

## Transgenic plants of creeping bent grass harboring the stress inducible gene, *9-cis-epoxycarotenoid dioxygenase*, are highly tolerant to drought and NaCl stress

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### Abstract

Transgenic lines of creeping bent grass were generated by *Agrobacterium*-mediated transformation with the *VuNCED1* which was cloned from cow pea has a homology to *9-cis-epoxycarotenoid dioxygenase*, which is supposed to be involved in abscisic acid (ABA) biosynthesis. ABA, a cleavage product of carotenoids, is involved in stress responses in plants. The limiting step of ABA biosynthesis in plants is presumably the cleavage of *9-cis-epoxycarotenoids*, the first committed step of ABA biosynthesis. Molecular analyses of transgenic lines as performed by Southern hybridization genomic DNA-PCR revealed integration of the *VuNCED1*. Challenge studies performed with transgenic plants by exposure to salt stress (up to 10 dS m<sup>-1</sup>) and water stress (up to 75%) for 10 weeks, revealed that more than 50% of the transgenic plants could survive NaCl and drought stress whereas wild-type was not. ABA levels were measured under drought and normal conditions, endogenous ABA was dramatically increased by drought and NaCl stress in transgenic plants. These results indicate that it is possible to manipulate ABA levels in plants by over expressing the key regulatory gene in ABA biosynthesis and that stress tolerance can be improved by increasing ABA levels.

### Introduction

Increased tolerance against various osmotic stresses is one of the major objectives of plant biotechnology. Among them salinity and drought are the most important factors limiting crop productivity. According to the United Nations

environment program, nearly 20% of the world's agricultural lands and about 10 million ha of irrigated land are abandoned because of excess salt deposition each year (Nelson et al. 1998). Plants respond to water deficit and adapt to drought conditions by various physiological changes including transition in gene expression during water deficit. The mechanisms of drought response have been investigated most extensively in a model

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plant, *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 1999).

Higher plants from several families have evolved mechanisms such as the accumulation of osmoprotectants including amino acids, ammonium compounds and polyols/sugars to protect themselves from salt and drought stress conditions. This provides tolerance to the cells under stress by stabilizing the quaternary structure of the complex proteins and adjusting the osmotic potential in their cytoplasm to maintain water content. Recent progress has been made in analyzing the complex cascades of gene expression in drought and cold stress responses, especially in identifying specificity and cross talk in stress signaling (Shinozaki et al. 2003; Siobhan et al. 2003).

A well known response of plants to water stress is accumulation of ABA, which is caused by *de novo* synthesis. The limiting step of ABA biosynthesis in plants is presumably the cleavage of 9-*cis*-epoxycarotenoids, the first committed step of ABA biosynthesis. The gene encoding the cleavage enzyme was first cloned from the ABA-deficient mutant *vp14* of maize (Tan et al. 1997). The *VP14* protein cleaves 9-*cis*-epoxycarotenoids at the 11–12 double bond and produces xanthoxin and C25-apocarotenoids (Schwartz et al. 1997). Therefore, this enzyme is named 9-*cis*-epoxycarotenoid dioxygenase in bean (Qin and Zeevaart 1999). Consistent with the predicted role of the cleavage reaction in the regulation of ABA biosynthesis, the *NCED1* gene of bean was strongly induced at both the mRNA and protein levels in response to water stress. This induction preceded the accumulation of ABA. Similar results have been found in tomato (Burbidge et al. 1997), avocado (Chernys and Zeevaart 2000), *Arabidopsis* (Iuchi et al. 2001) and tobacco (Qin and Zeevaart 2002). The gene *VuNCED1* we have used in this study was isolated from cowpea which has a homology with the 9-*cis*-epoxycarotenoid dioxygenase, which is involved in ABA synthesis and is targeted in to plastids and functions in the plastid to produce ABA, mainly function under drought and high salt conditions (Iuchi et al. 2000).

Bent grass (*Agrostis palustris* L.) is widely used as commercial plant, golf course and tennis court. Large tracts of salt affected and dry lands can be utilized for golf courses provided suitable high quality grasses are available with traits such as tolerance to drought and salt stress. Stable

transformation of creeping bent grass via particle bombardment has been reported by Zhong et al. (1993), Hartman et al. (1999), Xiao and Ha (1997), Guo et al. (2003) and Dai et al. (2003). Only few reports on *Agrobacterium*-mediated transgenic turf grass are available (Zilinskas, 2001).

In the present study, we report *Agrobacterium*-mediated transformation of bent grass cv. Penn-cross with stress inducible *VuNCED1* gene isolated from cowpea and its functional characterization in bent grass (Accession no. AB030293). The over expression lines showed a 3- and 4-fold increase in biomass production compared with wild type and increase in tolerance to drought and salt concentration. Based on the results from these experiments, we contend that *VuNCED1* plays a regulatory role in drought and high salt conditions. A large number of morphologically normal transgenic plants were obtained. Detailed molecular, biochemical, and physiological analyses were carried out with selected transgenic plants.

## Material and methods

### *Selection and regeneration of phosphinothricin resistant calli from seeds*

Mature seeds of Penn-cross creeping bent grass were used for callus induction. The seeds were surface sterilized in 70% ethanol for one min and then in 40% commercial bleach with 0.1% tween-20 for 20 min. The seeds were washed four times with sterile distilled water. About 500 seeds were placed in 9 cm Petri dish containing 25 ml of the MS + 2,4-D 5  $\mu$ M + 3% sucrose and 0.8% agar. The Petri dishes containing seeds were sealed with serene wrap and incubated at 26 °C in the dark for 4 weeks. The resulting embryogenic calli were subcultured in same media and incubated again for another 2 weeks. The callus was then used for transformation.

### *Vector construction and transformation*

The PCR fragment of *VuNCED1* inserted in the *EcoRV* site of pBluescript II SK+ cloning vector was obtained from Plant Science Center of RIKEN (The Institute of Physical and Chemical Research, Japan). The fragment was cloned in the

sense orientation at the polylinker of transcriptional fusion vector pCAMBIA 3301 with *Sma*I and *Kpn*I between a CaMV 35S promoter containing the phosphinothricin gene under the control of cauliflower mosaic virus promoter and NOS terminator.

A single colony of bacteria was inoculated into a liquid YEP medium containing 50 mg l<sup>-1</sup> kanamycin and incubated for more than 24 h at 28 °C with reciprocal shaking (150 cycles min<sup>-1</sup>). Cultured bacterial cells were collected by centrifugation (2000 × g 10 min) and suspended to a final OD 600 of 20 ml liquid medium containing MS + 2,4-D 5.0 μM + 30 g<sup>-1</sup> sucrose with 100 μM acetosyringone (Sigma Aldrich, USA). Six-week-old callus were segmented into small pieces of 3 mm and immersed in bacterial suspension for 5 min, and blotted on sterile filter papers. Twenty explants were placed onto 25 ml aliquots of above medium with 7 g l<sup>-1</sup> agar in 9 cm Petri dishes, the plates were sealed with serene wrap and incubated at 25 °C in dark for 3 days.

After 3 days of co-culture callus was transferred to MS + 2,4-D 5 μM + phosphinothricin 10 mg l<sup>-1</sup> + cefotaxime 300 mg<sup>-1</sup> as the step I selection. Here transformation efficiency was recorded at the end of 7th week as the number of callus clumps producing embryogenic calli. As a step II selection, during the 8th week, the callus was sub cultured in MS + 2,4-D 5 μM + phosphinothricin 20 mg l<sup>-1</sup> in order to reduce the number of escaped untransformed calli and improve the selection efficiency, here the transformation efficiency was counted as the number of callus clumps proliferated at the end of 9th week.

In the step III selection during the 10th week the embryogenic sectors from resistant calli were bisected and transferred to the regeneration media, MS + phosphinothricin 20 mg l<sup>-1</sup> for induction of plantlets, the transformation efficiency was counted as number of plantlets formed in each callus clumps at the end of 13th week. In the IV step selection the developed plantlets were transferred to 1/2 MS + phosphinothricin 20 mg l<sup>-1</sup> at 14th week. The transformation efficiency was counted as number of plantlets formed roots at the end of 17th week. The plantlets which were rooted were transferred to pots containing the media soil:sand:coopeat in 2:1:1 ratio.

For negative control the callus was inoculated with an *Agrobacterium* strain free of any plant

selectable marker. For positive control, the callus without co-culture was transferred to respective growth hormone media without any phosphinothricin.

#### Genomic analysis

Total genomic DNA was isolated from leaf tissues of the control and putatively transformed plants according to CTAB method (Kim et al. 2002). A 347 bp fragment of *phosphinothricin* (*ppt*) gene and 800 kb fragment of *VuNCED1* were amplified with set of specific primers 5'-GGATCTACCAT-GAGCCAGAAC-3' and 5'-GACTTCAGCAGGTGGGTGTAGA-3', and 5'-ATACACCTCTTCTCATTCCACC-3' and 5'-TTGGGGATTTTTCCGACCACCG-3' respectively. PCR for *VuNCED1* and was performed using a programmed temperature controlled system. (2400 r, Perkin Elmer, USA) under the following conditions: 5 min at 94 °C, 1 min at 94 °C, 1.5 min at 60 °C (for amplifying *VUNCED1*), 1 min at 57 °C (for amplifying *ppt*) and 2 min at 72 °C with 30 cycles. Amplified products were analyzed by electrophoresis in 1% w/v agarose gel.

For Southern blotting, genomic DNA (about 10 μg) was restricted with *Eco*RI enzymes, separated on a 0.8% agarose gel, and transferred onto Hybond-N nylon membranes (Amersham Pharmacia, UK). Filters were hybridized with *VuNCED1* specific probe obtained from a 800 bp *Sma*I and *Kpn*I restriction fragment with alkaline phosphates direct labeling using alkphos direct labeling reagent (Amersham Pharmacia, UK). Hybridization, washing and detection were performed according to the instruction manual of Alkphos direct labeling and detection system with CDP star (Amersham Pharmacia, UK).

#### Salt and drought stress test

For salt stress test, transgenic plants which were rooted and grown on *phosphinothricin* selection medium, were hardened for 8 weeks. They were clipped uniformly to a height of 2.5 cm for all the treatments and planted in 4" size pots and was watered twice a week at 100 ml having salt concentration of 0.2, 0.4, 0.6 and 0.8% to create EC levels of 2.5, 5, 7.5 and 10 dS m<sup>-1</sup>. The

control plants were given normal water. After 10 weeks exposure to salt stress, growth observations were determined and recorded.

For drought stress test, transgenic plants, which were rooted and grown on phosphinothricin selection medium, were hardened for 8 weeks. They were clipped uniformly to a height of 2.5 cm for all the treatments and planted in 6" size pots. They were then exposed to drought stress by giving different quantities of water. Control plants were irrigated with 100 ml of water every 3 days while the plants with 25, 50 and 75% stress was given 75, 50 and 25 ml water respectively. After 10 weeks exposure to drought stress growth observations were determined and recorded.

Both experiments were designed as a randomized block layout with three replications. Each salinity and drought treatments consisted of 15 pots (5 pots per replication) with 5 plants per pot. Leaf firing percentage was determined by visually estimating as total percentage of chlorotic leaf area. Shoots were harvested, dried at 70 °C for 48 h for dry weight. ABA measurements were performed on crude extract of the youngest shoots by employing ELISA with laboratory raised polyclonal antibodies (Weiler 1982).

## Results

### *Selection and regeneration of phosphinothricin resistant calli from seeds*

Callus formation from seed could be observed after 4–5 days of culture. Three morphologically different callus types could be easily distinguished, a compact type, a white and nodular type with no apparent structures, a watery and transparent type. In the further sub cultures callus became

swollen and enlarged considerably. For transformation, only compact and friable callus was used.

As early as one week after the transfer of callus to regeneration medium plantlets were formed, this readily formed roots upon transfer to half-strength MS medium without growth regulators. Plants continued to grow after transfer to pots. In the first step selection, (Table 1) *phosphinothricin* resistant colonies continued to grow, a total of 3.3% resistant colonies were obtained. The resistant colonies were subsequently transferred to fresh medium with 20 mg l<sup>-1</sup> *phosphinothricin* (step II selection), since most of the untransformed callus was filtered earlier, 30.3% clumps were proliferating further. In the step III selection in regeneration media percentage of callus forming plantlets was 22.5% (Figure 1A, B). In the step IV selection percentage of plantlets forming roots were only 30%. In the negative control, few clumps of callus continued to grow in the steps I–III, however 100% mortality was observed in the last selection. In positive control, it was frequently observed that as the structures enlarged, additional shoot meristem regions were produced which gave rise to many more shoots than would be expected from the initial number of embryogenic structures within the culture (data not shown). An average of 3.47 shoots per callus clump was observed in both transformed and non-transformed treatments.

### *Genomic analysis of VuNCED1 in transgenic plants*

The *VuNCED1* gene from bean was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 2) and introduced into wild bent grass. Presence and integration of the introduced bar gene and *VuNCED1* in

Table 1. Effect of *Agrobacterium* transformation on survival, proliferation and regeneration of creeping bent grass callus under different steps of selections.

	Selection steps*			
	I % callus survived	II % callus proliferated	III % callus forming plantlets	IV % plantlets forming roots
Transformed	3.3b	30.3b	22.5b	30.0b
Negative control	1.5c	2.1c	0.1c	0.0c
Positive control	95.5a	70.5a	45.5a	85.5a

\*Mean separation in column by Duncan's multiple range tests. Within the column figure followed by same letter do not differ significantly at  $p \leq 0.05$ .

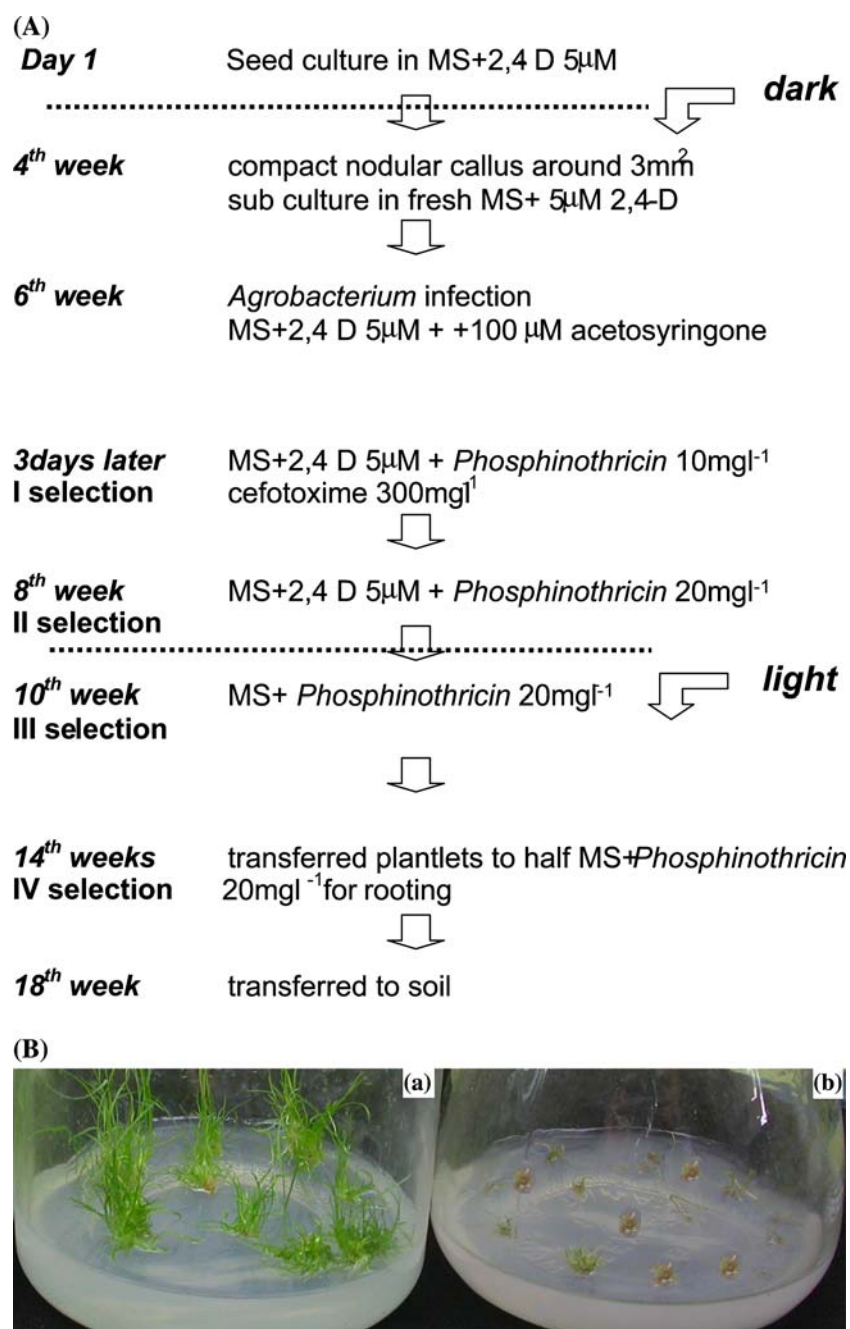
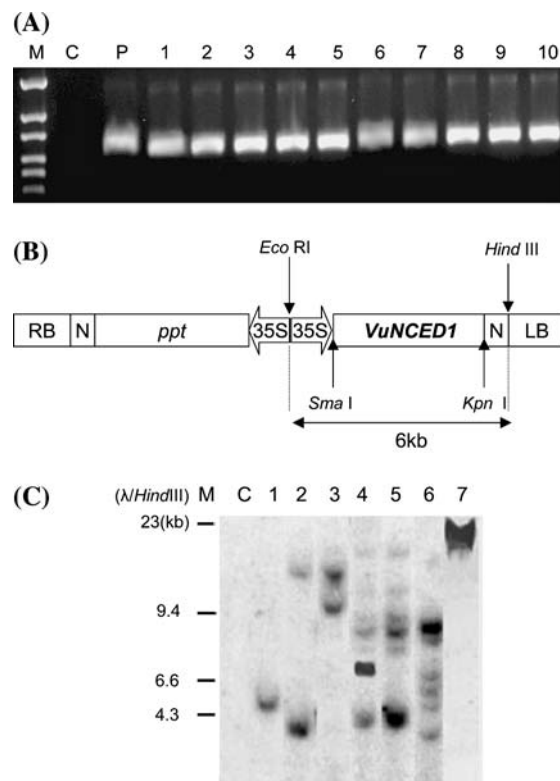


Figure 1. Diagram of the transformation method for bent grass. (A) The time schedule is indicated on the left. (B) Regeneration of plantlets under 20 mg l<sup>-1</sup> *phosphinothricin* in step III selection. (a) Transgenic plant; (b) control.

genomic DNA of transgenic plants were analyzed using PCR. A predicted 347 and 800 bp internal fragment for the *phosphinothricin* gene (data not shown) and *VuNCED1* were amplified in all of

them, suggesting that the *VuNCED1* is integrated into the genomes (Figure 2A).

The genomic DNA from a selected few putative transgenic shoots was subjected to gel blot analysis



**Figure 2.** PCR analysis of DNA isolated from leaves of independent transgenic plants and Southern hybridization. Agarose gel electrophoresis of PCR amplification was performed with primers for *VuNCED1* (A). A. lane M: molecular size marker (100 bp ladder), lane C: DNA from untransformed plant DNA, Lane P: plasmid DNA, Lanes 1–10: DNA from transgenic lines. (B) Constructs used for plant transformation. RB, Right T-DNA border; LB, left T-DNA border; 35S, CaMV 35S promoter; *ppt*, *phosphotransferase*; N, NOS terminator. (C) Southern-blot analysis of transgenic lines expressing *VuNCED1*. C lines with wild-type. Ten micrograms of genomic DNA was digested with *EcoRI*. Blots were probed with full-length *VuNCED1* (~6 kb) at high stringency.

under high stringency. Hybridization with *VuNCED1* specific probe confirmed foreign DNA integration in the transgenic lines analyzed (Figure 2B). Since there is no *EcoRI* site within the construct, these results suggest that the gene was integrated in to the genome at different sites. Further, hybridization of the probe to DNA greater than 23 kb in length in undigested DNA (lane 7) indicates that the *VuNCED1* is incorporated in to the genomic DNA and not maintained as independent plasmid. Genomic DNA from the wild type did not give any signal. The Southern blot (Figure 2C) shows insertion of the *VuNCED1* gene in the transgenic lines.

Expression of the *VuNCED1* transgene was monitored by RT-PCR. All over-expressing lines showed expression of the transgene (data not shown).

#### *Characteristics of transgenic plants under NaCl and drought stress*

To examine whether altered expression of *VuNCED1* affected drought tolerance in transgenics, the *VuNCED1* transgenic plants were grown for 10 weeks under normal conditions, then exposed to drought stress. The growth and development of wild and transgenic plants in soil in the greenhouse in the absence of stress appeared normal (data not shown). Under conditions of soil water deficit, the number of wilting plants and the number of plants that recovered after water was given again were quantified. These results indicate that wild type plants had substantially increased sensitivity to water deficit stress. Compared with wild type plants, Growth decreases when wild type and transgenic plants are grown in drought and NaCl

stress conditions (Tables 2 and 3), exhibiting reduced vigor grown at higher drought stress condition and at NaCl concentrations (Tables 2 and 3 and Figure 3). To determine the relative contribution of physiological traits to these decreases plant shoot and root of fresh and dry weight were investigated. Plants were grown in greenhouse, and irrigated with a complete nutrient solution supplied with 0 (control), 2.5, 5.0, 7.0 and 10 EC ( $\text{dS m}^{-1}$ ) NaCl and 0 (control), 25, 50 and 75% of drought condition. NaCl and drought stress reduced plant dry weight, height and tillers of leaves. Increasing of stress extent led to both morphological changes (reduction of shoot and root growth, Tables 2 and 3) and physiological changes (increase of endogenous ABA content, Table 4). Thus a correlation occurred between drought tolerance and NaCl stress, and the levels of expression of *VuNCED1* and endogenous ABA.

## Discussion

*Agrobacterium*-mediated transformation of creeping bent grass variety Penncross has been

accomplished. Although the transformation efficiency obtained with *Agrobacterium* was less than that achieved with gene gun, we could still obtain several transgenic plants for analysis of abiotic stress tolerance. This work, together with earlier reports of Iuchi et al. (2000) for Cowpea and Iuchi et al. (2001) for *Arabidopsis*, conclusively proves that engineering for abscisic acid, as accomplished by *VuNCED1* encoding for the enzyme *9-cis-epoxycarotenoid dioxygenase*, is an effective way of imparting stress tolerance to non-accumulators such as bent grass. In addition, this work has been carried out utilizing a popular and economically important variety Penncross. Although transgenic turf grass plants were already obtained using selection marker genes and other genes, this is first paper demonstrating the integration of *VuNCED1* gene and production of transgenic plants for drought resistance gene using *Agrobacterium*.

*De novo* organogenesis in tissue cultures is a useful system for studying regulatory mechanisms of plant development in addition to its application in plant biotechnology (Prakash and Kumar 2002). It is important that regeneration be maintained over relatively long period, in which calli

Table 2. Effect of NaCl stress on the shoot and root growth characters of wild and transgenic plants of creeping bent grass.

EC ( $\text{dS m}^{-1}$ )	Leaf firing (%)		No. of tillers		Shoot length (cm)		Root length (cm)		Shoot fresh weight (mg)		Shoot dry weight (mg)		Root fresh weight (mg)		Root dry weight (mg)	
	W	T	W	T	W	T	W	T	W	T	W	T	W	T	W	T
0	0	0	18.3a	20.4a	18.3a	20.4a	17.7a	19.3a	5735a	6131a	2674b	3845a	147a	184a	42b	77a
2.5	47.3a	16.3b	10.9a	12.6a	10.4b	15.1a	14.6b	20.4a	2402b	3790a	1310a	1758a	89b	124a	35a	52a
5.0	52.3a	36.7b	6.1b	9.8a	9.3b	13.3a	7.3b	16.3a	1710b	3010a	629b	1204a	64b	109a	21b	45a
7	87.3a	61.9b	4.1b	8.3a	6.3b	11.4a	4.2b	10.4a	750b	2185a	298b	874a	45b	73a	22a	30a
10.0	95.5a	78.9b	4.1b	7.6a	4.2b	8.9a	4.6b	8.9a	896b	1944a	323b	867a	38b	74a	21b	34a

Mean between wild and transgenic plants by Duncan's multiple range tests. Within the row figure followed by same letter for each character do not differ significantly at  $p \leq 0.05$ .

Table 3. Effect of drought stress on shoot and root growth characters of wild and transgenic plants of creeping bent grass.

Drought (%)	Leaf firing (%)		No. of tillers		Shoot length (cm)		Root length (cm)		Shoot fresh weight (mg)		Shoot dry weight (mg)		Root fresh weight (mg)		Root dry weight (mg)	
	W	T	W	T	W	T	W	T	W	T	W	T	W	T	W	T
0	0	0	22.2a	23.7a	21.2a	22.0a	20.8a	18.8a	9845a	9417a	4248a	3487a	126a	144a	50a	45a
25	14.5a	10.0a	18.4a	20.3a	19.2a	18.5a	16.8a	18.3a	7160b	8675a	3064a	3470a	102b	124a	40b	52a
50	45.5a	30.5b	11.3b	15.1a	12.3b	15.1a	10.3b	13.1a	3210b	5267a	1344b	2106a	62b	84a	25b	37a
75	65.5a	40.0b	8.1b	11.1b	9.0b	12.3a	8.6b	10.6a	1683b	3153a	673b	1261a	52b	89a	24b	35a

Mean between wild and transgenic plants by Duncan's multiple range tests. Within the row figure followed by same letter for each character do not differ significantly at  $p \leq 0.05$ .

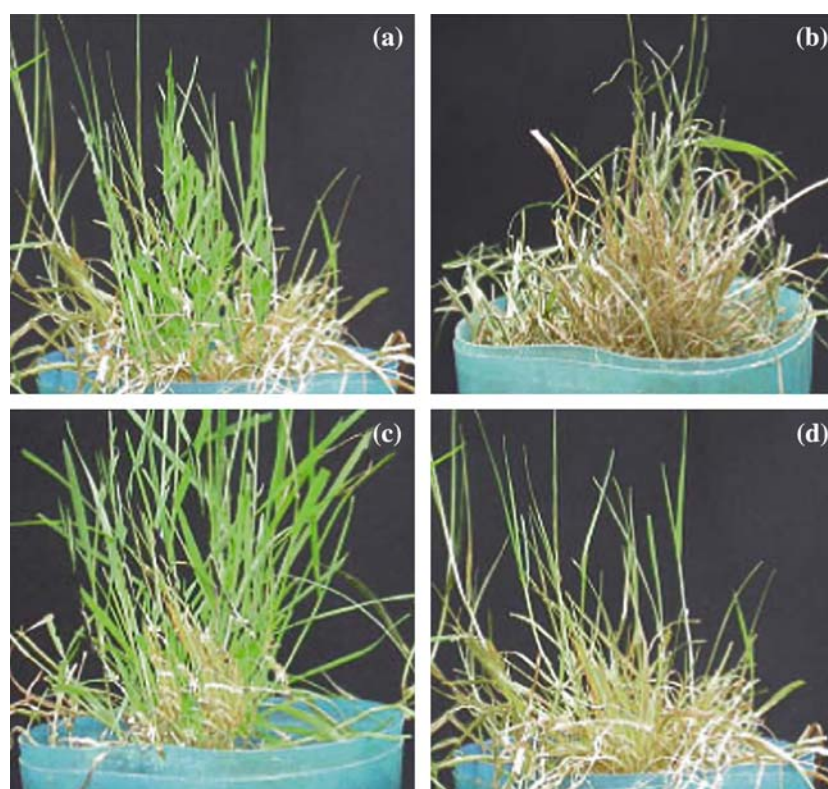


Figure 3. Enhanced drought and NaCl tolerance in wild bent grass plants overexpressing *VuNCED1*. Tolerance of plants under 75% drought stress. (a) transgenic plant, (b) wild type. NaCl tolerance under EC 7.5 dS m<sup>-1</sup>, (c) transgenic plants, (d) wild type.

are first produced from single transformed cells, after which shoots can be regenerated from the calli in indirect regeneration many non-transformed buds were formed next to transformed cells and these transformed cells might protect and nurse untransformed cells for regeneration (Orlikowska and Nowak 1997). Therefore we allowed the co-cultured callus for 4 weeks to proliferate and then put into regeneration medium.

Creeping bent grass callus was sensitive to phosphinothricin, addition of 10 mg l<sup>-1</sup> to medium fully inhibited proliferation. In such sensitive

system the chances of escapes will be very low. Selection at high phosphinothricin concentrations (20 mg l<sup>-1</sup>) during the first week of culture minimized escaped callus, and continuous culture on medium containing 20 mg l<sup>-1</sup> phosphinothricin inhibited shoot regeneration, and transformed shoots were not obtained at all.

It has been proposed that ABA is synthesized from carotenoids (C40) in plants (Zeevaart and Creelman 1998). Three genes that participate in ABA biosynthesis have been isolated. They encode *zeaXanthin epoxidase* (ZEP; Marin et al. 1996);

Table 4. Relative ABA content (ng/gDW) in wild and transgenic plants with different drought and NaCl stress conditions.

	EC(dS m <sup>-1</sup> )				Drought (%)			
	0	2.5	7.5	10	25	50	75	
Wild	18a	125b	145b	160b	120b	150b	185b	
Transgenic	25a	180a	220a	285a	190a	280a	300a	

Means separation in column by Duncan's multiple range tests. Within the column figure followed by same letter do not differ significantly at  $p \leq 0.05$ .



*9-cis-epoxycarotenoid dioxygenase* (NCED; Schwartz et al. 1997); and *abscisic aldehyde oxidase* (AAO; Seo et al. 2000). ZEP catalyses the epoxidation of zeaxanthin to produce epoxycarotenoid; NCED catalyses the cleavage reaction of epoxycarotenoids to produce xanthoxin (the first C-15 intermediate); and AAO catalyses the final step of ABA biosynthesis, which converts ABA aldehyde to ABA. Biochemical studies indicate that a key step in ABA biosynthesis is the cleavage of *9-cis-epoxycarotenoid* (Kende and Zeevart 1997). At least seven genes are homologous to *NCED* in the *Arabidopsis* genome, but little is known about their roles in the accumulation of ABA in various processes during plant growth and stress responses. The expression of several *NCED* genes induced by drought stress in maize and tomato was reported (Burbidge et al. 1997; Schwartz et al. 1997).

The cDNA of *VuNCED1* which we have used here was cloned from cow pea by Iuchi et al. (2000) revealed sequence homology with the *9-cis-epoxycarotenoid dioxygenase*, which is involved in ABA biosynthesis. The recombinant *VuNCED1* protein showed *9-cis-epoxycarotenoid dioxygenase* activity and consistent with the activity of maize VP14 (Schwartz et al. 1997). They demonstrated that the N-terminal region of the *VuNCED1* protein functions as a transit peptide for plastid targeting. Epoxycarotenoids localized in plastids and the oxidative cleavage reaction of epoxy-carotenoids is supposed to occur in plastids (Zeevaart and Creelman 1998). Their findings indicate that *9-cis-epoxycarotenoid dioxygenase* is targeted into plastids and functions in the plastid to produce ABA. They analyzed the effects of various environmental stresses on the expression of the *VuNCED1* gene, and found that the gene was strongly induced under a high-salt condition, but not by cold or heat stress. The induction of the *VuNCED1* gene was not detected by exogenous ABA application or water treatment.

The agronomic performance of our transgenic plants under stress is significant. More than 50% transgenic plants survived on exposure to salt and drought stress. The other remarkable changes in transgenic plants were increased biomass of plant body with increase number of tillers even under stress conditions. The increase in biomass refers to high efficient carbon fixation and consequent exchange of growth. We found 50% firing under EC of 2.5 dS m<sup>-1</sup> for control while it was as high as 7.5

in transgenic plants, in *Zygosiza* grass 50% firing was reported under EC of 3.0 dS m<sup>-1</sup> (Qian and Engelke 2000). Due to drought and salt stress, the root/shoot ratio significantly increased which is recognized to be a general morphological adaptation to decreased water potentials (Romero-Aranda et al. 2002). This suggests that growth reduction is possibly related more to the average stress in the shoot than to the average root zone stress (Munns 2002). These results confirm that osmotic stress alters the pattern of allocation of photosynthates to different plant parts. Similar results were reported in tomato by De Pascale et al. (2003b).

Although correlation between production of ABA and stress cannot unequivocally assess a cause-effect relationship, it is at least consistent with the hypothesis that ABA activates the metabolic signaling between stress perception and adaptation (Munns 2002). ABA may be the root signal for stomatal closure in the leaves (Davies et al. 2002), thus enabling high turgor maintenance in saline environments. ABA levels we obtained for transgenic plants were twenty times higher than that of control, which is very low compared to the level of ABA in 10-h dehydrated plants of cow pea which was 140 times higher than that in unstressed control plants (Iuchi et al. 2000), however it was more than that reported earlier for transgenic tobacco (Qin and Zeevaart 2002), tomato (De Pascale et al. 2003a) and even this much ABA was capable of imparting a high level of salt tolerance as revealed by challenge studies. Accumulation of ABA in the transgenic plants when exposed to drought/salt activated ABA signal transduction pathways and stomatal closure, resulting in enhanced drought tolerance. De Pascale et al. (2003a) observed salt-induced increase of the free ABA in tomato leaves was associated with reduced stomatal conductance, suggesting that under osmotic stress conditions, the ABA acts as a stress messenger from the roots to the shoots. ABA-deficient mutants of tobacco and ABA-insensitive mutants of *Arabidopsis* have reduced stomatal closure in response to water deficit, and show a wilted phenotype. Conversely, ABA-hypersensitive mutants of tobacco show a reduced transpiration rate and enhanced drought tolerance (Iuchi et al. 2000). Current models suggest that the stress is first perceived by cells as plasmalemma perturbations. This is caused by loss in turgor pressure, followed by an increase in cytosolic and apoplastic ABA due to *de novo* synthesis and/or

release of the hormone sequestered in organelles (De Pascale et al. 2003b; Kuklev et al. 2003). Same type of response was reported by Qin and Zeevaart (2002) when detached bean leaves were water stressed; ABA accumulation was preceded by large increases in *PvNCED1* mRNA and protein levels. Conversely, rehydration of stressed leaves caused a rapid decrease in *PvNCED1* mRNA, protein, and ABA levels. In bean roots, a similar correlation among *PvNCED1* mRNA, protein, and ABA levels was observed. However, the ABA content was much less than in leaves, presumably because of the much smaller carotenoid precursor pool in roots than in leaves. Ectopic expression of a tomato *NCED* gene caused overproduction of ABA in tomato and tobacco (Thompson et al. 2000), which also suggests a key regulatory role of *NCED* in ABA biosynthesis. Iuchi et al. (2001) also observed same response in over expression of *AtNCED3* *Arabidopsis* plants. *AtNCED3* sense transgenic plants were more resistant to drought stress than wild-type plants. By contrast, *AtNCED3* antisense transgenic plants and T-DNA-tagged mutant plants were more sensitive to drought stress than wild-type plants. This provide evidence in support of the long-standing hypothesis that drought-induced ABA biosynthesis is regulated by the 9-*cis*-epoxycarotenoid cleavage step at the transcriptional level, assuming that the abundance of *NCED* mRNA after dehydration is attributable to increased transcription. In plants, recent research has proved that intricate stress response mechanisms and ‘cross talk’ between stress responses exist (Siobhan et al. 2003).

In conclusion, gene engineering of *VuNCED1* in transgenic creeping bent grass plants could control endogenous ABA. Accumulation of ABA in the transgenic plants activated ABA signal transduction pathways resulting in enhanced NaCl and drought tolerance. These results point the way to molecular breeding of drought/NaCl tolerant crops using a key gene involved in ABA biosynthesis. We established an efficient and reproducible system for generating transformed bent grass using highly regenerative tissue derived from mature seed embryogenic callus.

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