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Identifying key genes involved in accumulation of ABA during drought in rice

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A B S T R A C T

This study was undertaken to identify homologs of NCED gene family involved in ABA accumulation in rice in response to drought stress. Results showed rapid accumulation of ABA in rice peduncles during drought and its faster degradation during rewatering. BLAST analysis using the NCED sequences of Arabidopsis led to the identification three NCED family member in rice located on chr. 3, 7 and 12. Semi-quantitative RT-PCR analysis revealed *OsNCED1* is induced in rice leaves in response to drought stress and their expression level reaches to normal upon rewatering. Further, transcript abundance of *OsNCED1* was found to be correlated with ABA levels in rice leaves. The findings need further confirmation by developing over-expression/knockout mutants.

Introduction

The plant hormone ABA (abscisic acid) has been demonstrated to be involved in many plant growth processes including seed development, dormancy, germination, vegetative growth and environmental stress responses. ABA plays important role in regulating stomatal opening and closing during water deficit conditions and prepare the seed for dormancy and germination (McCarty, 1995). In plant system, ABA accumulates during seed development as well as under stress conditions drought, salinity, cold etc. and helps in mediating stress responses (Ingram andBartels, 1996). Further, ABA act as signaling element and regulates the expression of corresponding downstream genes involved in various biochemical and physiological processes that helps the plant to overcome the stress conditions (Rock, 2000; Söderman *et al.*, 2000). To perform these diverse functions a complex regulatory mechanisms involved in signal perception, controlling its production, degradation, and transduction is required.

Since the discovery of ABA in the early 1960s, various genetic and biochemical studies has been conducted to elucidate the biosynthetic pathway of ABA in higher plants leading to identification of all major genes involved in the biosynthetic pathway (Schwartz *et al.*, 2003). Most of the ABA biosynthetic steps occurs in plastids but last

two steps involved in conversion of xanthoxin to ABA (Marin *et al.*, 1996). The first step in biosynthesis of ABA is the conversion of zeaxanthin to trans-violaxanthin which involves a two-step epoxidation catalysed by zeaxanthin epoxidase (Marin *et al.*, 1996). Then further all-trans-violaxanthin in converted to 9-cis-violaxanthin or 9-cis-neoxanthin by an unknown enzyme. In the next step 9-cis-violaxanthin and/or 9-cis-neoxanthin is cleaved by 9-cis-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin. Cleavage of step 9-cis-violaxanthin and/or 9-cis-neoxanthin to xanthoxin by NCED is considered to be rate-limiting in ABA biosynthesis and occurs in plastids (Qin and Zeevaart, 2002). Thereafter xanthoxin is exported to the cytosol and converted to abscisic aldehyde by a short-chain dehydrogenase/reductase (Cheng *et al.*, 2002). Abscisic aldehyde is then finally oxidized to ABA by aldehyde oxidase (Seo *et al.*, 2004). AO needs the sulphurylated form of a molybdenum cofactor for its activity (Bittner *et al.*, 2001).

Now the new challenge is to understand how the genes involved in biosynthetic pathway of ABA are regulated under different environmental conditions. Recent molecular genetic analyses indicated that members of the Arabidopsis 9-cisepoxycarotenoid dioxygenase (*AtNCED*) gene family play distinct roles in the regulation of ABA biosynthesis during seed development and germination (Lefebvre *et al.*, 2006; Seo *et al.*, 2004). Hence, this study was aimed to identify the key members of NCED gene family involved in ABA accumulation during drought conditions.

Materials and Methods

Plant material and Growth Conditions

The seeds of rice genotype IR64 and Moroberekan was obtained from the Department of Rice, Tamil Nadu Agricultural University, Coimbatore, India. Plants were grown in pots filled with 2 kg of field soil mixed with required amount of fertilizer [1.25 g of (NH4)2SO4, 0.08 g Muriate of potash (KCl), and 0.08 g single superphosphate (SSP)] and maintained at 28±2°C under ≈12h light/12h dark at natural day light conditions with a relative humidity of 80±5% under greenhouse conditions at Tamil Nadu Agricultural University. Drought stress was imposed to a set of plants at vegetative stage (40 days after sowing) by withholding watering 4 days. Leaf sample were collected from both drought stressed as well as control plants when the soil moisture reached around 20% in drought stressed plants and snap-frozen in liquid nitrogen for RNA extraction. The plants were further revived by rewatering and leaf sample were collected 3 days after rewatering and snap-frozen in liquid nitrogen for RNA extraction.

RNA extraction and cDNA synthesis

For isolating total RNA frozen leaf samples were ground in liquid nitrogen and total RNA was extracted using One Step RNA Reagent (Biobasic Inc., Canada) as per manufacturer's protocol. The integrity of RNA was assessed by separating the RNA on 1% formaldehyde agarose gel containing 0.5µg/ml ethidium bromide at 80 volts for one hour and examining the separated RNA under UV light in Gel documentation system (BioRad, USA). The quantity of isolated RNA was assessed using Nanodrop ND-1000 VIS spectrophotometer (Thermo Fisher Scientific, USA). Only the samples having the 260/280 and 260/230 ratios around 1.9- 2.1 were selected for further analysis.

Total RNA isolated from leaves of control, drought stressed and rewatered plants was used for cDNA sysnthesis. Three micrograms of total RNA was treated with DNase (Thermo Scientific, USA) and incubated at

37°C for 30 min and reaction was stopped by treating with 1 µL of 50 mM EDTA and followed by incubation at 65°C for 10 m. DNase treated total RNA was converted into single stranded cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) as per manufacturers protocol.

Semi-quantitative RT-PCR

In order to identify the members of NCED gene family involved in ABA biosynthesis in rice during drought stress, putative homologous genes in rice were identified by BLAST analysis in the TIGR (www.tigr.org) using Arabidopsis NCED genes as a query sequence. Gene specific primers were designed to amplify the transcripts of various members of NCED gene family members of rice. Semi-quantitative RT-PCR was performed using 50ng of each cDNA sample in a final reaction mixture (20 µl) containing PCR buffer (10 mMTris-HCl pH 8.0, 50 mMKCl, 1.5 mM MgCl2, 0.1% gelatin), 0.2 mMdNTPs (Thermo Scientific, USA), 120 ng of each primers and 1 unit of Taq DNA polymerase (Thermo Scientific, USA). The thermal cycling conditions were composed of an initial denaturation step at 95°C for 5 min, 27 cycles at 95°C for 30 sec, then 58°C for 30 sec and 72°C for 30 sec. PCR product was resolved on a 2.5% agarose gel, stained with ethidium bromide and visualized under Quantity One GelDoc (Biorad, USA).

Results and Discussion

ABA's accumulation during drought and their involvement in stress responses has been reported in several plant species(Qin and Zeevaart, 1999; Iuchi *et al.*, 2001; Iuchi *et al.*, 2000).Abscisic acid (ABA) regulates drought stress response in plants by affecting transpirational water loss, stomatal closure, photosynthesis, water use efficiency, seed development and maturation, leaf senescence and cell membrane protection, and so on. The degree of biosynthesis and accumulation of ABA in a crop cultivar is a possible indicator of drought tolerance. In all ABA-dependent physiological and developmental processes, regulation of ABA signaling is central to develop drought tolerance in plants.

The accumulation of ABA under water deficit may result from enhanced biosynthesis. Drought stress-regulated ABA biosynthesis depends on a key enzyme, 9-*cis*-epoxycarotenoid dioxygenase (NCED) involved in catalyzing a rate limiting step of ABA biosynthesis i.e. conversion of 9-cis-violaxanthin and/or 9-cis-neoxanthin to xanthoxin. In order to identify the members of 9-cis-epoxycarotenoid dioxygenase (NCED) gene family involved in ABA biosynthesis in rice during drought stress, putative NCED homologous genes in rice were identified by BLAST analysis in the TIGR (www.tigr.org) using Arabidopsis NCED as a query sequence. Results of BLAST analysis revealed that there are 3 homologous genes in rice *viz.*,*OsNCED1* is located on chromosome 3 (LOC_Os03g44380), *OsNCED2* is located on chromosome 12 (LOC_Os12g42280) where as *OsNCED3* is located on chromosome7 (LOC_Os07g05940).

With an aim of identifying gene members of NCED family involved in ABA accumulation in rice leaves during drought and rewatering, we designed gene specific primers for all three members of NCED family in rice and their expression level was checked in RNA samples extracted from leaves of IR64, Moroberekan under well watered, drought stressed and then rewatered conditions.

Fig.1 Semi-quantitative RT-PCR analysis of NCED family members during drought and rewatering in the leaf tissues of rice. Where 1 is IR64 well watered ; 2 is IR64 drought stressed; 3 is IR64 rewatered; 4 is Moroberekanwell watered; 5 is Moroberekan drought stressed ; 6 is Moroberekan rewatered

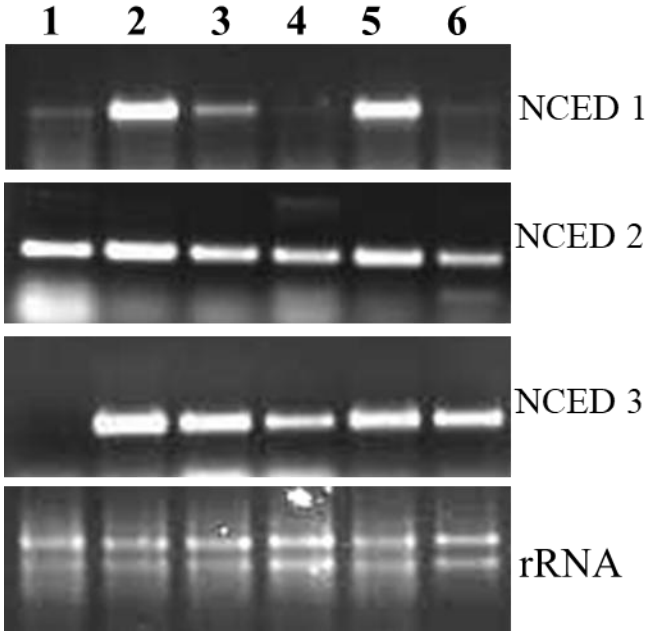

Table.1 Information on the primer sequences and physical location of different members of *NCED* family in rice genome

Gene name	Locus ID	Physical location	Primers sequence (5'-3')
OsNCED1	LOC_Os03g44380	Chr.3; 24959107 – 24961777 bp	Fow- actgcttctgcttccacctc Rev- gctccctctggtcacttcct
OsNCED2	LOC_Os12g42280	Chr.12; 26268230 – 26270794 bp	Fow- ggctacatcctctccttcgtc Rev-caccctcagtctctccctaa
OsNCED3	LOC_Os07g05940	Chr.7; 2870686 – 2872832 bp	Fow- cggagaagttcatctacg Rev- aaaatcagtagtgcatgacc

Among 3 NCED members of rice only *OsNCED1* was found to be drought responsive and was found to be over-expressed in response to drought in both the rice genotypes where as there expression was reduced further after rewatering. Overexpression was found to be much higher in tolerant rice genotype moroberekan as compared to susceptible rice genotype IR64. Whereas *OsNCED3* has not shown any difference in its expression pattern in response to drought stress in both rice genotypes. Arabidopsis homologue of NCED2 gene has shown slight upregulation in response to drought stress in rice in both the genotypes. This clearly indicates that *OsNCED1* is majorly involved in ABA accumulation in response to drought stress in rice leaves whereas *OsNCED2* may also be playing some role in accumulation of ABA in rice leaves during drought.

NCED gene is found to be over expressed in drought stress condition in maize (Tan *et al.*, 1997), tomato (Burbidge *et al.*, 1999), *Phaseolus vulgaris* (Qin andZeevaart, 1999), Arabidopsis (Iuchi *et al.*, 2001) cowpea (Iuchi *et al.*, 2000) etc. A remarkable rise in *OsNCED1* transcript levels provides an evidence for their activation in response to dehydration and their probable role in drought responsive ABA accumulation and thereby providing stress tolerance through ABA dependent pathway. Results of this study indicated that *OsNCED1* can serve as a putative candidate for improving drought stress tolerance in rice through genetic engineering.

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