BIOCHEMICAL AND MOLECULAR CHANGES IN BUCKWHEAT LEAVES DURING EXPOSURE TO SALT STRESS

Ž. S. JOVANOVIĆ1*, VESNA R. MAKSIMOVIĆ1 and SVETLANA R. RADOVIĆ2

1 University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, 11010 Belgrade, Serbia
2 University of Belgrade, Faculty of Biology, 11000 Belgrade, Serbia

Abstract - In spite of the great nutritive and pharmacological potentials of buckwheat, data about the abiotic stress tolerance of this plant species are very limited. The aim of this work was to analyze the biochemical and molecular response of buckwheat plants in the middle vegetative phase against short- and long-term salt stress. Changes in relative water content, level of lipid peroxidation, content and localization of H2O2 as well as changes in antioxidative enzyme activity and expression of ubiquitin and dehydrins, were investigated. Reasons for observed buckwheat salt stress sensitivity as well as possibilities for enhancing stress tolerance are discussed.

Key words: Salt stress, buckwheat, Fagopyrum esculentum Moench, antioxidative enzymes, dehydrins, ubiquitin.

INTRODUCTION

Salinity in soil or water is of increasing importance to agriculture because it causes stress to crop plants. Plants exposed to an excess amount of salts such as NaCl underwent osmotic stress, water deficit and ionic imbalances. The production of reactive oxygen species (ROS) is also increased under saline conditions (Hasegawa et al., 2000). Excess amounts of ROS are harmful to many cellular components, including membrane lipids, photosynthetic pigments, proteins and DNA.

Higher plants possess very efficient enzymatic and non-enzymatic antioxidative defense mechanisms that allow the scavenging of ROS and protection of cellular components from oxidative damage. The enzymatic defense mechanisms include the activities of enzymes regenerating the reduced forms of antioxidants, such as ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), and ROS-interacting enzymes (ROS scavengers) such as superoxide dismutase (SOD EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), non-specific soluble guaiacol peroxidases (POD, EC 1.11.1.7) etc.

In addition to this, abiotic stresses provoke the expression of the other mechanisms that protect cells from detrimental structural and functional changes. It is predicted that plants under stress conditions activate protein turnover machinery in order to degrade stress-damaged and environmentally-regulated proteins. Despite the fact that plants possess ubiquitin-independent machinery for degradation (Kurepa and Smalle, 2008), ubiquitin–26S proteasome-mediated proteolysis still remains the main mechanism. In light of this, the changes in the free ubiquitin level and appearance of ubiquitin conjugates might reflect the effect of stress on protein turnover (O’Mahony and Oliver, 1999).
Besides ubiquitin, there are many proteins, e.g. chaperons, LEA (late embryogenesis abundant) and LEA-like proteins, which are involved in protein quality control. Dehydrins (DHNs-LEA2; D11 family) are a group of thermo-stable, highly hydrophilic proteins ubiquitously produced in plants in response to water stress (Close, 1997; Cellier et al., 1998; Rinne et al., 1999). They are believed to play an important role in drought protection, although there is no direct evidence of their function. Dehydrins may also act as free radical scavengers and may sequester ions (Collet et al., 2004; Hara et al., 2004).

Buckwheat (Fagopyrum esculentum Moench) is a crop plant with high grain nutritive value and other useful properties (Pomeranz, 1983). The cultivation of this species is dependent on many environmental factors, e.g. temperature, salinity, nutrients etc, affecting the yield and reproductive potential of the plant (Slawinska and Obendorf, 2001; Taylor and Obendorf, 2001; Matsuura et al., 2005). The aim of this study was to investigate some of the biochemical and molecular changes in buckwheat plants subjected to salinity in order to better understand the mechanisms of plant response to this specific environmental stress. For this purpose, buckwheat plants in the middle vegetative phase were exposed to enhanced salinity conditions and the salinity effects were monitored in terms of relative water content (RWC), ROS accumulation, level of lipid peroxidation, activity of antioxidative enzymes (SOD, CAT, PODs, APX, GR), as well as through the expression analysis of ubiquitin and dehydrins.

MATERIALS AND METHODS

**Plant materials and stress treatments**

Buckwheat (Fagopyrum esculentum Moench, cv. Darja) has sown and grown in a greenhouse at the Institute of Molecular Genetics and Genetic Engineering in Belgrade under optimal conditions. Plants in the phase of 4 formed leaves, assigned as the middle vegetative phase, were picked out and further grown hydroponically either in 1/2 Murashige-Skoog nutrient solution (Murashige and Skoog, 1962) supplemented with 10, 25 and 100 mM NaCl for salt stress treatment, or in a nutrient solution without added salt as a control. Leaves assigned as second (2, in order of appearance) were harvested from the treated as well as the control plants after 2 and 7 days.

**Estimation of water content**

The relative water content (RWC) in the leaves was determined for each salt treatment and calculated according to the formula (Barrs and Weatherly, 1962):

$$\text{RWC} (%) = \left(\frac{\text{fresh weight} - \text{dry weight}}{\text{saturated weight} - \text{dry weight}}\right) \cdot 100$$

The leaf dry weight was measured after oven drying at 105°C for 24 h, and the saturated weight was measured after incubating the leaves in moist filter paper for 24 h in Petri dishes at room temperature.

**Lipid peroxidation assay**

The level of lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction as described by Heath and Packer (1986). The crude extract was mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice-bath. After centrifugation at 3500xg for 10 min, the absorbance of the supernatant was monitored at 532 nm. The MDA content was calculated per mg of leaf dry weight and the level of lipid peroxidation was expressed in respect to the control (100%).

**In situ histochemical detection of hydrogen peroxide**

**In situ** histochemical localization of hydrogen peroxide (H$_2$O$_2$) in buckwheat leaves was performed according to Thordal-Christensen et al. (1997) and the accumulation of H$_2$O$_2$ was visually detected. This method is based on H$_2$O$_2$-catalyzed polymerization of 3,3-diaminobenzidine (DAB). Leaves from each of the treated plants as well as the control were cut with
Buckwheat Response to Salt Stress

A razor blade and supplied with a 1 mg/ml solution of 3,3-diaminobenzidine (DAB) for 4 h. The leaves were then immersed in boiling ethanol (96%) for 20 min and DAB polymerized in the presence of H₂O₂ was visualized as a brown precipitate.

Protein extract preparation

Frozen leaves were ground in liquid nitrogen and the powder was suspended in an extraction buffer containing 50 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA. The homogenates were centrifuged at 15000xg for 20 min and the supernatant fraction was used for the assays of enzyme activity as well as for Western blot analysis. All steps were carried out at 4°C. The protein concentration in the extracts was determined according to Bradford (1976) using a Bio Rad assay kit and bovine serum albumin as the standard.

Enzyme assays

Catalase activity was assayed in a reaction mixture (1.5ml) composed of 50mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 2 mM H₂O₂ and 20 µl crude extract. The reaction was started by adding H₂O₂ and the activity was followed by monitoring the decrease in absorbance at 240 nm (Aebi, 1984). Catalase activity was expressed as ΔAbs₂₄₀ min⁻¹mg⁻¹ protein. Ascorbate peroxidase activity was assayed as described by Nakano and Asada (1981) using a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 10 mM H₂O₂ and 20 µl crude extract. Ascorbate peroxidase activity is expressed as ΔAbs₂₉₀ min⁻¹mg⁻¹ protein.

Superoxide dismutase activity was measured according to Beauchamp and Fridovich (1971). Crude extract (50 µl) was added to the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 13 mM methionine, 2 µM riboflavin and 75 µM nitro blue tetrazolium (NBT). The reaction was started by exposing the mixture to cool white fluorescent light for 15 min. SOD activity was expressed as ΔAbs₅₆₀ min⁻¹mg⁻¹ protein.

Glutathione reductase was measured according to Foyer and Halliwell (1976). The assay medium contained 0.025mM phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH-Na₄ and 50µl of extract. NADPH oxidation was determined at 340 nm. Activity was expressed as ΔAbs₃₄₀ min⁻¹mg⁻¹ protein.

Western blot analysis of stress-related proteins

Proteins (50 µg) were resolved by 12% SDS-PAGE according to Laemmli (1970) and then transferred for 40 min at room temperature to a PVDF membrane (Millipore) using a Biometra semi-dry transblotter at a constant current of 5 mA/cm² membrane. The membranes were then blocked overnight with 5% dried non-fat milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blots were used for the immunochemical detection of ubiquitin and dehydrins.

Ubiquitin was detected using rabbit anti-ubiquitin antiserum (Calbiochem), according to the instructions of the manufacturer. Dehydrins were detected according to Close et al. (1993). After incubation with the primary antibodies, the membranes were incubated for 1 h in a 1:15 000 dilution of secondary antibody (goat anti-rabbit IgG whole molecule, alkaline phosphatase conjugate - Sigma) in TBST. The signals were detected using an AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and NBT/BCIP as a substrate.

Statistics

The level of lipid peroxidation and enzyme activities were determined in three independent experiments.
with three replications each. All data obtained were subjected to statistical analysis using the Sigma stat program. Comparisons with P<0.05 were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard errors of the means.

RESULTS AND DISCUSSION

Plant response to salt stress occurs in two phases: the first is the osmotic, and the second is the ionic phase (Munns and Tester, 2008). In order to distinguish the osmotic and ionic effects of salt stress on buckwheat, biochemical and molecular changes in buckwheat leaves were monitored after short exposure (2 days, osmotic phase) as well as longer exposure (7 days, ion-specific phase). According to salinity-induced visible foliar changes and the behavior of the plants during continued salt exposure, a threshold level for buckwheat salt tolerance was determined at 25 mM NaCl. Based on this observation buckwheat can be considered as salt-sensitive plant species.

The disruption of water potential, as well as redox homeostasis, is among the deleterious effects of salinity stress on plants. The present study shows that salt treatments of buckwheat plants was accompanied by a reduction of water content in the buckwheat leaves (not shown). Treatments with 10 and 25 mM NaCl during two days caused a moderate decrease of the RWC relative to the control (decreasing of 20% and 22%, respectively). However, 100 mM NaCl caused a dramatic lowering of RWC. After 7 days of exposure, the RWC was 60% and 50% for 10 and 25 mM NaCl treatment, respectively, while after exposure to 100 mM NaCl the plants were almost completely dehydrated (RWC 4%). Wilting at 100 mM NaCl was the typical visible sign of foliar damage. The exclusion of excess salt in the form of observed salt crystals on leaf blades was a consequence of the high transpiration rate. These observations suggest a low capacity of buckwheat leaves for water retention and osmotic adjustment.

Lipid peroxidation is often used as an indicator of oxidative stress (Hernandez and Almansa, 2002). NaCl induced oxidative stress in buckwheat leaves in a dose-dependent manner, as evidenced by the increase in the MDA content (Fig. 1). The high level of lipid peroxidation suggests that a significant elevation of ROS in the buckwheat leaves occurred during exposure to salinity.

To see if salinity induced the formation of H$_2$O$_2$, buckwheat leaves were stained with DAB for in situ detection of H$_2$O$_2$. In the leaves of salt-treated buckwheat plants, increased H$_2$O$_2$ accumulation was visualized in response to all applied salt concentrations after 2 days of exposure (Fig. 2), suggesting that the salt-shock induced a rapid dose-independent production of H$_2$O$_2$. Longer exposure (7 days) did not enhance the staining intensity, which was decreased in comparison to the intensity visualized after 2 days of exposure. Furthermore, longer exposure restricted...
In situ detection of hydrogen peroxide accumulated in buckwheat leaves under salinity treatments. A. DAB staining of hydrogen peroxide in buckwheat leaves after two days of exposure to 0 mM NaCl (a), 10 mM NaCl (b), 25 mM NaCl (c), 100 mM NaCl (d). B. DAB staining of hydrogen peroxide in buckwheat leaves after seven days of exposure to 0 mM NaCl (a), 10 mM NaCl (b), 25 mM NaCl (c), 100 mM NaCl (d).

The staining to vascular tissue, whereas during shorter exposure H$_2$O$_2$ was accumulated in leaf tissue in the form of localized foci. Localized accumulation of H$_2$O$_2$ could represent a possible protective mechanism enabling the majority of leaf tissue to properly perform photosynthesis and other metabolic processes. Vascular localization of H$_2$O$_2$ during longer salt exposure could suggest a protective function ensuring the maintenance of the photosynthetic competence in leaf tissue, as well as a possible signal...
function in the coordination of stress response on a whole-plant level. It was shown that the accumulation of hydrogen peroxide preceded the phase of senescence initiation in the veinal tissue (Niewiadomska et al., 2009). The observed pattern of hydrogen peroxide accumulation in the leaves of 25 mM
NaCl-treated plants indicated a state of senescence initiation. Further salt exposure caused senescence process progression, enabling the survival of younger leaves. The lack of DAB staining in response to a higher salt concentration (100 mM NaCl), may be an indication of vascular tissue collapse, which is in agreement with the observed accelerated premature senescence and abscission of leaves.

In addition to the absence of correlation between applied salt concentration and hydrogen peroxide accumulation, there was no correlation between H₂O₂ accumulation and level of lipid peroxidation. This observation suggests the build-up and role of other ROS in cell damage as well as the existence of efficient H₂O₂ detoxifying systems. Among antioxidative enzymes and ROS scavengers, only SOD activity

---

Fig. 3. Effect of salinity on antioxidative enzyme activity in buckwheat leaves. Values are mean ± S.E. based on three replicates and shown as a percentage relative to the control (100%)
k-control (untreated) plant.
was decreased compared to the control (Fig. 3). Such a decrease could be due to some stress-induced damage of the enzyme and may lead to the accumulation of superoxide anion (O$_2^-$). Moreover, hydrogen peroxide could be formed by other activities (Kranner and Birtic, 2007; Halliwell and Gutteridge, 1999; Rea et al., 2002). All of these activities could be responsible for the observed level of lipid peroxidation and H$_2$O$_2$ accumulation after 2 days of salt exposure (Fig. 1 and Fig. 2).

Hydrogen peroxide generated during stress exposure is decomposed by the activities of CAT, PODs and APX. The activity of CAT was unaffected, whereas the activities of PODs and APX were induced by salinity treatments, especially by 10 mM NaCl during 2 days of exposure (Fig. 3). A further increase of NaCl concentration decreased the activity of PODs, APX and GR, compared to the activity of 10 mM NaCl. After 7 days of exposure, the highest increase in the activities of these antioxidative enzymes was at 25 mM NaCl. The increased activity of PODs and APX indicated their important role in H$_2$O$_2$ detoxification. The prominent increase in the activity of APX and GR suggests the involvement of ascorbate and glutathione in the antioxidative response of buckwheat. Ascorbate and glutathione are important in the regulation of senescence (Vanacker et al., 2006). Also, the GSH/GSSG ratio can influence the ubiquitin-dependent protein degradation pathway (Theriault et al., 2000).

The data presented here show that the expression of ubiquitin and ubiquitin conjugates were in-
duced by salt treatments and that the highest level of free ubiquitin as well as ubiquitin conjugates was observed at 25 mM NaCl after 2 days of exposure, and at 100 mM NaCl after 7 days of the treatment (Fig. 4). This could be an indication of stressful conditions, suggesting a high level of ubiquitin-dependent protein turnover. However, the accumulation of ubiquitin conjugates may indicate the low level of proteolysis. It was also shown that longer exposure to salinity alleviated the salt-induced expression of free ubiquitin, suggesting the higher rate of protein ubiquitination (10 and 25 mM NaCl after 7 days). The ubiquitin profile observed at 100 mM NaCl after 7 days suggests the reduction of the ubiquitination process and proteolysis, which is in agreement with the general metabolic reduction under conditions of severe water loss.

Dehydrins as stress-protective proteins are induced in vegetative tissues in response to different kinds of stress including dehydration, salinity, cold and heat stress (Khedr et al., 2003). Western blot analysis of dehydrins in buckwheat leaves revealed a salt-enhanced expression of 36 kDa dehydrin in a dose-dependent manner during 2 days of exposure (Fig. 5). The pre-existence of 36 kDa dehydrin and its quantitative regulation during stress indicate that this protein is required for normal metabolism and its up-regulation by stress should provide more amounts required during stress. It was shown in barley that one dehydrin was present in well-hydrated seedlings, but that under water stress, many others were newly synthesized (Close et al., 1993). The observed dehydrin-like protein in buckwheat leaves and its quantitative regulation suggest that buckwheat stress protection relies on the action of an individual protein instead of the coordinated action of a whole set of dehydrins. It may be one of the reasons for buckwheat sensitivity to salt stress.

Fig. 5. Effect of salt stress on the expression of dehydrin-like proteins in buckwheat leaves. Proteins from buckwheat leaves (50 μg) were resolved by SDS-PAGE and dehydrin-like proteins were detected by western blot using anti-dehydrin antibodies. 1-4: buckwheat plants treated for 2 days with 0 (1), 10 mM NaCl (2), 25 mM NaCl (3), 100 mM NaCl (4). 5-8: buckwheat plants treated for 7 days with 0 (5), 10 mM NaCl (6), 25 mM NaCl (7), 100 mM NaCl (8). MW is a molecular mass standard (Fermentas).

Abbreviations:
APX - ascorbate peroxidase; CAT - catalase; DAB - 3,3-diaminobenzidine; DNA - deoxyribonucleic acid; EDTA - ethylene diamine tetra acetate; GR - glutathione reductase; MDA - malondialdehyde; MW - molecular weight; NBT - nitro blue tetrazolium; POD - peroxidase; ROS - reactive oxygen species; RWC - relative water content; SE - standard error; SOD - superoxide dismutase.
In conclusion, the observed sensitivity of buckwheat plants to salt treatments at the middle vegetative developmental stage could be attributed, at least in part, to the failure of osmotic adjustment, uncoordinated activity of CAT, PODs, APX and GR with SOD activity resulting in significant oxidative stress, as well as to the reduction of stress-specific protectants, such as dehydrins. It is evident that salt treatment induced rapid osmotic and oxidative stresses which are later followed by increased leaf senescence as a consequence of ionic stress. According to our results, by demonstrating the very detrimental multi-level effects of salinity in buckwheat plants, we believe that new strategies for improving buckwheat stress tolerance can be developed.

Acknowledgments - This work was supported by the Ministry of Science and Technological Development of Republic of Serbia, as a part of research project 143017. The authors of this paper wish to thank Dr Timothy Close, Department of Biochemistry, University of California, Riverside, for kindly providing anti-dehydrin antibodies.

REFERENCES


