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Assessment of salt tolerance in transgenic tobacco (*Nicotiana tobacum* L.) plants expressing the AUX gene

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Transformation of plants using *Agrabacterium rhizogenes* may affect secondary metabolite production as well as morphological changes. In this study, T-DNA from Ri plasmid in *A. rhizogenes* carrying pRi15834-PRT35S-GUS was introduced into tobacco leaf segments to initiate development of transformed hairy roots. Plant regeneration from transgenic roots used MS medium, and plants regenerated from transgenic roots were subjected to 300 mM NaCl. Transgenic plants showed higher levels of salt tolerance compared to non-transgenic plants. This could be due to over expression of the AUX gene in transformed hairy roots and plants regenerated from transgenic roots. © 2011 Progress in Biological Sciences, Vol. 1, No.1, 17-23.

KEY WORDS: Tobacco, Auxin, salt tolerance, transgenic tobacco plant

INTRODUCTION

Agrobacterium rhizogenes is responsible for abundant hairy root production at the site of inoculation in many dicot plants. Such roots can be grown *in vitro*, in the absence of the inciting bacterium, and they generally grow better than normal roots of the same species. The underlying mechanism of hairy root formation is the transfer of bacterial genes to the plant genome. Hairy root cultures exhibit a typical phenotype with a lack of geotropism and high incidence of lateral branching as compared to normal roots (Guern et al., 1988).

In both natural and agricultural conditions, plants are exposed to environmental stresses. Stresses, including biotic and abiotic factors, are usually defined as external factors that they cause disadvantageous effects on plants, negatively affecting plant survival, growth and development (Taiz and Zeiger, 2002). Sodium chloride, the predominant salt in saline soils, constitutes an environmental stress that affects plant growth and productivity (Munns, 2005; Arturi et al., 2009). Salinity can reduce growth, germination levels, radicle and hypocotyl length and dry weight, and seedling fresh and dry weight (Akbari et al., 2007). Inhibition of growth and development results from reduction in photosynthesis, respiration, and protein synthesis in sensitive species (Desingh and Kanagaraj, 2007).

Adverse effects on plant growth may also be related to induction of other stresses such as osmotic effects due to decrease in the soil water potential, oxidative stress, and reduction in mineral uptake (Arora et al., 2002; Flowers and Flowers, 2005; Altunlu et al., 2007; Kaymakanova and Stoeva, 2008). Ionic stress can result from salinity leading to a reduction in K^+ and Ca^{+2} content and an increase in Na^+, Cl^-, and SO_4^{2-} content (Kaymakanova and Stoeva, 2008). Various strategies to cope with ion stress, including limited salt absorption, salt compartmentation, excluding salt from the plant, and the control of its transport to the shoots (Ashraf, 2004). The initial plant response to salt stress is in the pattern of ion uptake, since this determines the means whereby plants maintain water balance and avoid Na^+ and/or Cl^- toxicity under saline conditions. Control of Na^+ uptake by cells and long distance Na^+ transport is considered an important adaptation of plants to saline substrate.

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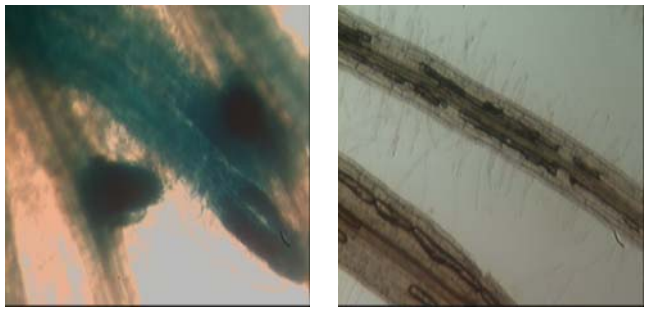
Fig. 1. Transformed hairy roots from tobacco leaf segments on MS medium containing kanamycin.

For example, salt tolerance in most plants is associated with low uptake and accumulation of Na^+, which is mediated through the control of influx and/or by active efflux from the cytoplasm to the vacuoles and also back to the growth medium (Munns, 2005; Ashraf and Qasim 2006).

It is thought that adverse effect of salinity on growth could be related to endogenous levels of plant growth regulators. Phytohormones exchange signals between root and shoot responses to external stresses (Bano et al., 2009). Salt stress reduces gibberellin and auxin production in plants. It has been reported that treatment of rice plants under salt stress with gibberellin was associated with an increase in growth (Prakash and Prathapasenan, 1990). Application of exogenous phytohormones such as auxin and cytokinin are variously implicated in decrease of salinity effects (Angrish et al., 1997). Since transformed plants carrying T-DNA from Ri plasmid produce high levels of auxin in hairy roots, it is speculated that they may show different responses to salt stress compared to untransformed roots. The aim of this study was to evaluate salt tolerance in regenerated tobacco plants with transformed hairy roots expressing the AUX gene.



a b

Fig. 2. GUS assay, a: transgenic roots. b: non-transgenic roots.

MATERIAL AND METHODS

Stem segments with an auxiliary bud from *Nicotiana tabacum* L. cv. Wisconsin were used in this experiment. Explants were cultured and propagated in MS medium (Murashige and Skoog 1962). Cultures were maintained at 25°C in the culture room under a 16:8 h L:D photoperiod.

Inoculation and root culture

Leaf segments of tobacco plants were submerged in an *Agrobacterium rhizogenes* strain carrying pRi15834-PRT35S-GUS linked to the AUX gene for 15-30 min and subsequently transferred to MS medium for 3-5h. Explants were then transferred to MS medium supplemented with 700 mg l^{-1} cefazoline and 30 mg l^{-1} kanamycin. Transformed hairy roots were induced on leaf segments after 2-3 weeks.

Evidence of transformation

β-Glucuronidase enzyme activity (GUS-assay) and PCR were used to confirm transformation. Several pieces of hairy roots, as well as non transformed roots, were transferred to x-gluc substrate and incubated at 37°C overnight (8-10 h). Blue color appeared on transformed roots following incubation; roots were photographed under the light microscope.

Total genomic DNA was isolated from roots using a C-TAB method based on a method described by Ehsanpour and Twelv (2005). PCR was performed using NPTII (Neomycin phosphotransferase II) primers (FW: 5′-GAGGCT

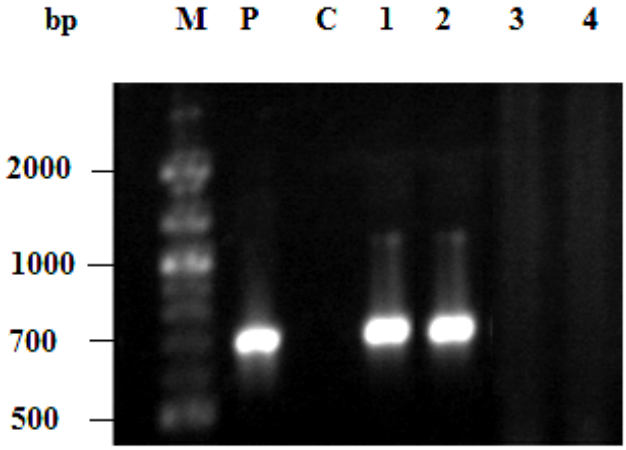


Fig. 3: PCR amplification of the NPTII gene in transgenic roots. M marker; P plamid; C negative control; 1,2 transgenic roots; 3,4 non-transgenic roots.

ATT CGG CTA TGA GTG-3′, RV: 5′-ATC GGG AGC GGC GAT GTA-3′). DNA amplification was performed by an initial cycle at 94°C for 2 min, 39 cycles at 94°C for 1 min, 55°C for 30 sec, and 72°C for 30 sec.

Plant regeneration

Segments of transgenic roots were cultured in callus formation medium [MS medium containing 2 mg l^{-1} naphthalene acetic acid (NAA), 0.25 mg l^{-1} kinetin, 700 mg l^{-1} cefazolin and 30 mg l^{-1} kanamycin]. Calluses were produced after 2 weeks and were transferred to the regeneration medium [MS medium containing 1 mg l^{-1} benzyleaminopurine (BAP), 0.1 mg l^{-1} NAA, 700 mg l^{-1} cefazolin and 30 mg l^{-1} kanamycin]. Plant regeneration from non transgenic roots was carried out in the same medium.

Salt treatments

Regenerated plants from transgenic and non-transgenic roots were transferred to liquid MS medium containing 0 and 300 mg l^{-1} NaCl. After 4 weeks, fresh and dry weight of roots and shoots, relative chlorophyll content of leaves, and sodium and potassium in roots and shoots were measured. All experiments were carried out with 3 replications.

RESULTS

Transgenic roots appeared in 80% of explants 15-20 days after inoculation with *A. rhizogenes*. Transgenic roots were thicker, shorter, and had more branches than control roots and could grow in medium containing kanamycin (Fig. 1).

The result of GUS assay (blue color) on transgenic roots confirmed that roots were transformed with T DNA from Ri plasmid. No blue color was observed on non-transgenic roots (Fig. 2).

Transgenic roots were analyzed by PCR (Fig. 3). Amplified DNA fragment with 700 bp indicated the presence of NPTII gene in the transgenic plants.

Regenerated plants (transgenic and non-transgenic) transferred to the medium with 300 mM NaCl plants showed different patterns of growth. Transgenic plants were healthy and grew well under salt stress, with a small amount of yellowish color observed on the surface of the leaves. Non-transgenic plants showed dramatically lower general growth. Results after 4 weeks with and without exposure to 300 mM NaCl are illustrated in Fig. 4.

Fresh and dry weight

Analysis of fresh and dry weight showed root fresh and dry weight to be increased in transgenic plants in medium with or without treatment with 300 mM NaCl compared to non-transgenic plants. In saline medium, shoot fresh and dry weight of non-transgenic plants were decreased compared with controls (Fig. 5).

Relative chlorophyll content of leaves of transgenic and non transgenic plants was measured. No significant difference between transgenic and non-transgenic plants at 0 and 300 mM NaCl was observed (Fig. 6).

Na^+ and K^+ content

Sodium and potassium were measured in plants following salt treatment. There was no significant difference between Na^+ content in transgenic and non-transgenic plants either with or without 300 mM NaCl. However, the Na content of plants (shoot and root) under salt treatment was

a b c d

Fig. 4. Growth pattern of (a) Transgenic plant in MS medium; (b) Non-transgenic plant in MS medium; (c) Transgenic plant in MS containing 300 mM NaCl; (d) Non-transgenic plant in MS containing 300 mM NaCl.

significantly higher than that of untreated plants (Figs 7 a, b). Potassium content of transgenic roots was significantly higher than non-transgenic, while there was no significant difference between K content of shoots in transgenic and non-transgenic roots. In contrast, the root and shoot K content of transgenic plants was significantly higher than that of non-transgenic plants. (Figs 7 c, d).

K^+/Na^+ ratio

The K^+/Na^+ ratio in transgenic roots was significantly higher in comparison with non-transgenic roots while this ratio in shoots of transgenic plant showed no significant difference. The ratio of K^+/Na^+ in transgenic plants treated with 300 mM was very low, with a significant difference between transgenic and non-transgenic shoots (Figs 8 a, b).

DISCUSSION

Agrobacterium rhizogene is a soil organism inducing hairy root formation. The observed morphogenic effects in plants with infection have been attributed to the transfer of part of a large plasmid, known as the root-inducing (Ri) plasmid, containing the auxin production gene (AUX gene). The symptoms observed with *A. rhizogenes* infection are due to mostly from auxin effects (Angrish et al., 1997). Similar to our findings, production of transformed root has been reported in *Rubia tinctorum* for condensed anthraquinone biosynthesis (Gülhan Ercan et al., 1999). A number of other plant species have also been transformed with *A. rhizogenes* (Arturi et al., 2009). In our study, whether or not transformed hairy roots produce more or different alkaloid (such as nicotine) substances was not clear.

Under salt stress plant hormones (i.e. free cytokinin) may be decreased (Ahmed et al., 1998; Irfan *et al*., 2005), or salt stress may be associated with an increase of plant hormones such as Abscisic acid (ABA) (Bohnert and Thomas, 1993). However, in the present study, hairy roots increased auxin levels in the cells (unpublished data), and, consequently, it is possible that increased salt tolerance in transgenic tobacco plant might be related to auxin functions.

The morphology of transgenic plants regenerated from transformed roots was altered in comparison to the non-transgenic plants. In transgenic plants roots were thicker, shorter, and more branched than were control roots. Leaves were also smaller, and growth was slower than in non-transgenic plants. The morphological alterations may have been affected by auxin produced from transgenic roots. It has been reported that, in the wild type of *A. rhizogenes*, the *rol*B gene is one of the genes transferred to the host in the infection process considered to be responsible for rooting and some morphological variations (Domingos et al., 2003). On the other hand, a higher degree of root formation can cause more cytokinin biosynthesis, and higher cytokinin concentrations induce thicker roots. This is in accordance with Bohnert and Thomas (1993) who reported that the morphology of root grown with increased cytokinin was different from untreated roots.

Root fresh and dry weight was increased in transgenic plants under saline conditions, while fresh and dry weight of non-transgenic plants was decreased. This is in accordance with Bano et al. (2009), who reported that wheat plants exposed to 150 mM NaCl with ABA, benzyladenine (BA), or cycocel (CCC) showed increased root and shoot weight.

Sodium content of roots was increased with 300 mM NaCl in transgenic and non transgenic plants. In agreement to our findings, Bano et al. (2009) found increasing levels of NaCl to significantly increase Na^+ concentration in the wheat

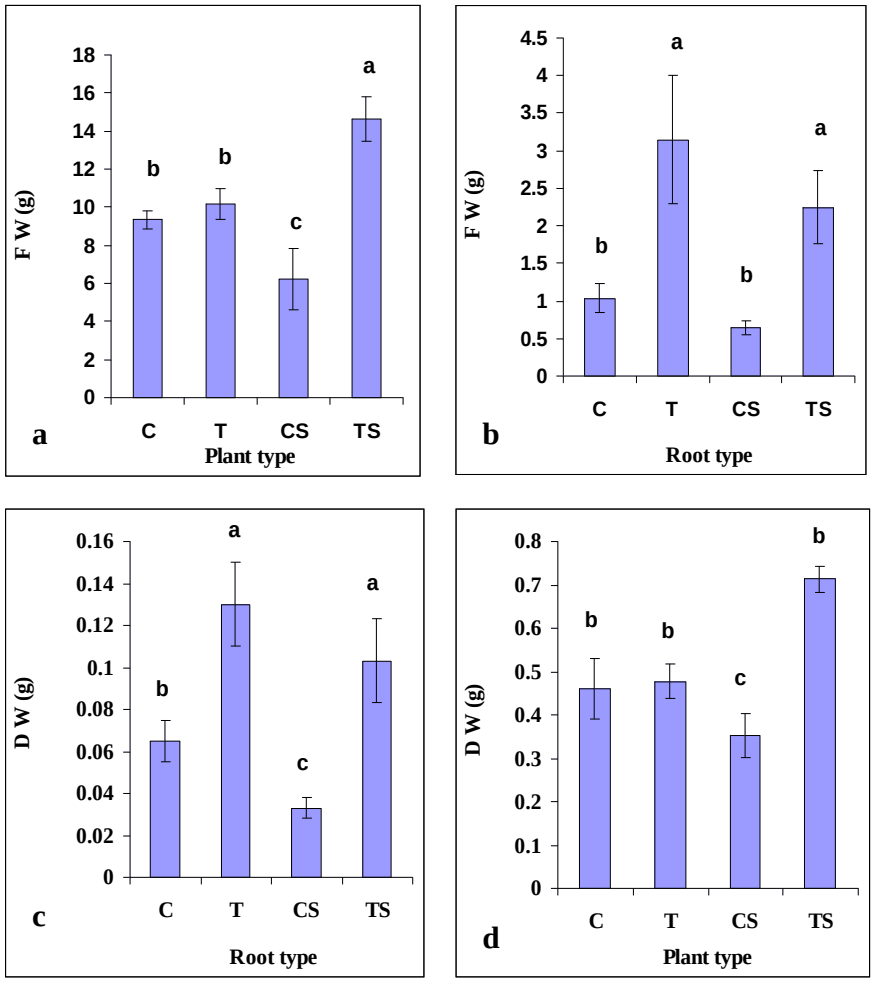


Fig. 5. (a) root fresh weight, (b) shoot fresh weight, (c) root dry weight, (d) shoot dry weight. C non-trangenic plant without salt; T transgenic plants without salt; CS non-transgenic plants in 300 mM NaCl; TS transgenic plants in 300 mM NaCl. Data are means ± SD.

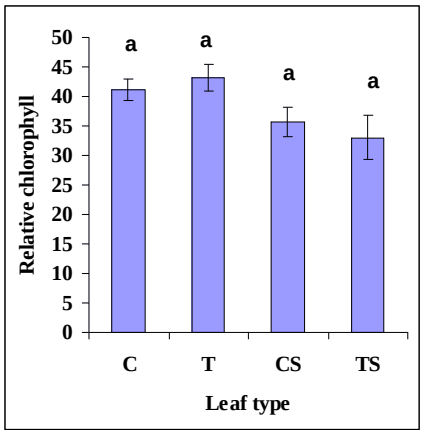


Fig. 6. Relative chlorophyll content in plants. C, non-transgenic plant without salt; T, transgenic plant without salt; CS, non-transgenic plant in 300 mM salt; TS, transgenic plant in 300 mM salt. Data are means ± SD, common letters are not significant ($P < 0.05$).

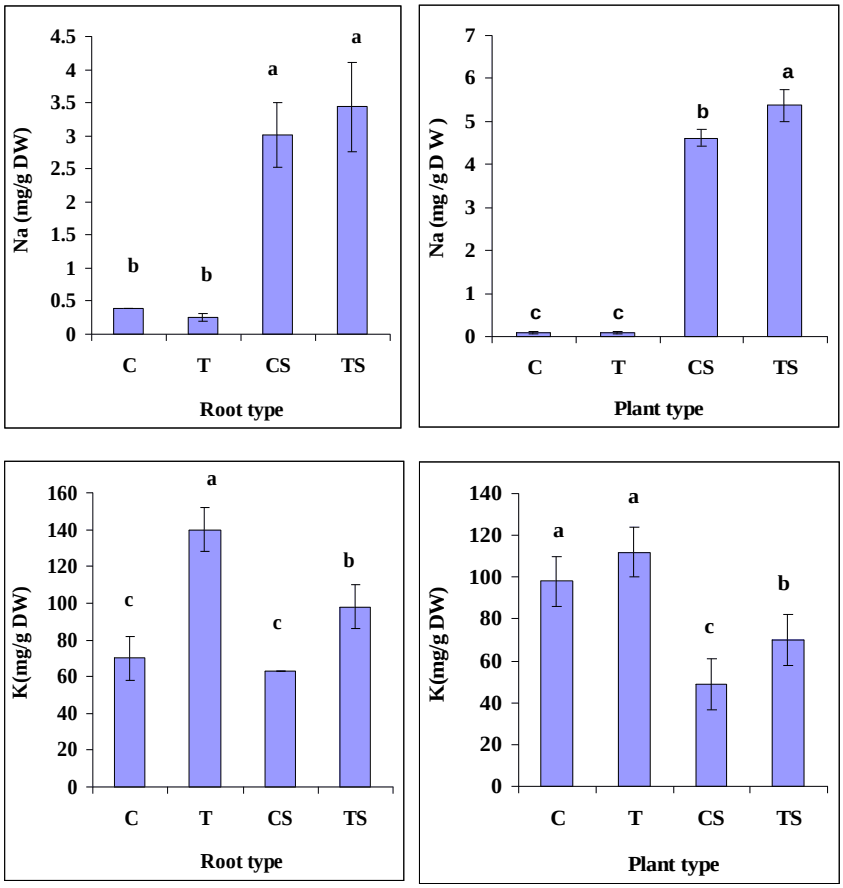


Fig.7. (a) Na^+ in root, (b) Na^+ in shoot, (c) K^+ in root, (d) K^+ in shoot. C, non-transgenic plant without salt; T, transgenic plants without salt; CS, non-transgenic plant in 300 mM NaCl; TS, transgenic plant in 300 mM NaCl. Results are means ± SD, common letters are not significant ($P < 0.05$).

cultivars tested. However, when wheat cultivars were treated with ABA, BA, or CCC, the Na^+ level was decreased dramatically.

The presence of NaCl decreased potassium in roots and shoots. It is generally accepted that competition exists between Na^+ and K^+ leading to a reduced level of internal K^+ with high external NaCl concentration (Altunlu et al., 2007). Potassium content was increased in transgenic roots in medium both with and without 300 mM NaCl. Results showed that 300 mM NaCl induced a significant decrease in K^+ uptake, while T-DNA containing AUX gene expression and auxin production in transgenic plants increased K^+ uptake. It has been documented that K is a compatible solute for plant cells, and increasing levels of K influx will improve salt tolerance of the plant cells (Aghaei et al., 2009). However, in the present study, tobacco as a glycophyte plant did not significantly increase the level of K inside the cell. When the ratio of K/Na content measured the K^+/Na^+ ratio was decreased in roots under saline conditions. The K^+/Na^+ ratio was decreased in shoots in 300 mM NaCl and increased in transgenic shoots with 300 mM NaCl (Fig. 8). The relatively high level of K^+/Na^+ in transgenic plants might be interpreted as the result of transformation of the AUX gene and expression of auxin in the cell. This is in accordance with Bano et al. (2009) who reported that K^+/Na^+ ratio decreased with salt stress and was increased by ABA, BA, or CCC in wheat plants.

Acknowledgments

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