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Gene Cloning and Expression of the Pyrroline-5-carboxylate Reductase Gene of Perennial Ryegrass (*Lolium perenne*)

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Abstract

Salt and drought limit the range of applications of perennial ryegrass (*Lolium perenne* L.), which is one of the important turf and forage grasses. Previous studies have suggested that pyrroline-5-carboxylate reductase (P5CR) might play a central role in proline accumulation in plants that are responsive to stresses. In the present study, the *Lolium perenne* L. pyrroline-5-carboxylate reductase (*LpP5CR*) gene was cloned from leaves of the cultivar 'Derby' using the RACE technique. The full-length *LpP5CR* gene was 1 047 bp in length, which comprised an open reading frame (ORF) of 840 bp in size. Sequence alignment revealed that the putative LpP5CR had a 94.3% similarity to TaP5CR. qRT-PCR displayed that the mRNA levels of the *LpP5CR* gene were almost the same as that in the roots, stems, and leaves of perennial ryegrass seedlings subjected to normal condition or NaCl treatment for 1 h. Moreover, the transcription level of *LpP5CR* was up- or down- regulated with NaCl, polyethylene glycol (PEG), cold, or abscisic acid (ABA) treatment for 3 to 48 h. In addition, confocal microscopy localized the GFP-LpP5CR fusion protein to the cytoplasm of onion epidermal cells. These findings suggest that *LpP5CR* encodes a cytoplasmic P5CR protein that plays an important role in the response of perennial ryegrass to various stresses.

Keywords: *Lolium perenne*; pyrroline-5-carboxylate reductase; proline; subcellular localization; gene expression

1. Introduction

As one of the most important turf and forage grasses, perennial ryegrass has been extensively used for virescence in parks, airdromes, and golf courses (Salminen et al., 2003). This grass exhibits various excellent traits, including high efficiency for livestock digestion and high frequency for grazing. However, its application is strongly restricted by salt and drought (Ma et al., 2006; Mhadhbi et al., 2011). In contrast to traditional breeding methods that are time-consuming and laborious, genetic engineering is an alternative way of modifying stress-related traits in perennial ryegrass (*Lolium perenne* L.)

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It is well known that proline plays an important role in protecting protein and cellular redox homeostasis from stress-induced damage (Verbruggen and Hermans, 2008; Szabados and Savoure, 2010). Δ1-pyrroline-5-carboxylate synthetase (P5CS) and Δ1-pyrroline-5-carboxylate reductase (P5CR) are the key enzymes of proline biosynthesis in plants. P5CS reduces glutamic acid to γ-glutamic semialdehyde (GSA), whereas P5CR spontaneously converts into Δ1-pyrroline-5-carboxylate (P5C). Finally, the conversion of P5C to proline is catalyzed by P5CR (Szabados and Savoure, 2010).

P5CS genes in various plant species have been induced by various stresses, including drought, salt, cold, or ABA (Savoure et al., 1995; Szekely et al., 2008; Xue et al., 2009). Additionally, the *p5cs* mutant is sensitive to drought and salt stresses in *Arabidopsis thaliana*, whereas P5CS-overexpressed transgenic potatoes or *Arabidopsis* plants display a stress insensitivity phenotype (Hmida-Sayari et al., 2005; Mattioli et al., 2009; Su et al., 2011). In contrast to P5CS, some studies have indicated that the level of P5CR transcripts is not induced by these stresses (Szoke et al., 1992; Delauney et al., 1993; Verbruggen et al., 1993; Hua et al., 1997; Sharma and Verslues, 2010), and the overexpression of P5CR does not lead to enhanced proline content (Szoke et al., 1992), which indicates that P5CS rather than P5CR is a rate-limiting enzyme in plants. However, the overexpression of P5CR affects the level of proline in soybean (De Ronde et al., 2004a, 2004b). These findings imply that there could be species- and stress-specific differences in P5CR function in various plants. To date, studies on P5CR have been mainly restricted to plant and crop models such as *Arabidopsis*, rice, and soybean; whereas cloning and function analyses of P5CR from *Perennial ryegrass* have not yet reported.

Recently, Funck et al. (2012) localized *AtP5CR* to the cytosol of *Arabidopsis* protoplast. Other previous studies have also revealed that *P5C* from the mitochondria could be transported into the cytosol and re-reduced to proline by cytosolic P5CR (Szabados and Savoure, 2010), which indicates that P5CR plays an important role in proline synthesis and in cycling proline and *P5C* between cellular compartments. However, it is unclear whether the localization pattern of P5CR in other species varies.

Despite the functional analysis of this gene in model plants such as *Arabidopsis*, it has not been reported for *P5CR* expression and sub-cellular localization of its encoding protein in *Lolium perenne*. In this study, a full-length P5CR has been isolated using the RACE technique. The sequence of this gene as well as the encoding protein was analyzed. The expression of *LpP5CR* and cellular localization of its putative protein were investigated using qRT-PCR and particle gun-mediated transformation, respectively. This study will provide a candidate gene and a theoretical basis for modifying proline biosynthesis.

2. Materials and methods

2.1. Perennial ryegrass growth conditions and stress treatments

Perennial ryegrass ‘Derby’ seeds were sterilized with a 15% sodium hypochlorite solution and germinated in Petri plates. The seedlings were transferred onto boxes (5 cm × 5 cm) that contained a substrate (nutrient soil : vermiculture : perlite = 4 : 1 : 1). The thermo- and photoperiods in the culture room were 25 ℃ /18 ℃ and 18 h/6 h, respectively. To detect the tissue-specific expression pattern of *LpP5CR*, 0.2 g of respective fresh roots, stems, and leaves of 4-week-old seedlings grown under normal conditions or under 200 mmol · L^{-1} NaCl treatment for 1 h were used for RNA extraction.

For various stress treatments, 4-week-old seedlings were treated with 1/2 Hoagland’s solution containing 200 mmol · L^{-1} NaCl, 20% PEG4000, and 1 μmol · L^{-1} ABA. For the cold treatment, seedlings in 1/2 Hoagland’s medium were kept at 4 ℃. At the same time, the plants in 1/2 Hoagland’s solution at room temperature were used as control. The beginning of the treatments was designated as time point 0 h. Subsequently, at the certain time points, 0.2 g of leaves was collected for RNA extraction. For all samples, materials from three independent seedlings were collected as one replicate. Three biological replicates were conducted for each treatment.

2.2. Molecular cloning of LpP5CR

Total RNA was extracted using TRIzol (Invitrogen, USA) from 10-day-old ryegrass leaves and synthesized into cDNA using OligodT17 and a reverse transcriptase (Invitrogen, USA). The conserved DNA region was amplified by using two degenerate primers, *P5CR* For and *P5CR* Rev. The PCR conditions were as follows: 1 cycle at 94 ℃ for 5 min; followed by 33 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min; and a final cycle of 72 ℃ for 10 min. The PCR product was ligated onto a PMT-19T vector for subsequent sequencing. Based on the sequencing results, three specific primers (*P5CR* GSP1, *P5CR* GSP2, and *P5CR* GSP3) were designed to amplify the 5'- and 3'-nucleotide sequences of the gene by using the smart RACE kit (Clontech, USA). After sequencing, the full-length sequence of the *LpP5CR* gene was retrieved by assembling using the DNAMAN software. Then, the ORF of the *LpP5CR* gene was amplified using the primers (*LpP5CR* FL For and *LpP5CR* FL Rev) and subcloned into PMD-19T for sequencing and further analysis. The primers used in gene cloning are listed in Table 1.