Influence of Water Regimes on Reactive Oxygen Species Activity in Rice (Oryzasativa L.) Under Aerobic and Wetland Condition

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Abstract - Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples include peroxides, superoxide,hydroxyl radical, and singlet oxygen.In a biological context, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. Rice is the only crop can grow under different condition at that time in order to overcome these stressful condition plant can produce Strong oxidants like the various ROS enzymes like super oxide dismutase, peroxidase and catalase.The ROS's activity was estimated by using five days old seedling and at the initial stage ROS's enzyme activity was more as the time increases the activity was decreased.Primers specific to genes associated with ROS activity were designed in order to study the association of these markers with the plant. Out of 21 markers only three markers namely 0s05g0323900 (rmsod1) Os08g0513700 and Os07g0677300 (POXgX9) were polymorphic remaining others were monomorphic.

Key words: ROS- Reactive oxygen species, rmsod : rice marker super oxide dismutase, POX-peroxidase.

I. INTRODUCTION

Rice is mostly grown under submerged soil conditions and requires much water compared to other crops. The declining availability and increasing costs of water threaten the traditional way of irrigated rice production. Moreover uncertainty of rainfall is one of the major production constraints in rain fed areas where poor rice farmers' live. Hence, an efficient use of water is a critical factor to safeguard food security in water scarce areas.The rice crop is affected by various abiotic and biotic stress, thus resulting in huge yield losses wherever rice is grown. Among the abiotic stresses, drought or water stress most important. It will causes due to insufficiency of water during the plant growing period. ROS are chemically reactive molecule containing oxygen. ROS are formed as a natural by-product of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis.During the time of environmental stress ROS level increases resulting in significant damage to cell structure. Normally cellsdefend themselves against ROS damage with antioxidants. Under oxidative stress the free radicals will occur, if they are in small quantity there will not be any problem for plant to balance if they are large in number means those will imbalance the normal metabolism of the plants. gene-specific markers identification can be done if DNA polymorphism could be detected and assessed DNA based molecular markers clearly allow the direct comparison of genetic material of two individual plants avoiding any environmental influence on gene expression.In order to quench the free radicals ROS's enzymes are necessary. Byusing candidate genes andReactive oxygen species (ROS) is known to have detrimental effect on plant growth and yield and they are usually scavenged in plant by enzymes like peroxidase, catalase, super oxide dismutase and the genes which are responsible for countering ROS. Rice which is a wetland crop continuously experiences the stress condition which directly has an impact on growth and yield. Therefore, the present study was designed to discern the ROS activity in rice varieties were grown under aerobic and wetland conditions and to discern monomorphism/polymorphism for markers associated with reactive oxygen species (ROS).

II. MATERIAL AND METHODS

For Biochemical analyses of the seed for metabolites and discern monomorphism/polymorphism for markers associated with reactive oxygen species (ROS).

The varieties used for the study: BI-33 and JAYA and along with these varieties for marker validation work along with four genotypes another eight genotypes were also analyzed

1. AM-72 (RPHP-7)

2. AM-143 (RPHP-11)

3. AM-1 (RPHP-12)

4. AM-65 (RPHP-16)

5. AM-94 B (RPHP-21)

6. Vandana (RPHP-51)

7. SEBATI (RPHP-52)

8. Jeerigesanna

The RPHP stands for Rice Project Harvest Programme

Peroxidase activity

The peroxidase activity of seed samples was estimated according to Sadasivam and Manickam (2013).

Enzyme extract

One gram of six-day-old germinated seedling was extracted in 3 ml of 0.1M Phosphate buffer with pH 7.0 by grinding with a pre-cooled mortar and pestle. The homogenate was centrifuged at 10,000 rpm at 50C for 15 minutes. The supernatant was used as enzyme source within 2-4h. The enzyme extract was stored in ice till the assay is carried out.

Preparation of reagents

1. Phosphate Buffer 0.1M (pH 7.0)
2. Guaiacol solution (20mM): dissolve 240mg Guaiacol in distilled water and make up the volume to 100 ml. It can be stored frozen for many months.
3. Hydrogen peroxide solution (0.042%): 12.3mM Dissolve 0.14ml of 30% hydrogen peroxide and make up the volume to 100 ml with distilled water. It should be prepared at the time of use

Estimation of peroxidase activity

Pipette out three ml of buffer solution to a clean dry cuvette. Add 0.1 ml enzyme extract and 0.05ml Guaiacol solution. Bring the mixture to 25 ^{0}C and then place the cuvette in the Spectrophotometer set at 436 nm. Then, add 0.03 ml hydrogen peroxide solution and mix immediately start the stopwatch. Read the initial absorbance at 436nm and note increase in the absorbance for 3 minutes at an interval of 30 seconds. Water blank is used in the assay. Enzyme activity is expressed in terms of rate of increasedabsorbance/3 min/g of seed.

Estimation of Catalase activity

The Catalase activity of seed samples was estimated according to Sadasivam and Manickam (2013).

Enzyme extract

Homogenize plant tissue in a blender with M/150 phosphate buffer (assay buffer diluted 10 times) at 1-4^{0}C and centrifuge stir the sediment with cold phosphate buffer, allow to stand in ice with occasional shaking and then repeat the extraction once or twice. the extraction should not take longer than 24hr. use the combined supernatants for the assay. The Catalase activity can change considerably on storage of the tissue.In comparative studies. Therefore always use the same conditions of extraction, storage and temperature.

Procedure

1. Wave length: 240 nm
2. Final volume: 3ml approximately
3. Room temperature
4. Read against a control cuvette containing enzyme solution as in the experimental cuvette but containing H_2O_2 free phosphate buffer.
5. Pipette into the experimental cuvette.
6. 3ml $H_2O_2PO_4$ buffer mix in 0.01-0.04ml sample with a glass or plastic rod flattened at one end .note the time required for a decrease in absorbance from 0.45-0.4. this value is used for calculations

Estimation of super oxide dismutase activity

The Superoxide dismutase activity of seed samples was estimated according to Sadasivam and Manickam (2013).

Enzyme extract

1g of fresh clean leaf tissue in 10ml ice cold 50mM potassium phosphate buffer pH 7.8 in pre cooled pestle and mortar. Centrifuge the homogenate at 10,000 rpm for 10 minutes at 4^{0}C in a refrigerated centrifuge. The supernatant is used as enzyme source within 12 hrs of extraction.

Preparation of reagents

Potassium phosphate buffer 250mM, pH 7.8

Solution A: potassium mono hydrogen phosphate, 250mM 100ml

Solution B: potassium di hydrogen phosphate, 250mM 100ml

Add solution A to B with constant stirring until pH 7.8 reached

- Methionine 100mM in distilled water
- Riboflavin 10mM in distilled water
- EDTA 5mM in distilled water
- Nitro blue tetrazolium (NBT) salt 750µM in distilled water

Procedure

Mix a 3ml reaction cocktail containing : 50mM potassium phosphate buffer pH 7.8, 13mM methionine, 2µm of riboflavin, 0.1mM EDTA, 75µm NBT and 50µl of crude enzyme extract in a duplicate make up the volume equal by adding double distilled water. Set a blank without enzyme

and NBT to calibrate the Spectrophotometer. Set another control having NBT but no enzyme as reference control. Expose all tubes to 400w bulb for 15 minutes read the absorbance immediately at 569nm calculate the percent inhibition. The 50% inhibition of the reaction between riboflavin and NBT in the presence of methionine is taken as 1 unit of SOD activity and the enyme activity is expressed as units/mg of protein

Isolation of genomic DNA for ROS activity

DNA extraction for twelve genotypes was done using CTAB method from young leaves tissues as the protocol described by Doyle and Doyle (1990) as below.

Primer study

Exploring the genes associated with ROS activity in rice

All the genes reported to be associated for ROS activity in rice have been explored. All the potential genes were enlisted. (Table 1 and 2).

Downloading the sequence of candidate genes

The genomic sequences of the explored candidate genes were downloaded by using the NCBI website (www.ncbi.nlm.nih.gov). Nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov/nuccore) was used to download the sequences of the genes reported to be associated with zinc content. Genomic DNA sequence of all the target genes was downloaded.

Primer design

Primers specific to genes associated with ROS activity were designed by using the "pick primer" tool of NCBI in collaboration with Primer3 (Ye *et al.*, 2012).

The whole sequence of the downloaded genes was uploaded into NCBI database. Then pick primer tools was used. Some of the primer designing criteria considered was 50% optimum GC content, less difference (0.02^{o}c) in melting temperature of forward and reverse primers, optimum length of 20-25 base pairs.

PCR reaction mixture

PCR for ROS markers was performed in a total volume of 20 µl containing 1X PCR buffer (contains 10 mMTris-HCl, pH 8.0 at 25 ^{0}C, 50 mMKCl, 1.5 mM $MgCl_2$), 0.1 µM of each forward and reverse primers (Sigma Aldrich, USA), 50 ng rice genomic DNA, 2mM dNTPs mix and 1.2 units of *Taq* polymerase (Bangalore Genei, India).

Agarose gel electrophoresis

Agarose gel (3.0%) was prepared using electrophoresis grade agarose (Bangalore Genei, India) in a volume of electrophoresis buffer (1X TBE) sufficient for constructing a gel (220 ml for 20 X 20 cm gel). Ethidium bromide was added at concentration of 10 mg/ml of gel. The gel was allowed to solidify fully before removing the combs and loading the sample. 5 µlof 3X loading dye was added to 10 µl of PCR products, and mixed well before loading into the well. Care was taken to prevent mixing of samples between the wells. A voltage of 5 V/cm was given for a time period of three hours for separation of PCR fragments. The gel was viewed under UV trans-illuminator and the DNA banding pattern was recorded directly and later with Alpha Innotech gel documentation instrument.

Screening of candidate markers for polymorphism

A total of 20 candidate gene markers designed based on the strategy described earlier, were screened to discern their amplification profiles.

III. RESULTS:

Scoring of generated bands

The bands generated by gene specific primers were scored as ‘1’ for bands of higher size bands, ‘3’ for lower size and ‘2’ for those showing both the bands.

The ROS’s activity was estimated by using five days old seedling and at the initial stage ROS’s enzyme activity was more as the time increases the activity was decreased. Three enzymes activity were analysed by using twelve genotypes, among the three enzymes peroxidase plays an activity for longer time than that of the Catalase and superoxide dismutase. The data on activity of enzymes are listed in the table 2,3, 4.The expected product size of the primers designed ranged from 368bp to 1064bp, and annealing temperature from 52^{0} C to 62^{0} C. All the primers were showing amplification in all the selected genotypes.

Screening genotypes with designed molecular markers

Out of the 21 primers pair only three markers namely 0s05g0323900 (rmsod1) Os08g0513700 and Os07g0677300 (POXgX9) were polymorphic remaining others were monomorphic (fig:1) and remaining marker showed monomorphism (fig:2).

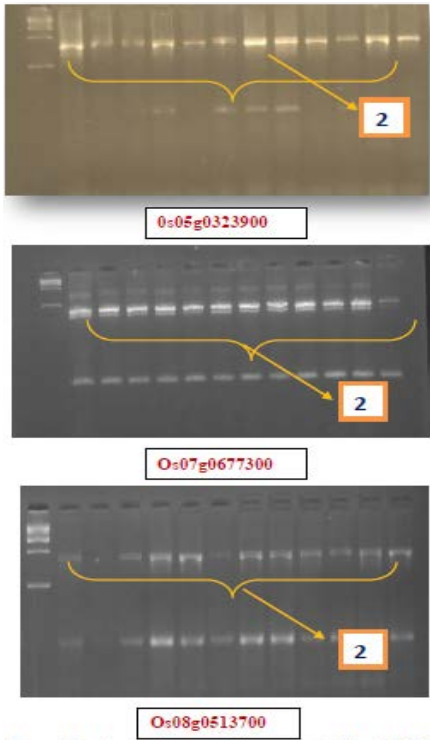

Fig. 1: Genes showing polymorphism associated with ROS activity (L, AM72, AM1, BI-33AEROBIC , JAYA AEROBIC, AM143, AM65, BI-33 WETLAND, JAYA WETLAND ,AM 94B, VANDANA, SEBATI, Jeerigesanna)

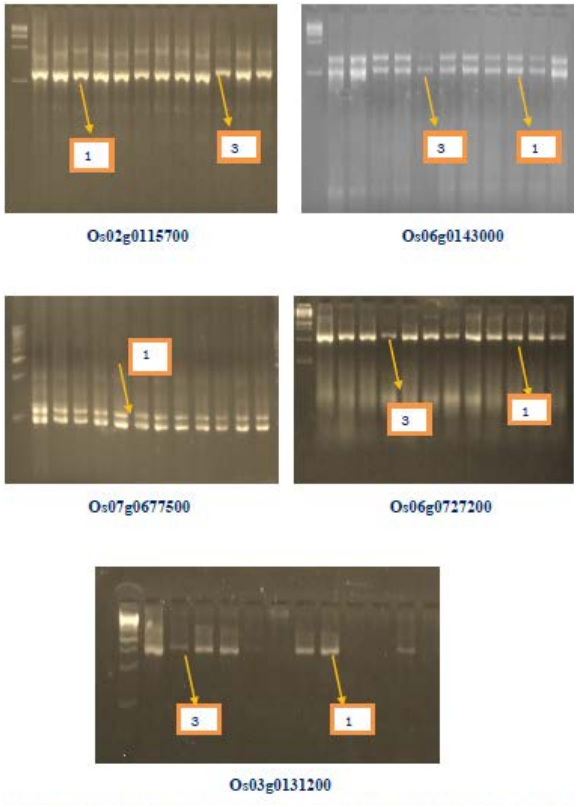

Fig. 2: Genes showing polymorphism associated with ROS activity (L, AM72, AM1, BI-33AEROBIC , JAYA AEROBIC, AM143, AM65, BI-33 WETLAND, JAYA WETLAND ,AM 94B, VANDANA, SEBATI, Jeerigesanna)

IV. DISCUSSION

Activity of Peroxidase, Catalase and Superoxide dismutase activity higher in the initial stage of plant growth in both aerobic and wetland condition (Fig.1,2 and 5.13).Over time genotypes response to environment the activity of these enzymes were noticed this may be due to the genotypic and large amount of ROS production at the initial seed germination stage. The similar findings were reported by Setter (1997) and Perata (1997).

To identify the ROS activity along with two genotypes and eight othergenotypes were also used. 42 primer pairs associated with ROS were designed, standardized and used in the study. All primers profiles are indicated in Plates 9 and 10.96 distinct bands were generated from forty two primers. Out of which 36 were polymorphic and 60 were monomorphic bands.

However, the primer 0s05g0323900 (rmsod1), Os08g0513700 (rmsod2) and Os07g0677300 (POXgX9) showed highest number of polymorphic bands and highest monomorphic bands were noticed in remaining all other primers.

All genotypes were expressed similar fashion to the DNA markers which were used in the study. This may be due to the same amount and activity may be found in all the genotypes and there was no difference found for all the alleles for ROS in the different methods of planting. This may be due to some extent plant feel stress under aerobic as well as under wetland condition and the activity will reduce during a period of time. Similar findings were reported byZhang and Khirkham ,1994, Setter *et al.*1996, Perataet *al.*1997.

V. CONCLUSION

ROS are chemically reactive molecule containing oxygen. ROS are formed as a natural by product of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. During the time of environmental stress ROS level increases resulting in significant damage to cell structure. Normally cells defend themselves against ROS damage with antioxidants. Under oxidative stress the free radicals will occur, if they are in small quantity there will not be any problem for plant to balance if they are large in number means those will imbalance the normal metabolism of the plants. In order to quench the free radicals ROS‘s enzymes are necessary. By using candidate genes and gene-specific markers identification can be done if DNA polymorphism could be detected and assessed DNA based molecular markers

clearly allow the direct comparison of genetic material of two individual plants avoiding any environmental influence on gene expression.

Activity of Peroxidase, Catalase and Superoxide dismutase activity higher in the initial stage of plant growth in both aerobic and wetland condition. Over time genotypes response to environment the activity of these enzymes were noticed this may be due to the genotypic and large amount of ROS production at the initial seed germination stage. To identify the ROS activity along with two genotypes and eight other genotypes were also used. 42 primer pairs associated with ROS were designed, standardized and used in the study. 96 distinct bands were generated from forty two primers. Out of which 36 were polymorphic and 60 were monomorphic bands. However, the primer 0s05g0323900 (rmsod1), Os08g0513700 (rmsod 2) and Os07g0677300 (POXgX9) showed highest number of polymorphic bands and highest

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Table 1.Details of the gene specific primers designed

Sl. No.	Primer name	Primer Sequence (5' to 3')	bp	Annealing temperature (oC)	Expected product size (bp)
1	Os06g0143000 aF	GTGGTGGATAGATAGCGCGG	20	60.39	1317
2	Os06g0143000 aR	GGGTTGCCGTTGTTGTATGC	20	60.39	
3	Os06g0143000 bF	GCTGGGTTTGGCTTGTCTGT	20	61.11	1038
4	Os06g0143000 bR	AGCATCAAGCGACATGCTCC	20	61.09	
5	Os06g0143000 bF	CTGTTGCTTCGGCTGGGTTA	20	60.61	698
6	Os06g0143000 bR	GACATGAGGACCACTTTGGGG	21	60.61	
7	Os07g0677500 aF	CGTCGAATGCCAACACACG	19	60.15	983
8	Os07g0677500 aR	GAGGAGAGTTCGAGACCAAGC	21	60.14	
9	Os07g0677500 bF	GACTCCGTCGTCGCTGTAAGA	21	61.86	1150
10	Os07g0677500 bR	GCCCCTCTGTCCTCTGTATTCC	22	61.87	
11	Os07g0677300 aF	CCCAGCTCCCCTCGTATGATT	21	61.38	872
12	Os07g0677300 aR	GGTGGAGTCCCTTCTTCCCAA	21	61.39	
13	Os07g0677300 bF	TCGTCGCGGTAAGATCACGG	20	62.31	762

14	Os07g0677300 bR	CCTTGGAGCAGTTGAGCCTGA	21	62.33	
15	Os02g0115700 aF	CCCAACGACTCATCACACTGC	21	61.53	1254
16	Os02go115700 aR	GCGTCAACACCTACACCTTCG	21	61.52	
17	Os02g0115700 bF	CCAGTGGAACTTGACGTACCTGG	23	62.96	680
18	Os02go115700 bR	ACGTGCTACCCATGCAGTTCC	21	62.98	
19	Os02go115700 cF	GGAACTGCATGGGTAGCACG	20	61.37	357
20	Os02go115700 cR	ACAAACCCCTCTCACTCCCAG	21	61.39	
21	Os06g0727200 aF	CACTGGATTCCTCCTCCTTGC	21	60.41	1480
22	Os06g0727200 aR	GCACTGGACCTATGGAGTGTG	21	60.41	
23	Os06g0727200 bF	CACACTCCATAGGTCCAGTGC	21	60.41	1277
24	Os06g0727200 bR	GCCTGTTAGAACACGAGGAGG	21	60.4	
25	Os06g0727200 cF	GGCTGTCGGGAAAAGGTGTG	20	61.52	585
26	Os06g0727200 cF	GGACGCATCACACTGCAACA	20	61.51	
27	Os03g0131200 aF	AGAGACCTGTCAGCCTGCATC	21	61.85	1005
28	Os03g0131200 aR	GGATCTTCTCCTACTCCGACACG	23	61.84	
29	Os03g0131200 bF	CGATGTTGCGGTTGAGCACC	20	62.51	829
30	Os03g0131200 bR	TCAGGCCCGATCCTTCTGGA	20	62.53	
31	Os03g0131200 cF	AGTCCTCCAGAAGGATCGGG	20	60.4	175
32	Os03g0131200 cR	TCAGCTAGGTCCAGACTCCG	20	60.39	
33	Os03g0131200 dF	GGAGTCTGGACCTAGCTGAGATG	23	61.56	749
34	Os08g0513700 aF	CCCTGTCCCATCCCATTTCA	20	59.37	
35	Os08g0513700 aR	ACTGCCCTGTTCAGGAGATG	20	59.38	1364
36	Os08g0513700 bF	CCAAGAGGAGTTTGGCCAGT	20	59.89	
37	Os08g0513700 bR	GGTTTCTCTGCTCCCCCAAA	20	59.89	999
38	Os05g0323900 aF	CCTTACCCCTTCCTCGCATAC	21	59.93	
39	Os05g0323900 aR	TAGCCCTGTCCTGAGGATACC	21	60.13	1623
40	Os05g0323900 bF	CTGTAGGATGGAGCTGCTGTG	21	60.47	
41	Os05g0323900 bR	GTAGTACGCATGCTCCCAGAC	21	60.54	1683

Table 2.Biochemical estimation of thecatalyse enzymein rice genotypes used for ROS's activity

Time in seconds	Enzyme activity (µm/ml) @ 240 nm
25	0.000882
30	0.00044
35	0.00038
40	0.00033
45	0.00029
50	0.00026
55	0.00024
60	0.00022

Table 3.Biochemical estimation of the peroxidase enzyme in rice genotypes used for ROS's activity

Time in seconds	Enzyme activity (µm/ml) @ 436 nm
30	995.304
60	497.652
90	331.768
120	248.826
150	199.0608
180	165.884
210	142.1863
240	124.413
270	110.5893
300	99.5304

Table 4.Biochemical estimation of the SOD enzyme in rice genotypes used for ROS's activity

Genotypes	Enzyme activity (µm/ml) @ 560nm
AM 72	2.426
AM 143	2.6539
AM 1	2.4583
AM 65	2.0336
AM 94 B	2.5048
Vandana	2.4791
Sebati	2.6139
JeerigeSanna	2.6834
BI-33 Aerobic	2.4062
BI-33 Wetland	2.5048
Jaya Aerobic	2.6118
Jaya Wetland	2.4886