATTGAGTGGTGGTA -3', R: 5'-CATGGTCCGGGTATTGTTTC -3'. The reaction for real-time PCR (included SYBR®Premix Ex Taq™ (2×) 12.5 μL, PCR Forward Primer 0.5 μL, PCR Reverse Primer 0.5 μL, cDNA solution 1 μL, ROX Reference DyeⅡ(50×) 0.5 μL, added H_2O up to 25 μL) was conducted by preheating at 95°C for 30 s, followed by 40 cycles of shuttle heating at 95°C for 5 s, 52°C for 30 s，72°C for 30 s. There were 3 replicates for each treatment (sample, *P5CS, δ-OAT* and β-actin). At the end of temperature reaction, the specificity of PCR reaction was judged by melting curve, the C_T quantitative value (average ± standard deviation) was calculated according to the standard curve and fluorescence curve. *P5CS* and *δ-OAT* abundance were detected by the comparative threshold cycle (CT) method established by Livak and Schmittgen (Livak & Schmittgen, 2001).

Results

Proline content of *Medicago sativa* cv. Pianguan under PEG stress: Under PEG stress, the proline content in *Medicago sativa* cv. Pianguan increased at the beginning and then reduced gradually with the time development. With the stress time lasting, proline content increased. When the PEG stress treatment lasted for 40 h, it reached the highest level at 3431.93μg g^{-1} FW, it was 14 times of the proline content compared with non-stressed *Medicago sativa* cv. Pianguan. And after 24 h, the proline content was significantly higher than that in non-stressed group (p<0.05) at each time point. However, after 40 h, with the stress time forward, the proline content decreased (p<0.05) (Fig. 1).

***P5CS* activity in *Medicago sativa* cv. Pianguan under PEG stress:** Under PEG stress, *P5CS* activity in *Medicago sativa* cv. Pianguan showed the trend of "up-down-up-down" (Fig. 2). Compared with the non-stressed group, *P5CS* activity is higher significantly at each time point ($p<0.05$), and it reached the highest up to 609.47 U mg^{-1} protein, which was 6.16 times more than that in the non-stressed group. After 32 h, *P5CS* activity decreased significantly at the detected time points ($p<0.05$).

***δ-OAT* activity in *Medicago Sativa* cv. Pianguan under PEG stress:** Compared with non-stressed group, *δ-OAT* activity in *Medicago Sativa* cv. Pianguan under PEG stress increased significantly ($p<0.05$) at 8 h and the *δ-OAT* activity reached the highest at 8h, about 752.87 U mg^{-1} FW compared with each other groups. However, *δ-OAT* activity declined significantly after 8 h ($p<0.05$). After 8 h treatment, PEG stress accelerated the decrease of *δ-OAT* activity in *Medicago Sativa* cv. Pianguan (Fig. 3).

Expression of *P5CS* and *δ-OAT*: Amplification of partial sequence of *P5CS* and *δ-OAT* gene in *Medicago Sativa* cv. Pianguan showed the sizes of amplified *P5CS* and *δ-OAT* sequences are 694 bp and 606 bp, respectively (Fig. 4). Similarity analysis by GenBank data showed the gene sequences of *P5CS* and *δ-OAT* in *Medicago Sativa* cv. Pianguan was more similar to other species. Among them, the similarity of *P5CS* sequence for *Medicago Sativa* cv. Pianguan, *Medicago sativa* subsp, *Medicago truncatula*, *Phaseolus vulgaris*, *Glycine max*, *Ricinus communis* were 98%, 96%, 87%, 87% and 76%, respectively; *δ-OAT* gene of *Medicago Sativa* cv. Pianguan and the similarity of *Medicago truncatula*, *isum sativum*, *Vicia villosa*, *Glycine max* were 97%, 91%, 90% and 89%, respectively.

Effects of PEG stress on abundances of *P5CS* and *δ-OAT* mRNA expression in *Medicago sativa* cv. Pianguan: The quantitative real-time PCR results showed *P5CS* and *δ-OAT* gene were expressed under PEG stress at all detected time points. Compared with the no PEG treatment group, *P5CS* gene expression level raised, 5 times more than that in the untreated group, then declined. However, at 32 h, *P5CS* gene expression level became larger, 7 times more than that in untreated group. It showed a trend like "up – down – up". The *δ-OAT* gene expression was lower in *Medicago sativa* cv. Pianguan without PEG treatment, but after PEG treatment, *δ-OAT* gene increased radically. At 8 h, it reached the highest level, which was 27 times more than that in untreated group (Figs. 5 and 6).

Discussion

P5CS gene had been cloned from many plants like sweet sorghum, *Arabidopsis thaliana*, sugarcane, tomato, bean, apocynum, xinmu No.1 alfalfa and so on (Su *et al.*, 2011; Kim & Nam, 2013). *δ-OAT* gene was also cloned/expressed in hairy vetch, soybean, alfalfa, *Arabidopsis thaliana* and other plants(Nada *et al.*, 2010). In this study, we cloned partial sequences of *P5CS* and *δ-OAT* genes in *Medicago Sativa* cv. Pianguan.

Under osmotic stress, the compatible penetrant such as proline, glycine betaine and carbohydrate were accumulated. Under high salt stress the proline accumulation was preponderantly (Qudsia *et al.*, 2013). A large number of studies showed that proline accumulation was positively correlated with adaptability of plants to drought and salt stress (Anjum *et al.*, 2012; Tabot & Adams., 2014). In this study, proline content increased gradually then declined after 40 h PEG stress treatment. This could be related to plant adaptabilities to stress conditions. It was reported that, the related gene expression levels were increased in *Arabidopsis thaliana* and rice to prevent from drought stress (Seki *et al.*, 2002; Rabbani *et al.*, 2003). *Arabidopsis thaliana* seedling under salt stress, proline content, *P5CS* and *δ-OAT* genes expression, *δ-OAT* activity were increased (Roosens *et al.*, 1998). In this study, *P5CS, δ-OAT* activities and gene expression levels became increasing trend. In the early stage of PEG stress, *δ-OAT* activity and gene expression level increased significantly than *P5CS* activity and gene expression level. However, with the stress time going forward, *δ-OAT* gene expression level was not decreased significantly but the *δ-OAT* activity declined significantly. In addition, the *P5CS* activity and gene expression level showed the trend of "down- up- down". At the early stage of PEG stress in *Medicago Sativa* cv. Pianguan, ornithine-proline was a main pathway of proline accumulation. As the stress continues, osmotic change in the early stage of PEG stress caused dehydration, leading to the lack of water, and the glutamate-proline pathway was the major source of proline accumulation. It was consistent with the former studies (Hervieu *et al.*, 1995; Wang *et al.*, 2007).

Proline may regulate the expression of salt-responsive gene (Oono *et al.*, 2003; Banu *et al.*, 2009). *P5CS* gene expression level was lower without PEG treatment in *Medicago Sativa* cv. Pianguan. After PEG treatment, *P5CS* gene expression level changed. With PEG treatment, the *P5CS* gene expression rised gradually and reached the highest at 8 h. It was 5 times than that in the unstressed group and then declined, but *P5CS* gene expression level increased after 24 h, the whole trend was "up- down- up". The cause of this phenomenon was probably the plant was not stimulated by drought because of enough water in the root at 0 h, consequently with less proline. After the PEG treatment, *P5CS* expression was induced and the relatively excessive *P5CS* was a catalytic of proline synthesis so that the resistance was improved. The proline was increased as well as the *P5CS* synthetic quantity, meanwhile the increased proline played a role in feedback regulation of *P5CS* synthesis. Consequently there was the phenomenon that *P5CS* was declined. *δ-OAT* gene expression level in *Medicago Sativa* cv. Pianguan was lower without PEG treatment. It's increased sharply after PEG treatment. It was 27 times than that in untreated group and it would reach the highest at 8 h, but then decreased modestly. It probably results from that the water in plant root was enough at 0 h, proline content synthesis was lower. *Medicago Sativa* cv. Pianguan suffered from drought stress after PEG treatment, in order to induce *δ-OAT* expression, synthesized of excessive *δ-OAT* catalytic proline, the plant needed a large number of proline to fight against stimulation of drought, used excessive proline expression to resist drought stimulation. The expression level of *δ-OAT* in a high level continuously could result in that *δ-OAT* was not feedback and regulated by proline.

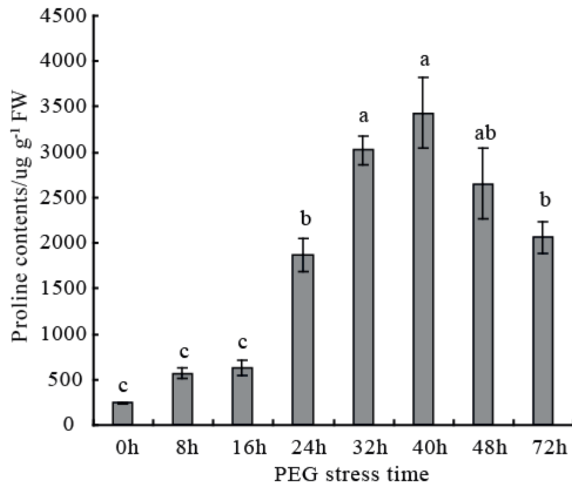

Fig. 1. Proline content of *Medicago sativa* cv. Pianguan under PEG stress.

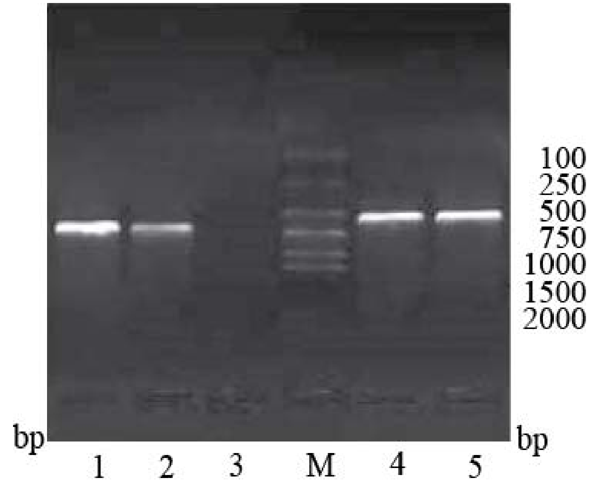

Fig. 4. Agarose gel electrophoresis patterns of PCR product.
M. DL2000 DNA ladder; 1, 2. *P5CS* PCR product; 3. Negative control; 4, 5. *ä-OAT* PCR products.

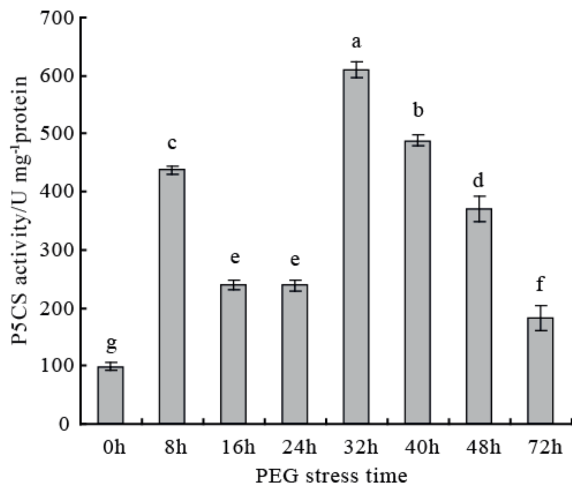

Fig. 2. *P5CS* activity of *Medicago sativa* cv. Pianguan under PEG stress.

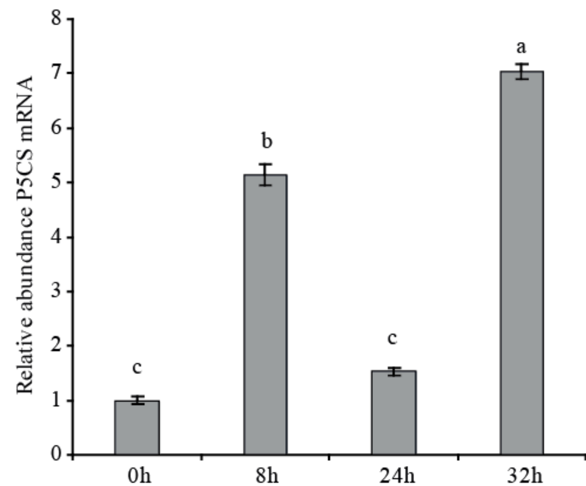

Fig. 5. Relative *P5CS* mRNA abundance in *Medicago sativa* cv. Pianguan under PEG stress.

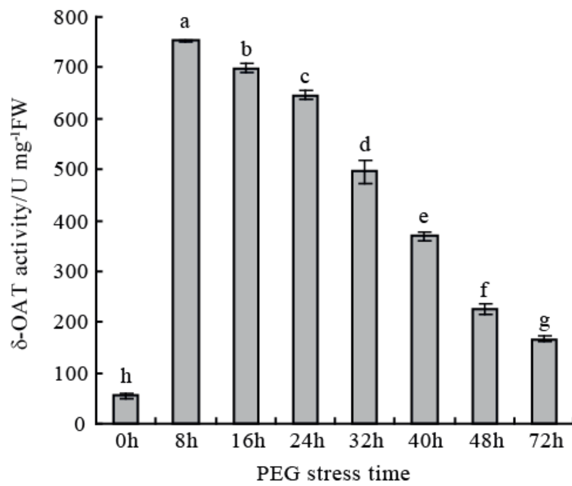

Fig. 3. *δ-OAT* activity of *Medicago sativa* cv. Pianguan under PEG stress.

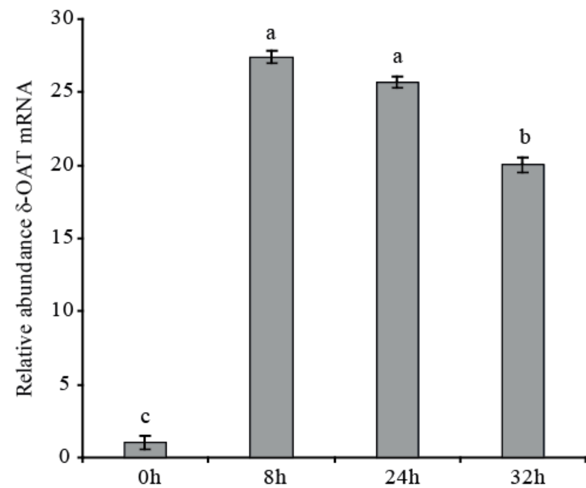

Fig. 6. Relative *δ-OAT* mRNA abundance in *Medicago sativa* cv. Pianguan under PEG stress.

Conclusions

In this study, we cloned the partial sequences of the *P5CS* and *δ-OAT* genes in *Medicago sativa* cv. Pianguan, and found the significantly differences at the proline content, the activity and mRNA levels of *P5CS* and *δ-OAT* in different time point under PEG stress. Our results provided theory supporting important role of *P5CS* and *δ-OAT* in drought resistance of *Medicago sativa* cv. Pianguan.

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