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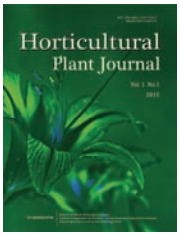

Gene Cloning and Expression of the Pyrroline-5-carboxylate Reductase Gene of Perennial Ryegrass (*Lolium perenne*)

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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.

Table 1 The primers used in this study

Primer	Sequence
P5CR For	5'-TTRCAGGAWTGGTCTGGTCA-3'
P5CR Rev	5'- ACCATVGTTGCAGCWCCWAG-3'
P5CR GSP1	5'-GCAGGAATGGTCTGGTCAGC-3'
P5CR GSP2	5'-CAGCAACTCCACCATCAGC-3'
P5CR GSP3	5'-TTGAGGCCATGGCTGATGGT -3'
P5CR FL For	5'-ATGGCCGCGCCTCCCCA-3'
P5CR FL Rev	5'-TTAATTTTTTGAGAGCT-3'
P5CR Realtime For	5'-GTAATGCCAAACACTCCCTC-3'
P5CR Realtime Rev	5'-CAGCAACTCCACCATCAGC-3'
eEF-1 For	5'-GAGAGGTCCACCAACCTTG-3'
eEF-1 Rev	5'-GGCTTGGTGGGAATCATC -3'

2.3. Sequence alignment and pholygenetic tree analysis of LpP5CR

Sequence of *Lp5CR* at the nucleotide level was analyzed through a BLAST on NCBI (http://www.ncbi.nlm.nih.gov). Multi-alignment of LpP5CR and other P5CR protein sequence were carried out using ClustalW2 (EMBL European bio-informatics institute). Finally, the pholygenetic tree was constructed using the Neighbor-Joining method by MEGA5.

2.4. Expression analyses of LpP5CS under different stresses by qRT-PCR

The relative expression levels of the *LpP5CR* gene were investigated by qRT-PCR using the primer pair, *P5CR* Realtime For and *P5CR* Realtime Rev. Each 25 μL reaction system contained 1 μL of cDNA, 2.5 μL of 10× PCR buffer, 0.5 μL of 10 mmol · L^{-1} dNTPs, 0.25 μL of 20 pmol · $μL^{-1}$ of each primer, 0.5 μL of 2 U · $μL^{-1}$ *Taq*, 1 μL of SYBR Green, and 19 μL of double-distilled water. PCR was performed using the following conditions: 1 cycle of 94 ℃ for 2 min; 35 cycles of 94 ℃ for 30 s, 59 ℃ for 30 s, and 72 ℃ for 30 s. The *e-EF* gene was used as loading control.

2.5. Arabidopsis transformation and investigation

The ORF of *LpP5CR* was digested by *Sam* Ⅰ/*Xho* Ⅰ and subcloned into pCAMBIA 1301 under the control of a *Cauliflower mosaic virus* (CaMV) 35S promoter. Then, the plasmid was introduced into *Agrobacterium tumefaciens* GV3101 via electroporation.

Arabidopsis thaliana was grown in a growth room with 12 h light at 23 ℃ and 12 h dark at 20 ℃ photoperiod under 60 μmol · m^{-2} · s^{-1} of light intensity. *Arabidopsis* transformation was conducted by using the flower dip method. The seeds were harvested and the transgenic plants were screened on the medium containing hygromycin B (50 μg · mL^{-1}). The leaves of two-week-old T2 generation transgenic plants or wild-type *Arabidopsis* plants under normal conditions were used for the investigation of *LpP5CR* expression by qRT-PCR.

2.6. Subcellular localization of LpP5CR in onion epidermal cells

The ORF of *LpP5CR* on vector PMD19T was subcloned into PUC18-GFP by using the restriction enzymes, *Sma* Ⅰ/*Xho* Ⅰ. The recombinant plasmids were then introduced into the onion epidermal cells by particle gun-mediated transformation as described by Scott et al. (1999). After culturing for 16 h, the onion epidermal cells were observed under a confocal laser scanning microscope (Nikon, TE2000-E).

3. Results

3.1. Cloning and sequence analyses of the LpP5CR gene

The full-length *P5CR* gene was retrieved from perennial ryegrass leaves and registered as KC896628 in GenBank. Using the sequence of LpP5CR as query, BLAST analysis indicated high identities between LpP5CR and other P5CRs (HvP5CR/AK372176.1, 91%; TaP5CR/ AY880317.1, 91%; and SbP5CR/ XM_002459045.1, 88% at > 85% query cover level) at the nucleotide level.

The *LpP5CR* gene had a 61 bp of 5'-UTR, 146 bp 3'-UTR, and an 840 bp of ORF that encoded a 279 amino acids polypeptide. Multiple alignment indicated that the putative LpP5CS (AGS78291.1 in GenBank) was highly similar to well-studied proteins such as *Triticum aestivum* P5CR (94.3%), *Oryza sativa* P5CR (87.8%), and *Arabidopsis thaliana* P5CR (67.3%) at the amino acid level. Based on the alignment between LpP5CR and *Streptococcus pyogenes* P5CR whose crystal structure has been reported (Nocek et al., 2005), LpP5CR was determined to consist of two main functional domains, one NADP-binding domain and one dimerization domain. The former domain comprised five alpha helixes and four beta strands that were responsive to NADP binding, whereas the latter had six alpha helixes that formed a dimer (Fig. 1).

3.2. Phylogenetic tree analysis of putative LpP5CR

To elucidate the relationship between LpP5CR and other known P5CRs, a phylogenetic tree was constructed using the full length amino acid sequences by using the ClustalW and MEGA5 software. The P5CRs could be divided into two groups, namely, monocots and dicots. LpP5CR belonged to the monocot group and was closely related to *Hordeum vulgare* P5CR and *Triticum aestivum* P5CR (Fig. 2).

The *LpP5CR* gene displayed a constitutive expression pattern under normal conditions, and no obvious differences in transcription levels were observed among the leaves, stems, and roots. When exposed to 200 mmol · L^{-1} NaCl for 1 h, the transcript levels of the *LpP5CS* gene were elevated in all three organs (Fig. 3).

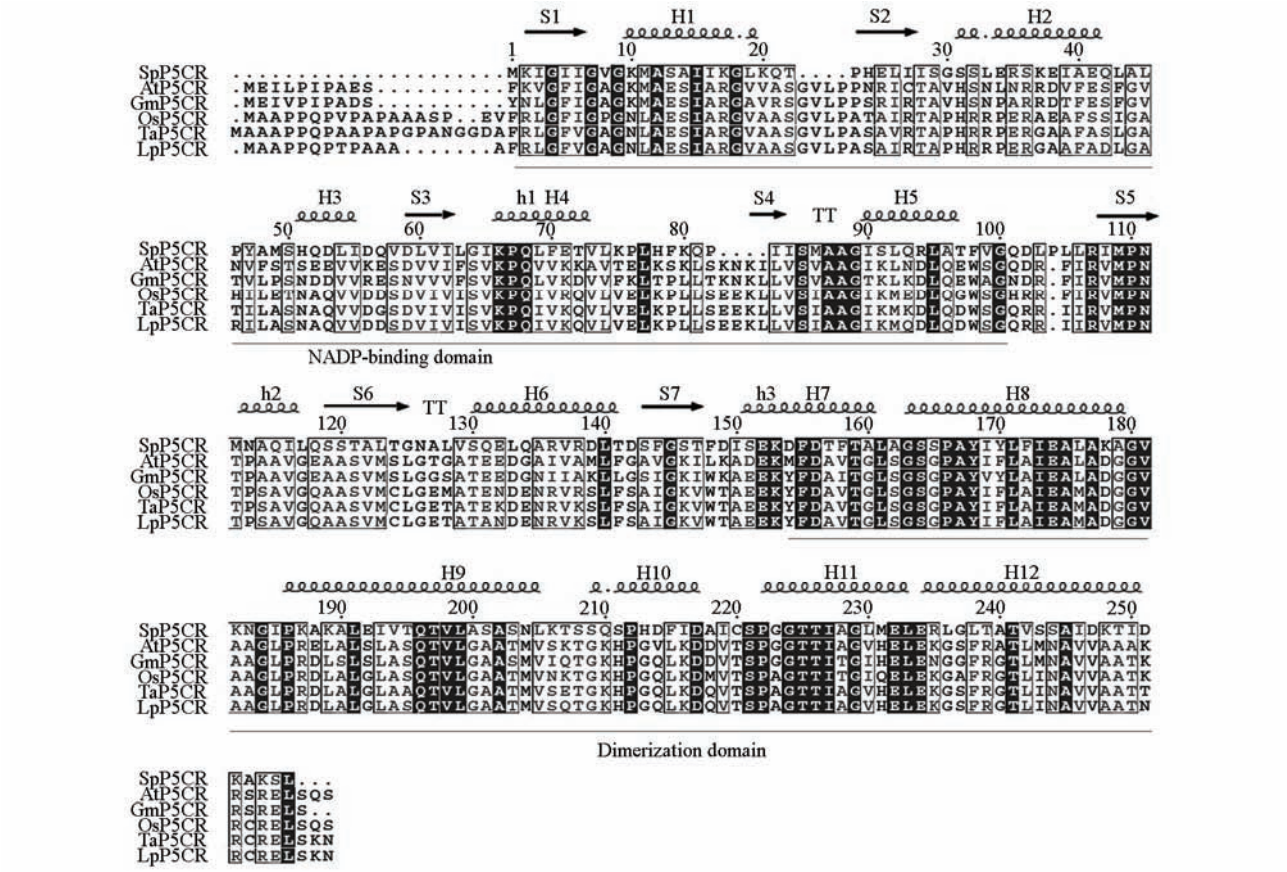

Fig. 1 Multiple sequence alignment and domain prediction of P5CRs

Sequence identities are marked by the red background and the similarities are highlighted in red letters. The secondary structure of P5CR from *Streptococcus pyogenes* is shown at the top. H, α-helix; h, β-helix; S, α-strand. Sp: *Streptococcus pyogenes*; At: *Arabidopsis thaliana*; Gm: *Glycine max*; Os: *Oryza sativa*;Ta: *Triticum aestivum*; Lp: *Lolium perenne*.

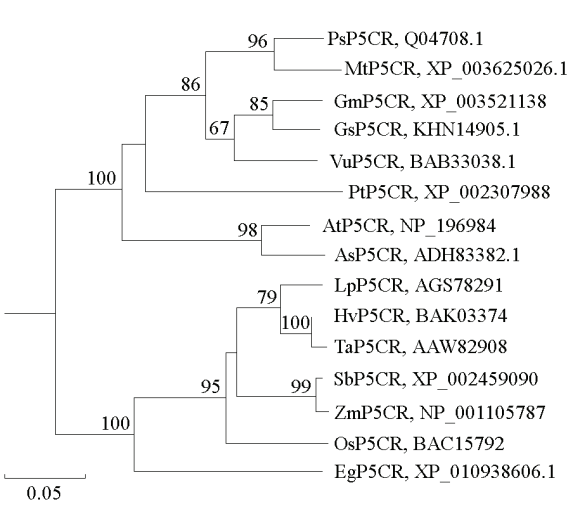

Fig. 2 Phylogenetic relationship between LpP5CR and other P5CRs

A rooted tree was constructed by using the MEGA5 software.

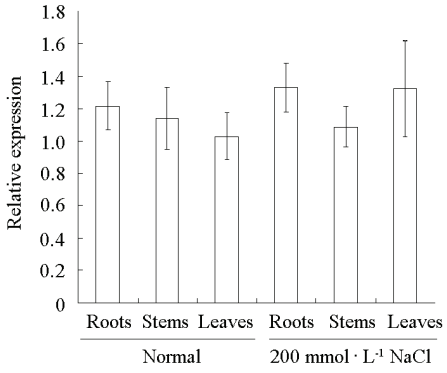

Fig. 3 Tissue-specific expression of *LpP5CR* under normal condition or NaCl stress

Levels of relative expression were calculated in comparison to the lowest one, which was designated as 1.0. All data were obtained from three independent biological replicates.

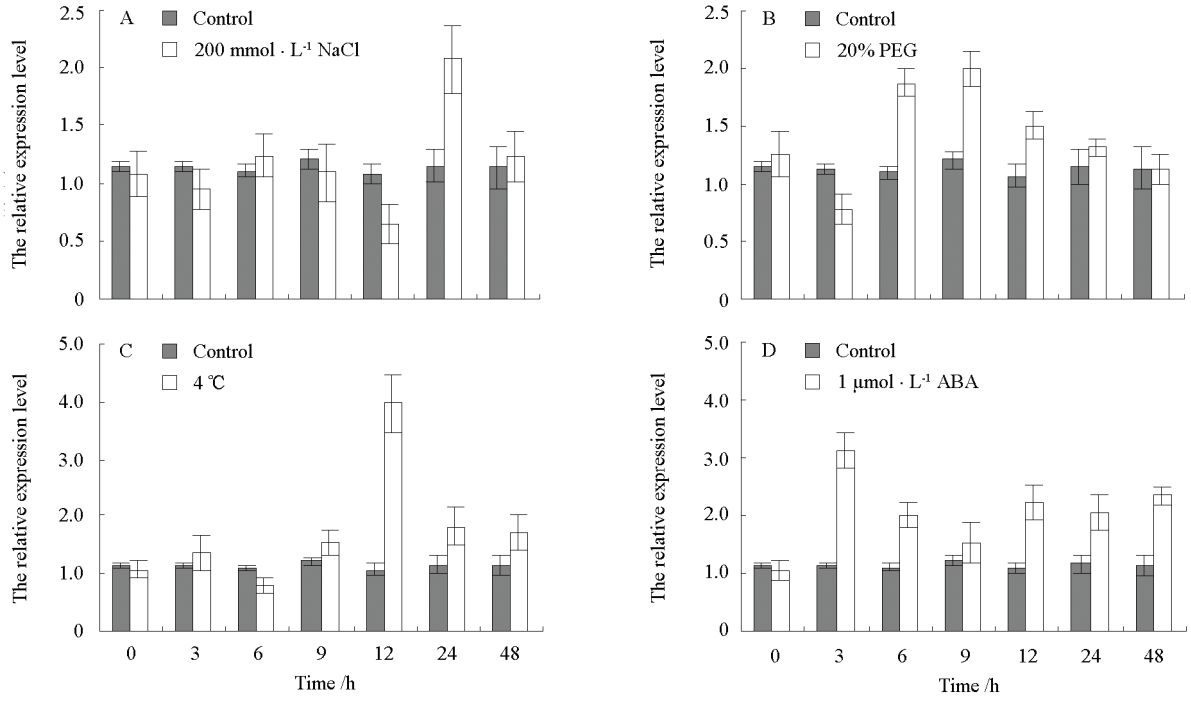

Fig. 4 Expression profile of the *LpP5CR* gene in leaves under various stresses and ABA

Relative expression levels were calculated in comparison to that of the control, which was designated to have an expression level of 1.0. All data were obtained from three independent biological replicates.

3.3. Expression pattern of LpP5CR under various stresses

The expression profile of the *LpP5CS* gene in perennial ryegrass leaves was investigated by qRT-PCR under NaCl, PEG, cold or ABA treatments (Fig. 4). For the control of only 1/2 Hoagland's solution, no differences in expression of the *LpP5CR* gene were observed at particular time points. Under 200 mmol · L^{-1} of NaCl, the expression levels of *LpP5CR* slightly decreased from 3 to 12 h, increased to 1.8-fold relative to that of the control at 24 h, and decreased to the control levels at 48 h (Fig. 4, A). Under 20% PEG, the expression levels of the *LpP5CR* gene decreased at 3 h, increased by 1.4-fold relative to that of the control at 12 h, and then gradually decreased to the control levels (Fig. 4, B). For cold treatment, the transcript levels of the *LpP5CR* gene were the highest, showing a 4-fold increase relative to that of the control at 12 h, and about 1.8-fold at 24 h and 48 h (Fig. 4, C). When exposed to 1 μmol · L^{-1} of ABA, the mRNA levels of the *LpP5CR* gene increased at 3 h by about 3-fold relative to that of the control and were higher than those of the control at all time points (Fig. 4, D).

3.4. Expression analysis of LpP5CR in transgenic Arabidopsis

The *LpP5CR* gene was detected in transgenic *Arabidopsis* plants (three independent lines: L5, L13, and L9), but not in the wild-type plants (Fig. 5). No distinct differences in growth between the wild-type and transgenic plants were observed (Fig. 6).

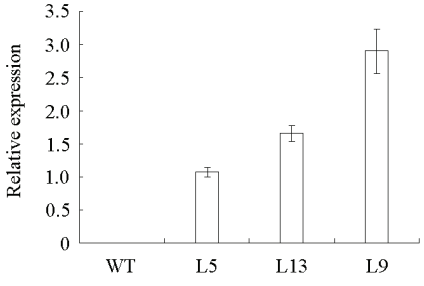

Fig. 5 Expression of the *LpP5CR* gene in transgenic plants

Relative expression levels were calculated in comparison to the lowest one, which was designated as 1.0.

Fig. 6 Transgenic *Arabidopsis* (L9) and wild-type (WT)

3.5. Subcellular localization of the LpP5CR protein in onion epidermal cells

To clarify the subcellular localization of the LpP5CR protein, the recombinant plasmid was transformed into onion epidermal cells by electroporation. PUC18-GFP alone was used as the control. Fig. 7 shows that a strong fluorescent signal of the GFP alone was observed in the nuclei, cytoplasm, and the cell membrane. However, the GFP-LpP5CR fusion protein was localized onto the cell membrane and in the cytoplasm. These results indicated that the LpP5CR is a membrane- and cytoplasm-localized protein.

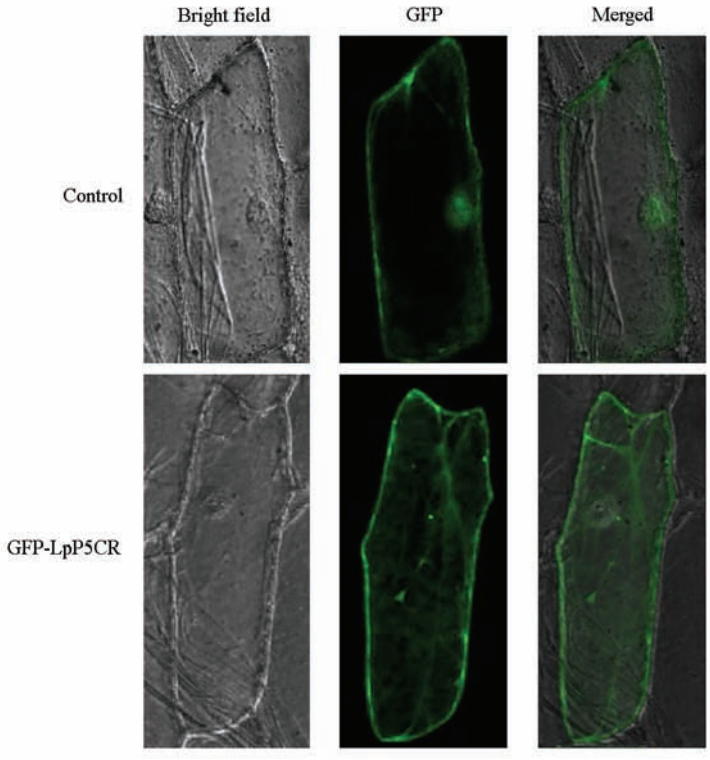

Fig. 7 Subcellular localization of the putative LpP5CR in onion epidermal cells

4. Discussion

In the present study, a full-length *P5CR* was isolated from perennial ryegrass leaves. BLAST analysis revealed that the *LpP5CR* gene was highly similar to the P5CRs of other plant species. The putative LpP5CR was predicted to have essential functional domains based on the alignment between this protein and *Streptococcus pyogenes* P5CR, whose crystal structure has been well established (Fig. 1), thereby indicating that LpP5CR might be a functional enzyme.

In the past several decades, the contribution of key enzymes such as P5CS and P5CR to proline accumulation and acquired tolerance in plants has been investigated (Szabados and Savoure, 2010). In contrast to P5CSs whose functions are relatively clear, the expression and subcellular localization of P5CR were ambiguous in different plant species.

In *Arabidopsis*, P5CR transcription is induced by stress (Delauney et al., 1993; Verbruggen et al., 1993; Hua et al., 1997; Sharma and Verslues, 2010), and over-expression of P5CR does not lead to enhanced proline content (Szoke et al., 1992), which indicates that P5CR might not be a rate-limiting enzyme in proline biosynthesis. However, the P5CR transcript was upregulated by osmostress in soybean root (Delauney and Verma, 1990). The expression levels of *Triticum aestivum* P5CR were also upregulated by salt, PEG, and ABA (Ma et al., 2008). The findings of the present study indicated that the *LpP5CR* transcripts in leaves could be induced by NaCl, PEG, cold, and ABA (Fig. 3, Fig. 4). It is possible that there were differences in P5CR expression between dicot and monocot plants under these stresses. Recently, AbdElgawad et al. (2015) reported that P5CR played a central role in proline metabolism in grass, including *Lolium perenne* and *Poa pratensis*, and changes in P5CR activity also matched closely with changes in P5CR transcript levels, which supports our results. In three independent lines overexpressing *LpP5CR*, *LpP5CR* transcripts were detected, indicating that the introduction of *LpP5CR* into *Arabidopsis* was successful. The growth of the transgenic *Arabidopsis* plants was not inhibited (Fig. 7). This phenomenon could be explained by the fact that P5CS, but not P5CR, is the limiting-rate enzyme, and overexpressed *LpP5CR* does not lead to enhanced proline levels in *Arabidopsis*. Functional analysis of LpP5CR in transgenic *Arabidopsis* should be done in future investigations.

Proline biosynthesis occurs in the cytosol and the chloroplast (Szabados and Savoure, 2010). In pea and soybean, P5CR activity was detected in plastids by co-sedimentation (Rayapati et al., 1989; Szoke, 1992). Recently, P5CR has been localized in the cytosol in Arabidopsis protoplast (Funck et al. 2012). In the present study, the GFP-LpP5CR fusion protein was observed in the cytosol of onion epidermal cells (Fig. 5). This finding implies that in perennial ryegrass cell, proline biosynthesis occurs in the cytosol, which is in agreement with the results derived from pea, soybean, and *Arabidopsis*.

The present study performed cloning and characterization of a novel *P5CR* from perennial ryegrass. Our data reveal that LpP5CR could be a cytosol-located protein whose transcript responds to various stresses such as NaCl, PEG, cold and ABA signals, which indicates that this gene may play an important role in proline accumulation in perennial ryegrass cytosol under adverse conditions. We conclude that *LpP5CR* might be a candidate gene for stress-associated molecular breeding in perennial ryegrass.

Acknowledgements

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