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Redox regulation of peroxiredoxin and proteinases by ascorbate and thiols during pea root nodule senescence

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Abstract **Redox factors contributing to nodule senescence were studied in pea. The abundance of the nodule cytosolic peroxiredoxin but not the mitochondrial peroxiredoxin protein was modulated by ascorbate. In contrast to redox-active antioxidants such as ascorbate and cytosolic peroxiredoxin that decreased during nodule development, maximal extractable nodule proteinase activity increased progressively as the nodules aged. Cathepsin-like activities were constant throughout development but serine and cysteine proteinase activities increased during senescence. Senescence-induced cysteine proteinase activity was inhibited by cysteine, dithiotreitol, or E-64. Senescence-dependent decreases in redox-active factors, particularly ascorbate and peroxiredoxin favour decreased redox-mediated inactivation of cysteine proteinases.**

Keywords: Senescence; Proteinase; Ascorbate; Peroxiredoxin; Redox signalling

1. Introduction

Nodule senescence, like leaf senescence [1] is a highly organised process, orchestrated in an age-dependent manner [2]. However, nodule longevity is also determined by environmental factors. High nitrate availability or stressful environmental conditions such as high temperatures, acid soil conditions, root pathogen infection or drought induce premature senescence [3–6]. Characteristic features of nodule senescence are loss of leghemoglobin and N_2 fixation and increased lytic activities [7–9]. The nodule cytosolic proteins, especially leghemoglobin, are considered to be early targets of senescence-induced proteinases [10–12].

Cysteine proteinases are involved in nearly every stage of plant development from the degradation of seed storage proteins to fruit ripening as well as in legume nodule development. They are important in stress responses as well as in programmed cell death. Some cysteine proteinases have specific characteristics such as a C-terminal KDEL motif, which is an endoplasmic reticulum retention signal for soluble proteins. In pea, transcripts encoding a cysteine proteinase denoted as *PsCyp15a*, accumulated first in wilting leaves [13,14] and then in nodules [15,16]. A second cysteine proteinase denoted as *PsCyp1* was reported to be more highly expressed in nodules than in shoots [15]. A nodule-specific and senescence-related cysteine proteinase belonging to the legumain family has been identified in soybean [17]. Three groups of cysteine proteinases were expressed in the senescing zone of white clover nodules [18].

Micro- and macroarray studies on mature *Medicago truncatula* nodules have indicated a complex picture of up- and down-regulated genes from the same proteinase families. In particular, the proteasome subunit β type and the ubiquitin-conjugating enzyme E2 were up-regulated while a cysteine proteinase (26S protease regulatory subunit 4) was down-regulated [19]. An increasing body of evidence suggests that the activity and function of the ubiquitin-dependent 26S proteasome protein degradation pathway is controlled by cellular redox status of the cell. A high cellular redox status, as defined for example by high GSH/GSSG ratios, impedes the binding of target proteins [20–23]. Nodule senescence is characterised by decreased levels of ascorbate and glutathione [2,6,24–27], a condition that would tend to favour the activation of the 26S proteasome protein and enhance protein degradation.

Peroxiredoxins constitute small gene families in all organisms including cyanobacteria and plants [28,29]. They have recently been identified as important components of the plant antioxidant defence network. They detoxify alkyl hydro peroxides, hydrogen peroxide and peroxinitrite. In addition to their role in antioxidant defence they appear to control or modulate redox dependent signalling. In *Arabidopsis thaliana*, four peroxiredoxins are targeted to the chloroplast (2-Cys Prx A and B, Prx Q and Prx II E), one to the mitochondrion (Prx II F), one to the nucleus (1-Cys Prx) and four remain in the cytosol (Prx II A–D). Plants lacking mitochondrial peroxiredoxin

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Abbreviations: Asc, ascorbate; DTT, dithiothreitol; Prx, peroxiredoxin; PMSF, phenylmethylsulphonylfluoride; E-64, L-*trans*-epoxysuccinyl-laucylamido(-guanidino) butane

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(Prx) II F have altered gene expression for example of antioxidant defence genes, and root growth is strongly inhibited under stress [30]. The role of the cytosolic peroxiredoxins remains uncharacterised, but expression analysis suggests that these isoforms function during optimal conditions as well as under stress [31,32]. To date, there have been no reports of nodule peroxiredoxins and their role in nodule development and function remains to be elucidated.

We have previously reported that the activities of many of the pea nodule antioxidant enzymes as well as the GSH/GSSG ratio are not decreased greatly at the time of nodule senescence [27]. Hence, here we have concentrated in the present experiments, on key nodule antioxidants particularly ascorbate, which show marked changes consistent with senescence. Since peroxiredoxins are essential contributors to the redox homeostasis of the plant cell, the occurrence of peroxiredoxins and their abundance was studied during nodule development. The following experiments were therefore undertaken to determine the extent to which redox factors influence pea root nodule senescence. We analysed nodule proteinase activities and antioxidants, particularly ascorbate and cytosolic peroxiredoxin. We show that pea redox-sensitive proteinases are inhibited by reductant and that the abundance of cytosolic peroxiredoxin is modulated by ascorbate. These results point towards the importance of redox factors in the control of nodule senescence.

2. Materials and methods

2.1. Plant material

Pea seeds (*Pisum sativum* cv. Phönix) were inoculated with a commercial *Rhizobium* strain (HiStick pea inoculants, Becker Underwood Ltd., USA). Plants were grown on vermiculite in controlled environment chambers with a 25 °C/19 °C, 70%/85% humidity day/night regime, under a 14-h photoperiod (600 μmol m^{-2} s^{-1}). Plants were supplied daily with nitrogen-free nutrient solution [33].

2.2. Chlorophyll content, total soluble protein and nitrogenase activity measurements

Chlorophyll was determined in leaf extracts prepared in 80% acetone according to [34]. For estimations of protein and nitrogenase activity tissues were homogenised in 50 mM Tris buffer (pH 8.0) and centrifuged for 15 min at 17000 × *g*. The total protein content of the supernatant was estimated with BioRad protein microassay (Bio-Rad, Richmond, CA, USA). Nitrogenase activity was assayed by the acetylene reduction method [35] using excised nodules as described by Groten et al. [27].

2.3. Nodule proteinase activities

Nodule proteinase activity was assayed by a standard fluorimetric microassay method [36–38] using specific substrates for cathepsin L (N-CB2-phe-arg-MCA, Sigma–Aldrich, UK), cathepsin B (Nα-CB2-arg-arg-MCA, Sigma–Aldrich, UK) and cathepsin H (L-arg-MCA, Sigma–Aldrich, UK). Azocaseinase activities were measured as described previously [9,39,40]. In-gel detection of proteinase activity was performed according to the method of Michaud et al. [41]. Proteins (5 μg) were separated by mildly denaturing gelatine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). In order to discriminate between cysteine and serine proteinases, protein extracts were pre-incubated for 15 min at 37 °C with 100 μM L-*trans*-epoxysuccinyl-laucylamido(-guanidino) butane (E-64, Sigma–Aldrich) and 1 mM phenylmethylsulphonylfluoride (PMSF, Sigma–Aldrich), class-specific inhibitors of cysteine and serine proteinases respectively. For activation of cysteine proteinases, cysteine (5 mM) and dithiothreitol (DTT, 1 mM), respectively, were added together with the inhibitors.

2.4. Immunoblot analysis of nodule peroxiredoxins

Extracted proteins (50 μg) were denatured with reducing SDS sample buffer, boiled for 10 min and separated by SDS–PAGE. SDS–PAGE and immunoblotting was performed as described previously [32]. Antisera directed against the recombinant *Arabidopsis* type II peroxiredoxins C and F were each used in a 1:10000 dilution.

2.5. Total ascorbate content and ascorbate feeding experiments

Three and 9-week-old nodules were vacuum-infiltrated (30 mbar) for 8 h at room temperature with 10 mM sodium phosphate buffer (pH 7.8) in the presence or absence of 20 mM ascorbate. Samples were then incubated in 10 mM sodium phosphate buffer (pH 7.8) and removed after 8 and 24 h for analysis of peroxiredoxin protein abundance. Total ascorbate and reduced ascorbate contents were measured in neutralised root and nodule extracts at the indicated time-points as described previously [42] by measuring the decrease in absorbance at 265 nm before and after the addition of ascorbate oxidase. For the quantification of total ascorbate, dehydroascorbate was reduced back to ascorbate using DTT. Samples were incubated with 1 mM DTT in the dark for 30 min prior to measurement.

3. Results

3.1. Senescence characteristics

Pea leaf chlorophyll contents (Fig. 1A) declined sharply 9 weeks after sowing. The plants started to flower at 6 weeks after sowing while both leaf and nodule total soluble protein content (Fig. 1B and C) decreased after 9 weeks. Nitrogenase activity declined gradually from 3 weeks after sowing but still maintained high levels in nodules up to an age of 9 weeks (Fig. 1D).

3.2. Proteinase activities increase during nodule senescence

Total proteinase activities, determined either by spectrophotometric assays (Fig. 2A) or by in-gel proteinase activity assays using gelatine as a substrate (Fig. 2B), increased during nodule senescence. Proteinase activities increased in nodules 5–7 weeks after sowing but highest activities were observed in senescent nodules. The in-gel-proteinase assay revealed two major activity bands at approximately 45 kDa and slightly more than 160 kDa. The assay was performed at pH 8 and pH 5. The activity pattern was fairly similar under both conditions (data not shown).

Class-specific proteinase inhibitors were used to discriminate between the types of proteinases induced during nodule senescence. PMSF, an inhibitor of serine proteinases, inhibited the activity of the high molecular weight isoform (Fig. 3A) but not that of the 45 kDa isoform. The activity of the 45 kDa isoform declined following the addition of E-64, an inhibitor of cysteine proteinases (excluding legumain-type proteinases). It is interesting to note that there is also a cysteine proteinase activity that appears early in nodule development with activity at pH 8 (Fig. 3A), suggesting that this is a cytoplasmic enzyme form.

The assays with E-64 routinely contained 1 mM DTT or 5 mM cysteine because cysteine proteinases are generally considered to be activated under reducing conditions. We therefore measured nodule proteinase activities in the presence or absence of DTT and with or without E-64. Surprisingly, DTT strongly decreased proteinase activity in 9-week-old nodules (Fig. 3B). Moreover, nodule cysteine proteinase activities were completely inhibited by the simultaneous application of DTT and E-64 (Fig. 3B).