International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 4, Issue 4, 2013, pp 481-487
http://www.bipublication.com

# IDENTIFICATION OF GENES CONTROLLING ABA ACCUMULATION IN RICE DURING DROUGHT STRESS AND SEED MATURATION

**Ashok Kumar K[1], Suresh Kumar M[2], Sudha M[3], Vijayalakshmi D[4], Vellaikumar S[3], Senthil, N[3] and M. Raveendran[3]***

[1]Post Doctoral Fellow, Canada.
[2]DEMIC, Tamil Nadu Agricultural University, Coimbatore, India-641 003.
[3]Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India – 641 003.
[4]Dept. of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore, India – 641 003.
*Corresponding author Email: raveendrantnau@gmail.com Tel: +91-8148095400 Fax: +91-422-6611462

[Received-24/09/2013, Accepted-12/11/2013]

**ABSTRACT**

Phytohormone abscisic acid (ABA) is critical for many plant growth and developmental processes including seed maturation, germination and response to environmental factors. ABA biosynthesis and degradation contribute to ABA homeostasis during drought stress and seed development. In this study, attempts were made to identify putative candidate genes involved in ABA biosynthesis and catabolism in rice during drought and seed maturation. Results revealed that rapid accumulation of ABA in rice peduncles during drought and its faster degradation during re-watering. The pattern of ABA accumulation at various stages of panicle development showed that ABA accumulation started at 10 days after heading and reached a maximum at 25 days after heading. Expression pattern of various members of abscisic aldehyde oxidase (AAO) and ABA-8' hydroxylase gene families were studied in the maturing seeds and drought stressed rice tissues through semi-quantitative RT-PCR analysis. Semi-quantitative RT-PCR analysis revealed that AAO3-1 and AAO3-2 are induced during drought stress and OsCYP707A5 is significantly down regulated in rice peduncles during drought and in rice panicles towards maturation. The ABA levels in peduncles and maturing rice panicles was thus found to be correlated with transcript abundance of OsCYP707A5.

**Key words**: *Oryza sativa, abscissic acid, biosynthesis, catabolism, gene expression, drought, seed maturation*

## INTRODUCTION

The phytohormone abscisic acid (ABA) plays an important role in a number of physiological processes such as seed maturation, seed dormancy and adaptive responses to abiotic stress [20]. ABA regulates stomatal responses, stress tolerance responses and prepares the seed for

dormancy and germination [2]. ABA accumulation happens during seed development or under stress conditions such as water deficit, salinity and cold mediated stress responses [3]. ABA induces or regulates corresponding gene expression in the biochemical and physiological processes which will help the plant to overcome the stress conditions [9,10].

ABA is found in all photosynthetic organisms and its amount is determined by the dynamic balance between biosynthesis and degradation. 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 [18]. AtNCED6 and AtNCED9 play a major role in ABA biosynthesis during seed development and germination. Apart from NCEDs, another key rate limiting step in the ABA biosynthesis involves the role of Abscisic Aldehyde Oxidase (AAO) gene family. Recent findings revealed that NCEDs are located in plastids and the product of NCED reaction, Xanthoxin is converted into ABA by AAO in the cytosol by two oxidation steps via the formation of abscisic aldehyde [21]. In rice, very little information is available with regard to the role of AAO gene family member involved in ABA biosynthesis during stress conditions.

ABA catabolism in plants takes place through two major pathways viz., i) glucose conjugation and ii) enzymatic conversion of ABA into Phaseic acid mediated by a Cytochrome P450 namely ABA-8'-hydroxylase which belongs to CYP707A family in rice **[6]**. In Arabidopsis, four CYP707A gene families (CYP707A1 to CYP707A4) encodes for ABA 8'-hydroxylases and CYP707A2 plays a major role in controlling ABA accumulation during seed development [22]. In rice, CYP707A family includes two members viz., CYP707A5 and CYP707A6 [23].

Two of the best-characterized ABA responses are developmental events during seed maturation and response to environmental stress. ABA biosynthesis and signalling genes have been well studied in non-seed tissues but little is known about the situation in seeds [14]. However, a comprehensive knowledge of ABA biosynthesis and catabolism events involved in the regulation of ABA levels in different grain tissues and their regulation during water stress is still lacking. Hence, the present study was aimed to understand i) The pattern of ABA accumulation and degradation during drought and re-watering of rice plants ii) To study the temporal regulation of ABA accumulation during panicle development in rice and iii) To identify the key members of AAO and CYP707A gene family involved in ABA accumulation and degradation during drought and panicle development stages.

## MATERIALS AND METHODS

### Plant material and Growth Conditions

Rice seeds (*Oryza sativa ssp. japonica* cv. Moroberekkan) were obtained from the Department of Rice, Tamil Nadu Agricultural University, Coimbatore, India. Plants were grown in pots under natural conditions ($28^{o}$ C) until panicle development stage. One batch of plants was drought-stressed for 3 days starting from 3 DBH and the severity of drought was monitored by measuring Relative Water Content (RWC %) of leaves as described by Weatherly [1]. A set of drought stressed plants were re-watered and peduncle (uppermost internode) tissues were collected from rice plants grown under all three conditions viz., control, drought stressed and re-watered conditions. Panicle tissues were collected at different stages of panicle development

(starting from 4 DBH to 25 days after heading (25 DAH)) from the well watered plants for ABA quantification.

**ABA measurements**

ABA accumulation pattern in rice peduncles (collected under control, drought stressed and rewatered conditions) and developing panicles were analyzed by HPLC as described by Krochko [6]. Tissue samples (three biological replications of all tissues) were ground in liquid nitrogen and the powder was freeze-dried and the dry weight was determined. ABA was extracted from lyophilized plant material using ethyl acetate (100%). Extraction was carried out twice each with 1 ml of ethyl acetate at 4$^{o}$C. The supernatant collected after centrifugation (13 000 g, 10 min, and 4$^{o}$C) was evaporated using a vacuum concentrator until dryness at room temperature. The dried samples were re dissolved in acetonitrile: methanol (1:1 v/v) and filtered using a 0.8 lm filter. The filtrate was used for subsequent quantification using HPLC (Varian 1200L) [25]. Chromatographic separation was carried out on a reverse-phase C 18 column by isocratic elution with a 75:25 (v/v) mixture of aqueous 1% acetic acid and acetonitrile and UV detection at 262 nm. The ABA fraction can be identified by retention time and comparison with known ABA standards, using 25 µl as injection volume and 1ml/min as flow rate. The peak areas were measured and the ABA concentration quantified using the standard curve obtained from ABA. All the data were analysed statistically and significance was tested by calculating the standard error.

**RNA extraction and semi-quantitative RT-PCR.**

Total RNA was prepared using TRIZOL reagent (Invitrogen, USA) method from the peduncles of control, drought stressed and rewatered plants of (*Oryza sativa ssp. japonica* cv. Moroberekkan). The RNA was treated with DNAse and normalized based on rRNA intensity. Expression levels of genes belonging to Abscissic AldehydeOxidase (AAO1-6) and ABA 8'hydroxylase (CYP707A) families were analyzed using semi-quantitative reverse transcription (RT)-PCR. The *japonica* sequence of each gene was obtained from the TIGR and NCBI rice database. Exonic sequences from each gene were used for the design of gene specific primers. GAPDH was used as a control gene. About 0.5 microgram of total RNA from each sample was used for RT reactions using the Invitrogen one step-RT PCR kit (Invitrogen USA). The thermal cycling conditions were composed of 52°C for 30 min (reverse transcription) followed by an initial denaturation step at 95°C for 5 min, 35 cycles at 95°C for 30 s, then 58°C for 1 min and 72°C for 1 min. The experiments were performed in duplicate for each gene. The RT-PCR products were resolved by 1.2% Agarose gel electrophoresis and visualized under Quantity One GelDoc (Biorad, USA).

**RESULTS AND DISCUSSION**

Drought stress had significant effect on ABA content of rice peduncles. ABA is known to be involved in stress responses and its accumulation during drought was reported in several plant tissues [7,15,16]. The ABA contents of the drought stressed peduncles (0.97µg/g) were ten fold higher compared to control (0 µg/g). In maize, Jia and others [13]; Zhang and others [5] also observed reduced ABA degradation under water stress. ABA accumulation was degraded very rapidly and reached its original level upon re-watering (Fig. 1).

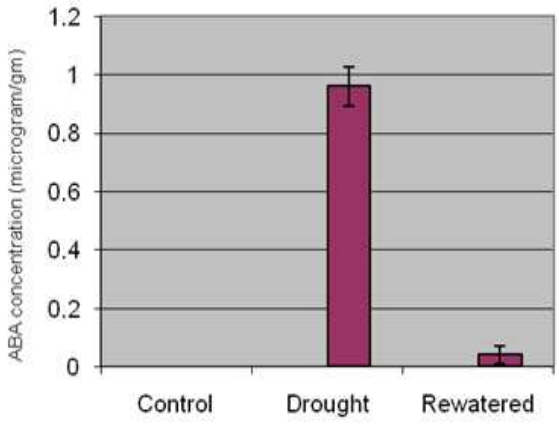


**Fig. 1** ABA accumulation and degradation pattern in rice peduncles during drought and re-watering.

In cassava also the leaf ABA content usually rapidly decreases after rehydration to the level observed in unstressed control plants [8]

The accumulation of ABA under water deficit may result from enhanced biosynthesis and/or a decrease in breakdown [4]. The final step in ABA synthesis is catalysed by the abscisic aldehyde oxidase (AAO3) [11]. In order to identify the members of Abscissic aldehyde oxidase (AAO) gene family involved in ABA biosynthesis in rice during drought stress, putative AAO homologous genes in rice were identified by BLAST analysis in the TIGR (www.tigr.org) using Arabidopsis AAO as a query sequence. Different sequence comparisons (BLASTN, BLASTX, tBLASTX) were performed against the Arabidopsis and rice genome sequences to derive homologous sequences of ABA biosynthesis and inactivation pathways in barley (Seiler and others 2011). Results of BLAST analysis revealed that there are 6 homologous genes in rice viz., two on chromosome 3 (LOC_Os03g57690 (AAO3-1) and LOC_Os03g57680 (AAO3-2), three on chromosome 7 (LOC_Os07g07050 (AAO7-1); LOC_Os07g18120 (AAO7-2) and LOC_Os07g18160 (AAO7-3) and one on chromosome 10 (LOC_Os10g04860 (AAO10-1). Semi-quantitative RT-PCR analysis of AAO gene family members revealed that both the genes located on chromosome 3 (AAO 3-1 and AAO 3-2) were found to be induced during drought and reversed back to the original abundance within 24 hrs after re-watering (Fig. 2).

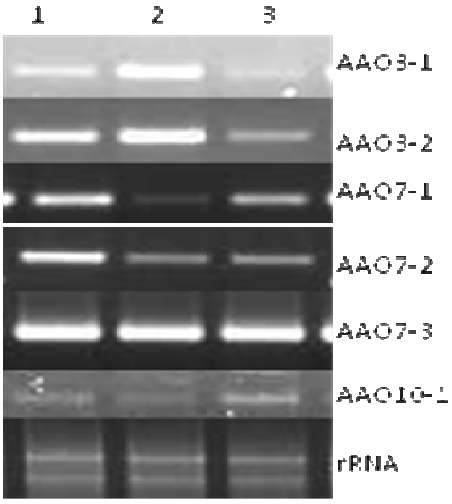


**Fig. 2** Semi-quantitative RT-PCR analysis of AAO family members during drought and re-watering in the peduncle tissues of Rice.
**Lane 1:** Control, **Lane 2:** Drought stress, **Lane 3**: Re-watered

This clearly indicated that these two members may be involved in elevated accumulation of ABA during drought. This is similar to reports from Seo and others [11] and Taylor and others [12] who found that the AAO3 mRNAs are upregulated by water stress in Arabidopsis.

A variety of pathways exist for ABA catabolism, but the 8'-hydroxylation of ABA to form phaseic acid appears to be the predominant pathway for ABA inactivation. This reaction is catalysed by the Cytochrome P450 8'-hydroxylase (CYP707A) (Kushiro and others 2004). Gene

specific primers were designed for both the members of OsCYP707A family in rice and used for RT-PCR analysis (Table 1).

| S. No | Gene name | Locus | Ch. No. | Distance (cM) |
|---|---|---|---|---|
| 1 | ***OsCYP707A5*** | **LOC_Os02g47470** | **2** | **~123cM** |
| 2 | ***OsCYP707A6*** | **LOC_Os08g36860** | **8** | **~91cM** |

**Table 1.** Information on the physical location of different members of *CYP707A* family in rice genome.

Semi-quantitative RT-PCR analysis of OsCYP707A5 and OsCYP707A6 genes using total RNAs isolated from control, drought and re-watered peduncles revealed that OsCYP707A5 is significantly down regulated during drought and the transcript abundance reached its original level within 24 hrs of re-watering (Fig. 3)

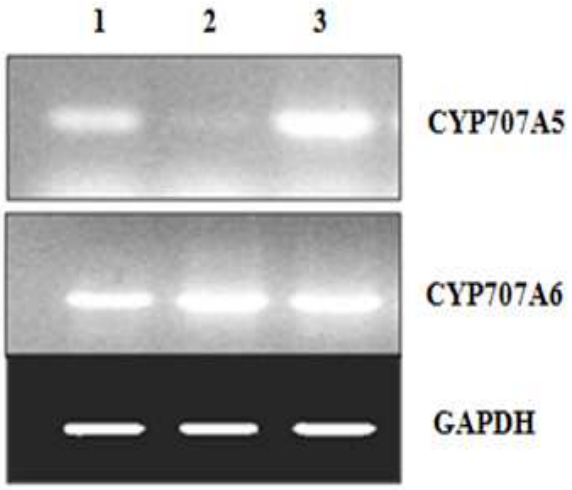


**Fig. 3** Semi-quantitative RT-PCR analysis of CYP707A family members during drought and re-watering in the peduncle tissues of rice.
**Lane 1:** Control, **Lane 2:** Drought stress, **Lane 3**: Re-watered

Down-regulation of this OsCYP707A5 may be associated with increased accumulation of ABA and its recovery during re-watering may be associated with faster degradation of accumulated ABA. These results are in agreement with the other authors who have found that the transcript levels of all four CYP707A's in arabidopsis are induced by abiotic stress, dehydration, and exogenous ABA treatment [17,19].

ABA accumulation pattern was analyzed in the panicle tissues of rice (*Oryza sativa ssp. japonica* cv. Moroberekkan) at various stages of panicle development starting from 4 DBH to 25 DAH. The ABA contents varied from 0.67 to 42.62 µg/g. In maturing seed, ABA-regulated genes include those required for the acquisition of desiccation tolerance (Rock, 2000). Results revealed that ABA accumulation started increasing at 10 DAH (1.53 µg/g) and reached its peak at 25 DAH (42.62 µg/g) (Table 2).

| Stages | 4 DBH | JH | 5 DAH | 10 DAH | 15 DAH | 20 DAH | 25 DAH |
|---|---|---|---|---|---|---|---|
| Quantity (µg/g) | 0.78 | 0.67 | 0.83 | 1.53 | 1.81 | 3.59 | 42.62 |
| SD | 0.25 | 0.014 | 0.014 | 0.39 | 0.54 | 0.88 | 6.25 |

**Table 2.** ABA accumulation pattern in (*Oryza sativa ssp. japonica* cv. Moroberekkan) at various stages of panicle development
**DBH** – Days Before Heading; **JH** – Just Heading;
**DAH** – Days After Heading

Endogenous production of ABA is likely to take place in both the endosperm and the embryo during seed maturation as reasoned by the activation of ABA biosynthesis genes in barley was found by (Sreenivasulu and others [24]). Semi-quantitative RT-PCR analysis of OsCYP707A5 and OsCYP707A6 transcripts using total RNAs isolated from various growth stages of rice panicles 4DBH to 25DAH revealed that OsCYP707A5 was significantly down regulated towards the end of panicle maturation (Fig. 4).

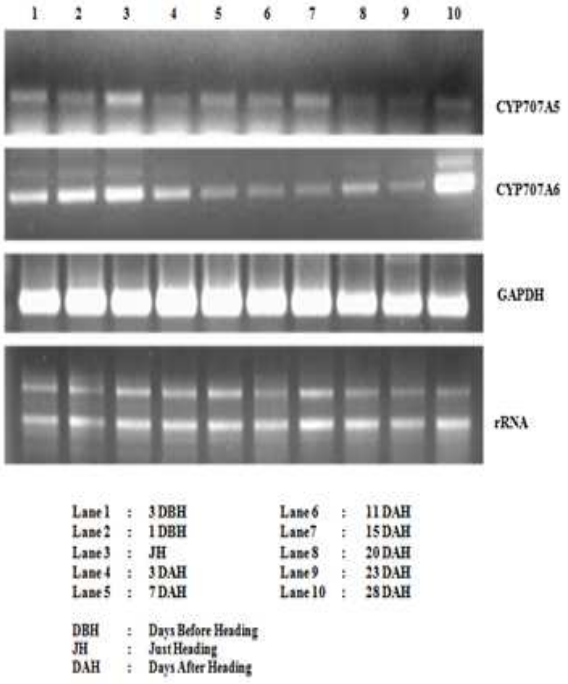


**Fig. 4** Semi-quantitative RT-PCR analysis of CYP707A family members at various stages of panicle development in rice

Concomitant with the above findings, Seiler and others 2011 have concluded that ABA catabolic gene is not activated during the early stages of seed development in barley. Similarly, Okamoto and others 2006 have observed ABA catabolism during seed development is catalyzed essentially by two CYP707As, CYP707A1 at the mid-maturation stage and CYP707A2 at the late maturation stage. Down-regulation of this OsCYP707A5 may be associated with increased accumulation of ABA in maturing rice panicles. In Arabidopsis up – regulation of CYP707A2 was found to plays a key role in the rapid decrease in ABA levels [21].

## CONCLUSION

In conclusion, the final picture of the experiments related here is that, it was understood that rapid accumulation of ABA in rice peduncles takes place during drought and its faster degradation during re-watering. Further, the expression pattern of putative candidate Abscissic Aldehyde Oxidase members namely, AAO3-1 and AAO3-2 were found to be correlated with ABA accumulation pattern during drought and re-watering. The pattern of ABA accumulation started at 4 DBH in rice panicles and reached the maximum at 25 DAH. Among the candidate genes involved in ABA catabolism, OsCYO707A5 may be involved in controlling ABA levels during drought in peduncle tissues of rice and also in maturing rice panicles.

## REFERENCES

1. Weatherley PE (1950) Studies in the water relations of cotton plant - The field measurements of water deficit in leaves. New Phytol 49: 81-97.
2. McCarty DR (1995) Genetic control and integration of maturation and germination pathways in seed development. Ann Rev Plant Physiol Plant Mol Biol 46: 71-93.
3. Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants**.** Ann Rev Plant Physiol Plant Mol Biol 47: 377- 403.
4. Bray EA (1997) Plant responses to water deficit. Trends in Plant Sci 2: 48-54.
5. Zhang J, Jia W, Zhang D (1997) Re-export and metabolism of xylem-delivered ABA in attached maize leaves under different transpirational fluxes and xylem ABA concentrations. J Exp Bot 48: 1557-1564.
6. Krochko JE, Abrams GD, Loewen MK, Abrams SR, Cutler A (1998) (+)-Abscisic Acid 8'-Hydroxylase is a cytochrome P450 monooxygenase. Plant Physiol 118: 849-860.
7. Zeevaart JAD, Creelman RA (1999) Metabolism and physiology of abscissic acid. Ann Rev Plant Physiol Plant Mol Biol 39: 439-473.
8. Alves AAC, Setter TL (2000) Response of cassava to water deficit: leaf area growth and abscisic acid. Crop Sci 40: 131-137.

9. Rock CD (2000) Pathways to ABA regulated gene expression. New Phytol 148: 357-396.
10. Söderman E, Brocard I, Lynch T, Finkelstein R (2000) Regulation and function of the Arabidopsis *ABA-insensitive4* (*ABI4*) gene in seed and ABA response signaling networks. Plant Physiol. 124: 1752–1765.
11. Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll A, Zeevaart JAD, Koornneef M, Kamiya Y, Koshiba T (2000) The Arabidopsis aldehyde oxidase 3 ($AAO_3$) gene product catalyzes the final step in ABA biosynthesis in leaves. Proceedings of the National academy of Sciences USA 97: 12908-12913.
12. Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscissic acid biosynthesis. J Exp Bot 51: 1563 – 1575.
13. Jia W, Zhang J, Liang J (2001) Initiation and regulation of water deficit-induced abscisic acid accumulation in maize leaves and roots: cellular volume and water relations. J Exp Bot 52: 295-300.
14. Nambara E, Marion-Poll A (2003) ABA action and interactions in seeds. Trends in Plant Science 8: 213–217.
15. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6: 410-417.
16. Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot 55: 225-236.
17. Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The Arabidopsis cytochrome P450 CYP707A encodes ABA 8'hydroxylases: key enzymes in ABA catabolism. EMBO J 23: 1647–1656
18. Seo MA, Koiwai H, Kamiya Y, Nambara E, Koshiba T (2004) Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of $AAO_3$ in ABA biosynthesis in seeds. Plant Cell Physiol 45: 1694–1703.
19. Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata K, Mizutani M (2004) Arabidopsis CYP707As encode (1)-abscisic acid 8' hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiol 134: 1439–1449
20. Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56: 165–185
21. Lefebvre V, North H, Frey A, Sotta B, Seo M, Okamoto M, Nambara E, Marion- Poll A (2006) Functional analysis of Arabidopsis *NCED6* and *NCED9* genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. Plant J 45: 309–319
22. Okamoto M, Kuwahara A, Seo M, Kushiro T, Asami T, Hirai N, Kamiya Y, Koshiba,T Nambara E (2006) CYP707A1 and CYP707A2, which encode Abscisic Acid 8'Hydroxylases, are indispensable for proper control of seed dormancy and germination in Arabidopsis. Plant Physiol 141: 97–107.
23. Yang SH, Choi, D (2006) Characterization of genes encoding ABA 8'-hydroxylase in ethylene-induced stem growth of deepwater rice (*Oryza sativa L)*. Biochemical and Biophysical Research Communications 350: 685–690.
24. Sreenivasulu N, Radchuk V, Alawady A (2010) Deregulation of abscisic acid contents causes abnormal endosperm development in the barley mutant seg8. Plant J 64: 589 – 603.
25. Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N (2011) ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. J Exp Bot 2: 1-18.