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Effect of overexpression of citrus *9-cis-epoxycarotenoid dioxygenase 3* (*CsNCED3*) on the physiological response to drought stress in transgenic tobacco

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Genet. Mol. Res. 16 (1): gmr16019292
Received September 15, 2016
Accepted February 16, 2017
Published March 30, 2017
DOI http://dx.doi.org/10.4238/gmr16019292

ABSTRACT. *9-cis-epoxycarotenoid dioxygenase* (*NCED*) encodes a key enzyme in abscisic acid (ABA) biosynthesis. Little is known regarding the regulation of stress response by NCEDs at physiological levels. In the present study, we generated transgenic tobacco overexpressing an *NCED3* ortholog from citrus (*CsNCED3*) and investigated its relevance in the regulation of drought stress tolerance. Wild-type (WT) and transgenic plants were grown under greenhouse conditions and subjected to drought stress for 10 days. Leaf predawn water potential (Yw_{leaf}), stomatal conductance (*gs*), net photosynthetic rate (*A*), transpiration rate (*E*), instantaneous (*A/E*) and intrinsic (*A/gs*) water use efficiency (WUE), and *in situ* hydrogen peroxide (H_2O_2) and abscisic acid (ABA) production were determined in leaves of irrigated and drought-stressed plants. The Yw_{leaf} decreased throughout the drought

stress period in both WT and transgenic plants, but was restored after re-watering. No significant differences were observed in *gs* between WT and transgenic plants under normal conditions. However, the transgenic plants showed a decreased ($P \leq 0.01$) *gs* on the 4th day of drought stress, which remained lower ($P \leq 0.001$) than the WT until the end of the drought stress. The *A* and *E* levels in the transgenic plants were similar to those in WT; therefore, they exhibited increased *A/gs* under drought conditions. No significant differences in *A*, *E*, and *gs* values were observed between the WT and transgenic plants after re-watering. The transgenic plants had lower H_2O_2 and higher ABA than the WT under drought conditions. Our results support the involvement of *CsNCED3* in drought avoidance.

Key words: Drought tolerance; NCED; Abscisic acid (ABA); *Citrus limonia*

INTRODUCTION

Adverse impacts on crop productivity are caused by drought, the major environmental stress affecting agricultural production worldwide. The mechanisms of drought response have been most extensively investigated in the model plant *Arabidopsis* (Todaka et al., 2015). These studies have demonstrated that the phytohormone abscisic acid (ABA) is a major regulator that mediates stomatal closure and signal transduction during the response to drought stress for controlling the transpiration rate and the expression of genes involved in drought resistance, respectively.

A key enzyme involved in ABA biosynthesis is 9-*cis*-epoxycarotenoid dioxygenase (NCED), which catalyzes the rate-limiting step in the regulation of ABA biosynthesis (Iuchi et al., 2001). *NCED*s belong to a small multigene family containing five members (*NCED2*, *3*, *5*, *6*, *9*) in *Arabidopsis thaliana* (Tan et al., 2003). *NCED3* is mainly responsible for ABA accumulation under drought stress (Iuchi et al., 2001), whereas *NCED6* and *NCED9* have been associated with ABA synthesis during embryo and endosperm development (Lefebvre et al., 2006). More recently, *NCED5* was demonstrated to act together with *NCED6* and *NCED9* in the induction of seed dormancy, and with *NCED3* in the drought stress tolerance (Frey et al., 2012). The overexpression of *NCED* orthologs from tomato (Thompson et al., 2000), cowpea (Aswath et al., 2005), peanut (Wan and Li, 2006), gentian (Zhu et al., 2007), and *Stylosanthes* (Zhang et al., 2009) has been demonstrated to increase ABA synthesis and to enhance drought tolerance in transgenic plants. These data suggest that genetic manipulation of *NCED*s is an effective approach for engineering drought resistance in transgenic plants.

Although *NCED* genes have been well characterized, especially in *Arabidopsis*, the available data on their functions at the physiological level are still fragmentary, precluding the exploitation of the potential of this gene family for improving stress tolerance in plants. A reduction in the transpiration rate of leaves under normal growth conditions was observed in transgenic *Arabidopsis* plants overexpressing *AtNCED3* (Iuchi et al., 2001). Reduced stomatal conductance under unstressed conditions was observed in transgenic tomato overexpressing *LeNCED1* (Thompson et al., 2000). More recently, significant decreases in net photosynthetic rate (*A*), transpiration rate (*E*), and stomatal conductance (*gs*) were reported in drought-stressed transgenic petunia plants expressing *LeNCED1* under the control of stress-inducible promoter

rd29A (Estrada-Melo et al., 2015). However, further evidence supporting the relevance of *NCEDs* in the physiological functions of plants, grown not only under normal conditions but also under environmental stress conditions, such as drought, remain to be demonstrated. Such evidence would render them a potentially more valuable target for engineering drought tolerance in plants.

Citrus fruits are the most economically important fruit crops cultivated in many tropical and subtropical areas of the world, where drought is a major environmental stress limiting their growth and productivity. Most citrus rootstocks are intolerant to drought, and therefore, efforts have been made to improve their drought tolerance (Gong and Liu, 2013). The relationship between ABA accumulation and expression of putative *NCED*s in vegetative and reproductive tissues of drought-stressed mandarins has been investigated, and a correlation between the expression of the orthologs of *NCED2*, *3*, and *5* and the pattern of ABA accumulation has been observed in their leaves and roots (Agustí et al., 2007; Neves et al., 2013). The overexpression of citrus *NCED5* was also shown to be correlated with the ABA levels in ripening fruits (Agustí et al., 2007). A citrus *NCED3* ortholog, *CrNCED1*, was isolated from 'Cleopatra' mandarin (*Citrus reshni*) and overexpressed in transgenic tobacco plants, which exhibited 1.3-3.0-fold higher levels of ABA under normal growth conditions and enhanced the ABA synthesis and tolerance under dehydration, drought, salt, and oxidative stresses when compared with WT (Xian et al., 2014). However, the effects of *CrNCED1* expression on physiological parameters of transgenic plants were not evaluated in the afore-mentioned study. In the present study, we investigated the effect of overexpression of *CsNCED3*, the *NCED3* from *Citrus* species, in the physiological response of transgenic tobacco plants to drought stress conditions.

MATERIAL AND METHODS

CsNCED3 cloning and generation of transgenic tobacco plants

The coding sequence of *CsNCED3* was amplified from the roots of drought-stressed 'Rangpur' lime (*Citrus limonia* Osbeck) by RT-PCR, using the primers 5'-ATGGCGGCAGCAACTACTACTT-3' and 5'-TTAGGCCTGCTTGGCCAAATC-3', and the product was purified and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). The identity of the cDNA fragment was confirmed by sequencing. The *CsNCED3* cDNA was 1,821 bp in length and contained an open reading frame (ORF) encoding a deduced protein of 606 amino acid residues, with a calculated molecular weight of 67.0 kDa and a theoretical isoelectric point of 6.37, corresponding to the identical sequence of locus 'Ciclev10019364m' in the citrus reference genome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Cclementina). The protein is predicted to contain a N-terminal chloroplast transit peptide (cTP) of 66 amino acids in length. The *CsNCED3* fragment was removed from pGEM-T by digestion with *Eco*RI and was subcloned into the same restriction site of a modified pCAMBIA 1390 binary vector (CAMBIA, Brisbane City, Australia) containing *CaMV 35S* promoter. Clones containing the fragment in sense orientation to *CaMV 35S* promoter were identified by restriction enzyme digestion and subsequently introduced into *Agrobacterium tumefaciens* strain EHA105 by direct DNA uptake. Preparation of leaf-disk explants of wild-type (WT) tobacco (*Nicotiana tabacum* cv. Havana), *Agrobacterium* transformation, shoot regeneration, and rooting were performed as described previously (Cidade et al., 2012). Primary transgenic plants (T_0 generation), representing distinct events of

genetic transformation, were micropropagated *in vitro* and transferred to soil and grown under standardized greenhouse conditions.

Molecular characterization of transgenic plants

Genomic DNA of T_0 transgenic and WT plants was extracted from young leaves using the cetyltrimethyl ammonium bromide (CTAB) method. The PCR amplifications were carried out as described previously (Cidade et al., 2012), using the primers 5'-CTATTTCTTTGCCCTCGGACGAG-3' and 5'-ATGAAAAAGCCTGAACTCACCGC-3' for amplification of the *hptII* gene fragment. The amplified DNA fragments were electrophoresed on 1.0% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Water treatments and physiological analysis

The WT and transgenic plants (T_0) were transplanted to 20-L pots, containing a mixture of Oxisol and washed sand in 2:1 ratio, and maintained in a greenhouse under controlled humidity (70 to 80% relative humidity) and air temperature (25° to 30°C) for 70 days. During this period, the plants were irrigated daily to soil field capacity. After 70 days, the irrigation was withheld for 10 days, by which time most of the soil moisture had been consumed, and subsequently restored. Four replicates of each WT and transgenic lines were used. Stomatal conductance (*gs*) was determined using a portable porometer (Model SC1 Decagon Devices, Pullman, WA, USA). The measurements were performed under conditions of light and CO_2 environments on alternate days between 9 and 11 am. Gas exchanges were measured weekly in fully expanded leaves, between 9 and 11 am, with a LI-6400 apparatus (Li-Cor, Lincoln, NE, USA) under artificial saturating light of 800 mmol photons$\cdot m^{-2} \cdot s^{-1}$, to determine *A* and *E*. Instantaneous efficiency (*A/E*) and intrinsic (*A/gs*) water use was calculated from the obtained values of *A*, *E*, and *gs*. Leaf water potential (yw) was measured in mature leaves from the middle part of the plants. The measurements were made weekly during predawn using a pressure-type pump Scholander (M670, PMS Instrument Co., Albany, OR, USA).

Accumulation of hydrogen peroxide (H_2O_2)

In situ H_2O_2 was detected using histochemical 3,3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997). Five leaf disks from each plant were exposed to vacuum infiltration with 1 mg/ml DAB solution for 20 min. After this period, the leaf discs were immersed in 96% ethanol solution, boiled for 5 h, and rinsed twice with 50% ethanol solution. Subsequently, they were photographed in a magnifier to detect the brown color caused by the production of H_2O_2.

ABA measurement

The ABA concentration was determined in a pool of mature leaves of control (irrigated) and drought-stressed (10 days after the suspension of irrigation) WT and transgenic plants, as described previously (Cidade et al., 2012). The extracts were analyzed by HPLC, using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS, Shimadzu, Kyoto, Japan). The ABA concentration was determined using a UV-VIS detector (Shimadzu) at 254 nm.

Statistical analysis

Statistical analysis was carried out using the software BIOESTAT (Universidade Federal do Pará, Brazil), which tested the experiments in a completely randomized design. Statistical differences were assessed based on analysis of variance (ANOVA) and the means were separated by the Student's *t*-test, with a critical value of $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$. All data were calculated as means of four biological replicates (N = 4).

RESULTS AND DISCUSSION

Transgenic tobacco plants overexpressing *CsNCED3* were generated and 17 transgenic lines, representing distinct transformation events, were randomly selected in hygromycin-containing medium and were screened for the presence of the transgene by PCR. A single band of the expected size (1,026 bp) for *hptII* was observed in 14 out the 17 analyzed putative transgenic lines (**Figure S1**). Screening for *CsNCED3* was not used for the assessment of genetic transformation because of the problems of cross-amplification with the highly homologous tobacco gene. Two independent transgenic lines, designated as N1 and N2, were selected for further analysis, in the present study. These plants were transplanted to soil and grown in a greenhouse; they exhibited a normal phenotype, with no differences in growth when compared to the WT plants.

No significant differences were observed in the leaf water potential (Yw_{leaf}) between the WT and transgenic plants under normal (irrigated) growth conditions and during the drought stress and subsequent recovery (**Figure S2**). The Yw_{leaf} was reduced to values between -1.0 and -1.5 MPa under drought conditions and was subsequently increased again to similar values as in the control (irrigated) treatment (-0.2–-0.3 MPa) after rewatering. No significant differences were observed in the stomatal conductance between the WT and transgenic plants under normal growth conditions (Figure 1). However, the transgenic plants exhibited a significant decrease in the stomatal conductance 4 days after water was withheld, in comparison to WT. The stomatal conductance of the transgenic plants remained significantly lower than that of WT up to 10 days after water was withheld, by the time when most soil moisture had already been consumed (Figure 1). These results indicate that the overexpression of *CsNCED3* promotes stomatal closure in response to both partial and complete soil drying. Thus, an ABA-induced drought-avoidance strategy was designed to prevent serious water loss under conditions of low water potential (Fang and Xiong, 2015). No significant differences in the stomatal conductance between WT and transgenic plants were observed after rewatering (Figure 1). More interestingly, the gas exchange analysis performed 8 days after water was withheld revealed no significant differences in the *A* between the WT and transgenic plants (Figure 2), despite the significantly decreased stomatal conductance showed by the latter (Figure 1). Hence, *A/gs* increased in the transgenic plants under drought conditions (Figure 2). In contrast, no significant differences in *E* and *A/E* values were found between the WT and transgenic plants. These results contrast with those reported recently for the tomato *NCED1* (*LeNCED1*), the overexpression of which in petunia plants under the control of stress-inducible promoter *rd29A* (*rd29A:LeNCED1*) significantly decreased *A*, *E*, *gs* under drought conditions when compared to the respective values in WT (Estrada-Melo et al., 2015). These contrasting results reflect not only the intrinsic differences between the promoter sequences used, but also the potentially non-redundant gene functions of *LeNCED1* and *CsNCED3*. The

analysis of gas exchange parameters after rewatering showed that all plants were recovered from drought stress within five days after re-watering with no significant differences in *A*, *E*, *A/gs*, and *A/E* values between the WT and transgenic plants (Figure 3). Thus, these data indicate that the overexpression of *CsNCED3* did not affect the capacity of the plants to recover from the drought stress conditions that were tested. A significantly improved ability to recover from severe drought stress conditions (14 days after water was withheld) was reported in *rd29A:LeNCED1* transgenic petunia and was associated with its reduced water loss and increased proline content in comparison to the corresponding values in WT (Estrada-Melo et al., 2015).

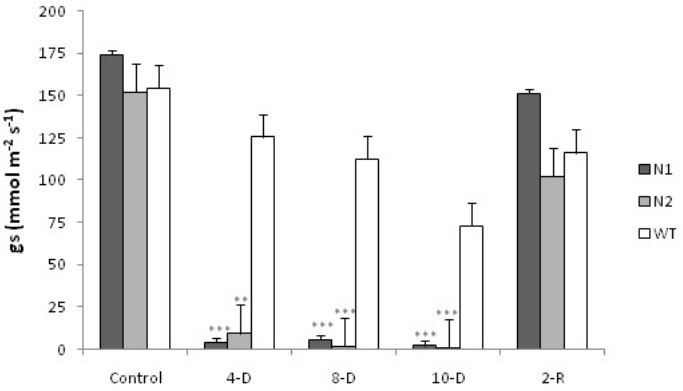

Figure 1. Stomatal conductance under control conditions of irrigation during the period of drought (4, 8, and 10 days) and 2 days after rewatering (2-R). The data are represented as means ± SE of four biological replicates (N = 4). **.***Denote significant differences from WT in the respective treatment at $P \leq 0.01$ or $P \leq 0.001$, respectively, according to Student's *t*-test.

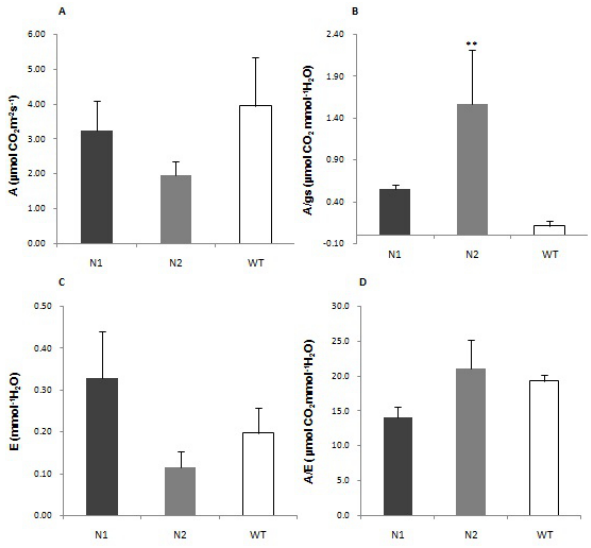

Figure 2. Gas exchange of WT and transgenic plants 8 days after water was withheld. **A.** Photosynthetic rate (*A*), **B.** Intrinsic efficiency water use (*A/gs*), **C.** Transpiration rate (*E*), **D.** Instantaneous efficiency water use (*A/E*). **Denotes significant difference from WT at $P \leq 0.01$, according to Student's *t*-test.

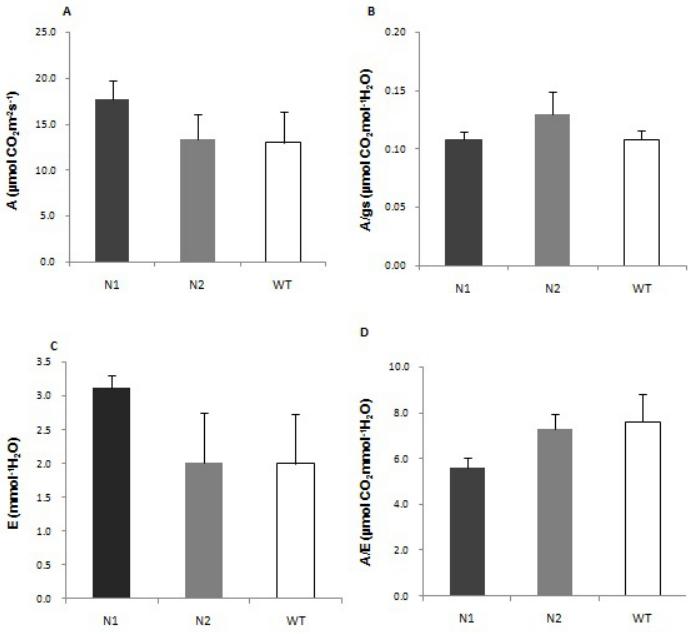

Figure 3. Gas exchange of WT and transgenic plants 5 days after rewatering. **A.** Photosynthetic rate (*A*), **B.** Intrinsic efficiency water use (*A/gs*), **C.** Transpiration rate (*E*), **D.** Instantaneous efficiency water use (*A/E*).

A decrease in the reactive oxygen species (H_2O_2 and O_2^-) accumulation was previously reported in the *CrNCED1*-overexpressing transgenic tobacco plants subjected to dehydration and salt treatments (Xian et al., 2014). In the present study, analysis of H_2O_2 accumulation in leaf disks of the WT and transgenic plants, carried out 10 days after water was withheld, revealed a similar result (**Figure S3**). Leaf disks of transgenic plants exhibited a minor browning caused by H_2O_2 accumulation in comparison to that in WT, confirming the previous findings about the role of *CsNCED3* in the activation of the antioxidant defense system (Xian et al., 2014). It accomplishes this by increasing the transcript levels of several genes associated with ROS scavenging, osmotic adjustment, and water maintenance, as well as the activities of the antioxidant enzymes SOD and CAT (Xian et al., 2014).

ABA concentration was analyzed in leaves of the WT and transgenic plants under control and drought stress conditions, in order to check the relationship between the observed phenotypes and ABA levels. Under control conditions, only one of the transgenic lines (N1) showed higher levels of ABA compared to the levels in WT (Figure 4). Drought stress increased the ABA concentration in all the plants, including WT. However, only the ABA concentration in N1 was higher than that in WT (Figure 4). The ABA levels of N2 were lower than those of WT under both control and drought stress conditions. Because N2 showed a phenotype of gas exchange similar to N1 upon soil-water deficit, which was significantly different from that of the WT (Figures 1 and 2), the putative increase in ABA might be counteracted by an increased ABA catabolism in this transgenic line. Such observation has also been reported in transgenic tobacco plants overexpressing *Gentiana lutea NCED1* gene, which exhibited an ABA-related phenotype of delayed seed germination even though their ABA levels were not significantly

different from that of WT (Zhu et al., 2007). These findings are supported by the fact that some ABA catabolites, such as 8′-hydroxy ABA, are known to contain substantial biological activities (Nambara and Marion-Poll, 2005).

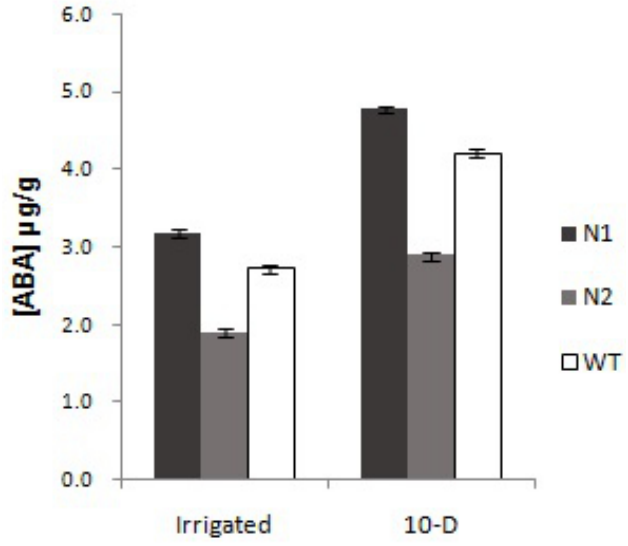

Figure 4. ABA concentration in mature leaves of control (irrigated) and drought-stressed (10 days after water was withheld) WT and transgenic plants.

CONCLUSION

In conclusion, our results demonstrate that overexpression of *CsNCED3* decreases the stomatal conductance without noticeable negative effects on the photosynthetic rates, providing evidence for its relevance and applicability in physiological strategies of drought-avoidance and increased intrinsic WUE in a water-limiting environment. These findings make *CsNCED3* an alternative target gene related to ABA biosynthesis pathway that may be useful for the genetic manipulation of drought resistance in citrus and other crop plants. Further studies will be required to examine whether this gene could enhance drought resistance under field conditions.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by research grants from Embrapa (Macroprograma 2), CNPq (Brasília, Brazil), CAPES (Brasília, Brazil) and FAPESP (São Paulo, Brazil). We gratefully acknowledge the master's degree scholarship to A.M. Pedrosa by FAPESB (Bahia, Brazil).

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Supplementary material

Figure S1. Genomic PCR analysis of the transgenic plants using specific primers for *hptII*. M, 1-kb molecular marker. C+, positive control (pCAMBIA 1390 plasmidial DNA). C-, negative control (WT tobacco). 1 to 17: independent transgenic lines 35S::*CsNCED3* (N1 = 1; N2 = 8).

Figure S2. Effects of the water treatments on leaf water potential (y_w) of WT and transgenic plants. 10-D: 10 days after water was withheld. 6-R: 6 days after rewatering. The data are represented as means ± SE of four biological replicates (N = 4).

Figure S3. Accumulation of H_2O_2 in leaf disks of WT and transgenic plants 10 days after water was withheld, as revealed by 3,3'-diaminobenzidine (DAB) staining. Representative photographs showing staining of H_2O_2 in leaf disks.