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Isolation and expression analysis of proline metabolism-related genes in *Chrysanthemum lavandulifolium*

Mi Zhang, He Huang, Silan Dai *

Beijing Key Laboratory of Ornamental Plants Germplasm Innovation & Molecular Breeding, Beijing 100083, China
National Engineering Research Centre for Floriculture, Beijing 100083, China
College of Landscape Architecture, Beijing Forestry University,Beijing 100083, China

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A B S T R A C T

Proline plays a significant role in plant resistance to abiotic stresses, and its level is determined by a combination of synthesis, catabolism and transport. The primary proteins involved are Δ^1-pyrroline-5-carboxylate synthetase (P5CS), proline dehydrogenase (PDH) and proline transporter (ProT). To utilise proline metabolism to improve the stress resistance of *Chrysanthemum* × *morifolium*, we isolated two *P5CS*-homologous genes (*ClP5CS1* and *ClP5CS2*), one *PDH* gene (*ClPDH*) and four *ProT*-homologous genes (*ClProT1-4*) (GenBANK accession numbers: KF743136–KF743142) from *Chrysanthemum lavandulifolium*, which is closely related to chrysanthemums and exhibits strong resistance to stresses. Expression analysis of these genes in different organs and under various stresses indicated that *ClP5CSs* showed substantial constitutive expression, while *ClPDH* was only strongly expressed in the capitulum and was inhibited under most stresses. The expression patterns of four *ClProT* genes presented characteristics of organ specificity and disparity under stresses. Above all, the expression of *ClProT2* was restricted to above-ground organs, especially strong in the capitulum and could be obviously induced by various stress conditions. Promoters of *ClPDH* and *ClProTs* contained many *cis*-acting regulatory elements involved in stress responses and plant growth and development. High levels of free proline were found in flower buds, the capitulum under the non-stress condition and later periods of stress conditions except cold treatment. Interestingly, organ specificity and disparity also exist in the level of free proline under different stress conditions. Our study indicates that ClProTs play significant roles in proline accumulation and stress responses, and that *ClProT2* could be used to genetically modify the stress resistance of chrysanthemums. In addition, proline metabolism might be closely related to plant flowering and floral development.

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1. Introduction

Because plants are sessile by nature, their growth and development depend on optimal environmental conditions. Abiotic stresses, such as drought, high salinity and extreme temperatures, have been severely impacting the normal growth and development of plants, which primarily results in the decrease of crop yield and quality (Todaka et al., 2012). Plants have adapted to endure and defend themselves from these adverse environmental conditions through many effective and adaptive responses at the physiological and biochemical levels as well as on a cellular level and molecular level (Yamaguchi-Shinozaki and Shinozaki, 2006).

The accumulation of compatible osmolytes (soluble sugar, proline, glycine betaine, etc.) is one of the most common responses to stresses in plants (Ashraf and Foolad, 2007; Verbruggen and Hermans, 2008). Among these compatible osmolytes, proline exists widely in plants and is one of the most beneficial osmolytes in osmotic adjustment (Armengaud et al., 2004; Miller et al., 2005). Apart from functioning as a compatible osmolyte, proline has also been proposed to contribute to stabilising the structure of proteins and enzyme activity, scavenging ROS and balancing cellular homeostasis during the process of stress, supplying energy for resumed growth after stress, regulating plant growth and development and functioning as a signalling molecule to influence other metabolic pathways (Hayat et al., 2012; Szabados and Savouré, 2010). Among the regulatory roles of proline metabolism in plant growth and development, the most valuable one is its function in flowering. Though its mechanism has not been clearly determined, a positive role of proline synthesis in flowering has been suggested in

Abbreviations: 3′ RACE, 3′ rapid amplification of cDNA ends; AAAP, amino acid/auxin permease; ABA, abscisic acid; APC, amino acid-polyamine-choline; ATF, amino acid transporter family; CAT, cationic amino acid transporter; CBF, C-repeat binding factor; *ClTUA*, α-tubulin gene in *Chrysanthemum lavandulifolium*; ESTs, expressed sequence tags; GSA, glutamate-semialdehyde; LHT, lysine–histidine transporter; ORF, open reading frame; P5C, Δ^1-pyrroline-5-carboxylate; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; PCR, polymerase chain reaction; PDH, proline dehydrogenase; PRE, proline- or hypoosmolarity-responsive element; ProT, proline transporter; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, real-time quantitative PCR; δOAT, ornithine-δ-aminotransferase.

* Corresponding author at: College of Landscape Architecture, Beijing Forestry University, No. 35 Tsinghua East Road, Haidian, Beijing 100083, China. Tel.: +86 10 62336100; fax: +86 10 62338250.
E-mail address: silandai@sina.com (S. Dai).

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Arabidopsis (*Arabidopsis thaliana*) (Mattioli et al., 2008, 2009; Samach et al., 2000). Therefore, it is of great importance to improve the stress resistance of plants, especially ornamental plants, via targeted engineering of proline metabolism. Furthermore, understanding the mechanism of proline metabolism may provide supplementary data regarding the biology of flowering.

To utilise proline metabolism to improve plant stress resistance, it is necessary to perform a more thorough investigation of the regulatory mechanism of proline metabolism in higher plants (Kishor et al., 2005). In plants, proline can be synthesised through two pathways, either from glutamate or ornithine. The glutamate pathway is normally located in the cytosol and chloroplasts (Armengaud et al., 2004). Glutamate is reduced to glutamate-semialdehyde (GSA) by Δ^1-pyrroline-5-carboxylate synthetase (P5CS), and is spontaneously converted to Δ^1-pyrroline-5-carboxylate (P5C). P5C is then reduced to proline, which is catalysed by Δ^1-pyrroline-5-carboxylate reductase (P5CR). In an alternative pathway, proline can be synthesised from ornithine, which occurs in mitochondria. Ornithine-δ-aminotransferase (δOAT) converts ornithine to GSA and P5C, which is then transported to the cytosol and converted to proline by P5CR. The catabolism of proline also takes place in the mitochondria. Proline is oxidised via the sequential action of proline dehydrogenase (PDH) producing P5C and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH), which converts P5C to glutamate (Lehmann et al., 2010; Szabados and Savouré, 2010). P5CS and PDH are regarded as key enzymes in proline synthesis and catabolism, respectively. Plant genomes usually contain two homologous genes encoding P5CS and PDH, as is the case in *A. thaliana* (Funck et al., 2010; Strizhov et al., 1997), *Medicago truncatula* (Armengaud et al., 2004) and *Nicotiana tabacum* (Ribarits et al., 2007).

Early studies of proline metabolism established a “standard model” whereby increased synthesis and reduced degradation led to the accumulation of proline (Chaitanya et al., 2009; Miller et al., 2005, 2009; Parida et al., 2008; Ribarits et al., 2007; Sharma et al., 2011). Based on this model, genetic manipulation to improve plant stress tolerance by overexpressing the *P5CS* gene or decreasing *PDH* gene expression has achieved initial success (Verbruggen and Hermans, 2008; Mizoi and Yamaguchi-Shinozaki, 2013). Through further investigations, researchers came to realise that the dynamic transport and turnover of proline between different organs, rather than static cell-autonomous accumulation, are fundamental to the protective role of proline (Sharma et al., 2011). At present, proline transporter (ProT), which belongs to the amino acid transporter family (ATF) or amino acid/auxin permease (AAAP) family, has been shown to be localised at the plasma membrane and is likely involved in the intercellular and long-distance transport of proline (Rentsch et al., 2007). Complementation of yeast strain mutants and the analysis of kinetic properties revealed that the affinity of ProT was highest for glycine betaine, intermediate for proline and lowest for γ-aminobutyric acid, a stress-induced compound (Fujiwara et al., 2010; Grallath et al., 2005). However, the molecular mechanism of ProT-mediated transport of proline or other compatible solutes is poorly understood (Lehmann et al., 2011).

Chrysanthemum × *morifolium* has taken a leading role in the commercial production and landscape application of ornamental plants (Zhang and Dai, 2009). However, the frequent occurrence of abiotic stresses has deleteriously affected the production and application of chrysanthemums. As a wild species with a close relationship to cultivated chrysanthemums, *Chrysanthemum lavandulifolium* has been shown to be an ideal material for research on the mechanism of stress resistance and molecular breeding process of chrysanthemums because it has a relatively small diploid chromosome number ($2n = 2x = 18$) and is strongly resistant to stresses (Huang et al., 2012a). In our previous study, we found that *C. lavandulifolium* could accumulate relatively high free proline level under salt stress and that the proline level increased as the stress continued (Huang et al., 2012a). Moreover, multiple expressed sequence tags (ESTs) related to proline metabolism (especially proline transport) were isolated using RNA sequencing and bioinformatic analysis (Huang et al., 2012b; Wang et al., 2013). Based on these data, our study aims to isolate proline metabolism-related genes (*P5CS*, *PDH* and *ProT*) and comprehensively analyse the expression patterns of these genes under various stress conditions and in different organs in order to guide in-depth research on the function of some key genes and to shed light on the mechanism of proline metabolism in chrysanthemums. Furthermore, this study will provide excellent gene resources for molecular modification aimed at improving the stress resistance of chrysanthemums.

2. Materials and methods

2.1. Plant materials

To obtain a large number of uniform, healthy *C. lavandulifolium* seedlings, axenic cultured plantlets were propagated by cutting stems into fragments with a single node. These stem fragments were cultured on Murashige and Skoog medium. After 7 days (when roots reached 2 to 3 cm), sterile plantlets were transferred into a 1:1 mixture of nutrient soil and vermiculite and continuously cultured in a growth chamber with temperature of 21 ± 2 °C, on a 12 h light/12 h dark regime. Stress treatments were performed at the stage where the eighth pair of true leaves had emerged (about seven weeks after transference).

2.2. Stress treatments and sampling

As described in our previous study (Huang et al., 2012b), stress treatments were performed as follows. For drought stress, *C. lavandulifolium* seedlings were carefully removed from the soil, and the soil mixture was washed away. Seedlings were then left to dry in a growth chamber. Salt stress was applied by irrigating seedlings with incrementally increasing concentrations of NaCl solution to reach the final concentration of 300 mM. For cold and heat treatments, plants were transferred to 0 °C and 40 °C growth chambers, respectively. Seedlings were sprayed with 200 μM abscisic acid (ABA) to study the impact of ABA on proline metabolism-related genes. *C. lavandulifolium* seedlings without any treatments were used as controls. Treatment time differed among the different treatments based on the seedlings' stress tolerance to these treatments. Medium leaves (the third pair of leaves under the apical bud) were sampled for the expression analysis of genes under various stresses. To analyse organ specificity of gene expression, roots, old leaves (the seventh pair of leaves under the apical bud), stems, young leaves (the first pair of fully expanded leaves under the apical bud) and the capitulum were sampled in the unstressed condition and at 48 h after salt stress. All samples were snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.3. RNA extraction and first-strand cDNA synthesis

Total RNA was extracted according to the instructions for the Quick RNA Isolation Kit (Beijing HUAYUEYANG Biotechnology CO., LTD.). The first-strand cDNA was synthesised from 5 μg RNA with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega) in accordance with the manufacturer's instructions.

2.4. Isolation of proline metabolism-related genes

Primers (Supplementary Table 2) for the 3′ rapid amplification of cDNA ends (3′ RACE) were designed based on the obtained 5′-end ESTs of the proline metabolism-related genes *P5CS*, *PDH* and *ProT* (Supplementary Table 1). cDNA synthesised from mixed RNA samples related to various stress treatments was used as the template for amplification of 3′-end cDNA sequences by polymerase chain reaction (PCR). Full cDNA sequences were spliced by DNAMAN software. To verify the accuracy of the isolated cDNA sequences, specific primers (Supplementary

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