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Xanthine Oxidoreductase (XOR) in Pea (*Pisum sativum* L.) Leaves Under Abiotic Stress Conditions †

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Abstract: Plants are exposed to continuous environmental challenges and depending on the intensity and time of exposition to a specific environmental condition, plants either overcome the adverse situation or undergo a different level of damages such as a nitro-oxidative stress. Xanthine oxidoreductase (XOR) is an FAD-, molybdenum-, iron- and sulfur-containing hydroxylase enzyme involved in the purine catabolism pathway that catalyzes the conversion of hypoxanthine/xanthine to uric acid with the concomitant formation of either NADH or superoxide radical ($O_2^{\bullet-}$) which is then dismutated into H_2O_2. In fact, under oxidative stress conditions, XOR is considered to participate in the generation of reactive oxygen species (ROS). Using pea (*Pisum sativum* L.) plants exposed to six different environmental conditions including high light intensity, low and high temperature, continuous light, continuous dark and mechanical wounding, XOR was analyzed at protein and gene expression levels. The obtained data suggest that XOR is modulated differentially under the assayed stress conditions being the low temperature the situations with causes the highest differences of enzyme activity and gene expression.

Keywords: abiotic stress; enzyme activity; gene expression; low temperature; reactive oxygen species

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1. Introduction

Xanthine oxidoreductase (XOR) is an enzyme involved in the purine catabolism pathway that catalyzes the conversion of hypoxanthine and xanthine to uric acid which the concomitant formation of either NADH or superoxide radical ($O_2^{\bullet-}$). It plays an important role in nucleic acid degradation in all organisms being considered also a source of nitrogen in higher plants [1,2]. However, the involvement of XOR activity has been associated with other processes in higher plants such as nodule metabolism in legumes [3], leaf senescence [4,5], fruit development [6], as well as in the mechanism of plant response to pathogen microorganisms [7–9].

In previous studies, we showed that XOR activity was affected in pea plants under salinity and cadmium stress [10,11]. Consequently, using pea plants as a model, the present study has the goal to explore how the XOR activity, as well as protein and gene expression, is modulated by other stressful conditions including high light intensity, low and high temperature, continuous light, continuous dark and mechanical wounding. Taken together, the data indicate that among these assayed conditions, the low temperature was the environmental conditions that modulate positively the pea XOR activity and gene expression.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Pea (*Pisum sativum* L., cv. Lincoln) seeds were obtained from Royal Sluis (Enkhuizen, Holland). Seeds were surface sterilized with 3% (*v*/*v*) commercial bleaching solution for 3 min, and then washed with distilled water, and germinated in vermiculite for 3–4 days under growth chamber conditions (16 h light, 24 °C/8 h dark, 18 °C under a light intensity of 190 μE m^{-2} s^{-1}; 80% relative humidity). Healthy and vigorous seedlings of 3 weeks were selected and exposed to different stress conditions as previously described [12]. Briefly, these conditions were: (i). High temperature (HT): plants were sequentially exposed to 30 °C for 1 h, 35 °C for 1 h and finally, 38 °C for 4 h. (ii). Low temperature (LT): plants were exposed for 48 h at 8 °C; (iii). High light intensity (HL): pea plants were irradiated for 4 h at 1189 μE s^{-1} m^{-2}, using a lamp GE 300 W-230 V PAR 56/WFL (General Electric); (iv). Continuous light (CL): plants were continuously illuminated for 48 h at 190 μE s^{-1} m^{-2}; (v). Darkness (D): pea plants were kept in darkness in a growth chamber for 48 h; and (vi). Mechanical wounding (W): pea leaves were injured *in planta* by clicking them with a striped-tip forceps, and after 4 h damaged leaves were collected and analyzed [13,14]. In all cases, the control pea plants were kept in the growth chamber under optimal conditions being processed at the same time that plants subjected to the different stress conditions.

2.2. Crude Extracts of Pea Leaves

Pea leaf homogenates were prepared in 50 mM Tris-HCl buffer, pH 7.5 containing 0.1 mM EDTA-Na_2, 0.2% (*v*/*v*) Triton X-100, 1 mM $MgCl_2$, glycerol 10% (*v*/*v*) and 2 mM 1,4-dithiothreitol (DTT), homogenizing the sample with nitrogen liquid in mortar, and using a weight/volume ratio of 1: 4. Subsequently they were centrifuged at 27,000× *g* for 30 min at 4 °C. The supernatants obtained were used for further analysis. Protein concentration was determined with the Bio-Rad Protein Assay (Hercules, CA, USA), using bovine serum albumin as standard.

2.3. In-Gel XOR Activity Assay and Immunoblot Analyses

Non-denaturing PAGE was performed on 6% acrylamide gels according to [15]. Samples for electrophoresis were prepared in 10% (*v*/*v*) glycerol and gels were run at a constant current of 10 mA/gel. XOR activity was visualized by incubating the gels in a solution consisting of 50mM Tris–HCl, pH 7.6, 0.50 mM xanthine, 0.5 mM, NAD^+, 0.25 mM NBT, 30 mM TEMED [11,16]. The appearance of the blue formazan bands over a colorless background was monitored and the reaction was stopped by immersing the gels in 7% (*v*/*v*) acetic acid.

For immunoblot analyses under non-denaturing conditions, after PAGE protein samples were transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA, USA) using a semi-dry transfer system (Bio-Rad Laboratories) with 10 mM CAPS buffer, 10% (*v*/*v*) methanol, pH 11.0, at 1.5 mA · cm^2 for 2 h. For immunodetection of XOR, a polyclonal antibody against rat liver xanthine oxidase (XOD; [17], diluted 1/500, was used. As the secondary antibody, a goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad), diluted 1/10,000, was used. To detect the immunoreactive bands an enhanced chemiluminescence method with luminol was used [18].

2.4. RNA Isolation and Cloning of a Partial cDNAs of Pea Xanthine Oxidoreductase

Total RNA was isolated from pea leaves with the Trizol Reagent (GibcoBRL, Paisley, UK) as described in the manufacturer's manual, and RNA was quantified spectrophotometrically. Two micrograms of total RNA were used as a template for the reverse transcriptase (RT) reaction. It was added to a mixture containing 1.5 mM dNTPs, 1.6 μg polydT23 primer, 1 μL RT-Buffer (25 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl and 2 mM DTT), 0.9 U RNasin ribonuclease inhibitor and 20 U avian myeloblastosis virus RT

(FINNZYMES, Espoo, Finland). The reaction was carried out at 42 °C for 40 min, followed by a 5-min step at 98 °C and then by cooling to 4 °C for 10 min. Then, the polymerase chain reaction (PCR) was carried out as follows: 1 mL of each cDNA was added to 250 μM dNTPs, 1.5 mM $MgCl_2$, 1x PCR buffer, 2.5 U of Ampli Taq Gold (Roche, Mannheim, Germany) and 0.5 mM of specific oligonucleotide designated as FXOR (5′-CTAATTTTCCTAGCAACACTGC-3′) and RXOR (5′-CAATCCTGACCCATCTCC -3′) in a final volume of 20 μL.

Reactions were carried out in a Hybaid thermocycler (Ashford, UK). A first step of 2 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 65 °C, with a final extension of 10 min at 65 °C. Amplified PCR products were detected after electrophoresis in 1% (*w*/*v*) agarose gels stained with ethidium bromide, and the visualized band was cut and extracted from the gel (Qiaex II gel extraction kit; Qiagen, Madrid, Spain). The purified fragments were cloned into the pGEM-T easy vector (Promega, Madrid, Spain). A partial cDNA of pea XOR with 418 bp was obtained and confirmed by sequencing.

2.5. Northern Blot Analysis

Total RNA from leaves of control and treated pea plants were extracted with Trizol reagent (Gibco BRL, Life Technologies) according to the manufacturer and stored at -80 °C. RNA (10 μg) was prepared in MEN buffer, 33% (*v*/*v*) formamide and 10% (*v*/*v*) formaldehyde and denatured at 65 °C. Agarose gels at 1.5% (*w*/*v*) were prepared in DEPC-treated water containing 1.6% (*w*/v) formaldehyde and MEN buffer (20 mM Mops, pH 7.0, 5 mM sodium acetate, 2 mM EDTA) [19]. After electrophoresis, RNA was capillary transferred overnight onto a nylon membrane (Zeta-Probe, Bio-Rad). Then, the membrane was briefly rinsed with 2x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and the RNA was attached to the membrane with a vacuum pump for 30 min at 80 °C. The membrane was stained with a 0.04% (*w*/*v*) methylene blue solution prepared in 0.3 M sodium acetate, pH 5.6. Simultaneously, the gel was stained with ethidium bromide 0.025% (*w*/*v*) for 15 min to check the efficiency of the transfer.

The probe used for mRNA detection was a 180 bp fragment of the XOR obtained from cDNA by PCR with the oligonucleotides FXOR2 (5′-CACCTGCCTGGTTCATAA GC-3′) and RXOR2 (5′-TTGAGCACTGCCCTCTATCC-3′). The probe was labelled using the commercial Rediprime™ II method (Amersham Biosciences). 25 ng of the probe was prepared in 45 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), which was denatured at 95 °C for 5 min and subsequently kept on ice for another 5 min. This mixture was added to the reaction tube supplied by the commercial company (which contains the enzyme Klenow polymerase and all deoxynucleotides except cytosine) together with 5 μL of [^{32}P] dCTP (50 μCi, 3,000 Ci/mmol, Amersham), and was incubated for 1 h at 37 °C. The reaction was stopped by adding 5 μl 0.2M EDTA. Deoxyribonucleotides not incorporated into the probe were removed using Boehringer gel filtration columns (Mini Quick Spin Columns). The probe, once labelled, was renatured at 95 °C for 5 min, and then placed on ice until added to the hybridization buffer. Filters were auto-radiographed using hyperfilm (Hyperfilm MP, Amersham Pharmacia Biotech) with an intensifying screen (from the same company) at −80 °C. Signal intensities were estimated from autoradiograms using a Shimadzu CS-9000 densitometer.

3. Results and Discussions

Xanthine oxidoreductase (XOR) is a complex enzyme constituted by two main subunits which contain one molybdenum atom, one FAD group and two Fe_2S_2 centers. The enzyme is present in all organisms and it could appear in two forms: xanthine oxidase (XOD) and xanthine dehydrogenase (XDH) [20–23]. In plants, it has been considered that the predominant form is the XDH [24,25] which was mainly associated to root nodule metabolism [26,27] (Triplett, 1985; Triplett et al., 1986); however, there is evidence that

both forms of XOR are present in plants and can be involved in processes of stress conditions such as salinity [10] or heavy metal [11].

Besides the relevance of the XOD in the generation of $O_2^{\bullet -}$, both forms (XOD/XDH) produce uric acid which is an efficient peroxynitrite ($ONOO^-$) scavenger [28,29]. This peculiarity allows connecting XOR with the metabolism of reactive oxygen and nitrogen species (ROS/RNS) in the same reaction. Based on a previous study where we analyzed the metabolism of RNS in pea plants under different stress conditions, the main goal of the present study was to evaluate how the XOR is modulated in the same model plants and the same experimental conditions.

Figure 1 shows the in-gel XOR activity assay (panel a) and immunoblot analyses of leaf extracts from pea plants subjected to different abiotic stress conditions described in Section 2.1. The intensity of XOR activity band was higher under low temperature (LT) in comparison to the control plants (C); however, under other assayed conditions the intensity of the band activity was similar or even lower than the control. Additionally, the intensity of the immunoreactive band in the assayed conditions was very similar to that observed in the control plants.

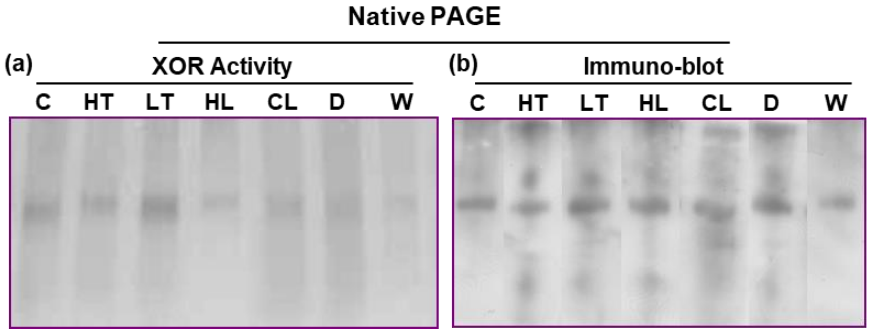

Figure 1. Xanthine oxidoreductase (XOR) in leaf extracts from pea plants subjected to different abiotic stress conditions. (**a**) Native PAGE (6% acrylamide gels) and in-gel staining of xanthine oxidoreductase activity. (**b**) Immuno-blot probed with an antibody against rat liver XOR (dilution 1:500). 80 μg of protein were loaded per lane. C, control; HT, high temperature (38 °C); LT, low temperature (8 °C); HL, high light intensity; CL, continuous light; D, continuous dark; W, wounding. Results are representative from at least three different experiments.

Likewise, Figure 2 depicts the mRNA-XOR content analyzed by Northern blotting using as a probe of 180 bp pair fragment obtained from cDNA (see Section 2.4). Again, a clear increase of mRNA-XOR levels was detected in the leaves of pea plants exposed to low temperature (8 °C for 48 h) as well as the leaves under mechanical wounding and, to a lesser extent, in those subjected to continuous darkness for 48 h. The levels of mRNA-XOD in the leaves treated with high light intensity and continuous light decreased in comparison to the levels in the control leaves. The increase of XOR activity and gene expression has been described in other plant species. For example, in tomato plants under drought stress [30], being this increase associated with a rise of ROS production throughout its NADH oxidase activity [31], but also in Arabidopsis where the XDH activity as part of the purine metabolism seems to play a role in the drought stress acclimatation [32].

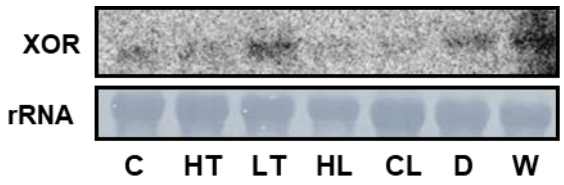

Figure 2. Northern blot analysis of XOR in in leaf extracts from pea plants subjected to different abiotic stress conditions. C, control; HT, high temperature (38 °C); LT, low temperature (8 °C);

HL, high light intensity; CL, continuous light; D, continuous dark; W, wounding. Results are representative from at least three different experiments.

4. Conclusions

The obtained data indicate that pea XOR is modulated differentially under the six assayed stress conditions being the low temperature the situations with causes the highest differences of XOR activity and gene expression in comparison to untreated pea plants. These data are in good agreement with those data reported previously on the metabolism of RNS in pea plants under the same experimental conditions [12] where the content of *S*-nitrosothiols and protein tyrosine nitration content, as well as S-nitrosoglutathione reductase and L-arginine-dependent NOS-like activities, were higher under low-temperature stress.

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