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DETERMINATION OF THE MULTIPLE ISOFORMS OF SOME ANTIOXIDANT ENZYMES IN *HABERLEA RHODOPENSIS*

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ABSTRACT. The multiple forms of several antioxidant enzymes were identified in leaves of the resurrection plant *H. rhodopensis*. Native PAGE showed the presence of six multiple Superoxide dismutase isoforms in the protein extract from fresh leaves, and the differential visualization revealed that four of them belonged to Cu,Zn-SOD isoforms, one belonged to Mn-SOD and one - to Fe-SOD. The same method showed one form of nonspecific guaiacol Peroxidase and two multiple isoforms of Ascorbate peroxidase.

INTRODUCTION

Water deficit in plants disrupts many cellular and organism functions, having a negative impact on plant growth and reproduction. In nature, a small group of angiosperms, so-called resurrection plants, have developed a variety of strategies for surviving the most severe form of water stress, air-dryness (Bartels et al., 1990; Ingram and Bartels, 1996). Their fully differentiated tissues possess the unique ability to maintain biological activity at very low levels (anabiosis) on prolonged dehydration and resume full activity (biosis) on rehydration.

The Balkan endemic plants Hab*erlea rhodopensis* Friv. and *Ramonda serbica* Panc. (Gesneriaceae) are tertiary paleophytic relicts that withstand a complete desiccation during the vegetative growth. The limitation of photosynthesis on prolonged desiccation (Markovska et al., 1994) could also bring about unbalancing between electron transport and CO_2 fixation rates. The enhanced electron leakage to oxygen, in turn, causes increased production of AOS that are considered as the ultimate consequence of many environmental stresses (Elstner, 1982; Smirnoff, 1993). A mechanism sensing the AOS initiates defensive responses on molecular, metabolic, physiological and developmental levels. The distinction between desiccation tolerance and intolerance has been suggested to be a function of the plant's ability to process AOS by maintaining defensive mechanism or amplifying them during desiccation and rehydration (Navari-Izzo and Rascio, 1999).

With this study we aimed to determine in *H. rhodopensis* the multiple isoforms of Superoxide dismutase (SOD), which constitutes the first line of defense against superoxide radicals, Peroxidase (PO) that scavenges the next downstream AOS, the hydrogen peroxide, and Ascorbate peroxidase (APX), which uses ascorbate as a hydrogen donor for detoxification of the hydrogen peroxide.

MATERIAL AND METHODS

Plant material

Plants of *Haberlea rhodopensis* Friv. were collected from their locality on shady rocks with northern exposure in the Rhodopa Mountain. The plants were adapted for two weeks in a greenhouse at a temperature 28-32ºC with a photoperiod of approximately 14 h of natural irradiation.

Enzyme assays

Leaf material (1g) was ground in liquid nitrogen to powder and the total proteins were extracted with 50 mM K-phosphate buffer (1 mM EDTA, pH 7.8, 0.1 % Triton X-100, 1 mM PMSF, 2 % PVPP). The homogenate was centrifuged at 12 000 x g for 30 min and the obtained supernatant was used for parallel tests of protein quantity and enzyme activity. The protein content was measured using Coomassie G-250 dye-binding assay (Bio-Rad) with bovine serum albumin as a standard. Proteins (20 µg per sample) were separated on a 10% non-denaturing polyacrylamide gel electrophoresis (Laemmli, 1970). The gels were run at 4°C (80 V).

SOD activity staining was done using nitroblue tetrazolium according to Beauchamp and Fridovich (1971). To determine the enzyme isoforms, 5 mM KCN was applied for inhibition of Cu,Zn-SOD while 0.4% H_2O_2 was used for simultaneous inhibition of both forms Cu,Zn-SOD and Fe-SOD (Sandalio et al., 1987).

PO activity staining was done according to Seevers et al. (1971) using a solution containing 1.3 mM each of diamino-benzidine hydrochloride and H_2O_2. Ax-PO activity staining was done by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium (Mittler and Zilinskas 1993).

RESULTS AND DISCUSSION

The identification of multiple isoforms of SOD, PO and AXP in *H. rhodopensis* leaves was performed by means of native PAGE on 10% gels. The elctrophoretic patterns of the enzymes in *Haberlea* were compared to those of *N. tabacum* since the antioxidant enzymes of the latter have been well characterized in biochemical and molecular aspects (Bowler et al., 1989; Van Camp et al., 1990; Slooten et al., 1995). This comparison is applicable only at the particular conditions of experiment such as plant physiological state and developmental stage.

SOD assays performed directly on a non-denaturing PAGE with pH 8.8 revealed six major bands of SOD activity in the protein sample extracted from fresh leaves of *Haberlea* while the protein extract of tobacco leaves, used on the same gel as a control, showed the expected three SOD isoforms (Fig 1). When the separation of protein extract was done on PAGE of pH 7.8, some of the upper bands became

stronger. Inhibition study with H_2O_2 and KCN demonstrated that one of the bands belonged to Mn-SOD (resistant to H_2O_2 and KCN), one to Fe-SOD (resistant to KCN and sensitive to H_2O_2) and four to Cu,Zn-SOD (sensitive to H_2O_2 and KCN).

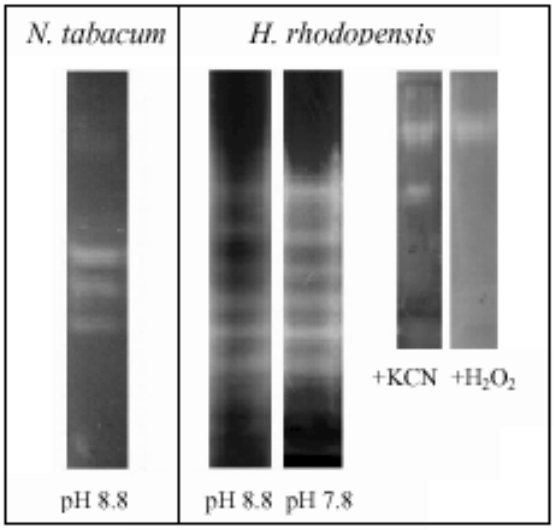

Fig. 1. *Non-denaturing PAGE of SOD isoforms of fresh leaves of H. rhodopensis collected at flowering stage. Protein extract of N. tabacum leaves was used as a control.*

The visualization of upper SOD bands was improved by performing electrophoresis at pH 7.8. Previously, Battistoni and Rotilio (1995) did protein separation at pH 7.8 for stabilizing bacterial Cu,Zn-SOD activity. Our result might indicate structural or functional similarities between some of bacterial SOD isoforms and those of *H. rhodopensis*, which is not unlikely since the latter species is a tertiary paleophytic relict with a limited area of spreading.

Depending on their physiological functions, PO can be separated in two main classes: PO, whose oxidized products carry out physiological function, and PO, which take part in hydrogen peroxide scavenging. Amino acid composition of PO shows that most of PO are acid proteins. The multiple isoforms of PO in *Haberlea* leaves were separated on 10% native gel with pH 8.8 – conditions that allow nonspecific guaiacol PO to be determined. Compared to the two multiple isoforms of PO in tobacco leaves, PAGE revealed in *Haberlea* leaves only one guaiacol isoform of PO of very low electrophoretic mobility (Fig. 2a). AXP is characterized by high substrate specificity and requires ascorbate in the extraction media and separating buffer. AXP activity staining of gels of *Haberlea* total soluble protein (Fig. 2b) identified two multiple isoforms of electrophoretic mobility higher than that of tobacco APX isoform.

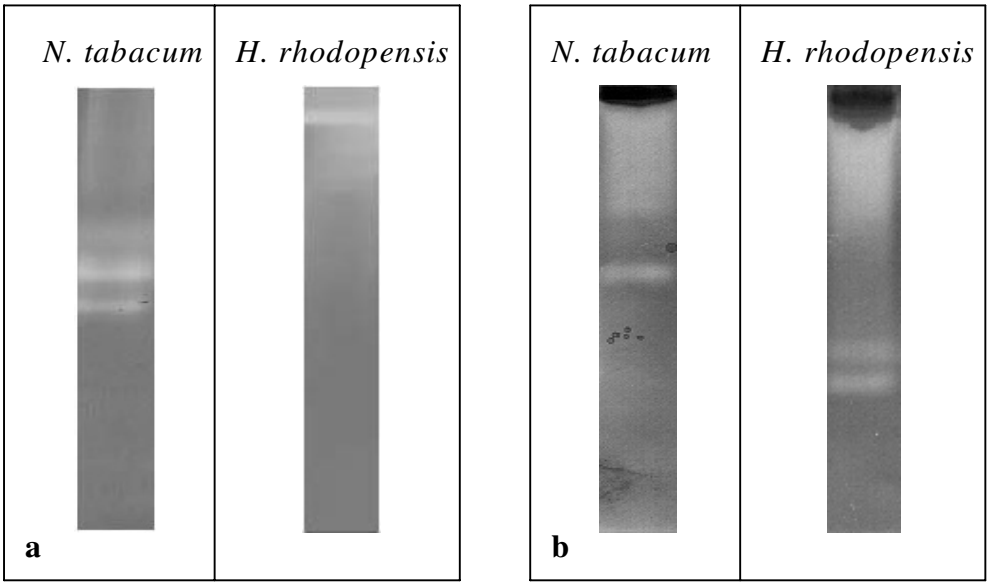

Fig. 2. *Non-denaturing PAGE of PO (2a) and APX (2b) isoforms of fresh leaves of H. rhodopensis collected at flowering stage. Protein extract of N. tabacum leaves was used as a control.*

In conclusion, the Balkan endemic and tertiary relict H. rhodopensis possesses all the three multiple isoforms of SOD as well as two isoforms of AXP which have most probably contributed for its survival of the fittest and the development of high drought resistance. The molecular analysis of the SOD multiple isoforms of H. rhodopensis is a subject of one of the ongoing projects of our research group.

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