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Differential tolerance to salinity in *Lotus japonicus* MG20 with choroplastic Mn-SOD and GR overexpression and parental genotypes of *Lotus* genera

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Lotus japonicus MG20 transgenic plants with choroplastic Mn-Superoxide dismutase (Mn-SOD) and Glutathione reductase (GR) overexpression were obtained by *Agrobacterium tumefaciens* mediated transformation. The plants were propagated by nodal cutting and maintained as clones. Since transgenic seeds were used, a system was adjusted for selection during seeds germination, for which Kanamycin 100 mg/l, Tween 20 0.01% and Triton X100 0.01 % were used. Root development inhibition and chlorosis increase were used as indicators for non transgenic seeds. Two levels of NaCl, 50 and 150 mM, were applied gradually in order to induce acclimatization responses. When inoculated plants were use, salinization started 48 h after *Mesorhizobium loti* inoculation. Shoot and root growth, biomass, nodule production apoplastic ROS production and antioxidative enzyme activities were evaluated.

Shoot length showed that SOD transgenic plants grew almost two times more than native plants whereas in GR transgenic plants, shoot length was significantly diminished. Growth in GR plants was stimulated with 50 mM NaCl respect to the control conditions; however, no differences were observed respect to native plants under treatment. SOD transgenic plants incubated under 50 mM NaCl did not show differences respect to the control condition, and maintained the differences respect to native plants under treatment. Under saline stress of 150 mM, shoot length of SOD transgenic plants was higher than GR transgenic and native plants.

Root length under control conditions was similar in SOD transgenic and native plants, whereas GR transgenic plants roots were shorter. Under 150 mM NaCl treatment, roots of transgenic SOD plants were shorter than in native plants. Although roots in GR transgenic plants were shorter than native plants, saline treatment-mediated stimulation of radical growth was observed in GR transgenic plants.

Biomass production was higher in SOD transgenic plants than in native plants at 0 and 50 mM NaCl without differences at 150 mM NaCl. In GR transgenic plants, biomass content was less than in native plants under control conditions, meanwhile, their production increased under salt treatment.

Nodule number did not show differences among genotypes under control and 50 mM NaCl treatment. Nodulation was affected in SOD transgenic plants under 150 mM NaCl whereas in GR transgenic plants, nodule number did not change respect to control values. The correlation of root growth and apoplastic superoxide production was assayed in native *L. japonicus* MG 20 plants under control and 50 mM NaCl conditions. Both culture conditions, which did not affect root growth, induced high levels of $\cdot O_2$ as verified by NBT reduction assay. Superoxide production was severely diminished under 150 mM NaCl, a culture condition that, as previously described, affected growth and reduced nodulation. Enzymes of Ascorbate-Glutathione cycle were analyzed by total activity, zymograms and Western blot. SOD and APX activities under 150mM were increased in transgenic SOD plants and diminished in GR transgenic whereas total GR activity was increased in both kinds of transgenic plants. These results suggest that redox intracellular state, which is differentially modified by SOD and GR enzymes overexpression, could modulate defense responses against adverse environmental conditions that generate oxidative stress.

On the other hand, a preliminary characterization of differential tolerance against saline stress was performed in parental genotypes of Lotus genera by growth parameters, ions and antioxidative enzymes. The assayed genotypes were *L. japonicus* ecotypes MG20 and Gifu, *Lotus filicaulis* and *Lotus burttii*. Shoot length showed that 50 mM NaCl promoted growth in shoots of all genotypes, except for *L. japonicus* Gifu, respect to the controls. Furthermore, 150 mM NaCl diminished shoot length in all genotypes. When comparing shoot length at the end of the treatment, it could be concluded that *L japonicus* MG20 and *L burttii* were the most tolerant genotypes, since they showed lower growth diminutions under saline stress. Root growth showed a similar behavior as shoot growth under salt treatment. *L. japonicus* MG20 and *L. burttii* showed tolerance, whereas in L burttii, root length diminution respect to their own control was higher than in *L. japonicus* MG20 under 150 mM treatment. *L. japonicus* MG20 was the unique genotype that increased root/shoot rate under 150 mM NaCl, mainly at the end of treatment. Such behavior has been described as a typical adaptative response to low hydric potential Antioxidant activity of SOD, GR and APX enzymes was determined in these material, and SOD and GR isoforms differences and specific protein content were determined by zymograms and Western blot respectively. In *L japonicus* MG20, total SOD activity was induced at 50 and 150 mM whereas in *L burttii* this induction was observed at 150 mM. GR activity was induced at 50 mM in all genotypes and diminished mainly in *L. japonicus* Gifu and *L filicaulis* at 150 mM NaCl. APX activity did not showed marked differences among genotypes under salinity although higher APX levels were observed in *L burttii*. SOD and GR zymograms correlated with total activity, in GR zymograms the changes were observed in both isoforms while in SOD zymograms Cu-Zn SOD was the isoform that showed higher differences. Western blot using anti Cu-Zn and anti GR antibody correlated partially with the activities, suggesting a regulation by specific protein content.

Conclusion

The main results can be summarized as follows

L. japonicus MG20 with GR overexpression showed higher weight and nodule number. -Both kinds of transgenic plants showed higher total and specific ARA at 0 and 50 mM NaCl. ARA was the process most affected by salinity.

Apoplastic ROS production, probably mediated by NADPH oxidase complex, correlated with root growth and nodule production in the different salt condition assayed.

Parental Lotus analysis under saline stress showed that *L. japonicus* MG20 and *L. burttii* were the salt tolerant genotypes. Both tolerant genotypes increased their SOD and GR activities under saline stress