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The insertion of an ancestral gene in *Nicotiana tabacum* plants reduces free radicals during saline irrigation

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Abstract: Transgenic organisms modified with ancestral genes for nitrogen metabolism are rare. Previously, it was reported that genetically modified *Nicotiana tabacum* with the *ARO4* gene of aromatic amino acid synthesis from the yeast *Debaryomyces hansenii* increases its growth during moderate salt stress. In this investigation, it was explored if the changes in the expression of the gene *DhARO4* in *Nicotiana tabacum*, during saline irrigation, are related to the chlorophyll content and the total reactive oxygen species production. Seedlings of transgenic and wild type *Nicotiana tabacum* germinated in standard conditions were divided into two irrigation groups, with 100 mM of NaCl and with tap water; and, after 50 days, in the non-senescent adult leaves of the plants, the total chlorophyll a and b and the total chlorophyll content were determined by spectrophotometry and the reactive oxygen species production (•OH, $^1O_2$, H_2O_2) was quantified by a 2',7'-dichlorodihydrofluorescein assay. The expression of the *DhARO4* gene was verified with a salt shock of 100 mM of NaCl for 24 hours in the transgenic and wild type plants in the tap water irrigation group. The *DhARO4* gene transcript increased ($P < 0.05$) in the transgenic plant; meanwhile, the average concentration of chlorophyll a increased ($P < 0.05$), and the average production of reactive oxygen species decreased ($P < 0.05$).

Keywords: gene *DhARO4*; gene expression; genetically modified organisms; salt stress; tobacco plant

One of the main problems in commercial crops is the salinisation of soils, resulting in a reduction in the plant and fruit size. Genetic improvement is one of the main strategies for obtaining plants resistant to high salinity in soils. Plant breeding research is extensive; it mainly involves the modification of plants with genes that encode ion transport proteins in the plasma membrane or in the vacuole, the synthesis of compatible organic compounds to compensate for the osmotic problem of the salts present in the soil or irrigation water, the antioxidants that neutralise the free radicals generated during salt stress and the regulators of the transcription of genes involved in the response to the increased salinity (Roy et al. 2014; Esmaelli et al. 2022). However, an alternative is the search for ancestral genes still shared by plants, prokaryotes, and yeast.

In the primordial environment of Earth, high temperatures prevailed with a reducing atmosphere, which facilitated the spontaneous synthesis of or-

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ganic compounds and led to their abundance. For example, it is believed that this environment was rich in the amino acids, alanine and aspartate, and that, on the contrary, other amino acids, such as methionine, histidine, and tyrosine, were less abundant and soon ran out. The scarcity of amino acids led to their synthesis by the primordial cells (Maeda & Fernie 2021). In the phylogenetic reconstruction of the first cells, it has been proposed that they were thermophilic autotrophs (Weiss et al. 2018) and that the amino acid synthesis pathways appeared before the last universal common ancestor (LUCA). One of the amino acid synthesis pathways shared by microorganisms and plants is the aromatic amino acids pathway, and the first gene of this pathway *ARO4* (*AROG*, *AROF*, *ARO4*, *DHS1/3*, *DHS2*; names they received in different organisms) encodes the 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase enzyme. This enzyme is crucial in the synthesis of chorismate, essential not only to produce phenylalanine, tyrosine, and tryptophan, which will be a part of proteins, but also a precursor of other compounds including ubiquinone and vitamin K essential in the transport of electrons in chloroplasts (Tzin & Galili 2010; Yokoyama et al. 2022) as well as phenolic compounds and flavonoids, that act as antioxidants. According to the phylogenetic reconstruction, DAHP synthase enzymes are classified into families I and II, the former including a DAHP synthase enzyme that was only present in LUCA (Tzin & Galili 2010), currently found in bacteria and yeasts, while family II is a characteristic of plants and has limited presence in bacteria and fungi. The comparison of the nucleotide sequence of this gene between different organisms of the two classes of DAHP synthase enzyme shows a similarity of only 10% (Light & Anderson 2013); paradoxically, the function of the enzyme encoded by this gene is the same, and it has been proposed that this function is preserved due to the similarity in the secondary conformation of the enzyme's amino acids (Webby et al. 2005).

In general, in organisms that live in adverse or extreme environments, amino acid changes in the catalytic site of their enzymes are associated with adaptations that ensure their function in these environments (D'Amico et al. 2002; DasSarma & DasSarma 2015). In addition, aromatic amino acids are of interest in the study of remediation of the negative effects on crops due to saline soils. In different plants growing in the presence of sodium, it has been reported that foliar irrigation with the amino acids, tyrosine and phenylalanine, improves the growth and increases the antioxidant capacity (Al-Mohammad & Al-Taey 2019; Atteya et al. 2022).

Tyrosine, a monophenolic amino acid, was reported as an antioxidant (Mooshmann & Behl 2000; Gülçin 2007; Dorta et al. 2018) that reacts with the radical peroxinytrite ($ONOO^-$) and produces the oxidised compound 3-nitrotyrosine (Radi 2004), a mark of oxidative stress. It was reported that the yeast *Debaryomyces hansenii* grown in media with 2M of NaCl overexpressed the *DhARO4* gene, and by means of a complementation assay on the null mutant aro4Δ of the yeast *Saccharomyces cerevisiae*, it was confirmed that the *DhARO4* gene was its functional orthologue (Calderón-Torres et al. 2006). The overexpression of the *DhARO4* gene during salt growth conditions leads to an increase in the specific activity of the DhAro4p enzyme, but no tyrosine was detected; it was 3-nitrotyrosine (Calderón-Torres et al. 2011); tyrosine oxidation prevents the damage to biomolecules.

One of the main problems of crops in saline soils is the elevated production of free radicals, for example, reactive oxygen species (ROS) with the consequent loss of the chlorophyll content (Taïbi et al. 2016; Lu et al. 2023), which, in turn, leads to the senescence of the leaves and the reduction of the size of the plant and its fruits. It was reported that *Nicotiana tabacum* transformed with the *DhARO4* gene had a significant increase in the morphometric parameters during salt growth conditions (Calderón-Torres et al. 2019); however, it was not evaluated whether the growth improvement is related to a decrease in the ROS production and, as a consequence, to less damage to the chlorophyll. Therefore, in this work, it was evaluated if the expression of the gene *DhARO4* in *N. tabacum* reduces the reactive oxygen species and increases the chlorophyll content during growth of *N. tabacum* with and without saline irrigation.

MATERIAL AND METHODS

Germination and growth of tobacco plants. The plant *Nicotiana tabacum* L. was germinated and grown in the Botanical Garden of Facultad de Estudios Superiores Iztacala, U.N.A.M. The seeds of the *N. tabacum* used in this work come from the research work of Calderón-Torres et al. (2019), who modified *N. tabacum* with the promoter + *DhARO4* gene; wild type and transgenic seeds were germinated in sterile soil, filtered with a 3 mm mesh sieve and

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incubated under standard growth conditions at 25 °C, 35% relative air humidity and 12-h light/dark cycles. After germination, the seedlings were transplanted into sterile soil of standard composition (Calderón-Torres et al. 2019).

The wild strain and *DhARO4* gene-modified plants were divided into two irrigation groups composed of five plants each: (*i*) tap water from Alcaldía Miguel Hidalgo County, Mexico City, and (*ii*) a 100 mM NaCl saline solution. After 50 days of growth with irrigation consisting of 15 mL every third day, samples of the non-senescent adult leaves were collected and placed in 1.5 mL Eppendorf tubes, frozen in liquid nitrogen, and stored at –70 °C until later use for the ROS and chlorophyll quantification of the complete batch of samples.

To evaluate the expression of the *DhARO4* gene, three wild-type plants and three plants from two transgenic lines (with the promoter + *DhARO4* gene) of the tap water irrigation group, were subjected to salt shock with 100 mM of NaCl for 24 h. Subsequently, samples of two non-senescent adult leaves were collected from each plant and the total RNA was obtained using the Amraee and Rahmani (2020) method, with minor modifications. In a 1.5 mL Eppendorf tube with 100–150 mg of leaves, 450 µL of a cetyltrimethylammonium bromide (CTAB) buffer [CTAB 2%, 20 mM EDTA, 1.4 M NaCl, 10 mM tris pH 8.0 and PVP-40 2%] (pre-warmed at 65 °C) and 9 µL of β-mercaptoethanol were added. The tissue was macerated with a polypropylene micropistil for Eppendorf tubes (KIMAX). The macerate was incubated at 65 °C for 10 min, allowed to cool to room temperature, and then 450 µL of chloroform/isoamyl alcohol (24 : 1) was added, vortexed for 30 s, and centrifuged at 12 470 × *g* for 10 min at 4 °C. The supernatant was transferred into a new sterile Eppendorf tube, 450 µL of acid phenol and 250 µL of chloroform/isoamyl alcohol were added, vortexed, and centrifuged at the same conditions. The aqueous phase was transferred to a new sterile Eppendorf tube and 0.2 volumes of 3M of sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol were added, allowing it to precipitate overnight at –20 °C. After centrifugation, the RNA pellet was solubilised in 40 µL of water previously treated with diethyl pyrocarbonate (DEPC). In all the RNA samples, the DNA remnants were removed with the DNA degradation enzyme of a commercial DNA-free system (Ambion RNA, life Technologies). The RNA integrity was verified by 1% agarose gel electrophoresis.

Real-time semi-quantitative reverse transcription and amplification reaction (RT-PCR). Oligonucleotides were designed for the amplification of a 125 base pair fragment of the gene encoding Elongation Factor 1-alpha (*EF-1-alpha*) from *Nicotiana tabacum* (NCBI accession number: NM_001326165) forward 5'-GTCATTGGCCACGTCGACT-3' and reverse 5'-GACCTCTTGTTCATCTCAGCA-3', and for a 120 base pair fragment of the gene *DhARO4* of the yeast *Debaryomyces hansenii* (NCBI accession number: XM_456529.1), forward 5'-GCCAGGTCCAAAAGAAGACA-3' and reverse 5'-CTTTCTTGCACGGCCTTACT-3'. The RT-PCR reactions were conducted with a commercial system: Power SYBR Green RNA-to-CT TM One-Step Kit (Applied Biosystems). Reactions were performed in triplicate with a total RNA concentration of 25 ng/µL and oligonucleotides in a concentration of 900 mM, in a final volume of 10 µL, added to a 0.1 ml 96-well plate, and processed in the StepOnePlus Real-Time PCR System (Applied Biosystems). The thermal cycling conditions were followed according to the supplier's recommendations. The dissociation curves and the standard curves of each gene were obtained in duplicate from the leaf samples of three plants.

The relative gene expression was calculated from the cycle threshold (Ct) values of the reference gene *EF-1-alpha* and of the gene of interest *DhARO4* according to Pfaffl (2001), and the standard deviation and standard error values were calculated according to Bakó et al. (2013).

Total cell extracts of *Nicotiana tabacum*. From each plant, two non-senescent adult leaves were selected to obtain a cell extract. One hundred (100) mg was taken from each leaf and placed in 1.5 mL Eppendorf tubes; 300 µL of 10 mM phosphate buffered saline (PBS) and 3 µL of 100 mM phenylmethylsulfonyl fluoride (PMSF) were added to each tube, and the samples were homogenised with a teflon micropistil at 4 °C. The tubes were then centrifuged at 12 470 × *g* for 5 min at 4 °C. The supernatant was transferred to a new Eppendorf tube and incubated at 4 °C. The protein concentration in the cell extract was determined by UV light spectrophotometry with a bovine serum albumin (BSA) protein standard curve.

Production of the reactive oxygen species (ROS). The ROS levels in the leaves were determined according to the modified method of Hempel et al. (1999). Briefly, an aliquot of each total cell extract described in the previous section was placed in a well of a 96-well plate for the following reaction: 15 µL of the total

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cell extract, 230 µL of 10 mM PBS, and 5 µL of dichlorodihydrofluorescein diacetate (DCFH-DA) (500 µM). Emission readings were recorded every 20 min for 1 h in a fluorescence spectrophotometer (FLX800 TB Microplate Fluorescence reader, Bio Tek Instruments, Inc., USA) at 485 nm excitation and 520 nm emission. The maximum fluorescence value obtained was used as an index for the ROS production per mg of the total protein.

Chlorophyll quantification. The total chlorophyll a and b content was measured by the method of Porra et al. (1989). One hundred to three hundred (100 to 300) mg of two non-senescent adult leaves were taken from each plant and placed in a 1.5 mL Eppendorf tube. Then, 1 mL of acetone (80%) was added, and the mixture was macerated with a teflon micro-pistil. The supernatant was transferred into a 15 mL Falcon tube. This step was repeated once again. Acetone (80%) was added to the final volume of 5 mL, the Falcon tube was vortexed and centrifuged at 500 × *g* for 10 min. In a quartz cell, 1 mL of the chlorophyll extract was added, and the absorbance reading was recorded in a spectrophotometer at 645 and 663 nm. The absorbance data obtained were used to calculate the chlorophyll concentration in µg/mL.

Statistical analysis. Each of the three groups of plant lines (wild-type, WT, and transcgenic lines L1, and L2) and each irrigation group (tap water and saline) were compared using an analysis of variance (ANOVA); this was conducted independently for the genetic expression data, ROS production, and chlorophyll a, b, and total content. When the ANOVA resulted in significant differences, Tukey's test was applied to identify the pairs of groups with meaningful differences. Significant differences were considered when $P < 0.05$. Analyses were performed using Tukey's HSD function of the R package.

RESULTS

Expression of the *DhARO4* gene in *Nicotiana tabacum*. In the transgenic and wild-type tobacco plants irrigated with tap water, salt shock was applied with a 100 mM of an NaCl solution for 24 h; and, in the leaves from each plant, the expression of *DhARO4* gene was quantified. The expression of the *DhARO4* gene increased up to an average value of 2.25 times compared to the expression of the *EF-1-alpha* reference gene (Figure 1).

Production of reactive oxygen species in *Nicotiana tabacum* modified with the *DhARO4* gene. To determine whether the expression of the *DhARO4* gene is related to the changes in the production of ROS in the total cell extracts of the non-senescent adult leaves of the wild-type and transgenic plants (L1 and L2) under two irrigation groups (tap water and saline solution), the production of ROS was evaluated. It was observed that with tap water irrigation, the average value of the ROS production from the wild-type and transgenic plants did not change. On the contrary, in the cell extracts of the leaves from the transgenic plants irrigated with 100 mM of an NaCl solution, the average ROS value was lower than in the cell extracts of the leaves from the wild-type plants. The ROS production was significantly lower for the transgenic plants of line L2 ($P < 0.05$) (Figure 2).

Quantification of the chlorophyll in *Nicotiana tabacum* modified with the *DhARO4* gene. In the leaves of the wild-type and transgenic plants irrigated with tap water, the average concentration of the chlorophyll content did not change. On the contrary, with the irrigation with 100 mM of the NaCl solution, it was observed that the chlorophyll a content increased significantly in the leaves of the transgenic plants L1 and L2 ($P < 0.05$) compared to the chlorophyll a content of wild-type plants. The total chlorophyll content increased significantly in the transgenic plants L1 ($P < 0.05$) and L2 ($P < 0.01$) (Figure 3). In the leaves of the wild-type and transgenic plants

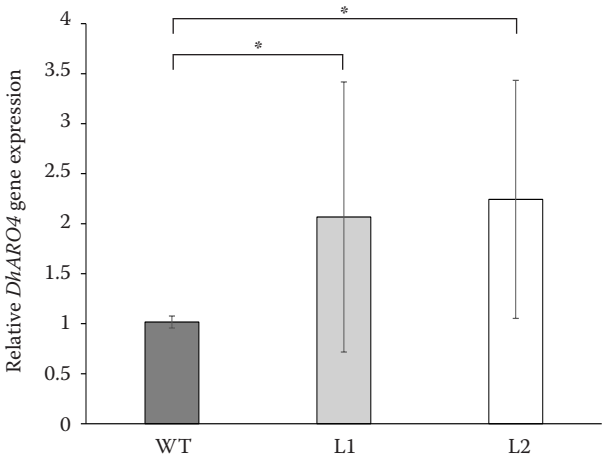

Figure 1. Relative expression of the *DhARO4* gene in the leaves of *Nicotiana tabacum* through real-time RT-PCR
The expression values were obtained with the Ct values of the *EF-1-alpha* reference gene and the *DhARO4* gene in two non-senescent adult leaves of each plant of the wil-type (WT; $n = 3$) and of the two transgenic plants (L1 and L2; $n = 3$ for each one) after salt shock with 100 mM of NaCl for 24 h; the error bars are the standard deviation; *$P < 0.05$

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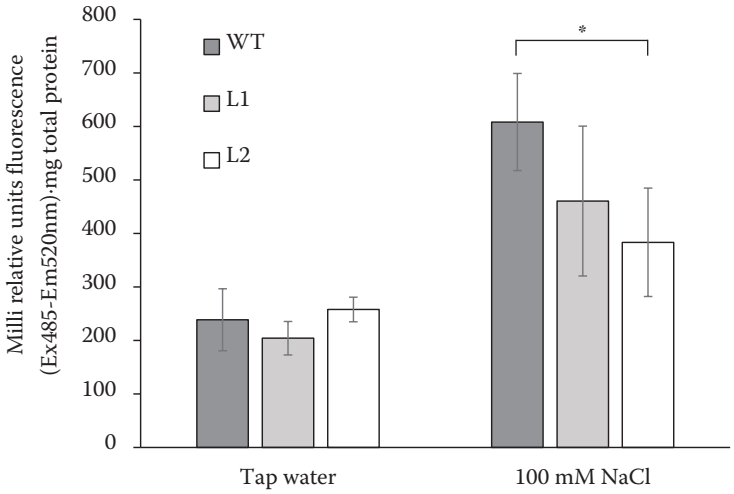

Figure 2. Production of reactive oxygen species (ROS) in *Nicotiana tabacum*

ROS was quantified by the assay of DCFH-DA in the total cell extract from two non-senescent adult leaves of each wild-type (WT) plant with tap water (n = 3) and with 100 mM of the NaCl solution (n = 3) irrigation, and also in the total cell extract from two leaves of each transgenic plant with tap water (L1, n = 3 and L2, n = 3) and with 100 mM of the NaCl solution (L1, n = 3 and L2, n = 3) irrigation; the error bars are the standard deviation; *P < 0.05

with the two types of irrigation, the average value of the chlorophyll b content remained unchanged.

DISCUSSION

The growth of plants in saline conditions leads to an increase in the free radicals and a loss of redox balance in the cells, resulting in the oxidation of proteins and lipids involved in different processes, such as chlorophyll synthesis, thereby leading to a decrease in the ability of photosynthesis (Lu et al. 2022). In general, plants respond to an increase in free radicals: reactive oxygen species (ROS) or nitrogen (RNS), activating an antioxidant response, which involves enzymatic systems such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) enzymes and non-enzymatic systems, such as phenolic compounds, flavonoids, and ascorbic acid (Jin et al. 2021). However, the antioxidant response may be lower or inhibited by the excess ROS production (Hajihashemi et al. 2022) due to the constant presence of salts in the cropland or irrigation water. The decrease in antioxidant systems leads to a loss in the chlorophyll content, then to chlorosis or yellowing of the leaves, and finally to leaf drop; when there is no leaf drop, the size of the plant may decrease. In transgenic plants with different genes, not only was there a decrease in the production of reactive oxygen species, but also an increase in the specific activity of antioxidant enzymes was reported (Jin et al. 2021). For example, in *Arabidopsis thaliana* modified with the gene *G6PD* encoding the enzyme, glycerol phosphate dehydrogenase, a reduction in the ROS was observed (Jin et al. 2021).

In this study, it was observed that, in transgenic tobacco plants irrigated with 100 mM of the NaCl

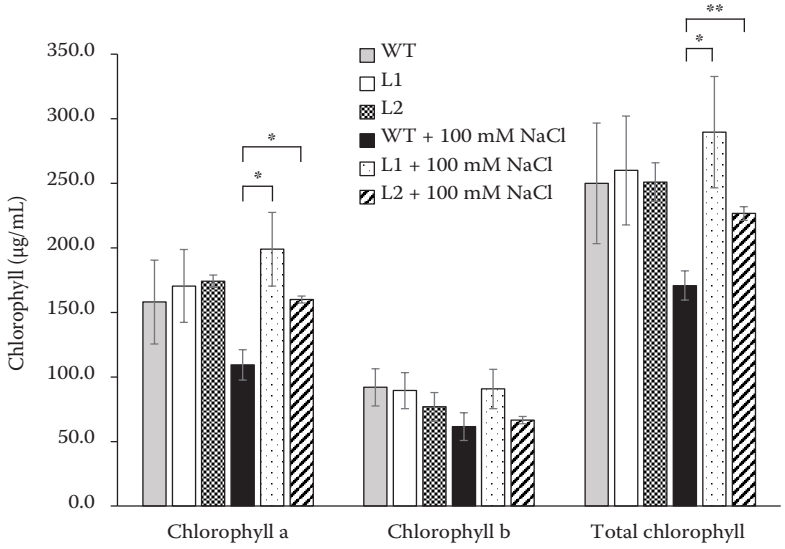

Figure 3. Chlorophyll content in the wild and transgenic strains of *Nicotiana tabacum*

In the non-senescent adult leaves of the wild-type (WT) and in two transgenic lines (L1 and L2) of the plant modified with the *DhARO4* gene, the chlorophyll a, b, and total chlorophyll content was measured; the chlorophyll content corresponds to the average value of the WT (n = 3), L1 (n = 3) and L2 (n = 3) plants for each type of irrigation (tap water and saline); the error bars are the standard deviation; *,**P < 0.05, 0.01

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solution, the ROS production was lower than in the wild-type plants. The lowering of the ROS levels reduces the biomolecule damage, and this, in turn, contributes to the increased growth reported previously in the presence of sodium (Calderón-Torres et al. 2019).

In plants exposed to high salinity, structural and morphological changes can be associated with biomolecule damage. For different plants, a decrease in the number and size of chloroplasts, the alteration of the structure of the lamellar cells in the collenchyma (which is a plant support tissue), as well as changes in the content of proteins and photosynthetic pigments have been reported, particularly a decrease in the chlorophyll concentration (Lu et al. 2022; Zahra et al. 2022). In the present investigation, only after irrigation with 100 mM of the NaCl solution did a significant increase in the chlorophyll content occur in the leaves of the two transgenic lines, but not in the leaves of the wild-type plants.

In plants, antioxidants derived through phenolic and flavonoid compounds are synthesised from three pathways: shikimic acid, acetic acid-malonic, and the acid-mevalonate pathways. It has been reported that in some varieties of *N. tabacum*, an enhanced content of phenolic compounds coincides with an increased antioxidant capacity (Zou et al. 2021). It would be expected that, in transgenic tobacco plants exposed to saline irrigation, the *DhARO4* gene expression leads to an increase in the specific activity of DhAro4p, and, as a consequence, to the increase in the monophenolic tyrosine levels, which react with the free radicals. Therefore, the expression of the *DhARO4* gene detected in transgenic plants after irrigation with sodium is a response to the oxidative stress that probably contributes to the reduction in the ROS levels observed in the leaf extracts and, in turn, diminishes the damage to proteins and photosynthesis pigments. In this approximation, chlorophyll a would be one of the main pigments that is protected from oxidation.

CONCLUSION

The growth of the tobacco plant modified with the *DhARO4* gene from the yeast *Debaryomyces hansenii* during saline irrigation is better than that of the wild strains due to the reduction in the reactive oxygen species content. Most likely, the reduction in the ROS has a positive impact on the increase in the chlorophyll a content.

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