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THE HETERELOGOUS EXPRESSION OF THE *Debaryomyces hansenii DhARO4* GENE IN *Nicotiana tabacum* IMPROVES GROWTH YIELD, EVEN AFTER INHIBITION BY SALINE STRESS

LA EXPRESIÓN HETERÓLOGA DEL GEN *DhARO4* DE *Debaryomyces hansenii* EN *Nicotiana tabacum* MEJORA EL CRECIMIENTO, INCLUSO DESPUÉS DE INHIBICIÓN POR ESTRÉS SALINO

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Abstract

It has recently been reported that the expression of the gene codifying for the first enzyme of tyrosine synthesis pathway Aro4p [3-deoxy-D-arabinoheptulosonate-7 phosphate synthase] is involved in the response of the extreme-tolerant yeast *Debaryomyces hansenii* to salt stress. In addition, and considering that microorganisms and plants share the pathway of synthesis of aromatic amino acids, the *DhARO4* gene codifying for the enzyme Aro4p was incorporated into the *Nicotiana tabacum* plant. It was hypothesized that the resulting plant would better face the salinity derived from irrigation with 100 mM NaCl as compared to pure water irrigation without affecting growth. The genetically modified plants were larger size than the wild type plants under both types of irrigation.

Keywords: *Debaryomyces hansenii*, *DhARO4*, salt stress resistance, transgenic tobacco.

Resumen

Se ha reportado recientemente que en la respuesta de la levadura extremo-tolerante *Debaryomyces hansenii* al estrés salino está implicada la expresión del gen de la primera enzima de la síntesis de tirosina Aro4p [sintasa de 3-deoxy-D-arabinoheptulosonato-7 fosfato]. Aunado a lo anterior y considerando que los microorganismos y plantas comparten la vía de síntesis de aminoácidos aromáticos, se incorporó el gen *DhARO4* de la enzima Aro4p a la planta *Nicotiana tabacum* con la hipótesis de que con ello la planta enfrentaría mejor las condiciones de salinidad mediante riego con NaCl 100 mM en comparación con agua sin sodio y sin afectar su crecimiento. Las plantas modificadas genéticamente tuvieron un tamaño mayor que las plantas silvestres en los dos tipos de riego.

Palabras clave: *Debaryomyces hansenii*, *DhARO4*, resistencia a estrés salino, planta de tabaco modificada genéticamente.

1 Introduction

One of the main problems of agriculture today is the increase of salinity in soils due to natural factors, such as changing cycles of rainfall, wind and drought, also as a consequence of human effects by inadequate soil management. For example, the practice of continuous cultivation cycles without proper land rest in addition to irrigation with untreated wastewater (Shirvastava and Kumar, 2015).

Soil is defined as saline when its salt levels reach or exceed 40 mM of NaCl (Munns, 2005). Plant cultivation in saline soils affects their growth (Azevedo Neto *et al*., 2004; Ferdous *et al*., 2017) due to hydric stress, ionic toxicity and increased production of reactive oxygen species (Sudhir and Murthy 2004; Nawaz *et al*., 2010).

Biotechnology research has been carried out for remediation of waste water (Martínez-Sánchez *et al*., 2017; Kuss *et al*., 2018), soils (Cornu *et al*., 2017) and to improve agriculture in saline soils.

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Recently, through genetic engineering, it has been possible to incorporate genes that confer resistance to salt stress in different plants such as rice, barley, lucerne and tomato (Munns, 2005; Roy *et al*., 2014).

The genes used are taken from salt resistant crop plants, mainly those encoding for monovalent cation transporters and those involved in the synthesis of compatible organic compounds such as mannitol and polyamines, that participate in osmoregulation and cell protection from oxidative damage (Pandey *et al*., 2011; Roy *et al*., 2014). However, there are few works on the application of genes from extremophile microorganisms, which have enzymes that enable them to survive under hard conditions (Guzmán-Rodríguez and Santos, 2017; Flores-Cosio *et al*., 2018).

Our group has determined that in the extremophile yeast *Debaryomyces hansenii* grown in 2M NaCl stress, there is an increase in the expression of *DhARO4* (Calderón-Torres *et al*., 2006), gene coding for the first enzyme of aromatic amino acid biosynthesis [3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase]. This protein is specific for the synthesis of tyrosine and it is feedback regulated by this amino acid. The increase in the expression of *DhARO4* correlated with a significant increase in the specific activity of the *DhARO4p* in the presence of 2M NaCl but without the apparent increase of tyrosine, because the amino acid is oxidized immediately to 3-nitrotyrosine (Calderón-Torres *et al*., 2011). Thus, we proposed that the overexpression of the *DhARO4* gene is a strategy of yeast to eliminate free radicals produced during saline stress. We also observed that an excess tyrosine in culture media containing 2M NaCl increases the growth of *D. hansenii*.

Considering that a) one of the problems of agriculture is the progressive increase of salinity in soils, b) the overexpression of the *DhARO4* gene improves the growth of *D. hansenii* during salt stress, and c) the pathway of synthesis of aromatic amino acids is similar in yeast and in plants (Maeda and Dudareva, 2012; Parthasarthy *et al*., 2018), we introduced the *DhARO4* gene to *Nicotiana tabacum*. In this paper we present the results of the growth parameters when these plants were irrigated with sodium. It was observed that the concentration of sodium and potassium in roots, stems and leaves of *N. tabacum* expressing the *DhARO4* gene were modified.

2 Materials and methods

2.1 *Verification of the presence of the DhARO4 gene in Nicotiana tabacum*

Genetic modification of *N. tabacum* was obtained in collaboration with Dr. Sergio Rosales Mendoza from the Facultad de Ciencias Químicas, U.A.S.L.P., México. The insertion of the gene was done with the bacterial transfection method of *Agrobacterium tumefaciens* (Horsh *et al*., 1985).

After obtaining the plants of *N. tabacum* modified with the *DhARO4* gen, they were grown in a culture medium of Murashige y Skoog (MS) with agar, which contains a mixture of macro and micro nutrients for plant growth, and the DNA was extracted by the hot phenol method (Schmitt *et al*., 1990). The presence of the *DhARO4* gene plus its promoter was assessed in *D. hansenii* (Fig. 1a), wild type *N. tabacum* (Fig. 1b) and *N. tabacum* expressing *DhARO4* (Fig. 1c) through the use of standard PCR. Amplicons for *DhARO4* promoter (lane 1), *DhARO4* gene (lane 2), promoter plus *DhARO4* (lane 3) show insertion of *DhARO4* in *N. tabacum* genome.

Previous to germination and transplantation of *N. tabacum* in soil, the quality and composition of the soil (Table I) was verified in the Edaphology Laboratory of the Unidad of Biotecnología y Prototipos (UBIPRO) of the Facultad de Estudios Superiores Iztacala U.N.A.M. Soluble cations were measured with the modified method of Cheng and Bray, total nitrogen was quantified by the method of Kjedahl modified by Bremner and phosphorus was measured by the Bray I method in Muñoz Iniestra *et al*. (2013).

Table I. Composition of the soil used in the growth of *Nicotiana tabacum*.

Parameter	Value
[a]**Exchangeable Ca^{++}**	3,626
[a]**Exchangeable Mg^{++}**	231
[a]**Exchangeable Na^{+}**	128
Total Nitrogen (%)	0.396
Assimilable Phosphorus (ppm)	2.93
[a]**Exchangeable K^{+}**	294

[(a)] Concentration values in mol/Kg of soil

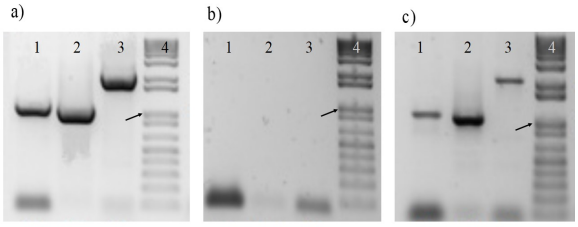

Fig. 1. Presence of the *DhARO4* gene in *Nicotiana tabacum* plant. PCR amplification of *DhARO4* gene: a) in yeast *D. hansenii*; b) in wild *N. tabacum* plant; and c) *N. tabacum* genetically modified. 1107 bp *DhARO4* gene band (lane 1), 850 bp promoter band (lane 2) and 1957 bp promoter and gen band (lane 3); lane 4 is the standard for DNA size in base pairs (bp), the arrows indicate DNA fragment of 1000 bp.

2.2 *Growth of Nicotiana tabacum in saline stress condition*

Seed germination and plant growth was achieved in the greenhouse of the botanical garden of the Facultad de Estudios Superiores Iztacala, U.N.A.M. Twenty seeds of each plant type were germinated, i.e. of wild type *N. tabacum* and of the plant modified with the *DhARO4* gene; they were given a treatment of chemical scarification with a solution of 4% NaClO, followed by a rinse with sterile distilled water in a 10 μm diameter filter and were placed on soil previously filtered with a 3 mm of diameter sieve and sterilized. The seeds were covered with soil and incubated in standard greenhouse conditions: a temperature of 25 ºC, 35% relative humidity and with a cycle of light and darkness of 12 hours.

After three weeks, germinated seedlings were transplanted in soil, which was previously sterilized and filtered on a 1 cm diameter sieve. Both wild type plants and the genetically modified plants were divided into two irrigation groups: *i*) tap drinking water (H_2O), and *ii*) tap drinking water added with 100 mM NaCl (H_2O + NaCl). Plants were allowed to grow for 53 days and were irrigated every four days with a volume of 10 mL. At the end of growth, the length and width of the largest leaf and the length of the stem were measured on all plants. A sample of leaf, stem and root was taken from each plant and placed in 1.5 mL Eppedorf tubes, that were frozen in liquid nitrogen and stored at -70 ºC.

2.3 *Quantification of Na^+ and K^+ soluble ions*

The determination of Na^+ and K^+ ions was made according to the method of González-Hernández *et al.* (2004). A sample of root, stem and leaf was taken from each plant, 200 mg were weighed and then dehydrated in a drying oven for 48 hrs at 80 ºC; then, each sample was placed in 50 mL conical tubes and 10 mL of milli Q distilled water was added. The tubes were incubated in boiling water bath for 1 hour and centrifuged at 4000 rpm for 10 min. The supernatant was recovered in a 15 mL conical tube and a volume of 1 mL was taken for reading in a flame photometer (Zeiss PF5), a solution a NaCl 1 mM y KCl 1 mM was used as a reference solution. The concentration of sodium and potassium in mol/L was obtained from the results of the reading.

2.4 *Analysis of results*

The average, standard deviation (SD) and standard error of the mean (SEM) were calculated from morphologic data and soluble ion concentration and were compared using the Student-*t* test considering significant differences when $P < 0.05$.

3 Results

3.1 *Nicotiana tabacum growth under salt stress conditions*

Wild type and genetically modified plants showed significant differences on leaf average size (Table II) when they were irrigated with H_2O (length 5.0 cm vs 8.2 cm, width 2.6 cm vs 3.9 cm), also in the length of the stem (11.6 cm vs 15.4 cm). Significant differences were also observed on average leaf size when the plants were irrigated with water and 100 mM NaCl (length 2.6 cm vs 3.9 cm), as well as the average length of the stem (10.0 cm vs 14.4 cm), although the size of both plants decreased when compared with the values observed under control conditions (Table II).

3.2 *Quantification of sodium and potassium*

The concentration of Na^+ y K^+ was measured in the root, stem and leaf of either wild type or modified *N. tabacum* plants. Significant differences in the Na^+ concentration were observed with H_2O irrigation.

Table II. Morphological measurements of stem and leaf of wild type and genetically modified plants in two irrigation conditions.

	H_2O irrigation		**H_2O+NaCl irrigation**	
	Wild plants	Genetically modified plants	Wild plants	Genetically modified plants
Leaf width (cm)	3.1 ± 0.21	4.1 ± 0.25*	2.6 ± 0.12	3.9 ± 0.25**
Leaf length (cm)	5.0 ± 0.32	8.2 ± 0.42**	3.6 ± 0.61	7.5 ± 0.65**
Stem length (cm)	11.6 ± 1.85	15.4 ± 0.66*	10.0 ± 1.15	14.5 ± 0.56*

Average values ± standard deviation; $n = 3$. The asterisks indicate significant differences compared with wild type plant by Student-t test, (*) when $P < 0.05$, (**) when $P < 0.01$.

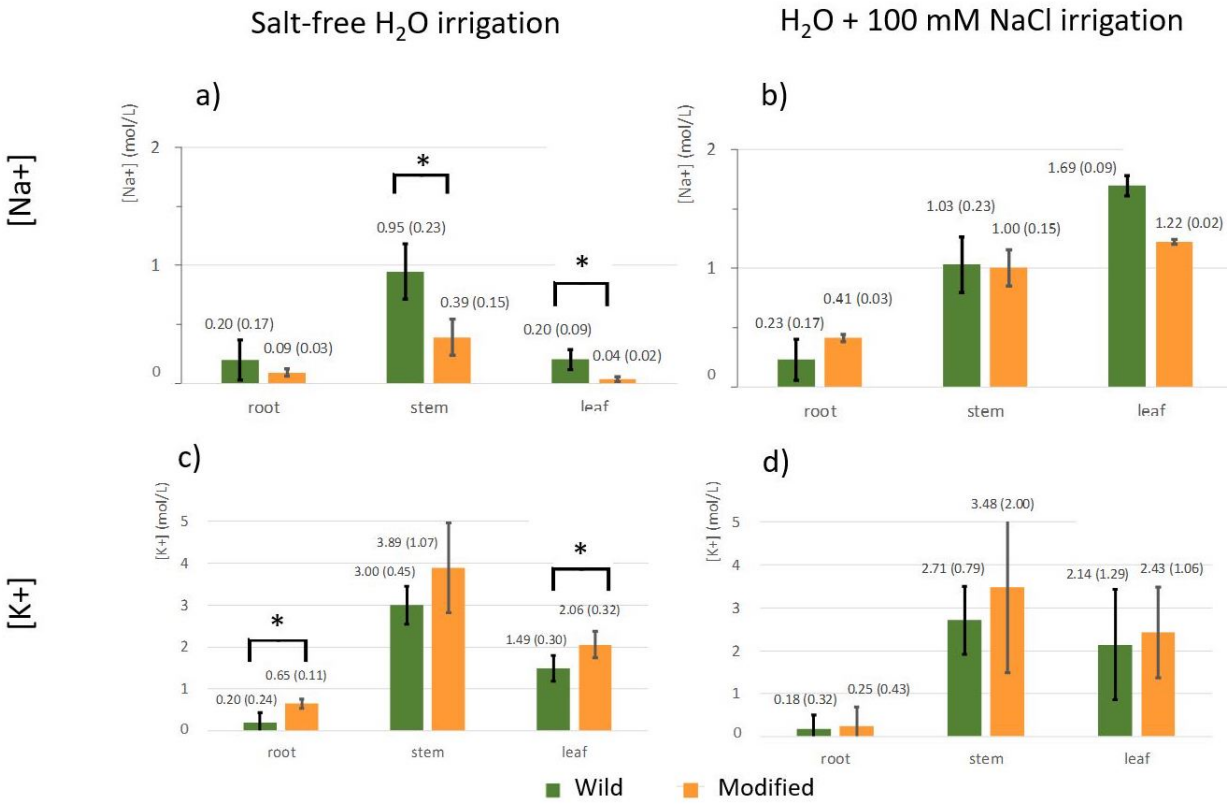

Fig. 2. Soluble ions Na^+ and K^+ in root, stem and leaf of *Nicotiana tabacum* wild and modified with *DhARO4* gene, in two irrigation conditions. Concentration of sodium (a) and potassium (c) of plants with fresh water irrigation, concentration of sodium and potassium of plants with H_20 + 100 mM NaCl irrigation (b,d). Average values ± (standard error); * $P < 0.05$.

Genetically modified plants accumulated a lower sodium concentration, particularly in leaf and stem the sodium concentration was lower ($P < 0.01$; Figure 2a). Na^+ concentration in wild type plant stems was more than double that of the modified plants (0.95 mol/L vs 0.39 mol/L), while in the leaves the average was five times higher (0.2 mol/L vs 0.04 mol/L). No statistically significant differences were observed at the roots.

Although no statistically significant differences were observed in Na^+ concentration in any of the three parts of the plant when irrigated with H_2O

+ NaCl, there was a clear tendency to accumulate sodium in the leaves and the stem in both types of plants (Figure 2b), as has been demonstrated in plants with saline irrigation (Hussain *et al*., 2016). Regarding the average concentration of potassium ions in plants irrigated only with H_2O, it was observed that the stem is the part of the plants with the highest concentration, but with no significant difference between both types of plants.

Significant differences were observed in the average potassium concentration in the root and leaf (Figure 2c). In the root of genetically modified plants, the average concentration of potassium was higher ($P < 0.01$) than in wild type plants (0.65 mol/L vs 0.22 mol/L). Similarly, the leaves of the modified plants maintained an average K^+ concentration higher compared to that of wild type plants (2.06 mol/L vs 1.49 mol/L).

When the plants were irrigated with H_2O + NaCl, no significant differences were found in the K^+ content within any of the analyzed parts. The modified plants showed a slightly higher potassium concentration than the wild type plants, and again it was observed that in both types of plants, potassium was preferably stored in the stem (Figure 2d).

4 Discussion

The growth of plants in saline soils has been described mainly in two phases: an initial one, which occurs in the first hours, when the plant activates signaling pathways in response to salt stress (Munns, 2002; Roy *et al*., 2014) and observed effects range from stoma closure and growth delay, to inhibition of leaf apparition and expansion (Mena *et al*., 2015). The second phase comprises days or weeks and the plant accumulates ions in toxic concentrations, especially in the leaves. This leads to its premature ageing or to its death, and decreases the number of flowers and fruits (Assia *et al*., 2014; Orlovsky *et al*., 2016).

In this work we observed that the growth of the wild type *N. tabacum* plants is reduced when irrigated with H_2O + NaCl and that the leaves accumulate Na^+ without a significant increase of K^+. Similar results were reported in the growth of wild type tobacco plants irrigated for 30 days with water + 250 mM NaCl (Ben-Romdhane *et al*., 2017) and for 42 days with water + 200 mM NaCl (Yan *et al*., 2008) and it was observed that in the leaf there is a lower uptake of potassium, which is probably what affects its growth. Bahrami-Rad and Hajiboland (2017) reported in *N. tabacum* exposed to drought stress, that the reduction in root and leaf size is reverted after a potassium treatment is applied.

In the plants modified with *DhARO4* gene it was observed that the parameters of growth such as the width and length of the leaf, and also the length of the stem are higher than those of wild type plants, either with irrigation of H_2O or H_2O + NaCl. Previously in *Arabidopsis thaliana* the gene *AROG* of bacteria was inserted, which also codifies for the 3-deoxy-7-phosphoheptulonate synthase (DAHPS) but it is feedback regulated by phenylalanine, the insertion of the modified *AROG* gene improved the growth of *A. thaliana* in presence of tryptophan analogue 5-methyltrypthopan (Tzin *et al*., 2012), so it was proposed that DAHPS enzymes are a key point between primary and secondary metabolism.

It is probable that plants modified with *DhARO4* and irrigated with salt-free H_2O showed a greater size because the expression of *DhARO4* results in the synthesis of tyrosine and in absence of salt stress the plant uses it for obtaining energy through tyrosine assimilation pathways (Hildebrandt *et al*., 2015) and this energy will be useful for *i*) activating mechanisms to expel sodium and accumulate potassium, this could explain the significant differences of the two types of plants in terms of the content of Na^+ and K^+ ions, and *ii*) accumulate carbohydrates that result in increased plant growth. While upon irrigation with H_2O + NaCl modified plants reduce their size but remain larger than the wild type plants. However, the significant difference in size can not be explained by the Na^+ and K^+ content in root, stem and leaf, because in both types of plants the Na^+ and K^+ concentrations are similar. It has been reported that salt stress in plants leads to oxidative stress (Kawano *et al*., 2002; Mittal *et al*., 2012; Ahanger and Agarwal, 2017), so we consider that in the plant modified with the *DhARO4* gene, the salt stress leads to the over expression of the *DhARO4* gene with the increase of tyrosine, which functions as an antioxidant to reduce free radicals just as in the yeast *Debaryomyces hansenii* exposed to salt stress by 2M NaCl (Calderón-Torres *et al*., 2011).

Although it is evident that the plant *N. tabacum* modified with the *DhARO4* gene has a higher growth than the wild type plant during salt stress, research on production of free radicals, changes in photosynthesis and the content of chlorophylls, as well as determination of their antioxidant capacity is still needed.

Conclusions

This work presents for the first-time evidence that the transfer of the *DhARO4* gene to *Nicotiana tabacum* plants confers resistance to salt stress, since during irrigation with water plus sodium, the size of the stem and leaf are larger compared to the values of wild type plants. It is probable that resistance of the genetically modified plants to high soil salinity is due to the fact that the expression of the *DhARO4* gene produces an antioxidant effect.

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