ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

cDNA- RAPD analysis in *Oryza sativa* and *Brassica juncea* under Arsenic stress

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Manuscript Info

Manuscript History:

Received: 15 July 2013
Final Accepted: 21 July 2013
Published Online: August 2013

Key words:
Arsenic, cDNA-RAPD, *O ryza sativa, Brassica juncea*

Abstract

Arsenic (As) is an important environmental pollutant and As toxicity has emerged as one of the important health concerns for human. For plants, As is nonessential and toxic element. There is little information available regarding the enzymes and their encoding genes involved in As metabolism and detoxification especially in crop plants. In crop plants like rice, significant genetic variation has been reported especially with regard to As accumulations. Identification and documentation of such variations would help in understanding the mechanism of differential As accumulation as well as detoxification. In present work *Oryza sativa* (IR-64) and *Brassica juncea* (Pusa bold) varieties have been screened for differential gene expression in As stress. Three cDNA-RAPD strategies have been employed to identify differential gene expression. The cDNA from As treated rice and Brassica plants were prepared using oligo (dT) primers, Smart oligos and random hexamers before subjecting to RAPD. Interestingly the maximum polymorphisim was observed with cDNA prepared from random hexamer primers.

Introduction

Exploitation of As-containing groundwater in large areas of Asia causes problem for millions of people via consumption of As-contaminated drinking water and food (Zhao et al 2010). Inorganic arsenate (V) and arsenite (III) are the main soluble As species found both in soil and water. As (V) predominates under aerobic conditions and considered as an analogue of phosphate, and As (III) is the dominant form under anaerobic conditions, which mainly reacts with -SH groups and is an effective inhibitor of enzymes requiring free sulfhdryl group and interrupts several morphological, physiological, biochemical and molecular processes in plants (Tripathi et al., 2007; Zhao et al., 2010). Random Amplified Polymorphic DNA (RAPD) is a PCR based method, different from conventional PCR as it needs only one primer for amplification. The size of primer is normally short (8-12 nucleotides), and therefore, less specific. The primers can be designed without the experimenter having any genetic information of the organism being tested. Genomic DNA normally has complimentary sequences to RAPD primers at many locations. If two of these locations are close to each other (<3000bp), and the sequences are in opposite orientation, the amplification will be established. This amplified region is said as a RAPD locus. Normally, a few (3-20) loci can be amplified by one single RAPD primer**.** RAPD marker is a dominant marker. DNA bands of different sizes are assumed to be amplified products from different RAPD loci. In cDNA- RAPD we use cDNA as template instead of genomic DNA so that we can detect the differential expression of genes. This technique is less expensive as compared to other techniques like microarray for detecting differential gene expression. Until now it has been widely used in genotoxicity studies to evaluate, detect and identify changes in DNA, caused due to environmental stressors (Gupta et al., 2009; Korpe et al., 2011).

Rice, a staple food crop for over half of the world's population, is a significant dietary source of inorganic arsenic, a class I non threshold carcinogen. Rice is an efficient accumulator of arsenic and thus irrigation with arsenic-contaminated groundwater and soil may induce human health hazard via water-soil-plant-human pathway (William et al., 2010). India is

the world's second and Bangladesh is the fourth largest rice producer, due to this reason most of the rice of the world consumed in Bangladesh and West Bengal state of India contributes to about half of the total arsenic intake. Variation in DNA bands detected by cDNA- RAPD is due to the loss of RAPD loci. The loss of RAPD loci may be due to the change of sequence at primer annealing site due to mutation so there will be no expression of that gene where RAPD primer anneals or large insertion in between two primer annealing sites. Therefore, with cDNA RAPD we can analyze the differentially expressed gene in control and stressed conditions in plant.

In the present work *Oryza sativa* (IR-64) and *Brassica juncea* (Pusa bold) varieties have been screened for differential gene expression in As stress. At least three different cDNA-RAPD strategies have been employed to identify differential gene expression in both these crops.

Material and Methods

A) Plant material and stress treatment

Rice (*Oryza sativa* L. indica cultivar group var *IR64*) and *Brassica* juncea (var *Pusa bold)* were grown in growth chamber (SCILAB instrument, Taiwan) hydroponically at 28°C with 16h light and 8h dark period for 14 days in 1/10th of the Hoagland solution. For Arsenic stress, different concentrations (150µM and 300 µM) of sodium meta-arsenite were prepared by dissolving in water and added to the Hoagland solution, so that the final concentration of Hoagland solution became 5% in which plants were grown. The plant samples were harvested after 96h of treatment by snap freezing in liquid N_2 and stored in −80°C for further analysis.

B) cDNA synthesis and RAPD analysis

First, cDNA (single stranded) have been synthesised from total RNA of As treated/control varieties of *O. sativa* and *B. juncea* rice using oligo(dT) primers. Total 200 random decamer primers (RAPD) have been employed to screen the four cultivars under As stress using cDNA as template. Next, double stranded cDNA was synthesized using SMART cDNA synthesis strategy (Clontech). A combination of RAPD primer and 5' SMART PCR/ 3' adapter primer (specific to adapter sequence of oligo (dT) primer used for first strand cDNA synthesis) were used for PCR to screen the same set of As treated varieties. In third strategy, single stranded cDNA was synthesized from total RNA of As treated plants using random hexamer primers instead of oligo (dT) primers. This cDNA was used in PCR reactions using different RAPD primers. Two hundred random primers obtained from Bio Basic Inc, including s1-s100 and s101-s102, were used to amplify cDNAs from control and treated plant roots and shoots. The PCR condition used was one cycle of 95^{o}C for 3min and 44 cycles of 95^{o}C for 30 sec, 36^{o}C for 30 sec and 72^{o}C for 90 sec. This was followed by a final extension at 72^{o}C for 5 min.

C) Cloning and sequencing of polymorphic bands

The PCR amplified products were gel purified and cloned in products were cloned in pJET1.2/blunt Vector. The resulting constrct were transformed in *E.Coli* (strain DH5α). The transformants were screened for the presence of inserts. The positive clones were confirmed by colony PCR. The plasmid was isolated from transformants using Alkaline Lysis Method (Sambrook and Russel, 2001) and subjected to sequencing. The sequencing was carried out with automated DNA sequencer ABI3730xl DNA analyzer. The sequence obtained were analyzed using BLAST program.

Result and Discussion

O. sativa and *B. juncea* plants growing under controlled condition in growth chambers were subjected to different concentrations (0, 150 µM and 300 µM) of As stress treatment in hydroponic culture. First, cDNA (single stranded) have been synthesised from total RNA of As treated/control varieties of *O. sativa* and *B. juncea* using oligo(dT) primers. Total 200 random decamer primers (RAPD) have been employed to screen the two cultivars under As stress using cDNA as template. However, there was no significant successful amplification observed using this strategy (data not shown).

Next, double stranded cDNA was synthesized using SMART cDNA synthesis strategy as mentioned in materials and methods section. The quality of cDNA synthesized was checked by performing a PCR amplification of actin genes from both the plants. The subsequent PCR amplification for polymorphism analysis due to As treatment was performed using a combination of RAPD primer and 5' SMART PCR/ 3' adapter primer (specific to adapter sequence of oligo (dT) primer used for first strand cDNA synthesis) were used. Unfortunately no polymorphism was observed when 5' SMART PCR (data not shown) was used, while 3' adapter primer exhibited some polymorphism (Fig. 1). RAPD in this combination resulted in amplification with slight variations especially in terms of intensity of a few amplicons.

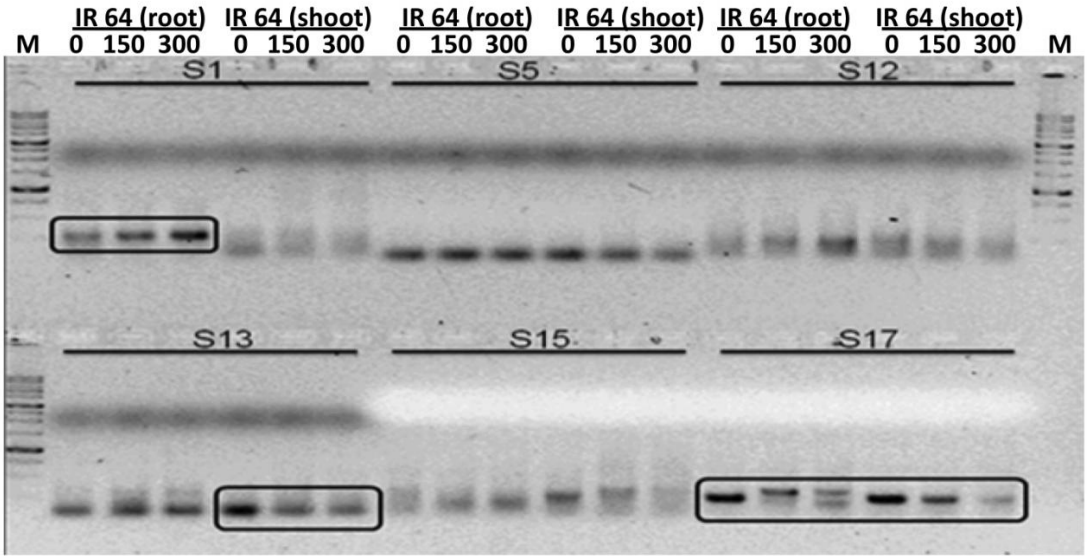

Fig 1. RAPD profile of Arsenic treated rice IR64 variety. cDNA was synthesised using smart cDNA synthesis strategy. A combination of decamer RAPD primer and 3' adapter primer were used for PCR amplification.

In the third strategy, single stranded cDNA was synthesized from total RNA of As treated plants using random hexamer primers instead of oligo (dT) primers. This cDNA, after checking the amplifciation of actin genes was used for PCR reactions using different RAPD primers. This led to successful amplifications both rice and Brassica varieties as evident from amplicons of different size on agarose gels. RAPD screenings of the As treated rice and brassica varieties led to identification of variations in terms of intensity of the bands as well as presence and absence of the bands. The representative polymorphism obtained in case of rice is shown in Figure 2, while that of Brassica is shown in Fig. 3.

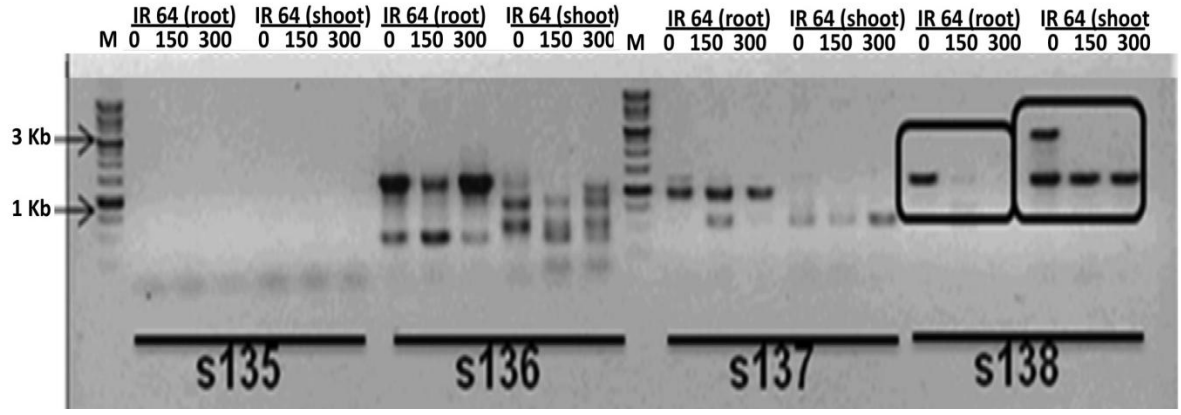

Fig 2. RAPD profile of Arsenic treated rice. cDNA was synthesised using random hexamer primers. Decamer RAPD primers were used for PCR amplification.

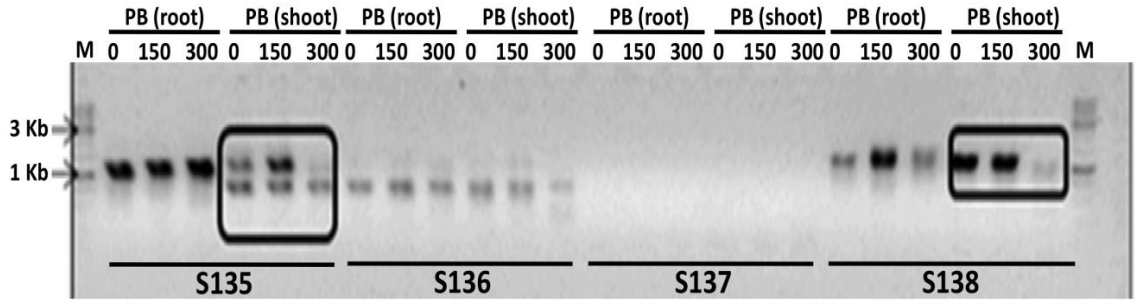

Fig 3. RAPD profile of Arsenic treated brassica. cDNA was synthesised using random hexamer primers. Decamer RAPD primers were used for PCR amplification.

From the differentially expressed (polymorphic) amplicons, 6 bands from IR-64 and 10 bands from Pusa bold were eluted and cloned in pJet1.2 vector and subsequently sequenced to identify the differentially expressing genes/ESTs. The length of these cDNAs ranged from 480 to 980 bp. Annotations of ESTs was done after searching individual sequence against the GenBank by Blastx and Blastn. A majority of the cloned cDNA inserts showed unknown functions thus suggesting that it could be the potential novel candidate genes having role in producing responses in As stress. A few ESTs showed similarity to proteins involved in transcription, metabolism, signal transduction etc. These ESTs could be used characterization to establish their involvement in producing responses to As stress.

Conclusion

These results show application of a simple and cost effective strategy in the form cDNA-RAPD to identify differentially expressing genes. The efficiency of cDNA-RAPD seems to be less over other existing methods of EST generation/gene identification; nevertheless this approach would be of great use for several orphan crop plants where genomics study is in neonatal stage.

Acknowledgement

The work is financially supported by University Grant Commission (UGC), India.

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