American Journal of Plant Physiology

ISSN 1557-4539

Academic Journals Inc.

www.academicjournals.com

American Journal of Plant Physiology 6 (3): 144-156, 2011
ISSN 1557-4539 / DOI: 10.3923/ajpp.2011.144.156

A Rapid Method for Estimation of Abscisic Acid and Characterization of ABA Regulated Gene in Response to Water Deficit Stress from Rice

[1]Kishwar Ali, [1]Ranjit Singh Gujjar, [2]Ram Niwas, [2]Madhuban Gopal and [1]Aruna Tyagi
[1]Divisions of Biochemistry, Indian Agricultural Research Institute, New Delhi, 110012, India
[2]Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi, 110012, India

Corresponding Author: Aruna Tyagi, Divisions of Biochemistry, Indian Agricultural Research Institute, New Delhi, 110012, India

ABSTRACT

Due to key role of ABA in drought tolerance, it was envisaged to develop a method for extraction and quantification of Abscisic Acid (ABA) and to isolate a ABA responsive and water deficit stress inducible gene from rice. A new analytical method which ensures the integrity of ABA during extraction, clean-up and estimation, was developed. ABA was quantified at different developmental stages of immature seeds and leaves. ABA content in leaves increases when a plant is exposed to water deficit stress but it decreases as the leaves approach maturity. ABA concentration also increases as developing seeds attain maturity. An ABA responsive gene of 264 bp was isolated which shows induced expression under water deficit stress. The gene has two domains, one belonging to aspartate aminotransferase super family of pyridoxal phosphate dependent enzymes and the other showing similarity with Major Facilitator Super family (MFS) of secondary transporters that include uniporters, symporters and antiporters. ABA content, extracted by a simplified methodology and estimation by HPLC, is found to be correlated with the degree to which the stress alters plant water status which results in the expression of the identified gene.

Key words: Drought, water deficit stress, abscisic acid (ABA), rice (*Oryza sativa*), RWC (relative water content)

INTRODUCTION

Rice, a monocot plant and a cereal crop which supplies food for more than half of the world's population is highly sensitive to salt and drought (Wang *et al*., 2008). According to FAO forecast during 2010, global paddy production has been lowered by 60 million tones mainly on account of drought, one of the most important abiotic stress factors which limit the growth and productivity of crop plants. Like other seed producing crops, rice is more susceptible to damage from water deficit stress at particular growth stages (Sangtarash, 2010). Plant responses to water deficit are complex, involving the co-ordination and integration of multiple biochemicals pathways leading to the expression of a number of genes encoding proteins which contribute to drought adaptation. A variety of genes are induced under water deficit stress in diverse plants (Rabbani *et al*., 2003). The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress responses. Phytohormones play a

key role in the growth and development of the plants. A major change in response to water deficit stress is increased synthesis of ABA which in turn induces a range of physiological and biochemical effects (Lian *et al*., 2006; Kumari, 2010). Abscisic Acid (ABA) also known as abscisin II and dormin is a plant hormone which plays an important part in plant response to environmental stress and plant pathogens (Zhu, 2002; Seo and Koshiba, 2002). After perception of water stress ABA expression increases, this helps in signal transduction. There are two types of signal transduction pathways; ABA dependent and ABA independent. Both the pathways function side by side in a parallel manner in response to drought. In ABA independent pathway, AP2/ERF family transcription factors bind to DRE, a cis-acting element in the promoter region. In ABA dependent pathway some of the important transcription factors belonging to MYB/MYC and bZIP bind to ABRE at the promoter region to induce gene expression for drought stress (Jakoby *et al*., 2002; Rabello *et al*., 2008; Ren *et al*., 2010). However, there is a cross talk between these two pathways. ABA accumulation during drought stress causes changes in many physiological processes e.g., stomatal closing, root elongation, inhibition of shoot growth etc. (Setter and Parra, 2010).

In the present study, a new method for extraction of ABA from seeds and leaves of rice using reverse phase HPLC was standardized. Further, the present study was undertaken with the objective to quantify ABA from developing seeds and leaf tissue of drought tolerant cultivar N-22 at different developmental stages of growth in control, as well as under Water Deficit Stress (WDS) and to isolate a ABA responsive WDS inducible gene from rice cv. N-22. Rice cultivar N-22 is an early maturing, deep rooted, drought tolerant and adapted to upland conditions.

MATERIALS AND METHODS

Plant material: Drought tolerant genotype (N22) was procured from Genetics Division, I.A.R.I., New Delhi. The seeds were surface sterilized by soaking in 0.1% mercuric chloride for five minutes, rinsed with 1N NaOH and thoroughly washed with distilled water and were grown in growth chamber under controlled conditions of 30°C/28°C (day/night) temperature, 85-90% RH and the light intensity of 600 μmol/m^2/sec PPFD (Photosynthetic photon flux density) and 45 days old seedlings were gradually subjected to water stress by withholding the water supply. Relative Water Content (RWC) of leaf tissues was estimated according to method of Weatherley, 1950. Rice plants having attained 70-80% RWC after withholding water supply were used as water stressed samples. ABA treatments were given by spraying the seedlings with 20 μM ABA solution on two alternate days.

ABA isolation, purification and quantification: ABA was isolated from mature seeds and also from leaves of rice cultivar N22 at different stages of growth. Rice seeds were powdered and representative sample (10 g) in triplicate was extracted by homogenizing with 40 mL of 80% aqueous methanol for 30 min at 4°C. The mixture was filtered in a separate conical flask using Whatman filter paper No. 1. The filtrate was vacuum evaporated in a lyophilizer (Model Christ α-2-4, Germany) and the vacuum dried residue was redissolved in 10 mL of 0.5M phosphate buffer (pH 8) by stirring for 30 min. The suspension was washed with 20 mL of light petroleum sprit. The pH of sample was adjusted to 2.8 using dil HCl and extracted four times with ethyl acetate (4×10 mL). The extract was lyophilized and redissolved in 5 mL of 0.5 M phosphate buffer (pH 8). Sample was then purified by passing through sephadex G10 column. For preparing Sephadex column, 1 g Sephdex was swollen in 10 mL double distilled water over night and packed in a glass column (1 cm diameter and 5 cm length) up to 2 cm length and the column was

equilibrated with the phosphate buffer. The eluted solution was again lyophilized and the residue was finally dissolved in 1mL of acetonitrile and analyzed by HPLC. A HITACHI Model L 7490 HPLC system with a UV detector and auto-injector was used for analysis. Stainless-steel column (250×4.6 mm I.D.) packed with LiChrosphere RP-18e (5 μm) was applied for the separation of standard and real field sample. A mixture of 0.5% acetic acid (40 mL) and acetonitrile (60 mL) was used as mobile phase at a flow rate of 0.3 mL min^{-1}. Samples were analyzed using UV wavelength at 254 nm. The retention time of ABA was 8.78 min. The reproducibility and linearity of the chromatographic system was estimated by five consecutive injections of different concentrations of standard ABA as well as spiked samples of rice seeds and leaves. Stock solution (1000 μg mL^{-1}) of pure ABA was prepared by dissolving 25 mg of ABA in 25 mL of HPLC grade acetonitrile. The calibration standards of concentration 1, 5, 10, 50 and 100 μg mL^{-1} were prepared by successive dilutions of the above working stock solution. The analytical method was validated by using single-laboratory validation approach (Thompson *et al*., 2002). The recovery experiments were carried out on rice grains and leaves. Rice seeds (10 g) were fortified in six replicates with the analyte solution at 5, 10, 30, 40, 90, 120 and 200 μg ABA. Rice leaves (10) g were crushed and homogenized with 40 mL of 80% aqueous methanol for 30 min at 4°C. Further extraction was followed as used in the extraction of ABA from seeds. Rice leaves (10 g) were also fortified with 3, 5, 10, 15, 25, 50 and 60 μg ABA in six replicates. Quantification of recovery samples was performed by external calibration using matrix-matched standards.

Total RNA isolation: For RNA isolation, leaf samples were collected on the day of corresponding RWC from the control as well as water stressed plants and immediately frozen in liquid nitrogen and stored till RNA isolation. Total RNA was isolated by using guanidium isothiocyanate (GTC) method (Sambrook *et al*.,1989) and was subjected to electrophoresis on 1.2% agarose gel containing 20 mM GTC. mRNA was isolated from ~250 μg of total RNA of each sample using oligotex mRNA spin column following the protocol supplied with Qiagen poly A^+ RNA isolation kit (Qiagen Inc., USA).

cDNA synthesis and cloning: cDNA was synthesized from 1 μg of total mRNA using Smart™ cDNA library construction kit from BD Biosciences Clontech, USA. A 5 μL sample of single stranded cDNA product was checked on 1.2% agarose/ethidium bromide gel. The cDNA was cloned into PCR Script™ vector using the protocol provided with PCR-Script™ Amp Cloning Kit procured from Stratagene, USA. The ligated products were then transformed into *E. coli* strain DH5α and grown on LBA plates containing 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-gal and 0.1M Isopropyl Thiogalactoside (IPTG). The recombinants were selected by blue/white screening and transferred to fresh Luria Bertani Agar plates containing ampicillin 100 μg mL^{-1}. The clones were grown overnight at 37°C and stored at 4°C for further analysis.

Insert analysis: Randomly selected clones were analyzed for the presence of insert by PCR amplification using T_3 and T_7 universal primers. Amplified products were checked on 1.2% agarose gel.

Probe: Amplified product by RT-PCR was labeled with α^{32}p dATP using Hexalabel™ DNA labeling kit of MBI Fermentas Inc., USA following manufacturer's protocol.

Northern blotting: Northern analysis was done using 10 μg of total RNA from leaf tissue of control as well as of the plants grown under moisture stress having desired RWC. Standard protocol was followed for hybridization.

Sequencing of the clone identified: Automated sequencing of the putative clones was got done from DNA sequencing facility, South Campus, Delhi University, New Delhi.

Statistical analysis: Statistical analysis of data depicted in Table 3 and 4 were carried out by using Windostat version 8.5 in order to study whether the ABA content in leaves and seeds of rice observed at different developmental stages are significantly different from their respective control. Subjecting the data presented in Table 3 to find whether the ABA content in seeds on 20 and 30 days after flowering are statistically different from each other or not, T test value was 105.5892 with F ratio 11149.080 at 0.0000 probabilities.

The data in Table 4 was also subjected to t- test in order to compare ABA concentration in leaves on three different stages. T test value for mean value 2.63 and 4.67 μg g^{-1} was 28.4728 with F ratio 810.701 at lowest probability. Similarly on day 25 DAP T test value was 101.2124 with F ratio 10243.950 and on 40 DAP, T test value was 10.3862 with F ratio 107.872 at very low probability, implying that these concentrations are indeed significantly different from that of control samples where no water stress was applied.

RESULTS

Considering the role of ABA in various stress signal transduction pathways and the genes regulated by ABA during stress, ABA was quantified at different developmental stages of rice plants (N22) in leaves and seeds under control as well as water deficit stress conditions. A simple method for extraction, clean up and estimation of ABA in leaves and seeds is reported. One millilitre of the acetonitrile dissolved sample was finally quantified for ABA concentration using HPLC.

Standardization of HPLC protocol for ABA estimation: In the present study, a simple method for extraction and estimation of ABA from seeds and leaves of rice by reversed phase HPLC was standardized. The results show percent recovery in range 75.8-89.5 for fortified rice seeds (Table 1), recovery being higher for higher concentrations. Similar results were obtained for fortified rice leaves (Table 2) where recovery ranged from 78.9-96.6%. Percent recovery from fortified rice leaves was slightly better as compared to rice seeds even at low fortification level (<5 μg). Multiple regression analysis using Windostat version 8.5 employing levels of ABA (variable chosen for x-axis) to response (y axis) led to proof of satisfactory recovery from seeds (Table 1). It had high correlation as reflected from R^2 0.9967 and F statistics 9456.817 at very low probability. In rice leaves (Table 2) also similar results were obtained. It also showed R^2 0.9960 and F statistics 4923.563 at the same very low probability.

ABA quantification in seeds: Ten gram of the seed samples taken from the rice (N22) field at 20 and 30 Days After Flowering (DAF) were quantified for the concentration of ABA. ABA concentration in seeds taken at 20 DAF and 30 DAF was 7.42 and 13.77 μg g^{-1}, respectively (Table 3). It was observed that ABA concentration increased as much as twice in the seeds at 30 DAF as compared to the seeds at 20 DAF. The statistical analysis proved that ABA accumulation increased significantly with the number of days after flowering.

Table 1: Recovery of abscisic acid from rice seeds

	Amount recovered (µg)							
ABA added (µg)	R1	R2	R3	R4	R5	R6	Average recovery (µg)	Recovery (%)
5	4.32	3.65	3.91	4.05	4.00	3.68	3.94	78.7
	4.22	3.70	3.81	3.99	4.15	3.75	3.94	78.7
	4.18	3.81	3.76	3.85	4.08	3.70	3.79	75.8
	4.25	3.61	3.79	3.87	4.03	3.69	3.87	77.5
10	8.24	7.63	7.78	8.26	7.69	7.75	7.89	78.9
	8.27	7.68	7.73	8.36	7.61	7.79	7.91	79.1
	8.32	7.71	7.80	8.29	7.70	7.77	7.93	79.3
	8.29	7.67	7.79	8.25	7.71	7.69	7.90	79.0
30	23.50	23.70	22.87	23.55	24.00	23.87	23.58	78.6
	23.59	23.75	22.80	23.57	23.90	23.97	23.60	78.6
	23.80	23.64	22.91	23.65	23.88	23.92	23.63	78.8
	23.75	23.82	23.02	23.78	23.99	23.79	23.69	79.0
40	27.26	31.06	29.06	35.57	35.11	34.77	32.14	80.3
	27.65	31.24	28.88	37.05	35.56	35.21	32.60	81.5
	28.19	30.76	28.85	35.00	35.25	35.21	32.21	80.5
	27.68	31.00	29.01	35.45	35.49	35.16	32.30	80.7
90	72.90	71.96	72.11	71.04	71.76	71.89	71.94	79.9
	72.81	72.10	72.34	70.06	71.65	70.21	71.53	79.5
	72.85	71.75	72.75	67.80	71.99	70.06	71.20	79.1
	69.13	72.05	74.35	74.23	72.01	71.33	72.18	80.2
120	97.04	101.69	99.46	100.05	93.80	92.66	97.45	81.2
	97.24	102.13	101.07	102.71	92.66	90.42	97.71	81.4
	95.64	100.52	95.05	102.73	97.63	99.02	98.43	82.0
	95.05	100.74	97.80	99.93	93.38	92.34	96.54	80.5
150	124.18	139.92	128.03	138.72	134.25	139.65	134.18	89.5
	125.08	139.52	128.53	138.63	134.56	139.03	134.23	89.5
	124.88	139.12	128.59	139.22	134.57	139.10	134.25	89.5
	124.98	139.62	128.63	139.02	134.28	138.95	134.25	89.5
200	170.41	159.15	177.14	190.14	174.92	183.81	175.93	87.9
	171.11	160.05	176.46	191.01	175.02	183.92	176.26	88.1
	171.31	160.33	176.20	190.31	175.32	184.10	176.26	88.1
	170.41	159.15	175.96	190.94	174.87	183.77	175.85	87.9
Control	0.65	0.77	0.78	0.97	0.87	0.76	0.80	

Statistical analysis of Table 1

VAR-COVAR MATRIX Individual regressors

Yi VARIABLE = Resp Y1 = -283116.2369+3422.10361X

Pooled n = 33		Lowest	Highest	Kurtosis	Skewness	Mean	Std. Dev.	Std. Error	C.V.%	Jarque bera	$X^{2\,Prob}$
Level	X1	0.0000	200.0000	-1.1097	0.5033	78.1818	68.1888	11.8702	87.2183	3.0862	0.2137
Resp	Y1	0.8000	176.2600	-1.0093	0.5996	66.3609	60.2332	10.4853	90.7661	3.3780	0.1847

Statistic	Value	Statistic	Value
R^2	0.9967	R^2adj	0.9966
F	9456.8170(1,31)	Probability	0.0000
RMS error	3.4981	AIC	2.5631

	Beta Wt.	Simple R^2	Reg. Coeff.	Std. Error	t-value	t Prob.	Partial R^2
Multiple regression on response							
INTERCEPT a		0.0000	-2.58645	0.9346	2.767	0.009**	0.198
Level	0.9984	0.9967	0.88188	0.0091	97.246	0.000***	0.997

Table 2: Recovery of abscisic acid from rice leaves

	Amount recovered (µg)							
ABA added (µg)	R1	R2	R3	R4	R5	R6	Average recovery (µg)	(%) recovery
3	2.97	2.67	2.73	2.99	2.68	2.75	2..80	93.3
	2.88	2.82	2.80	2.85	2.86	2.79	2.83	94.4
	2.92	2.85	2.79	2.90	2.88	2.76	2.85	95.0
5	4.38	4.03	3.91	4.38	4.03	3.91	4.11	82.1
	4.35	4.00	3.81	4.28	4.09	3.95	4.08	81.6
	4.36	4.05	3.92	4.25	4.05	3.96	4.10	81.9
10	8.26	7.62	7.79	8.24	7.65	7.77	7.89	78.9
	8.15	7.68	7.83	8.20	7.73	7.81	7.90	79.0
	8.19	7.71	7.77	8.21	7.69	7.85	7.90	79.0
15	14.08	13.19	13.64	13.88	13.28	13.64	13.62	90.8
	14.06	13.25	13.70	14.03	13.26	13.75	13.68	91.2
	14.12	13.23	13.68	14.01	13.24	13.73	13.67	91.1
25	24.50	25.70	22.87	24.00	24.70	22.87	24.11	96.4
	24.27	25.62	23.17	24.22	24.65	23.07	24.14	96.5
	24.44	25.65	23.08	24.12	24.69	23.01	24.17	96.6
50	44.95	40.50	41.29	44.05	42.50	41.19	42.41	84.8
	44.75	41.20	41.21	44.01	42.05	41.25	42.41	84.8
	44.88	41.55	41.25	43.95	42.61	41.09	42.56	85.1
60	50.19	45.93	55.19	50.10	46.03	54.89	50.37	83.9
	50.11	45.90	55.25	50.10	46.15	54.09	50.27	83.8
	50.21	45.83	55.33	50.10	45.93	54.29	50.28	83.8
Control	0.71	0.81	0.83	0.82	0.73	0.72	0.77	

Statistical analysis of Table 2

VAR-COVAR MATRIX

Individual regressors

Yi VARIABLE = Resp

Y1 =-7515.3473+328.91619X

Pooled n = 22		Lowest	Highest	Kurtosis	Skewness	Mean	Std. Dev.	Std. Error	C.V.%	Jarque bera	$X^{2\,Prob}$
Level	X1	0.0000	60.0000	-1.0279	0.7451	22.9091	21.4984	4.5835	93.8423	3.0040	0.2227
Resp	Y1	0.7700	50.3700	-1.1507	0.6531	19.8236	18.1544	3.8705	91.579	2.7776	0.2494

Staistic	Value	Statistic	Value
R^2	0.9960	R^2adj	0.9958
F	4923.5630(1,20)	Probability	0.0000
RMS error	1.1832	AIC	0.4230

	Beta Wt.	Simple R^2	Reg. Coeff.	Std. Error	t-value	t Prob.	Partial R^2
Multiple regression on response							
INTERCEPT a		0.0000	0.51712	0.3733	1.385	0.181	0.088
Level	0.9980	0.9960	0.84274	0.0120	70.168	0.000***	0.996

Table 3: Concentration of ABA in the seeds of *Oryza sativa* cultivar N22 at 20 and 30 days after flowering

	ABA Concentration in N22 seeds (µg g^{-1} plant tissue)				
Days After Flowering (DAF)	R1	R2	R3	R4	Average
2	7.44	7.39	7.39	7.46	7.42
30	13.60	13.84	13.80	13.84	13.77

Table 4: Concentration of ABA in control as well as water stressed leaf tissues of *Oryza sativa* cultivar N22 at 10, 25 and 40 days after planting

	ABA Concentration in N22 leaves (μg g^{-1} plant tissue)									
	Control					Stress				
Days After Planting (DAP)	R1	R2	R3	R4	Avg.	R1	R2	R3	R4	Avg.
10	2.63	2.55	2.78	2.56	2.63	4.55	4.70	4.65	4.78	4.67
25	1.42	1.40	1.51	1.43	1.44	4.20	4.22	4.19	4.16	4.19
40	1.18	1.36	1.43	1.31	1.32	2.05	1.99	1.89	1.95	1.97

ABA can also be induced in vegetative tissues when exposed to WDS. Hence to correlate ABA synthesis with water deficit stress in vegetative tissues, ABA was isolated from leaves of N22 rice seedlings grown in phytotron at 10, 25 and 40 Days After Planting (DAP) under control (RWC > 90%) as well as water deficit stress (RWC = 65-85%) condition. ABA concentration in leaves under control and WDS at 10 DAP was 2.63 and 4.67 μg g^{-1}, at 25 DAP, 1.44 and 4.19 μg g^{-1} and at 40 DAP, 1.32 and 1.97 μg g^{-1}, respectively (Table 4). The results of HPLC analysis revealed that ABA concentration was more in leaves that were exposed to water deficit stress as compared to control leaves at different developmental stages of vegetative growth. One interesting observation was that ABA concentration was decreasing with the maturity of leaves. There was a marginal decrease in the concentration of ABA during the growth from 10 DAP to 25 DAP but a drastic decrease in ABA concentration was observed when the plant reached at 40 DAP growth stage. The analysis of ABA content in leaves under stress condition showed significant increase in amount of ABA as compared to control.

Isolation of differentially regulated gene: Total RNA was isolated from stressed leaf tissue of rice expressing maximum ABA. Further, cDNA was synthesized from mRNA purified from stressed leaf tissues. The cDNA was cloned into PCR-Script™ vector (SK^+). The ligated product was then transformed in *E. coli* strain DH5α. Randomly selected one of the recombinant clones was analyzed for the presence of insert using restriction enzyme *Eco*RI (Fig. 1a). Insert was also confirmed by PCR using T_3 and T_7 primers and the amplified product was checked on 1.2% agarose gel (Fig. 1b). This amplified product was used as probe for Northern analysis. Northern analysis of the RNA isolated from control as well as stressed leaves revealed induced expression with respect to water deficit stress as compared to RNA isolated from well watered control. (Fig. 1c).On sequencing, the cloned fragment was found to be 264bp long (Genbank ID-GQ395809, Fig. 2). Homology search using BLAST-X search analysis showed 90-93% homology with clones (accession nos. BAB19767 and BAD33906) aminotransferase like proteins of *Oryza sativa* (Japonica Group). Conserved domain search results reveal that the coding region of the gene possesses two conserved domains (Fig. 3). One of the domains belongs to Aspartate Aminotransferase (AAT) superfamily (fold type I) of Pyridoxal Phosphate (PLP)-dependent enzymes. Another conserved domain belongs to the Major Facilitator Superfamily (MFS), a large and diverse group of secondary transporters that include uniporters, symporters and antiporters. The base composition of cDNA showed 49% GC and 51% AT content.

Characterization of gene identified: Further analysis of the sequence revealed that it had an ORF of 186 bp which encodes for 61 amino acids (Fig. 2). The calculated molecular weight was

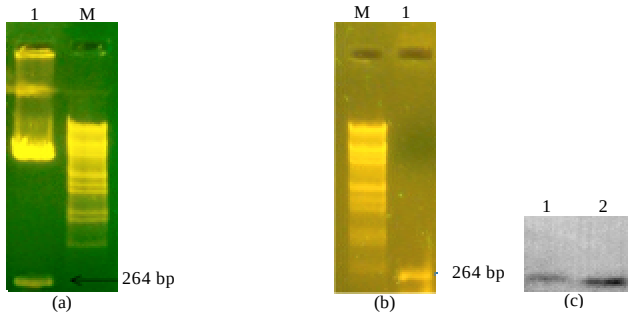

Fig. 1: The agarose gel (1.2%); (a) lane 1 showing randomly selected cDNA clone after restriction with *Eco*RI *restriction enzyme*; Lane M-showing lambda phage DNA Marker restricted with *Hin*dIII *and Eco*RI (b) lane I showing RT-PCR product amplified by using gene specific primers C; showing Northern hybridization using RNA isolated from control (lane 1) and stressed leaf tissue (lane 2) using insert as labeled probe

TAGGTCGTCGGATCAGTGGTTGGAGCAGGTGGACCATGGTGGCTACTGCAGACTTG
GCTGAATCTGGTAGTCATGAAAGCTGTGAATCGGCCATCTGTGACAGAAGCCGAATTCC
CAAGACTGGAGCTGATCACAGAAGACGATGGAGAAGAGTGCACCTGGACTGTCTACTGT
TGAATCAGCTGAATCACTTGTTGTCTTGGTGGCGTTTGCTGAACTGA**CATCGATTGAAC**
TGATCCGACGACCTAGTTAGCAATCGAATTC

Met V A T A D L A E S G S H E S C E S A I C D R S R I P K T G A D H R R R W R R V H L
D C L L L N Q L N H L L S W W R L L N Stop

Fig. 2: Nucleotide sequence of the gene and encoded amino acid sequence. Bold letters show 5' and 3' UTR regions (35 and 43 nts, respectively)

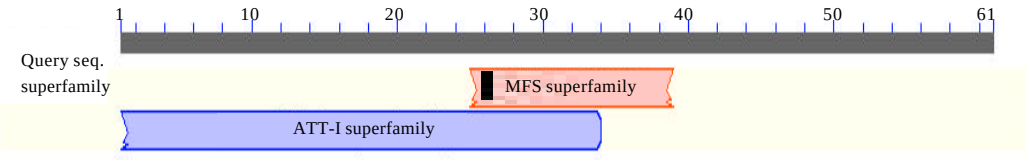

Fig. 3: The conserved domains search result of isolated cDNA. The figure is showing that cDNA has two conserved domains, MFS super family (cl11420) ,The Major Facilitator Superfamily (MFS) and AAT_I super family (cl00321), Aspartate aminotransferase (AAT)

7.1 kDa and pI was 8.68. The total number of negatively charged and positively charged residues was 7 and 9, respectively. With Open Reading Frame finder software analysis, it was observed

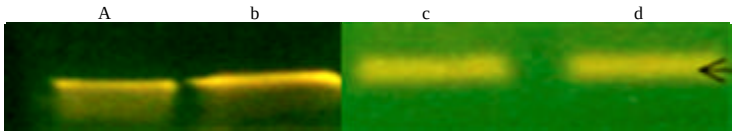

Fig. 4: The agarose gel (1.2%) showing RT-PCR product amplified using gene specific primers with, lane a: RNA isolated from leaves of control, lane b: RNA isolated from ABA treated plants lane c : actin gene with RNA isolated from leaves of control and lane d: actin gene with RNA isolated from leaves of ABA treated plants

that the open reading frame starts from 36 to 219. The start codon i.e. ATG is located at 36th nucleotide and stop codon at 219th nucleotide. The sequence contains a 35 nucleotides long 5' UTR and a 3' UTR of 43 nucleotides.

ABA inducible expression by RT-PCR analysis: To confirm the ABA inducibility of a drought inducible cDNA, the comparative expression of the gene and actin gene were checked by RT-PCR analysis using total RNA isolated from ABA treated and untreated (control) plants of *Oryza sativa* cultivar N22. To avoid bias, expression of the target gene is usually normalized relative to a reference gene which should not fluctuate in different (control and stressed) samples. The most widely used reference genes are those which belong to actin family (Zhang *et al*., 2009). Forward and reverse primers were designed from gene isolated and got custom synthesized. The specific primers of the gene isolated and actin primers were used to amplify cDNA using total RNA isolated from ABA treated and untreated (control) plants of *Oryza sativa* cultivar N22 by single step RT-PCR. The amplified products were checked on 1.2% agarose gel. The intensity of the bands of ABA treated sample was found to be more as compared to control sample (Fig. 4). This confirms the induction of the gene under high levels of ABA.

DISCUSSION

Drought stress is first perceived by cells as plasma lemma 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 (Bartels and Sunkar, 2005). Reported methods of ABA estimation in plant tissues have been time consuming and consist of a preparative and an analytical procedure (Ciha *et al*., 1976; Mapelli and Rocchi, 1983; Dobrev *et al*., 2005).

In the present study, a simple method for extraction and estimation of ABA from seeds and leaves of rice by reversed phase HPLC was optimized. Recovery of ABA from leaves (Table 2) was slightly better than those observed in seeds (Table 1) even at low fortification level (<5 μg). ABA content is influenced by an array of developmental and environmental cues and interplay with other phytohormones (Nambara and Marion-Poll, 2005). Multiple aspects of ABA metabolism may be involved in a homeostasis mechanism preventing excess ABA accumulation and matching ABA content to the type and severity of the stress to which the plant is exposed.

Considering the role of ABA in various stress signal transduction pathways and the genes upregulated by ABA during stress, ABA was quantified at different developmental stages of rice plants (N22) after flowering in young seeds. It was observed that ABA content at 30 DAF was almost 2 fold as compared to 20 DAF. Under stress, it has been observed that ABA content is correlated with the degree to which the stress alters plant water status as measured by changes in

turgor and RWC (Verslues and Bray, 2006; Verslues and Zhu, 2007). Increased ABA accumulation in seeds during maturation has been documented by various reports (Nambara and Marion-Poll, 2005; Zhu, 2002; Xiong and Zhu, 2003; Zhang *et al*., 2006; Angoshtari *et al*., 2009). ABA has been previously quantified in rice grains and it has been observed that during development of rice grains, ABA concentration increases till the grains attain full maturity (Kato *et al*., 1993). In the present study ABA increased as much as twice at 30 DAF as compared to 20 DAF.

ABA concentration in vegetative tissues increases as we apply water deficit stress. Similar findings have been reported by other workers (Lian *et al*., 2006; He *et al*., 2009). In a previous study in lab drought tolerant rice genotype showed more than 100% increase while susceptible genotypes showed negligible increase in ABA content under water deficit stress (Tyagi *et al*., 1999b). Differences in induction of genes that confer drought tolerance in tolerant cultivars as compared to drought sensitive plants have been observed which could possibly due to different accumulation of ABA (Jiang and Lafitte, 2007). In another study in lab expression of ABA responsive gene was studied in *Lathyrus sativus*, a hardy and drought tolerant crop which seemed to interpret changes in ABA in a way different from sensitive plants (Tyagi *et al*., 1999a). The possible reason for decrease in the ABA level in leaves (Fig. 2) along with maturity might be the degradation of ABA to Phaseic Acid (PA)/Dihydrophaseic Acid (DPA) and/or its transport from leaves to the reproductive parts that later produce seeds. ABA concentration in plant tissues depends mainly on its biosynthesis, catabolism (hydroxylation), transport and concentration of other hormones (Zeevaart, 1999; Bartels and Sunkar, 2005; Nambara and Marion-Poll, 2005).

An ABA as well as WDS inducible gene isolated in the present study showed homology to aminotransferase like protein of *Oryza sativa*. Plant aspartate aminotransferase plays a key role in primary nitrogen assimilation, transfer of reducing equivalents and the interchange of carbon and nitrogen pools between subcellular compartments (De la Torre *et al*., 2007). MFS proteins facilitate the transport across cytoplasmic or internal membranes of a variety of substrates including ions, sugar phosphates, drugs, neurotransmitters, nucleosides, amino acids and peptides. All permeases of the MFS possess either 12 or 14 putative or established transmembrane alpha-helical spanners and evidence is presented substantiating the proposal that an internal tandem gene duplication event gave rise to a primordial MFS protein prior to divergence of the family members. All 17 families are shown to exhibit the common feature of a well-conserved motif present between transmembrane spanners 2 and 3. The analyses reported, serves to characterize one of the largest and most diverse families of transport proteins, found in living organisms (Pao *et al*., 1998). Aminotransferses catalyze a reversible transamination reaction and play a key role in carbon and nitrogen metabolism during maturation of rice seeds and in the synthesis of seed storage proteins. A differentially expressed branched chain amino acid transferase gene by drought stress was isolated from *H. vulgare* (Malatrasi *et al*., 2006). Transcript levels of the branched chain aminotransferase increased in response to drought stress. It might have a role in degradation of branched chain amino acids which could serve as detoxification mechanism that maintains the pool of free aminoacids at low and non toxic levels during drought stress condition. Roosens *et al*. (1998) observed a close association between expression of ornithine amino transferase with salt stress and proline production. They observed an increase in proline content, O-aminotransferase activity and O-aminotransferase mRNA by salt stress treatment in young *Arabidopsis* plants. Seki *et al*. (2002) also observed up regulation of genes encoding aspartate aminotransferase and various transporter proteins by drought, cold and high salinity treatment in Arabidopsis.

To conclude, ABA was quantified at different developmental stages of immature seeds and leaves of *Oryza sativa* subsp. *indica* under control and water deficit stress conditions. ABA content in seeds increases with maturity. In leaves higher ABA content as compared to control was observed at different developmental stages. Further an ABA responsive gene of 264 bp associated with water deficit stress showing homology to aminotransferase like proteins of Oryza sativa was isolated.

REFERENCES

Angoshtari, R., R.A. Tavakkol, S. Kalantari and M. Omidi, 2009. Effects of abscisic acid on somatic embryogenesis and induction of desiccation tolerance in *Brassica napus*. Asian J. Plant Sci., 8: 276-284.

Bartels, D. and R. Sunkar, 2005. Drought and salt tolerance in plants. Crit. Rev. Plant. Sci., 24: 23-58.

Ciha, A.J., M.L. Brenner and W.A. Brun, 1976. Rapid separation and quantification of abscisic acid from plant tissues using high performance liquid chromatography. Plant Physiol., 59: 821-826.

De la Torre, F., M.F. Suarez, L. Santis and F.M. Canovas, 2007. The aspartate aminotransferase family in conifers: Biochemical analysis of a prokaryotic-type enzyme from maritime pine. Tree Physiol., 27: 1283-1291.

Dobrev, P.I., L. Havlicek, M. Vagner, J. Malbeck and M. Kaminek, 2005. Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography. J. Chromatogr. A, 1075: 159-166.

He, H., R. Serraj and Q. Yang, 2009. Changes in OsXTH gene expression, ABA content and peduncle elongation in rice subjected to drought at the reproductive stage. Acta Physiol. Plant., 31: 749-756.

Jakoby, M., B. Weisshaar, W. Droge-Laser, J. Vicente-Carbajosa, J. Tiedemann, T. Kroj and F. Parcy, 2002. bZIP transcription factors in *Arabidopsis*. Trends Plant Sci., 7: 106-111.

Jiang, W. and R. Lafitte, 2007. Ascertain the effect of PEG and exogenous ABA on rice growth at germination stage and their contribution to selecting drought tolerant genotypes. Asian J. Plant Sci., 6: 684-687.

Kato, T., N. Sakurai and S. Kuraishi, 1993. The changes of endogenous abscisic acid in developing grains of two rice cultivars with different grain size. Jpn. J. Crop Sci., 62: 456-461.

Kumari, S., 2010. Cellular changes and their relationship to morphology, abscisic acid accumulation and yield in wheat (*Triticum aestivum*) cultivars under water stress. Am. J. Plant Physiol., 5: 257-277.

Lian, H.L., X. Yu, D. Lane, W.N. Sun, Z.C. Tang and W.A. Su, 2006. Upland rice and lowland rice exhibited different PIP expression under water deficit and ABA treatment. Cell Res., 16: 651-660.

Malatrasi, M., M. Corradi, J.T. Svensson, T.J. Close, M. Gulli and N. Marmiroli, 2006. A branched-chain amino acid aminotransferase gene isolated from *Hordeum vulgare* is differentially regulated by drought stress. TAG Theor. Applied Genet., 113: 965-976.

Mapelli, S. and P. Rocchi, 1983. Separation and quantification of abscisic acid and its metabolites by high-performance liquid chromatography. Ann. Bot., 52: 407-409.

Nambara, E. and A. Marion-Poll, 2005. Abscisic acid biosynthesis and catabolism. Annu. Rev. Plant Biol., 56: 165-185.

Pao, S.S., I.T. Paulsen and M.H. Jr. Saier, 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev., 62: 1-34.

Rabbani, M.A., K. Maruyama, H. Abe, M.A. Khan and K. Katsura *et al.*, 2003. Monitoring expression profiles of rice genes under cold, drought and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol., 133: 1755-1767.

Rabello, A.R., C.M. Guimaraes, P.H.N. Rangel, F.R. da Silva and D. Seixas *et al.*, 2008. Identification of drought-responsive genes in roots of upland rice (*Oryza sativa* L.). BMC Genomics, 9: 485-485.

Ren, X., Z. Chen, Y. Liu, H. Zhang and M. Zhang *et al.*, 2010. ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis. Plant J., 63: 417-429.

Roosens, N.H.C.J., T.T. Thu, H.M. Iskandar and M. Jacobs, 1998. Isolation of the ornithine-δ´-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiol., 117: 263-271.

Sambrook, J., E.F. Fritich and T. Manitas, 1989. Molecular Cloning: A Laboratory Manual, Book 3. 2nd Edn., Cold Springer Harbor Laboratory Press, New York.

Sangtarash, M.H., 2010. Responses of different wheat genotypes to drought stress applied at different growth stages. Pak. J. Biol. Sci., 13: 114-119.

Seki, M., M. Narusaka, J. Ishida, T. Nanjo and M. Fujita *et al.*, 2002. Monitoring the expression profile of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using full-length cDNA microarray. Plant J., 31: 279-292.

Seo, M. and T. Koshiba, 2002. Complex regulation of ABA biosynthesis in plants. Trend Plant Sci., 7: 41-48.

Setter, T.L. and R. Parra, 2010. Relationship of carbohydrate and abscisic acid levels to kernel set in maize under postpollination water deficit. Crop Sci., 50: 980-988.

Thompson, M., S.L.R. Ellison and R. Wood, 2002. Harmonized guidelines for single-laboratory validation of methods of analysis. Pure Applied Chem., 74: 835-855.

Tyagi, A., I.M. Santha and S.L. Mehta, 1999a. Effect of water stress on proline content and transcript levels in *Lathyrus sativus*. Indian J. Biochem. Biophys., 36: 207-210.

Tyagi, A., N. Kumar and R.K. Sairam, 1999b. Efficacy of RWC, membrane stability, osmotic potential, endogenous ABA and root biomass as indices for selection against water stress in rice. Indian J. Plant Physiol., 4: 302-306.

Verslues, P.E. and E.A. Bray, 2006. Role of abscisic acid (ABA) and *Arabidopsis thaliana* ABA-insensitive loci in low water potential-induced ABA and proline accumulation. J. Exp. Bot., 57: 201-212.

Verslues, P.E. and J.K. Zhu, 2007. New developments in abscisic acid perception and metabolism. Curr. Opin. Plant Biol., 10: 447-452.

Wang, Q., Y. Guan, Y. Wu, H. Chen, F. Chen and C. Chu, 2008. Overexpression of a rice OsDREB1F gene increases salt, drought and low temperature tolerance in both *Arabidopsis* and rice. Plant Mol. Biol., 67: 589-602.

Weatherley, P.E., 1950. Studies in the water relations of the cotton plant. I. The field measurements of water deficit in leaves. New Phytol., 49: 81-97.

Xiong, L. and J.K. Zhu, 2003. Regulation of abscisic acid biosynthesis. Plant Physiol., 133: 29-36.

Zeevaart, J.A.D., 1999. Abscisic Acid Metabolism and its Regulation. In: Biochemistry and Molecular Biology of Plant Hormones, Hall, M. and K. Libbenga (Eds.). Elsevier Science BV, Amsterdam, pp: 189-207.

Zhang, J., W. Jia, J. Yang and A.M. Ismail, 2006. Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res., 97: 111-119.

Zhang, J.L., D.H. Liu, Z.H. Wang, C. Yu, J.H. Cao, C.T. Wang and D.M. Jin, 2009. Expression pattern of GS3 during Panicle development in rice under drought stress: Quantification normalized against selected housekeeping genes in real-time PCR. Asian J. Plant Sci., 8: 285-292.

Zhu, J.K., 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol., 53: 247-273.