

Overexpression of a 9-cis-Epoxy-carotenoid Dioxygenase Gene in *Nicotiana plumbaginifolia* Increases Absciscic Acid and Phaseic Acid Levels and Enhances Drought Tolerance¹

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The plant hormone absciscic acid (ABA) plays important roles in seed maturation and dormancy and in adaptation to a variety of environmental stresses. An effort to engineer plants with elevated ABA levels and subsequent stress tolerance is focused on the genetic manipulation of the cleavage reaction. It has been shown in bean (*Phaseolus vulgaris*) that the gene encoding the cleavage enzyme (*PvNCED1*) is up-regulated by water stress, preceding accumulation of ABA. Transgenic wild tobacco (*Nicotiana plumbaginifolia* Viv.) plants were produced that overexpress the *PvNCED1* gene either constitutively or in an inducible manner. The constitutive expression of *PvNCED1* resulted in an increase in ABA and its catabolite, phaseic acid (PA). When the *PvNCED1* gene was driven by the dexamethasone (DEX)-inducible promoter, a transient induction of *PvNCED1* message and accumulation of ABA and PA were observed in different lines after application of DEX. Accumulation of ABA started to level off after 6 h, whereas the PA level continued to increase. In the presence of DEX, seeds from homozygous transgenic line TN1 showed a 4-d delay in germination. After spraying with DEX, the detached leaves from line TN1 had a drastic decrease in their water loss relative to control leaves. These plants also showed a marked increase in their tolerance to drought stress. These results indicate that it is possible to manipulate ABA levels in plants by overexpressing the key regulatory gene in ABA biosynthesis and that stress tolerance can be improved by increasing ABA levels.

Absciscic acid (ABA) is necessary for seed maturation and dormancy and adaptation to a variety of environmental stresses. The role of ABA in these processes is mediated by changes in gene expression and stomatal closure. Mutants impaired in ABA biosynthesis display precocious germination and have a wilted phenotype (Zeevaart, 1999). Application of ABA results in delayed germination and increased tolerance to a variety of stresses.

It is now well established that ABA in higher plants is derived from C₄₀-carotenoids (Cutler and Krochko, 1999; Liotenberg et al., 1999; Taylor et al., 2000). Epoxidation of the C₄₀-carotenoid, zeaxanthin, gives rise to all-trans-violaxanthin, one of the xanthophylls in higher plants. This reaction is catalyzed by zeaxanthin epoxidase (ZEP). The ZEP gene was isolated from the *aba2* mutant of wild tobacco (*Nicotiana plumbaginifolia*) and the *aba1* mutant of Arabidopsis (Marin et al., 1996). The xanthophylls also include three other isomers, 9-cis-violaxanthin, all-trans-neoxanthin, and 9-cis-neoxanthin. Genes encoding neoxanthin synthase have been cloned from potato (*Solanum tuberosum*; Al-Babili et al., 2000) and tomato (*Lycopersicon esculentum*; Bouvier et al., 2000).

The first C₁₅ precursor of ABA is xanthoxin, which is a cleavage product of C₄₀-epoxy-carotenoids. Current evidence indicates that the key regulatory step in ABA biosynthesis is the oxidative cleavage reaction (Taylor et al., 2000). The gene encoding the cleavage enzyme was first cloned from the ABA-deficient mutant *vp14* of maize (*Zea mays*; Tan et al., 1997). The VP14 protein cleaves 9-cis-epoxy-carotenoids at the 11-12 double bond and produces xanthoxin and C₂₅-apocarotenoids (Schwartz et al., 1997). Therefore, this enzyme is named 9-cis-epoxy-carotenoid dioxygenase (NCED; Qin and Zeevaart, 1999). Consistent with the predicted role of the cleavage reaction in the regulation of ABA biosynthesis, the *PvNCED1* gene of bean (*Phaseolus vulgaris*) was strongly induced at both the mRNA and protein levels in response to water stress. This induction preceded the accumulation of ABA (Qin and Zeevaart, 1999). Similar results have been found in tomato (Burbidge et al., 1999), cowpea (*Vigna unguiculata*; Iuchi et al., 2000), and avocado (*Persea americana*; Chernys and Zeevaart, 2000).

The last two steps of ABA biosynthesis involve the conversion of xanthoxin to ABA-aldehyde and then to ABA. The ABA-aldehyde oxidase (AAO3) gene was cloned recently from Arabidopsis. AAO3 mRNA, but not the protein, was increased by water stress (Seo et al., 2000a, 2000b). As ABA accumulates, it is catabolized to phaseic acid (PA), or conjugated to ABA-Glc ester (Zeevaart, 1999). The enzyme that converts ABA to PA, ABA 8'-hydroxylase, is up-regulated by ABA (Cutler and Krochko, 1999).

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With the identification of genes encoding ABA biosynthetic enzymes, attempts to manipulate ABA levels in plants have been put into practice. First, the *ZEP* gene was constitutively overexpressed in wild-type tobacco and the *aba2* mutant. The transgenic plants showed delayed seed germination and increased ABA levels in mature seeds. Conversely, expression of an antisense *ZEP* led to rapid germination and reduced ABA levels in seeds. However, no difference in vegetative tissues was observed (Frey et al., 1999). Thompson et al. (2000) produced transgenic cultivated tobacco (*Nicotiana tabacum*) overexpressing *LeNCED1* driven by a tetracycline-inducible promoter. The primary transgenic plants showed various degrees of increase in ABA levels in leaves. The constitutive expression of *LeNCED1* in tomato showed increased guttation, and the seeds were more dormant than wild type. In a recent report, it was shown that in *Arabidopsis*, expression of *AtNCED3* is up-regulated by water stress. Plants that overexpressed *AtNCED3* accumulated ABA and showed a decrease in transpiration and enhanced drought tolerance (Iuchi et al., 2001).

In this paper, we engineered transgenic wild tobacco plants overexpressing the *PvNCED1* gene either constitutively or driven by a dexamethasone (DEX)-inducible promoter. Levels of ABA and its major metabolites were analyzed in time-course studies. The effects of increased ABA levels on seed germination and water relations were also investigated. Our results demonstrate that ABA levels can be altered by expression of the *NCED* gene. The elevated ABA levels affect both seed germination and water relations. These results provide direct evidence that the cleavage of C₄₀-epoxycarotenoids is the rate-limiting step in ABA biosynthesis.

RESULTS

Accumulation of ABA and Its Catabolites in Response to Water Stress in Wild Tobacco

We first determined the accumulation of ABA and its catabolites in detached leaves from wild-type tobacco. Immediately after detachment of the leaves, their fresh weight was reduced by 12% with a stream of warm air from a hair dryer. Figure 1 shows the accumulation of ABA, PA, and DPA over a 24-h period of stress. The initial levels of ABA, PA, and DPA were low, as shown at time zero. After stress, a concomitant increase of ABA and PA took place rapidly, whereas the DPA level remained low. These data indicate that the water stress-induced ABA is further converted to PA in tobacco, presumably because of the activation of ABA 8'-hydroxylase, the enzyme that converts ABA to PA. In the time frame studied, the DPA level was very low and did not appear to respond to water stress. As a consequence, the investigation of accumulation of ABA and its

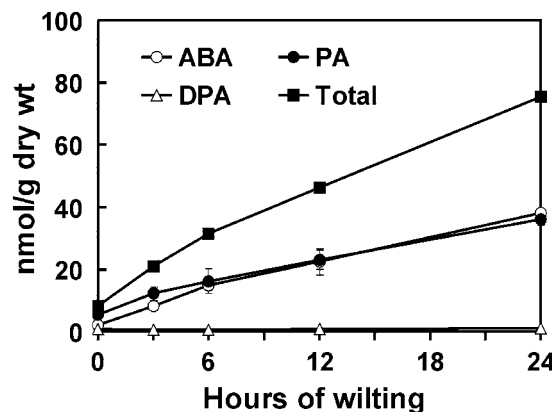


Figure 1. Accumulation of ABA, PA, and DPA in detached and wilted leaves. Fully expanded leaves were detached from wild tobacco plants and half-leaves were cut along the midrib. The fresh weight of the half-leaves was reduced by 12% under a stream of warm air from a hair dryer. The wilted leaves were stored in polyethylene bags until frozen in liquid N₂. Data are averages of measurements of two samples.

catabolites in transgenic plants was mainly focused on ABA and PA.

Constitutive Overexpression of *PvNCED1* Results in an Increase of ABA and PA

The previously identified *PvNCED1* gene from bean was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 2) and introduced into wild tobacco. The Southern blot (Fig. 3A) shows insertion of the *PvNCED1* gene in the four transgenic tobacco lines, BN2, BN11, BN13, and BN15. Except for the line BN11, which has two insertions, all lines have a single insertion (Fig. 3A). As expected, BN2, BN13, and BN15 showed a 3:1 segregation for kanamycin resistance in the F₁ generation (data not shown). The northern blot (Fig. 4B) shows high expression of *PvNCED1* mRNA in transgenic lines 2, 11, and 13. Line 15 had a weak expression even though the Southern blot did show a single insertion of *PvNCED1*. The low expression of the *PvNCED1* transgene in line 15 was correlated with only small increases in both ABA and PA levels in BN15 relative to those of wild-type plants or plants transformed with the pBI121 vector. For the three highly expressing *PvNCED1* lines, the ABA levels were about 3.5 times higher than in the control. Further analysis of ABA catabolites showed that the ABA oxidation product, PA, was increased 5- to 6-fold in these three lines over that in the control (Fig. 4A). The greater increase of PA relative to ABA levels in BN transgenic plants indicates that the constitutive expression of *PvNCED1* in the transgenic plants triggered ABA degradation. Hence, a higher accumulation of PA than of ABA was observed.

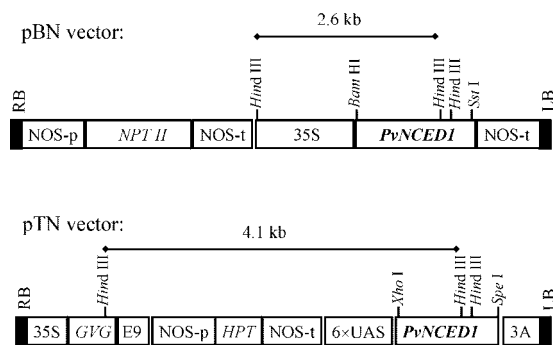


Figure 2. Constructs used for plant transformation. RB, Right T-DNA border; LB, left T-DNA border; 35S, CaMV 35S promoter; *NPT II*, neomycin phosphotransferase II; NOS-p, NOS promoter; NOS-t, NOS terminator; *HPT*, hygromycin phosphotransferase; GVG, glucocorticoid trans-acting element; 6×UAS, cis-acting element; E9, pea rbcS-E9 terminator; 3A, pea rbcS-3A terminator.

Inducible Expression of *PvNCED1* Leads to Accumulation of ABA and PA

The inducible promoter vector pTA7002 consists of the glucocorticoid receptor domain, the VP16 trans-activating domain, and the GAL4 DNA-binding domain (GVG) driven by the CaMV 35S promoter (Aoyama and Chua, 1997). The target gene *PvNCED1* was inserted downstream of the cis-elements (6×GAL4 UAS; Fig. 2). In the presence of glucocorticoid molecules, the trans-elements are activated and induce expression of the target gene. The control vector (pTA) or the vector carrying *PvNCED1* (pTN) was introduced into wild tobacco by *Agrobacterium tumefaciens*-mediated transformation. Four positive TN transgenic plants (T_0) were obtained (Fig. 3B). After infiltrating the leaves with 30 μ M DEX for 12 h, induction of *PvNCED1* transcript was detected by northern blot (Fig. 5B). The ABA level was increased

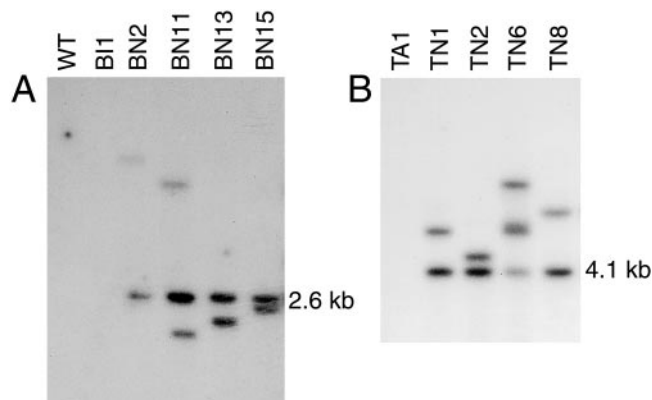


Figure 3. Southern-blot analysis of transgenic lines expressing *PvNCED1*. A, BN lines with wild-type and BI1 plants as controls; and B, TN lines with TA1 line as control. Ten micrograms of genomic DNA was digested with *Hind*III. Blots were probed with full-length *PvNCED1* at high stringency. The arrows on the right side indicate the expected 2.6-kb band from transgenic wild tobacco plants carrying the pBN construct (A) and the 4.1-kb band from transgenic plants carrying pTN (B).

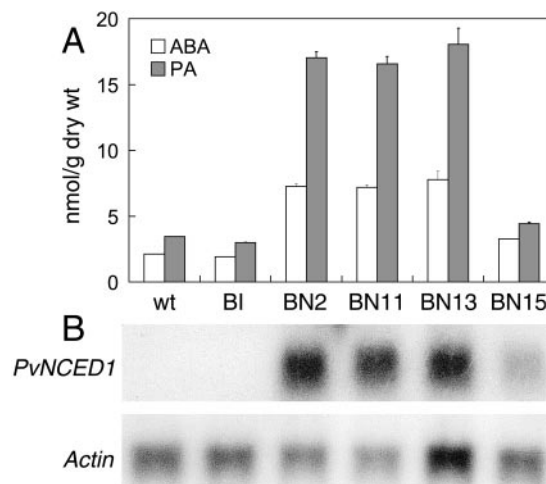


Figure 4. Accumulation of ABA, PA, and *PvNCED1* mRNA in wild tobacco lines that constitutively overexpress *PvNCED1*. A, ABA and PA levels. Data are averages of measurements of two samples. B, Northern blot. Ten micrograms of total RNA isolated from leaves was loaded. The blot was probed with *PvNCED1* or *Actin* from tobacco.

1.4- (TN8) to 7.5-fold (TN1) compared with that of the control plant (TA1) transformed with the empty vector. The level of PA increased to different degrees in the four transgenic lines (Fig. 5A).

Line TN1 was selected for a more in-depth study. Two homozygous lines of TN1 were obtained. These plants were used to analyze the time-course of DEX induction. Total RNA showed no apparent *PvNCED1* expression without the DEX induction (0 time point). However, after application of 30 μ M DEX, a strong induction of the *PvNCED1* message was seen after 3 h, which lasted for the remainder of the 48-h experiment (Fig. 6B). The ABA level increased strongly during the first 6 h of induction and then leveled off, whereas PA continued to increase throughout the time

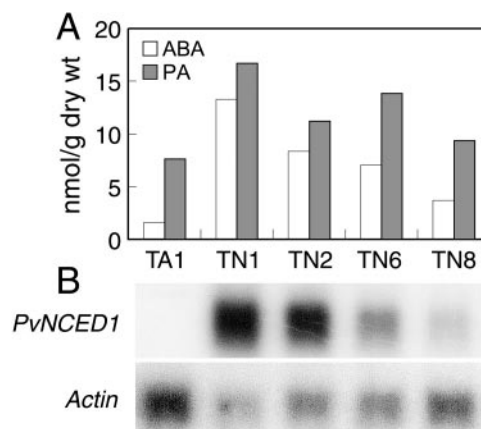


Figure 5. Accumulation of ABA, PA, and *PvNCED1* mRNA in wild tobacco lines that were transformed with TA or TN. Leaf samples were harvested 12 h after infiltration with 30 μ M DEX. A, ABA and PA levels. Results represent one sample for each line. B, Northern analysis of *PvNCED1* and *Actin*. Twenty micrograms of total RNA was loaded for each sample.

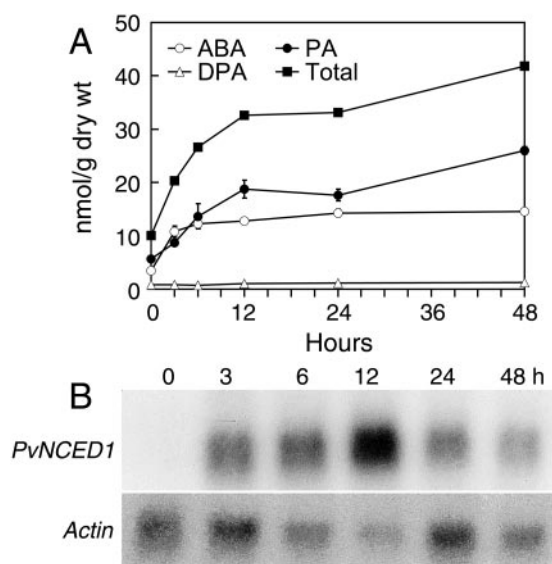


Figure 6. Accumulation of ABA, PA, DPA, and *PvNCED1* mRNA after treatment of line TN1 with DEX. Mature leaves were infiltrated with 30 μM DEX and harvested at the times shown. A, Accumulation of ABA, PA, and DPA. Data are averages of measurements of two samples. B, Northern blot. Twenty micrograms of total RNA was loaded. Blot was probed with *PvNCED1* or *Actin* gene from tobacco.

period analyzed. In agreement with the results in Figure 1, only small amounts of DPA were detected, even after a 48-h induction with DEX (Fig. 6A).

Delayed Seed Germination of TN1 by DEX

Wild-type tobacco seeds and seeds from a TN1 plant were harvested at the same time. These seeds were used to test germination under normal conditions or in the presence of 30 μM DEX. The results in Figure 7 show that DEX had no effect on the germination of wild-type seeds. Also, TN1 seeds had a germination rate similar to that of wild-type seeds on one-half-strength Hoagland medium. However, in the presence of DEX, germination was delayed for about 4 d. This result indicates that the ABA content

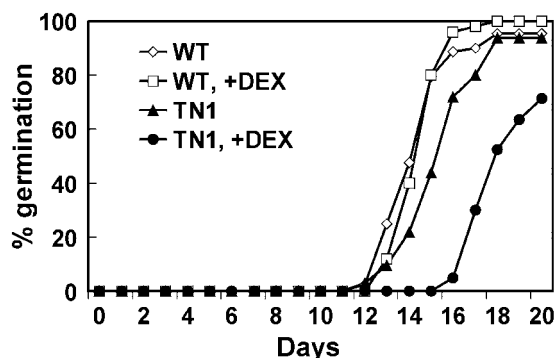


Figure 7. Effect of DEX on germination of wild tobacco seeds. Seeds from wild type or from plants homozygous for TN were sown on agar plates containing one-half-strength Hoagland nutrient solution with or without 30 μM DEX.

of TN1 seeds was probably increased by the DEX treatment and caused delayed germination. Moreover, a reduction in size was observed of TN1 seedlings growing on DEX-containing agar. This experiment was repeated twice with similar results.

Reduced Water Loss from Detached Leaves of Transgenic Plants

Three plants each of the TA2 and TN1 lines were either sprayed with 30 μM DEX in 0.01% (v/v) Tween 20 water solution or with 0.01% (v/v) Tween 20 only. The next day, one fully expanded leaf from each plant was detached and exposed in the open air to monitor water loss. For each treatment, the most rapid loss of water occurred during the first 30 min after detachment (Fig. 8). The leaves from control TA2 plants lost about 12% of their fresh weight in 2 h, regardless of whether they were treated with DEX. The leaves from TN1 plants had a similar weight loss when sprayed with 0.01% (v/v) Tween 20 water solution only. However, leaves from TN1 plants sprayed with a DEX solution had a much reduced water loss. These data indicate that the induction of ABA synthesis resulted in the closure of stomata of TN1 leaves and, as a result, in a decrease in water loss by transpiration. This was further confirmed by measurements of stomatal conductance of control and DEX-treated plants. The stomatal conductance showed marked differences between these two groups of plants, being 0.49 ± 0.02 ($n = 6$) and 0.14 ± 0.04 cm s^{-1} ($n = 4$) for water and DEX-treated plants, respectively. This difference in stomatal conductance persisted for at least 3 d in DEX-treated plants. Reduced stomatal conductance was also observed when *PvNCED1* was under the control of the 35S promoter. For example, the values for the BI (control), BN2, and

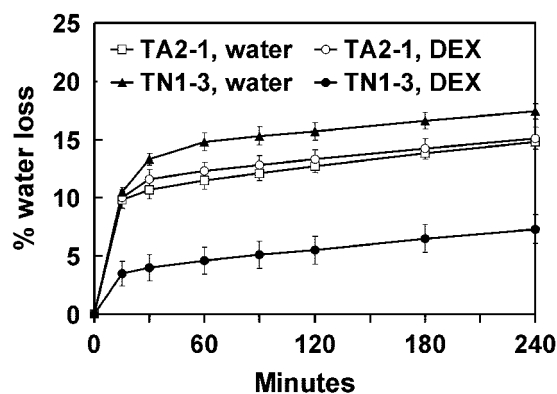


Figure 8. Weight loss of detached leaves of tobacco. Control plants TA2 or plants of the homozygous transgenic line TN1 were sprayed with either water or 30 μM DEX in 0.01% (v/v) Tween 20 and left overnight. Leaves were detached and exposed to air to monitor weight loss. Three leaves from each of three separate plants were used for each treatment. The average water loss in percentage $\pm$ SE is plotted.

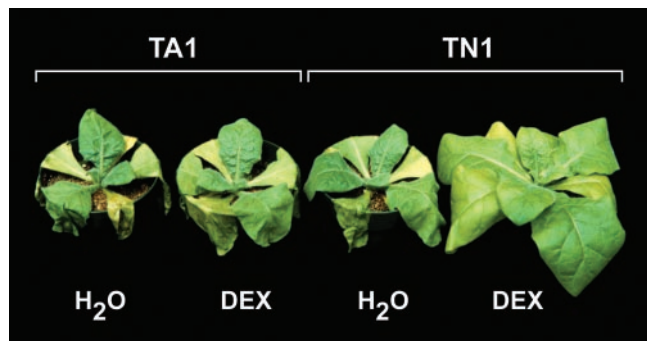


Figure 9. Enhanced drought tolerance in wild tobacco plants overexpressing *PvNCED1*. Homozygous TA1 or TN1 plants were sprayed with water or 30 μM DEX in 0.01% (v/v) Tween 20 and then kept in a growth chamber without further watering. The photograph was taken 1 week after the last watering.

BN13 lines were 0.35 ± 0.07 , 0.12 ± 0.06 , and $0.06 \pm 0.01 \text{ cm s}^{-1}$, respectively.

Plants Overexpressing *PvNCED1* Show Enhanced Drought Tolerance

Plants of the TA1 and TN1 lines that had developed approximately 10 leaves were sprayed with water or with a DEX solution; watering of the plants was then discontinued. Afterward, the plants were kept in a growth chamber maintained at low humidity. Two to 3 d later, the TA1 plants and the TN1 plants sprayed with water started to show wilting symptoms, which gradually increased in severity. The TN1 plants sprayed with DEX showed no obvious wilting for up to 10 d, but the color of the leaves changed to pale green, and expansion of young leaves and formation of new leaves gradually declined and ultimately ceased. Figure 9 shows representative plants of each treatment after 1 week without watering. A transient inhibition of leaf growth and a lighter green color than in control plants was also observed in TN1 plants, but not in TA1 plants, sprayed with DEX under well-watered conditions. BN plants in which the *PvNCED1* gene was constitutively expressed also showed an increase in drought tolerance, but this was less pronounced than in DEX-induced TN plants (data not shown).

DISCUSSION

Water-stressed leaves of wild tobacco accumulate large amounts of ABA and PA, but DPA is a minor metabolite (Figs. 1 and 6), as also found in some other species (Zeevaart, 1999). In addition, all three compounds are also present in conjugated forms, presumably Glc esters from which they can be released by mild alkaline hydrolysis (J.A.D. Zeevaart, unpublished data). These conjugates were not quantified in the present study. Thus, the total amounts of ABA and its catabolites in wild tobacco are actually larger than presented here.

Overexpression of the gene encoding the cleavage enzyme for ABA biosynthesis from bean in wild tobacco resulted in increased levels of ABA and PA, providing further evidence that cleavage of the 11-12 double bond of 9-cis-epoxycarotenoids is the key limiting step in ABA biosynthesis in leaves. Thus, manipulation of ABA levels can be achieved by altering *NCED* expression. However, our results also demonstrate that there are limits to the steady-state ABA level that is maintained in transgenic plants overexpressing *NCED*. More ABA+PA accumulated in water-stressed leaves (as a result of induction of the native *NCED*) than in transgenic plants expressing *PvNCED1* constitutively or in an inducible manner (compare results in Fig. 1 with those in Figs. 4 and 6). One possible explanation is that the promoter of the stress-regulated *NCED* gene causes a stronger expression than either the CaMV 35S promoter or the DEX-inducible promoter does with the *PvNCED1* transgene. Regardless of whether the ABA level is elevated by water stress or by the *NCED* transgene, a steady-state level is reached where the rate of ABA synthesis equals the rate of conversion to PA because of induction of 8'-hydroxylase by the increase in ABA (Cutler and Krochko, 1999; Zeevaart, 1999). Thus, a further increase in ABA level beyond what has been achieved so far will require repression of expression by antisense technology or a knockout of the ABA 8'-hydroxylase gene.

Previous work has demonstrated an important role for ABA in maintenance of dormancy in seeds of wild tobacco (Frey et al., 1999; Grappin et al., 2000). Transgenic plants overexpressing *ZEP* exhibited delayed seed germination and had increased ABA content of the seeds, whereas antisense *ZEP* had the opposite effect (Frey et al., 1999). In non-photosynthetic organs such as roots and seeds, the small pool of epoxycarotenoids may be limiting for ABA synthesis (Audran et al., 1998; Qin and Zeevaart, 1999). It is surprising, therefore, that DEX-induced *PvNCED1* also caused a delay in seed germination (Fig. 7). Assuming that the delay in germination was because of DEX-induced ABA synthesis, then this finding suggests that the cleavage step is limiting in seeds.

Well-watered transgenic plants overexpressing *PvNCED1* were indistinguishable from wild-type plants both in the vegetative and reproductive phase. However, there were clear-cut differences with respect to water relations. The transgenic plants had a low stomatal conductance and presumably as a result they remained much longer turgid than wild-type plants when deprived of water. *Commelina virginiana* leaves that had been treated daily with ABA under well-watered conditions (which might have an effect similar to that of overexpression of *NCED*) also showed a decreased stomatal conductance, but unaltered photosynthetic capacity (Franks and Farquhar, 2001). These findings suggest that water use effi-

ciency in plants can be improved by genetic manipulation of ABA levels.

It appears that to be effective in ameliorating stress the ABA level has to be elevated before the onset of stress. Non-DEX treated plants undoubtedly produced copious amounts of ABA once they lost turgidity, but this stress-induced ABA was obviously less effective than ABA induced by DEX before the onset of stress (Fig. 9). A similar observation was reported by Mizrahi et al. (1974). These workers found that spraying barley and wheat seedlings with ABA was most effective in prolonging survival of water-deprived seedlings when ABA was applied at a stage when soil moisture was close to maximal. One may conclude from this that the elevated ABA has to act in the turgid state for a certain duration to increase the plant's fitness under subsequent stress.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Seeds of wild tobacco (*Nicotiana plumbaginifolia* Viv.) were germinated in petri dishes containing one-half-strength Hoagland solution solidified with 0.9% (w/v) agar. Seedlings were transplanted into "soil," consisting of Baccto (Michigan Peat Co., Houston) and sand (4:1, v/v) in 325-mL containers. When the plants had developed 10 to 12 leaves, they were transplanted into 1-L containers in the same mixture. The plants were grown in a growth chamber with a 9-h photoperiod at a photoflux density of approximately $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 23°C and a night temperature of 20°C. Under these conditions, wild tobacco remains in the rosette stage for at least 3 months. For seed production, plants were induced to flower by extending the main light period with light from incandescent bulbs.

Construction of Plant Expression Vectors

The full-length *PvNCED1* cDNA was introduced into the pBI121 vector at the *Bam*HI and *Sst*I sites to replace the GUS gene (Jefferson et al., 1987). The resulting construct was named pBN (Fig. 2). The DEX-inducible promoter vector pTA7002 was obtained from Dr. Nam-Hai Chua (Rockefeller University, New York). The full-length *PvNCED1* was inserted at the *Xho*I and *Spe*I restriction sites as recommended by Dr. Chua. The corresponding construct is referred to as pTN (Fig. 2). All four vectors—pBI121 (pBI), pBN, pTA7002 (pTA), and pTN—were introduced separately into *Agrobacterium tumefaciens* strain LBA4404 (CLONTECH, Palo Alto, CA).

Transformation of Wild Tobacco

For transformation, leaves were surface sterilized and cut into small discs. Leaf discs were incubated with *A. tumefaciens* suspensions for 5 to 10 min and then transferred to Murashige and Skoog medium supplied with 2 mg L⁻¹ benzyladenine and 0.1 mg L⁻¹ naphthylacetic acid. After 3 d of coculture, leaf discs were transferred to selec-

tion medium (Murashige and Skoog, 2 mg L⁻¹ benzyladenine, 0.1 mg L⁻¹ naphthylacetic acid, and 100 mg L⁻¹ kanamycin, or 30 mg L⁻¹ hygromycin and 500 mg L⁻¹ carbenicillin). The developing shoots were transferred to Murashige and Skoog medium with 0.1 mg L⁻¹ indole-3-acetic acid, 100 mg L⁻¹ kanamycin, or 30 mg L⁻¹ hygromycin, and 500 mg L⁻¹ carbenicillin for inducing roots. The rooted plants (T₀) were transferred to soil and grown in a growth chamber under the conditions described above.

DEX Treatment of pTA and pTN Transgenic Plants

DEX (Sigma, St. Louis) was first dissolved in ethanol at a concentration of 30 mM. Before use, it was diluted to 30 μM with water. The same ratio of ethanol to water was used as control solution. To induce *PvNCED1* gene expression in transgenic plants, water or 30 μM DEX was infiltrated into mature transgenic tobacco leaves with a syringe pressed against the lower surface.

DNA and RNA Gel-Blot Analyses

Ten micrograms of genomic DNA from transgenic plants was digested with *Hind*III and hybridized with the full-length *PvNCED1* probe. Because of the two *Hind*III sites in *PvNCED1* at bp 1,797 and 1,876, and the *Hind*III sites in the pBI121 or pTA7002 vector (Fig. 2), the expected bands for tobacco transformed with pBN are 2.6 kb, with another band of unknown size in the case of a single insertion. The expected bands for pTN transgenic lines are 4.1 kb, with an additional band of varying size.

Total RNA was extracted with TRIzol reagent (Gibco-BRL, Rockville, MD) according to the manufacturer's description. Ten micrograms of total RNA was resolved in formaldehyde denaturing gel and transferred to Hybond N⁺ membrane (Amersham Pharmacia, Piscataway, NJ). Hybridization was carried out as described before with *PvNCED1*, or with an *Actin* gene from tobacco as a loading control (Qin and Zeevaert, 1999).

Measurement of ABA and Its Catabolites

The procedures for extraction, purification, and quantitation of ABA and its catabolites PA and DPA were as described (Cornish and Zeevaert, 1984) with some modifications. The lyophilized samples were extracted and homogenized in 80% (v/v) aqueous acetone with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Five milliliters of 1 M Pi buffer at pH 8.0 was added to the extract. After removal of the acetone on a rotary evaporator, lipids were removed by partitioning the aqueous concentrate twice with hexanes. The pH of the aqueous phase was adjusted to 2.5 with 6 N HCl and extracted three times with ethyl acetate. The acidic fraction was dried and subjected to reverse-phase HPLC (Cornish and Zeevaert, 1984). The fractions containing ABA, PA, and DPA were collected and dried in a centrifugal vacuum concentrator (Jouan, Winchester, VA). After methylation with diazomethane, the fractions were further purified by normal-

phase HPLC (Cornish and Zeevaart, 1984). The methyl esters of ABA, PA, and DPA were analyzed on a 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an automatic injector and electron capture detector. Methyl ABA was analyzed isothermally at 188°C, methyl PA and DPA at 190°C on an HP-5 capillary column (30-m $\times$ 0.32-mm i.d. $\times$ 0.25- μ m film thickness, Agilent Technologies, Palo Alto, CA). Endrin was used as an internal standard. All values reported in this paper have been corrected for recoveries of added ^3H -labeled compounds (Cornish and Zeevaart, 1984). All measurements of ABA and PA were repeated at least once with similar results.

Seed Germination

Seeds of wild tobacco and the homozygous transgenic line TN1 were spread on one-half-strength Hoagland agar plates (0.9%) or Hoagland agar containing 30 μM DEX. Three plates were used for each treatment, and 50 to 100 seeds were spread per plate. Plates were kept at 22°C with 16- to 8-h light/dark cycles. Germination was counted when two fully expanded green cotyledons had developed.

Water Loss Analysis

TA2 and TN1 plants that had developed approximately 10 leaves were sprayed with water or 30 μM DEX containing 0.01% (v/v) Tween 20 and left overnight in a growth chamber. The next day, three just fully expanded leaves from three plants for each treatment were detached and left on a bench. The leaves were weighed at different times to determine the rate of water loss. Stomatal conductance was measured with a LI-1600 steady-state porometer (LI-COR Inc., Lincoln, NE). Measurements were performed on two mature leaves on each plant for several days after treatment in a growth chamber during the light period. Similar measurements were performed on leaves of BN plants, but at a later stage of development.

Drought Tolerance

TA1 and TN1 plants, grown in 325-mL containers, with approximately 10 leaves were sprayed with water or 30 μM DEX containing 0.01% (v/v) Tween 20. They were kept in a growth chamber maintained at low humidity without further watering to evaluate their drought tolerance. Untreated BI and BN plants were subjected to the same treatment.

Availability of Material

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial purposes.

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