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Involvement of abscisic acid in the response of *Medicago sativa* plants in symbiosis with *Sinorhizobium meliloti* to salinity

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ABSTRACT

Legumes are classified as salt-sensitive crops with their productivity particularly affected by salinity. Abcisic acid (ABA) plays an important role in the response to environmental stresses as signal molecule which led us to study its role in the response of nitrogen fixation and antioxidant metabolism in root nodules of *Medicago sativa* under salt stress conditions. Adult plants inoculated with *Sinorhizobium meliloti* were treated with 1 μM and 10 μM ABA two days before 200 mM salt addition. Exogenous ABA together with the salt treatment provoked a strong induction of the ABA content in the nodular tissue which alleviated the inhibition induced by salinity in the plant growth and nitrogen fixation. Antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were induced by ABA pretreatments under salt stress conditions which together with the reduction of the lipid peroxidation, suggest a role for ABA as signal molecule in the activation of the nodular antioxidant metabolism. Interaction between ABA and polyamines (PAs), described as anti-stress molecules, was studied being detected an induction of the common polyamines spermidine (Spd) and spermine (Spm) levels by ABA under salt stress conditions. In conclusion, ABA pre-treatment improved the nitrogen fixation capacity under salt stress conditions by the induction of the nodular antioxidant defenses which may be mediated by the common PAs Spd and Spm that seems to be involved in the anti-stress response induced by ABA.

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1. Introduction

The response of plants to biotic and abiotic stresses is regulated by phytohormones and plant growth regulators that include: salicylic acid (SA), abscisic acid (ABA) and a group of polycationic compounds known as polyamines (PAs). These endogenous low molecular weight molecules regulate, via synergistic and antagonistic actions, the expression of different but overlapping suites of genes, which is referred as signaling cross-talk [1].

ABA is a phytohormone that plays an important role in plant tolerance and adaptation to a variety of stresses involving water deficit, cold, drought or salinity [2]. The content of ABA increases in various tissues, including root nodules of legumes such as *Phaseolus vulgaris* [3] and *Medicago ciliaris* under salinity stress [4], alleviating the damages caused in such conditions. One of the consequences of salinity in plants consists in the production of reactive/activated oxygen species (ROS), including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). In order to mitigate and repair damage produced by ROS, plants have developed a complex antioxidant system [5] which has been suggested to be involved in the redox balance and in the regulation of nodule metabolism. For instance, the diminution of nitrogen fixation under abiotic stress is correlated with a modification of the redox balance and a strong decline in the antioxidant defence system [6,7]. In this context, a strong antioxidant defence system may be crucial to allow an efficient nodule functioning.

ABA has been shown to increase the expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) in vegetables [8–10]. Moreover, in *Zea mays* and *Ipomoea batatas* genes of SOD, CAT and APX were induced by ABA treatment [11,12]. The role of ABA in the tolerance of rice against chilling stress by the induction of antioxidant defence systems, including enzymatic and non-enzymatic, has been also previously described [13].

Polyamines (PAs) are low-molecular-weight polycationic amines that have been involved in a variety of biological processes including the response against abiotic stresses [14]. The relation between the accumulation of ABA and the biosynthesis of PAs under stress conditions has been reported, suggesting a role for ABA as signal molecule involved in the maintenance of the cellular

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pool of PAs under salinity conditions [15]. In addition, ABA regulates the expression of several genes involved in PAs biosynthesis [16,17] and has been shown to have a stimulatory effect on the putrescine (Put) and spermidine (Spd) levels in common bean plants under salt stress [18]. Interestingly, correlation between concentration of PAs and nitrogenase activity in root nodules have been reported [19] and in addition to the common PAs (Put, Spd and Spm), uncommon PAs such as homospermidine (Homspd) has been detected in the nodular tissue [20]. Proline, considered one of the most common osmolytes in response to abiotic stresses [21], reflect a close relationship with the PAs biosynthesis since both metabolites share the common precursor glutamate [22] and should be therefore tightly regulated.

Due to the sensitivity of legumes to abiotic stresses and their importance in environmentally sustainable agricultural systems, is essential to gain further knowledge in the mechanisms involved in the nodular responses to salt stress conditions. In this work, we have studied the responses induced by exogenous ABA in the symbiotic nodules under salt stress conditions. We have found an alleviation of the negative effects of salinity on plant growth and nitrogen fixation which might be due to the induction of the nodular antioxidant metabolism and the accumulation of the nodular polyamines.

2. Materials and methods

2.1. Biological material and growth conditions

Seeds of *Medicago sativa* var. Aragon were surface-sterilized by immersion in 5% NaClO for 3 min and germinated in 0.8% water-agar plates at 25 °C in darkness. After two days, seedlings were transferred into individual pots of about 200 ml containing a sterilized vermiculite-perlite mixture (3:1), inoculated with 1 ml of a stationary culture of *Sinorhizobium meliloti* GR4 strain (ca. 10^9 cell ml^{-1}) and watered with N-free nutrient solution [23]. Plants were grown in a controlled environmental chamber with a 16/8 h light-dark cycle, 23/18 °C day-night temperature, relative humidity 55/65% and photosynthetic photon flux density (400–700 nm) of 450 μmol m^{-2} s^{-1} supplied by combined fluorescent and incandescent lamps.

2.2. Treatments and harvest

Abcisic acid (ABA) treatment (1 and 10 μM) was applied to the growth medium, 49 days after sowing. Two days after hormone treatment, NaCl (200 mM) was applied to the nutrient solution. Plants were harvested at 12 days after the saline treatment and 10 plants were randomly selected for each treatment and dried at 70 °C for 48 h to calculate dry weight. Nodules were detached and stored at −80 °C for the enzyme assays and analytical determinations.

2.3. Nitrogenase activity

Nitrogenase activity (EC 1.7.9.92) was measured as H_2 evolution in an open-flow system [24] using an electrochemical H_2 sensor (Qubit System Inc., Canada). For nitrogenase measurements, pots maintained in a controlled environmental chamber (as described above) were sealed and H_2 production was recorded. H_2 production under N_2:O_2 (80:20%) provides only a measurement of apparent nitrogenase activity (ANA) because only 25% of the electrons passing through nitrogense are used to produce H_2, while the others are used in the reduction of N_2 to ammonia. Total nitrogenase activity (TNA) was determined under Ar:O_2 (79:21%), in that way all the electrons that were previously allocated to N_2 reduction are used for H^+ reduction increasing the rate of H_2 evolution from the nodule. All the measurements were determined with a total flow of 0.4 l min^{-1}. The nitrogen fixation rate (NFR) was calculated as (TNA-ANA)/3. Standards of high-purity H_2 were used to calibrate the detector.

2.4. Antioxidant enzyme assays

For protein extract preparation, nodules were homogenized at 4 °C in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 10 mM β-mercaptoethanol and 20% (w/w) polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 15,000 × g for 20 min at 4 °C. The resulting extract was used to measure enzyme activities superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, E.C.1.11.1.11), glutathione reductase (GR, E.C.1.8.1.7), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and peroxidase (POX, E.C.1.11.1.7).

Superoxide dismutase activity was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm. The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA and 0.05 ml of enzyme extract. The reaction started when adding 2 μM riboflavin, and the mixture was incubated under fluorescent lamps for 15 min then kept in the dark to stop the reaction. The absorbance of the mixture was measured at 560 nm. The reaction mixture with no enzyme developed maximum color due to maximum rate of reduction of NBT. One unit of SOD was determined as the amount of enzyme that inhibits 50% NBT photoreduction. The activity was expressed as unit min^{-1} mg^{-1} protein [25].

Ascorbate peroxidase was assayed by recording the decrease in absorbance at 290 nm [26]. The reaction mixture contained 50 mM Tris–HCl (pH 7.8), 0.4 mM ascorbate and 0.3 mM H_2O_2.

Glutathione reductase was determined by measuring the decrease in absorbance at 340 nm due to NADPH oxidation as described by Edwards et al. [27]. The reaction mixture consisted of 0.1 mM HEPES-NaOH (pH 7.8), 3 mM $MgCl_2$, 0.25 mM oxidized glutathione, 0.2 mM NADPH and 1 mM EDTA.

Dehydroascorbate reductase was assayed according to Dalton et al. [28]. The activity was measured in 50 mM potassium phosphate (pH 6.5), 0.1 mM EDTA, 0.5 mM reduced glutathione (GSH), 0.5 mM dehydroascorbate (DHA), and 50 μl of extract.

Peroxidase was estimated by monitoring the guaiacol oxidation at 470 nm according to Kato and Shimizu [29]. The activity was assayed in 200 mM sodium phosphate buffer (pH 5.8) containing 7.2 mM guaiacol and 11.8 mM H_2O_2.

Catalase activity was assayed by the method of Aebi [30]. Nodules (0.2 g) were homogenized with a mortar and pestle using 2.5 ml 100 mM potassium phosphate buffer (pH 7), 100 mM EDTA, 0.1% (v:v) Triton X-100 and 10% (w:w) polyvinylpolypyrrolidone. The homogenate was centrifuged at 27,000 × g for 20 min at 4 °C, and the supernatant was used for the enzyme assay. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 0.1% (v:v) Triton X-100 and 10.5 mM H_2O_2. The decrease in the absorbance at 240 nm was recorded for 3 min by spectrophotometry.

2.5. Determination of non-enzymatic antioxidants glutathione and ascorbate

Fresh nodule samples (0.2 g) were homogenized in 2 ml of metaphosphoric acid 5% (w:v) at 4 °C and centrifuged at 13,500 × g for 15 min. Then, 0.1 ml of supernatant was used to determine total ascorbate according to Law et al. [31]. The assay is based on the reduction of Fe^{+3} by reduced ascorbate, followed by complex formation between Fe^{+2} and bipyridil, which absorbs at 525 nm. Total ascorbate was determined after reduction of dehydroascorbate to ascorbate through the reaction with dithiothreitol.

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