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REVIEW

Multifunctional proline rich proteins and their role in regulating cellular proline content in plants under stress

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Abstract

Proline rich proteins (PRPs), earlier famous as animal salivary proteins, have now been proven as indispensable plant proteins. They are highly rich in proline amino acid residues at the N-terminus whereas a characteristic eight cysteine motif is located at the C-terminus. The PRPs support a number of developmental processes from germination to plant death. Under normal environmental conditions, *PRP* genes express customarily in different plant parts depending on the specific function to be carried out. During abiotic stresses, *PRP* genes exhibit an uneven pattern of transcriptional regulation depending on the time and intensity of stress. Transgenic plants overexpressing *PRP* genes show an enhanced tolerance to abiotic stresses. This review focuses on contemporary functions of PRPs during stresses and proposes that PRPs are involved in the regulation of free cellular proline content during stress in a well synchronized manner.

Additional key words: abiotic and biotic stresses, *PRP genes*, transgenic plants

Introduction

Proline rich proteins (PRPs) were initially identified as salivary proteins of mammals. During 1960s, PRPs were reported in plants when hydroxyproline rich proteins were obtained by enzymatic degradation of cell wall (Lamport 1969). Later, it was confirmed that hydroxyproline rich proteins are derived by hydroxylation of PRPs on rough endoplasmic reticulum in the cytoplasm and transported to cell wall (Ridge and Osborne 1970, Pitzschke *et al.* 2016). The PRPs, having tandem repeats of a hexapeptide PPPVHL, were first identified and characterized in maize endosperm (Esen *et al.* 1982). Three proline rich proteins of soybean (SbPRP1, SbPRP2, and SbPRP3) were extensively studied for their function, localization, and differential expression (Hong *et al.* 1989, 1990, Francisco and Tierney 1990, Wyatt *et al.* 1992). The PRPs consist of two domains, a proline rich domain at the N-terminus (Dvorakova *et al.* 2007) and a typical hydrophobic eight cysteine motif (8-CM) at the C-terminus (Jose-Estanyol *et al.* 2004). The 8-CM C-terminal domain is a characteristic feature of lipid transfer protein (LTP) family/protease inhibitor/hydrophobic seed storage proteins (Jose-Estanyol *et al.* 2004), but a classical LTP is devoid of proline rich domain. The classification of PRPs is mainly based on the sequence and distribution of proline repeat motifs throughout the protein. Two broad classes of proline rich proteins are: simple PRPs that are devoid of a hydrophobic domain, and hybrid PRPs having a hydrophobic domain at the C terminus.

Preliminary research on plant proline/hydroxyproline rich proteins suggests their localization on cell wall (Lamport 1969, Deutch *et al.* 1995, Fowler *et al.* 1999) but recent reports show the subcellular localization of plant PRPs/hybrid PRPs (HyPRPs) at plasmalemma (Zhan *et al.* 2012, Qin *et al.* 2013, Li *et al.* 2014). The PRPs have

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Abbreviations: 8-CM - eight cysteine motif; LTP - lipid transfer protein; P5CS - pyrroline-5-carboxylate synthase; PRP - proline rich protein;
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been reported to play a crucial role in plant growth and developmental processes particularly during germination and flower development. Investigation during the past decade has confirmed that the expression of PRPs is modulated by biotic and abiotic stresses like chilling, water deficit, salinity, abscisic acid or salicylic acid treatments, and a viral inoculation. A specific localization of PRPs in different plant organs expedites the variability in their functions. This review focuses on a meticulous analysis of the functionality and molecular diversity of these multifunctional proteins. Further, participation of PRPs in both biotic and abiotic stresses has been discussed. There are two interesting facts that encouraged us to suggest a link between PRPs and free cellular proline. Firstly, as the name suggests, PRPs have a proline rich domain consisting of about 70 % of proline residues. Secondly, PRPs demonstrate irregular patterns of expression under stress conditions.

Sequence diversity in PRPs and their similarity with lipid transfer proteins

Both PRPs and LTPs belong to a common superfamily of proteins called prolamins (Edstam *et al.* 2011). An N-terminal proline rich domain (Fig. 1 Suppl.) and C-terminal 8-CM (Fig. 2 Suppl.) domains were aligned separately for the assessment of sequence diversity. Proline rich domains of PRPs portray different patterns of tandem repeats: PPVV, PPIV, PPYV, etc. Few PRPs have a continuous stretch of three or four proline residues: PPPIV, PPPYV, PPPVV, PPPS, PPPPIV, PPPPYV, and PPPPTP. Conversely, a C-terminal 8-CM domain is highly conserved in PRPs. The common feature of this domain is the presence of eight cysteine amino acid residues at specified positions and the abundance of leucine residues all over the stretch (Jose-Estanyol *et al.* 2004). There is a notable difference in number of leucine residues between the 8-CM domain of PRPs (12 - 13 L) and LTPs (9 L). Motif search along PRP sequences exposes the presence of three key motifs: a hydrophobic seed protein, probable LTP 2, and tryptophan α-amylase. Besides, some PRPs have histone H1-like nucleoprotein HC2, proteasome subunit, proteins of unknown function (DUF1409), a transposase-associated domain, and the SOCE-associated regulatory factor of calcium homoeostasis.

Though the 8-CM domain is functionally analogous in both PRPs and a classical LTP family (Dvorakova *et al.* 2007), there are many notable differences in the sequence. The 8-CM domain of PRPs has an average stretch of 82 amino acid residues from the cysteine residue placed at the first position to the cysteine residue at the eighth position compared to classical LTPs, which have 79 amino acid residues along this stretch (Fig. 3 Suppl.). Differences in structure between the 8-CM domain of PRP and LTP have been analysed by comparing their 3D structures generated by the *i-TASSAR* server (Yang *et al.* 2015). The 8-CM domains of both the proteins consist of four α-helices interconnected with three loops, representing a typical membrane protein structure. The fourth helix of LTPs is comparatively more extended than that of PRP. Comparison of 3D structures between the 8-CM domain of PRP and LTP is represented in Fig. 4 Suppl. Despite the significant difference in amino acid residues between both the domains, their 3D structure is almost the same, portraying the conservation of structure for carrying out the same functions at the cellular level.

Developmental functions of PRPs

The PRPs are key players in plant development from germination of seeds to morphogenesis of different organs and ultimately to cell death. Though there are many other factors inducing germination of seeds, PRPs have been identified as important regulators of germination in soybean (Hong *et al.* 1989), *Medicago truncatula* (Bouton *et al.* 2005), cotton (Qin *et al.* 2013), tobacco (Chen *et al.* 2014), rice (Mellacheruvu *et al.* 2016), and many more plants. During the course of germination, their expression is high in the apical region of hypocotyl (Hong *et al.* 1989) and embryo axis (Bouton *et al.* 2005). During the reproductive stage, PRPs are preferentially expressed in floral tissues (petals, pollen grains, pollen tubes, ovules, and zygotes) where they promote flower development and pollen tube growth (Gothandam *et al.* 2010, Qin *et al.* 2013, Chen *et al.* 2014). Under normal conditions, PRPs are constitutively expressed in roots and vegetative tissues (Bouton *et al.* 2005, Peng *et al.* 2015). The *PRPL1* gene of *Arabidopsis* is peculiarly expressed in root hairs and triggers their elongation (Boron *et al.* 2014). Involvement of PRPs in cell elongation is further supported by over-expression of the Hy*PRP* gene in tobacco BY-2 cell lines where calli size increases in suspension culture as well as on solid media (Dvorakova *et al.* 2012). The process of cell elongation may be due to loosening cell wall triggered by the hydrophobic 8-CM domain of PRPs. The hydrophobic 8CM domain of LTPs has been reported to interrupt hydrogen bonds between cellulose and hemicelluloses leading to non-hydrolytic loosening these cross-links (Nieuwland *et al.* 2005). Furthermore, a novel observation came out in Arabidopsis where a SICKLE protein (PRP) was found to be involved in microRNA biogenesis and degradation of spliced introns. SICKLE loss of function mutants exhibit pleiotropic developmental defects, such as reduction in plant height, delay in flowering, increase in serration at the leaf margin, and abnormal inflorescence phyllotaxy (Zhan *et al.* 2012). Recently, a hybrid PRP of *Glycine soja* (GsEARLI17) has been reported to influence cuticle formation as thicker cuticle asere observed in transgenic lines of *Arabidopsis* over-expressing this gene (Liu *et al.* 2015).

Puzzling expression of PRPs during abiotic stresses

Abiotic stresses modulate transcription of *PRP* genes remarkably. Some investigations support their down-regulation whereas other favour up-regulation in different plant species. Furthermore, a notable number of investigations demonstrated a temporal regulation of *PRP* genes depending on the period and intensity of abiotic stress. A microarray experiment performed in our laboratory showed a significant down-regulation of *PRP* gene expression in tomato under osmotic stress. Reverse transcription quantitative PCR analysis revealed down-regulation of an Sl*PRP* gene under drought stress in all tomato tissues: root (1314-fold), stem (11-fold), leaf (40-fold), and flower (2-fold) (Gujjar *et al.* 2014, 2018). Similar results were reported earlier in *Solanum tuberosum* (Menke *et al.* 2000) and in *Poncirus trifoliate* (Peng *et al.* 2015) where osmotic stress lead to gradual decrease in transcription of *PRP* genes.

On the other hand, contrasting observations favouring up-regulation of *PRP* genes at various abiotic stresses were reported in *Phaseolus vulgaris* (Battaglia *et al.* 2007), cotton (Qin *et al.* 2013), and *Glycine soja* (Liu *et al.* 2015). Temporal and spatial variations in expression of *PRP* genes were also reported in soybean (He *et al.* 2002) and *Poncirus trifoliate* (Peng *et al.* 2015). The expression of an *SbPRP* gene in soybean is specifically limited to leaf and epicotyle in response to multiple stresses. Upon exposure to salt stress, the *SbPRP* gene is up-regulated initially at 0.1 % NaCl reaching a maximum up-regulation at 0.4 % NaCl; then the expression decreases (He *et al.* 2002). No *SbPRP* transcription is detected at 2 % NaCl. It seems that expression of *PRP* genes is modulated under stress according to the requirement of a plant. In either way, PRPs project themselves as beneficial proteins under all types of environmental stresses. It has been confirmed by their over-expression and silencing in transgenic plants where over-expression of *PRP* genes undisputedly results in enhanced tolerance to multiple abiotic stresses (Barthakur *et al.* 2001, Gothandam *et al.* 2010, Priyanka *et al.* 2010, Liu *et al.* 2015, Mellacheruvu *et al.* 2016). Recently, promotor analysis of *PRP* genes in *Nicotiana tabaccum* and *Glycine soja* evidenced the presence of stress inducible elements, viz., W-boxes (WRKY-binding sites), a GATA box (the binding site for basic helix-loop-helix), and MYBs on it (Chen *et al.* 2014, Liu *et al.* 2015).

Proline rich proteins during biotic stress

Though implications of PRPs in biotic stress tolerance are limited to only few reports, their contribution has been well documented in providing tolerance against viral (He *et al.* 2002), bacterial (Li *et al.* 2014, Cecchini *et al.* 2015), and fungal (Mellacheruvu *et al.* 2016) pathogens. Treatments with salicylic acid and methyl jasmonate trigger the accumulation of PRPs in soybean (He *et al.* 2002) and *Arabidopsis* (Li *et al.* 2014). The soybean mosaic virus induces *SbPRP* expression during initial stages of inoculation, but its expression decreases thereafter (He *et al.* 2002). Treatments with virulent and avirulent strains also induce expression of a double hybrid PRP *AtDHyPRP1* in *Arabidopsis* (Li *et al.* 2014). Further, transgenic lines of *Arabidopsis* over-expressing the *AtDHyPRP1* gene exhibit an improved tolerance to bacterial suspension of virulent *Pst*DC3000 whereas silencing *AtDHyPRP1* by RNA interference confers susceptibility of plants to virulent *Pst*DC3000. (Li *et al.* 2014). Transgenic rice plants overexpressing a hybrid PRP from *Cajanus cajan* (CcHyPRP) exhibited enhanced resistance to a fungal pathogen *Magnaporthe grisea*, which causes blast disease (Mellacheruvu *et al.* 2016). However, research on *Capsicum annuum* and *Nicotiana benthamiana* showed PRPs as positive regulators of cell death. This is evidenced by pathogen induced cell death in *Nicotiana benthamian*a transgenic plants over-expressing an *HyPRP1* gene (Yeom *et al.* 2012). It was also suggested that cell death in these transgenic plants may be due to the down-regulation of reactive oxygen species scavenging genes and an enhanced pathogen susceptibility. Moreover, silencing the *HyPRP1* gene suppresses pathogen induced cell death and enhances disease resistance in tobacco signifying the role of *HyPRP1* as a negative regulator of basal defence against pathogens (Yeom *et al.* 2012).

Proline rich proteins and proline content during stress: is there any link?

Proline is an excellent osmolyte that protects subcellular structures and macromolecules under osmotic stress (Szabados and Savoure 2010, Meringer *et al.* 2016). Some of the core functions of proline during stress include reactive oxygen species scavenging activity during oxidative stress (Smirnoff and Cumbes 1989, Matysik *et al.* 2002), protection of nitrate reductase, ribonucleases, and proteases during heavy metal stress (Sharma and Dubey 2005, Mishra and Dubey 2006), and molecular chaperoning to protect integrity of proteins and enzymes during extreme temperatures (Rajendrakumar *et al.* 1994). After experiencing stress, oxidation of proline by proline dehydrogenase and P5C dehydrogenase provides electrons for mitochondrial respiratory chain and contributes to energy supply for resumed growth (Hare and Cress 1997, Kishor *et al.* 2005). Though proline has plenty of protective functions, its high content (40 - 50 mM) may inhibit growth and cell division in plants (Maggio *et al.* 2002, Hayat *et al*. 2012). Excess proline has been reported to inhibit seed germination (Hare *et al.* 2003), restrict growth of plants (Yamada *et al.* 2005), and arrest root growth (Kant *et al.* 2006). It may cause a feedback inhibition of pyrroline-5-carboxylate synthase (P5CS) which, in turn, blocks proline biosynthesis and affects the NADP+/NADPH ratio and redox balance in plastids leading to chlorophyll damage (Mani *et al.* 2002, Nanjo *et al.* 2003). Interestingly, transcript profiling in *Arabidopsis* revealed that about one third of rehydration-inducible genes are triggered by a high proline content (Oono *et al.* 2003). Thus, proline may be considered as an undisputed indicator of all types of stresses, and regulation of its content in plants is of an utter importance.

Till date, a well demonstrated process for proline accumulation in plants during stress is its synthesis through P5CS (Kishor *et al.* 1995, Perez-Arellano *et al.* 2010). Some results disapprove a hypothesis that proline accumulation is solely dependent on P5CS. The earliest evidence that an increase in free proline content during water stress is due to cell wall bounded protein degradation was given by Chen *et al.* (1964). The amount of free proline which accumulates during stress was quantified in the following years, and was found to be considerably higher than the content of bounded protein indicating de-novo synthesis of proline (Barnett and Naylor 1966, Thompson *et al.* 1966). In developing grapevine fruits, proline accumulation is independent and not associated with either an increase in amount of P5CS mRNA and proteins or a decrease in content of proline dehydrogenase (Stines *et al.* 1999). Furthermore, proline content does not correlate with a high amount of *P5CS* transcripts in roots of *A. thaliana* (Verbruggen and Hermans 2008). Alternatively, it was suggested that degradation of cell wall bound proteins and few PRPs may contribute to accumulation of proline during stress conditions (Chen *et al.* 1964, Barthakur *et al.* 2001).

Though the involvement of PRPs in various stresses has been well studied, their precise function in mitigating the stresses has not been investigated till date. However, increase or decrease in expressions of PRPs is largely advantageous to a plant in mitigating stress. Recently, we have found that transcription of a tomato *SlPRP* gene is reduced under drought stress in roots, stems, leaves, and flowers with the greatest reduction in roots. Simultaneously, proline content increases in all plant parts under drought stress with the highest increase in leaves (Gujjar *et al.* 2018). The interpretation may be that PRPs probably act as appendages to regulate cellular content of proline during and after the stress. Furthermore, it may be suggested that transcription of *PRP* genes during stress is sagaciously synchronized to equilibrate cellular proline content as per cellular requirement.

A suggested model for drought induced accumulation of proline

On exposure to drought stress, proline accumulates in all plant tissues to maintain the osmotic balance. Content of proline in cells may increase by its de-novo synthesis by P5CS (Verslues and Sharma 2010, Hayat *et al.* 2012) and degradation of cell wall bound PRPs (Chen *et al.* 1964, Barthakur *et al.* 2001). Besides, down-regulation of *PRP* genes under drought stress (Gujjar *et. al.* 2014, Peng *et al.* 2015, Gujjar *et al.* 2018) indirectly helps in building up a high cellular proline content to counteract drought (Fig. 1A). The earliest upshot of drought stress is perceived by root cells where a high amount of *P5CS* transcripts is noticed, and *de-novo* synthesis of proline sets in motion

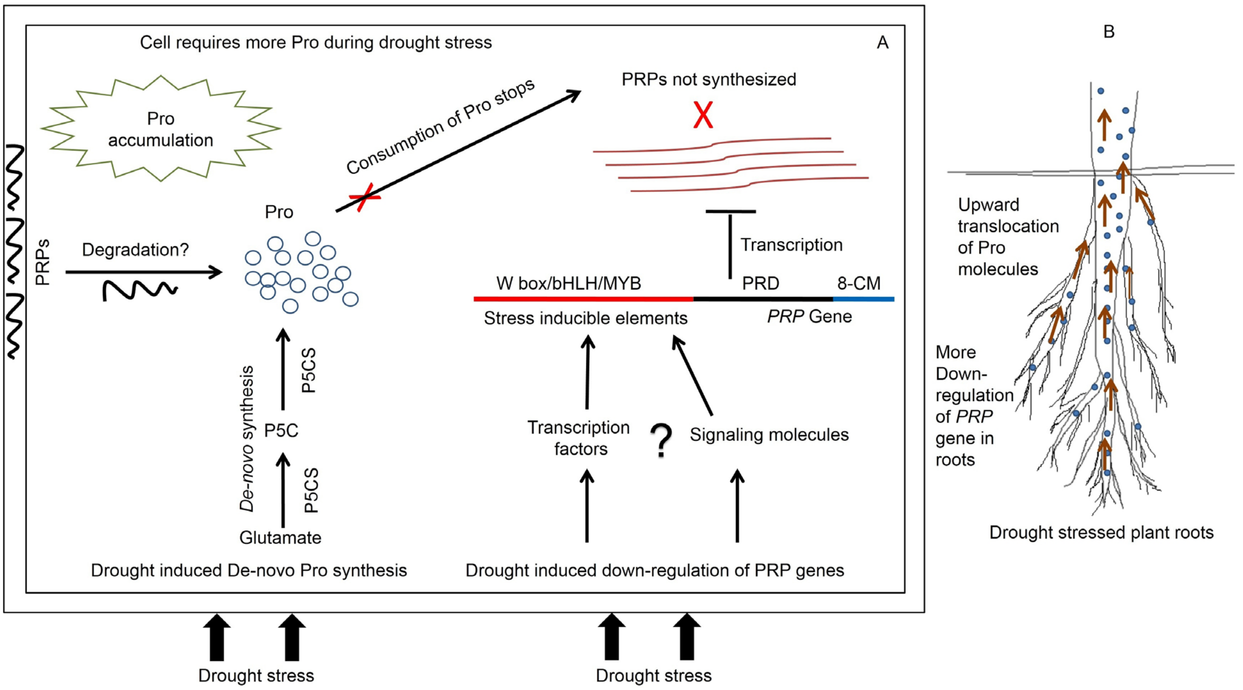

Fig. 1. *A* - Regulation of free cellular proline (Pro) content during drought stress in plants. Drought perception induces accumulation of Pro in plant cell by three ways: *1*) *de-novo* synthesis of Pro mediated through pyrroline-5-carboxylate synthase (P5CS) pathway whereby glutamate is converted into Pro; *2*) cell wall bound proline rich proteins (PRPs) are degraded to release free Pro inside the cell; *3*) transcription of a *PRP* gene is repressed or blocked *via* some unknown signalling cascade. This results in a decreased synthesis of PRPs so as to avoid consumption of free cellular Pro. *B* - Redistribution of proline from roots to shoots. Proline accumulated in roots during drought stress is translocated upwards through xylem to aerial plant parts resulting in a lesser content of Pro in roots (redrawn from Verbruggen and Hermans 2008).

instantly. Moreover, the highest down-regulation of a *PRP* gene in roots of tomato (Gujjar *et al.* 2018) also supports an enhanced synthesis of proline in roots. Interestingly, proline content of roots is relatively low and does not match with a high transcription of a P5CS gene in *Arabidopsis* roots (Hua *et al.* 1997, Verbruggen and Hermans 2008). This ambiguity may be attributed to the fact that proline is translocated upwards from roots to shoots (Fig. 1B) through xylem (Verbruggen and Hermans 2008). Besides this long distance translocation, intracellular as well as intercellular transport of proline is also evident during drought stress; it is carried out by specific proline transporters (Rentsch *et al.* 1996, Schwacke *et al.* 1999).

Concluding remarks and future perspectives

The PRPs have been studied under different environmental stresses where their expression is either increased, decreased, or transiently changed. Contemporary investigations suggest that the promoter region of *PRP* genes has drought inducible elements, and these cis-acting elements serve as binding sites for WRKY transcription factors, basic helix-loop-helix proteins and MYBs (Chen *et al.* 2014, Liu *et al.* 2015). This finding may fascinate the curiosity of researchers in future to investigate more about these proteins. It is obvious from literature that stress induces de novo synthesis of proline (the reaction catalysed by P5CS). Besides natural synthesis, two additional pathways may be suggested for proline accumulation in cells during exposure to abiotic stresses: 1) degradation of PRPs during osmotic stress to liberate free proline (Chen *et al.* 1964, Barthakur *et al.* 2001), and 2) down-regulation of *PRP* genes in plants at specific stages of drought stress (Peng *et al.* 2015, Gujjar *et al.* 2018). It may be proposed that transcription of *PRP* genes is discontinued or repressed by some unknown regulatory mechanism to substantiate the need of free proline in the cell during stress. However, the mechanisms underlying both of the above suggested events are yet to be discovered. Plenty of confusing questions are still left to be unravelled in the future, *i.e.*: Are PRPs degraded to liberate free proline or is their transcription blocked to substantiate a high content of free proline inside the cell during stress? After stress when cells do not require high content of proline, does transcription of *PRP* genes resume to normalize free proline content inside the cells? When proline content reaches toxic levels at any time during stress, does transcription of *PRP* genes increase to consume free proline residues from cytosol? The future research should focus to unravel missing links between accumulation/depletion of proline and PRPs during and after stress.

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