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Ectopic expression of rice *OsNCED3* in Arabidopsis increases ABA level and alters leaf morphology

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ABSTRACT

In higher plants, many genes are involved in the abscisic acid (ABA) biosynthetic pathway. Of these, 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) encodes what is considered to be the key enzyme. In the present study, we investigated the effects of ectopically expressing a monocot rice *NCED* gene, *OsNCED3*, in dicot Arabidopsis (*Arabidopsis thaliana*) plants. We showed that *OsNCED3* is functionally active in dicot Arabidopsis plants since the ectopic expression of *OsNCED3* successfully complements the 129B08/*nced3* mutant phenotype. Furthermore, overexpression of *OsNCED3* in wild-type Arabidopsis plants results in increased accumulation of ABA, reduced relative water loss, delayed seed germination, and greater drought tolerance relative to that of wild-type. Additionally, we observed that seed ABA content and germination patterns are similar between wild-type and the 129B08/*nced3* mutant, suggesting functional redundancy or differential spatial expression of other *NCED* gene family members. Transgenic Arabidopsis lines overexpressing the monocot *OsNCED3* gene in a wild-type background result in a smaller and rounder leaf shape and midvein. The data suggest that, in addition to ABA biosynthesis, the monocot *OsNCED3* gene may have additional functions in shaping leaf morphology and vascular bundle development in Arabidopsis.

1. Introduction

The plant hormone abscisic acid (ABA) plays a pivotal role in regulating seed development, dormancy, and stress tolerance [1–5]. The ABA biosynthetic pathway in higher plants has been thought to be an indirect pathway [6] in which plastid-localized 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) catalyze the cleavage of *cis*-epoxycarotenoids, 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin, to a C15 intermediate (xanthoxin) and a C25 by-product in plastids [7]. The xanthoxin is then transported to the cytosol and used as an intermediate for ABA biosynthesis. Nine-*cis*-epoxycarotenoid dioxygenase has long been considered to be the key enzyme in the ABA biosynthetic pathway since its overexpression in plants is associated with an increased ABA level and since its rapid induction is followed by ABA accumulation in leaves under water stress [8–10].

Plant *NCED* genes belong to a family of carotenoid cleavage dioxygenases (CCDs) that can cleave the carotenoid backbone of an epoxycarotenoid at diverse positions [11]. The first plant *NCED* gene to be cloned was *VP14* from maize [7,11]. In Arabidopsis, *NCED* genes belong to a multigene family with nine members; however, only five of them are functionally active in ABA biosynthesis [12]. Of these five genes, *AtNCED3* is thought to be the major player in leaves in response to stress [13]. In addition, Lefebvre et al. [3] showed that *AtNCED6* and *AtNCED9* are involved in ABA biosynthesis during seed development. More recently, Agusti et al. [14] proposed that *AtNCED5* might be involved in ABA biosynthesis in chromoplast-rich organs. Besides maize and Arabidopsis, *NCED* genes have been cloned in many other plant species, including peanut [*Arachis hypogaea* L. cv. YueYou 7; 4], *Gentiana lutea* [15], and *Stylosanthes guianensis* [5,16], and they have been characterized by ectopic expression in Arabidopsis and tobacco (*Nicotiana tabacum* L.). It is worth noting that all of the above mentioned *NCED* genes have been cloned from dicot plant species and that the plants transformed with these dicot *NCED* genes are all dicot plant species. It remains largely unknown what would happen if monocot *NCED* genes were ectopically expressed in dicot plant species.

On the other hand, Zhu et al. [15] studied two *G. lutea NCED* genes, *GlNCED1* and *GlNCED2*, and found that they are closely related to *AtNCED6* and *LeNCED1*/*AtNCED3*, respectively. The

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functions of *GlNCED1* and *GlNCED2* were further characterized by ectopically expressing these two genes in tobacco (*N. tabacum* L. cv. SR1) under the control of the 35S promoter. These same authors reported that *GlNCED1*, but not *GlNCED2*, exerts a physiological effect in transgenic tobacco plants, although both genes are transcribed in the transgenic tobacco plants. Furthermore, Chernys and Zeevaart [10] cloned three avocado (*Persea americana* Mill. cv Lula) *NCED* genes, *PaNCED1*, *PaNCED2*, and *PaNCED3*, and they found that *PaNCED1* and *PaNCED3* are highly expressed as the fruit ripens. *PaNCED2*, however, is constitutively expressed during the ripening process. They also discovered that *PaNCED1* but not *PaNCED3* is water stress-inducible and that both PaNCED1 and PaNCED3, but not PaNCED2, show 9-*cis*-epoxycarotenoid dioxygenase enzymatic activity in vitro. Results from these studies further support the notion made by Nambara and Marion-Poll [17] that not all genes with *NCED*-related sequences are functionally active in ABA biosynthesis.

Welsch et al. [18] reported that the rice carotenoid-cleaving dioxygenase (CCDs) family comprises 13 members. They analyzed the expression levels of the three members that show the highest sequence homology to maize *VP14* and named them *OsNCED3*, *OsNCED4*, and *OsNCED5*. These authors found that all three of these *OsNCED* genes are strongly induced under salt or ABA treatment. In current rice genomic databases more than three *NCED* gene sequences are listed, and it remains an open question as to whether these rice *NCED* genes are involved in ABA biosynthesis.

In the present study, we ectopically expressed the rice *OsNCED3* gene in Arabidopsis to investigate, (1) whether this gene is involved in ABA biosynthesis and exerts any effect on ABA-controlled physiological processes such as seed germination, relative water loss, and drought tolerance and, (2) what physiological effects may result from the ectopic expression of *OsNCED3*, a monocot rice *NCED* gene, in the dicot Arabidopsis. Our results demonstrate that ectopic expression of the *OsNCED3* gene in transgenic Arabidopsis plants not only restores the 129B08/*nced3* mutant phenotype to wild-type but also increases ABA accumulation, reduces relative water loss, delays seed germination, and strengthens the drought tolerance of transgenic plants relative to wild-type plants. Furthermore, a smaller rosette leaf, a rounder leaf shape, a smaller and rounder midvein, and smaller spongy mesophyll cells were found in transgenic plants overexpressing the monocot rice *OsNCED3* but not in transgenic plants overexpressing the dicot *AtABA2* gene. Since ABA levels in rosette leaves were significantly higher in all the aforementioned transgenic plants, the changes in leaf morphology cannot simply be attributed to the increased leaf ABA levels. Therefore, it is possible that monocot *NCED* genes may have additional functions (other than ABA biosynthesis) in shaping leaf morphology and altering vascular bundle differentiation of land plants such as Arabidopsis.

2. Materials and methods

2.1. Plant materials and growth conditions

The plants used in this study were Arabidopsis (*Arabidopsis thaliana*) of the Columbia ecotype. Seeds were sterilized and cold-treated at 4 °C for 4 days in the dark, and then grown on agar plates or in soil at 22 °C under long-day conditions (16-h light/8-h dark cycle) with a light intensity of ~80 μE/s m^2. For aseptic growth, sterilized and cold-pretreated seeds were transferred to modified MS medium composed of half-strength MS basal salts, B5 organic compounds [19] and 0.05% MES [2-(N-morpholino)ethanesulfonic acid monohydrate]. For experimental treatments, the media were supplemented with sucrose (Suc), NaCl, and glucose (Glc) at the concentrations provided in Section 3.

2.2. Transgenic plants and mutant isolation

The rice (*Oryza sativa* L. cv. TNG67) *OsNCED3* (NCBI accession no. AY838899) full-length coding sequence was amplified by PCR and cloned into the pGEM-T Easy vector (Promega, Madison, MI, USA), after which it was subcloned into a binary vector modified from pCAMBIA1281Z, where its expression is driven by a constitutive 35S promoter (*35S*::*OsNCED3*). After the sequence of the transgenic construct was confirmed by sequencing, it was subsequently transformed into 129B08/*nced3* mutant and wild-type (WT) (T0) plants using the floral-dip method [20]. The transgenic lines thus obtained were denoted N3C (for complementation) and N3OE (for overexpression), respectively. T1 seeds subjected to cold pretreatment were screened on 1% Suc agar plates containing 50 mg/L hygromycin B (InvivoGen, USA, Cat. no. ant-hm-1). To obtain homozygous lines for N3C and N3OE transgenic plants, more than 10 independent transgenic lines each were isolated and two to three homozygous lines were randomly chosen for further study. The *nced3* mutant used in this study is a T-DNA-insertion mutant obtained from the Nottingham Arabidopsis Stock Centre (NASC, #N412308). The mutant line was screened for a homozygous mutation by selection on 1% Suc agar plates containing 5.25 mg/L antibiotic, sulfadiazine (Sigma, USA, Cat. no. S-6387). Because this mutant has previously been denoted as 129B08/*nced3* by Wan and Li [4], we use the same name in this study.

2.3. Phenotypic comparisons and germination tests

For phenotypic comparisons, cold-pretreated seeds from WT, 129B08/*nced3*, two N3C (N3C-1 and N3C-2), and three N3OE (N3OE-1, N3OE-2, and N3OE-3) transgenic plants were grown on modified MS medium supplemented with 1% Suc or 1% Suc plus 200 mM NaCl for 7 days. Plants in another set of experiments were grown on modified MS medium supplemented with 6% Glc for 7 or 14 days or in soil for 21 or 33 days. For leaf shape comparisons, cold-pretreated seeds from WT, 129B08/*nced3*, N3C-1, N3C-2, N3OE-1, N3OE-2, N3OE-3, and transgenic plants overexpressing the Arabidopsis *ABA2* gene in a wild-type background (AtABA2OE-4-4 and AtABA2OE-5-1 [21]) were grown in soil for 23 days. After 23 days of growth in soil, the fifth leaves were sampled for leaf width/length ratio measurements or for anatomical analysis following the methods of Yang et al. [22]. For germination tests, seeds harvested from three different batches were cold-pretreated and then grown on water agar medium containing no sugar and MS.

2.4. ABA assay

Mature dry seeds or seedlings after growth on agar plates supplemented with 1% Suc for 12 days or the fifth and sixth leaves of 23-day-old soil-grown plants were harvested for ABA analysis. ABA extraction, purification, and subsequent quantification were carried out as previously described [21] except that the dry seeds and soil-grown leaf samples were first ground using an SH-48 tissue homogenizer (Kurabo) before treatment with extraction buffer.

2.5. Water loss tests

For measurement of relative water loss, cold-pretreated seeds from WT, 129B08/*nced3*, N3C-1, and N3C-2 plants were grown in soil for 26 days and cold-pretreated seeds from WT, 129B08/*nced3*, N3OE-2, and N3OE-3 plants were grown in soil for 23 days. The aerial parts of the plants were then excised and the detached rosette leaves were placed in plastic weigh boats and stored in an