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Evaluation of differential expression of miRNAs in response to oxidative stress in *Brassica juncea*

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ABSTRACT

Oxidative stress constitutes one of the most deterrent abiotic stresses that interfere with the agricultural potential of crop plants. To induce tolerance against such stresses the role of miRNAs (microRNAs) has been implicated time and again. MicroRNAs have been shown to play significant role in regulating various abiotic stress responses in plants. In this work we have evaluated the role of several conserved microRNAs that showed differential expression in the plants of Brassica juncea under various oxidative stresses: miR164c and miR169c are up-regulated in the plants whileas miR156a, miR391 and miR400 are down-regulated. The expression of selected conserved miRNAs responsive to different oxidative stresses was validated through Northern Blotting. Target transcripts of these conserved miRNAs were validated through qRT-PCR to ascertain the role of these miRNAs in the transcriptome of B. juncea. The expression of miRNAs was shown to be in conformity with the expression of their predicted targets.

Key words: Lipid peroxidation, microRNA, Oxidative stress, Post- transcriptional gene regulation, Transcriptome,.

1. INTRODUCTION

Oxidative stresses offer major physiological challenges to the productive capacity of plants. To overcome these challenges plants are seen to undergo major genetic regulations which can be sensed in their ability to undo the unfavorable environmental constraints. When plants are subjected to severe environmental constraints several mechanisms are turned on and off to combat such constraints and provide plants with an adaptive potential [1].Various abiotic stresses like oxidative stress form reactive oxygen species (ROS) within plant tissues. ROS which are formed under stress conditions can lead to cellular injury. Therefore plants must respond both at biochemical level as well as molecular level. So far as the biochemical immune responses in plants are concerned formation of antioxidant enzymes is the primary premise. Enzymes such as Superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase and mono-dehydroxyascorbate peroxidase play a fundamental role in

defence response of plants to oxidative stresses. These enzymes carry out detoxification in plants thus eliminating the threat of plant cellular damage. Likewise, mRNA regulating mechanisms are fundamentally employed to guarantee, a plant under stress, survival and well-being [2]. The response to stresses is such that specific genes or proteins are induced that help to resist adverse conditions and subside the severe cellular damages. miRNA-mediated post-transcriptional regulation of gene expression is one such mechanism that has quite recently been demonstrated to be in operation in plants subjected to various biotic and abiotic stresses [3]. In plants, miRNAs are significantly associated with biotic [4] and abiotic stresses [5].

MicroRNAs (miRNAs) are small regulatory RNA molecules of approximately 22 nucleotides that regulate the expression levels of target genes involved in mediating stress responses in plants. In the cellular cytoplasm, the mature miRNA is loaded into the RNA-induced silencing (RISC) complex and binds with near perfect complementarity on target mRNAs thus causing post-transcriptional gene silencing by mRNA cleavage or by translational repression [6]. The first role of miRNA regulation in plants was described independently by Jones-Rhoades et al. [7] and Sunkar et al. [8]. Following the footprints of these workers several subsequent studies lead to identification of stress responsive miRNAs. miR398 and miR395 have been shown by independent workers to regulate cellular responses in many abiotic stresses [9]. Similarly, miR169 and miR393 have been shown to help *Arabidopsis* survive under limited phosphorus and nitrogen conditions respectively [10]. miR394 has been seen to combat salinity and drought stress in Arabidopsis [11].

Brassica juncea (Czern) L., commonly known as Indian mustard, is one of the main oil seed crop in India. Like other crops this oil seed crop is also under severe pressure imposed by detrimental environmental stresses [12]. Present study has been undertaken to evaluate the role of some microRNAs and their predicted targets under oxidative stresses.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Brassica juncea (Czern) L., seeds were procured from Indian Agricultural Research Institute, Pusa, New Delhi. Seeds were sterilized with 1% sodium hypochlorite solution supplemented with 0.5 % Triton-X for 15 min. followed by after-washes with distilled water so much so that no further froth formation takes place. The seeds were then transferred to plastic beakers provided with muslin cloth for their successful germination. The beakers were previously autoclaved and provided with aluminum wrap to prevent any fungus from contaminating the hydroponic solution or the seeds. The beakers with the seeds were then transferred to growth chamber with long day conditions of 16hr day/8hr dark and maintained at a temperature of 26^0C.

2.2 Stress Conditions and Treatment

Two week old seedlings were subjected to two oxidative stresses. Oxidative stress with Methyl Viologen, MV (20 µM) for 1 h, 3 h, 6 h and 12 h by adding Methyl Viologen in proper concentration to hydroponic solution. Similarly, Oxidative stress with 200mM H_2O_2 was also given for the same time periods as that for MV. Seedlings without any treatment were taken as control.

2.3 RNA isolation and Northern Blotting of miRNAs

Total RNA was isolated as suggested by Chomczynski and Sacchi with some modifications [13]. In short 1g tissue was homogenized in liquid nitrogen and to it 10 ml GITC buffer with 1%β-mercaptoethanol was added. The homogenate was transferred to an oakridge tube and to it was added 1 ml of 3M sodium acetate. After treatment with Phenol-Chloroform the RNA was precipitated with chilled isopropanol. To obtain LMW (low molecular weight) RNA the total RNA was dissolved in 1ml 4M Lithium Chloride. LMW RNA was quantified using nano drop (Bio-Rad, Hercules, CA) and its integrity was checked by 15% Urea-PAGE.Forty micrograms of total RNA was loadedper lane and resolved on a denaturing 15% polyacrylamide gel and transferred electrophoretically to Hybond N+ membranes (Amersham Biosciences, Piscataway). Membranes were UV cross-linked for 2 h at60^{0}C. DNA oligonucleotides complementary to small RNA sequenceswere end labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (Invitrogen, USA).Blots were subjected to pre-hybridization for about 1 h and then hybridized over night using Perfect HYB Plus buffer (Sigma) at 40 ^{0}C. Blotswere washed four times (once with 5XSSC and 0.1% SDS for 2 min, once with 2X SSC and 0.1% SDS for 2 min, once with1X SSC and 0.1% SDS and once with 0.1X SSC and 0.5% SDS) at 50^{0}C. After each washing, counts were checked by a GM Counter. The membranes were air dried and then photographed by a phosphorimager (GE Healthcare).

2.4 Quantitative PCR of miRNA Target Genes

Total RNA was isolated from *Brassica juncea* plants by GITC reagent (Amresco). 10 µg of RNA was treated with 2 U of RNase free DNase I (Promega) followed by phenol-chloroform extraction and precipitation.2 µg of the treated RNA was polyadenylated with ATP by poly (A) polymerase (PAP) following the manufacturer's instructions for the Poly (A) Tailing Kit (Ambion). Subsequently, each sample was reversetranscribed using 0.5 microgram of Poly (T) adapter and Superscript III (Invitrogen, USA). miRNA target gene expression was determined by quantitative real-time PCR on a Realplex master cycler (Eppendorf, Germany) using Quantitec SYBR Green PCR kit (Qiagen). Actin was selected as the internal reference gene for PCR quantitation. CT values obtained through qPCR were analyzed using delta delta CT method to calculate relative fold change [14].

3. RESULTS

3.1 Analysis of chlorophyll content, relative water content and lipid peroxidation

The three parameters were evaluated to analyse the influence of oxidative stress on the natural biosynthetic pathways and to ascertain which biochemical mechanisms are being explored by the plants to combat stresses. Chlorophyll and relative water content were chosen to analyze the effect of oxidative stress on anabolic processes such as photosynthesis. The two parameters showed a constant decrease in their contents relative to the un-treated samples. Lipid peroxidation which could be employed to measure the immunity building mechanism of the plants against stresses was estimated by measuring the amount of malondialdehyde (MDA) which in turn was measured as described previously Heath and Packer [15]. We observed an increased level of lipid peroxidation in plants under oxidative stress relative to control samples. MDA content showed a constant increase with the increase in the time of treatment either with MV (20 µM) or with H_2O_2 (200mM). However, when the treatment was given for 12 hours the MDA content was seen to show a drastic decrease which is indicative of localized death in plants [T**able 1 and 2**].

Table 1: Changes in the total chlorophyll, Malondialdehyde (MDA) and relative water content in *Brassica juncea* under Methyl Viologen (20 µM)

Treatments	Total chlorophyll content (mg/gm fw)	Relative water content (%)	MDA content n mol/gm fw
T0	1.89±0.02a	96.16±1.5f	1.57±0.04b
T1	1.42±0.06c	88.49±0.43c	1.85±0.04b
T2	1.29±0.06c	88.21±0.60c	2.40±0.23b
T3	1.24±0.12f	84.99±1.95f	2.29±0.09a
T4	1.01±0.006a	79.37±0.41c	1.18±0.08a
LSD at 5%	0.00	0.00	0.00

Values are expressed as means ± standard errors of three replicates of two experiments. Different superscript letters (a–f) indicate significance at $p \leq 0.05$ level according to LSD. T1 = 1 hour; T2 = 3 hours; T3 = 6 hours; and T4 = 12 hours; T0=Control.

Table 2: Changes in the total chlorophyll, Malondialdehyde (MDA) and relative water content in *Brassica juncea* under Hydrogen Peroxide (200 mM)

Treatments	Total chlorophyll content (mg/gm fw)	Relative water content (%)	MDA content n mol/(gm fw)
T0	1.84±0.02a	98.26±0.61d	1.32±0.05c
T1	1.29±0.03b	83.86±0.24b	1.56±0.08d
T2	1.21±0.05c	81.83±0.31b	1.80±0.06a
T3	1.14±0.03b	82.83±0.37b	2.11±0.05a
T4	1.03±0.03b	81.20±0.57c	1.27±0.03a
LSD at 5%	0.00	0.00	0.00

Values are expressed as means ± standard errors of three replicates of two experiments. Different superscript letters (a–f) indicate significance at $p \leq 0.05$ level according to LSD. T1 = 1 hour; T2 = 3 hours; T3 = 6 hours; and T4 = 12 hours; T0=Control.

3.2 miRNAs show differential expression in Response to oxidative Stress

To analyze the influence of oxidative stresses on miRNA levels in *B. juncea* plants, we chose to evaluate the expression of some of the conserved miRNAs that have previously been analyzed to show differential expression patterns under a regime of abiotic stresses [8]. Thus based on the homology of mature miRNAs sequences in miRBase v19 between different plant species, miR156a, miR164c, miR 169c, miR391 and miR 400 were selected for in silico analysis. The selected miRNAs were searched for variant miRNAs in miRBase through BLASTn algorithm. Sequence homology indicated that four miRNA sequences (miR164c, miR169c, miR391 and miR400) were identical to those reported in *A. thaliana* whileas miR156a was identical to the one reported in *B. napus*. The predictable targets for the said miRNAs were evaluated by ‘‘psRNA target” software. The dataset of *Brassica napus* was used as the reference genome. The predicted target for miR156a is an *A. thaliana* homologue SBP (Squamosa Binding Protein). Similarly the target of miR164c is an *A. thaliana* homologue NAC. The predicted target for miR169 is an mRNA coding for CCAAT and HAP-2 whileas the target accession for miR 391 was not found. miR 400 is predicted to target a large number of genes of the pentatricopeptide repeat (PPR) protein family and targets a large number of genes which are targeted by other miRNAs like miR 160 [8]. This aspect of coordinated regulation has made it clear that several dissimilar miRNAs may target the same set of genes. Thus apart from PPR family miR 400 has been seen to target a homologue of *Arabidopsis thaliana* (ARF). To evaluate differential expression in the levels of miRNAs in Indian mustard, we performed RNA hybridization (**Figure 1**). We observed the over-expression of two miRNAs and under-expression of three miRNAs which were selected through bioinformatic approaches as previously described (Materials and Methods). We found that miR169c, miR391 andmiR400 showed

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the strongest response to oxidative stress in *Brassica* plants. miR164c showed slight up-regulation in response to said stress when compared with the corresponding expression in untreated plants while as miR169c showed 1.5-fold up-regulation when compared with control plants of the same category. miR391, miR400 and miR156a in general and miR400 in particular showed a strong down-regulation relative to the untreated control plants. miR400 showed 3-fold change in its expression and seems to negatively influence mRNA expression.

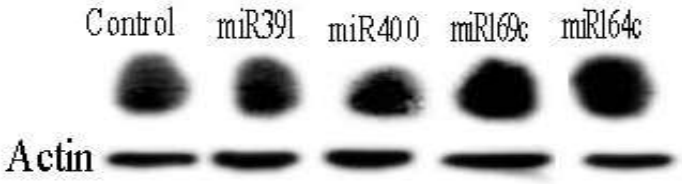
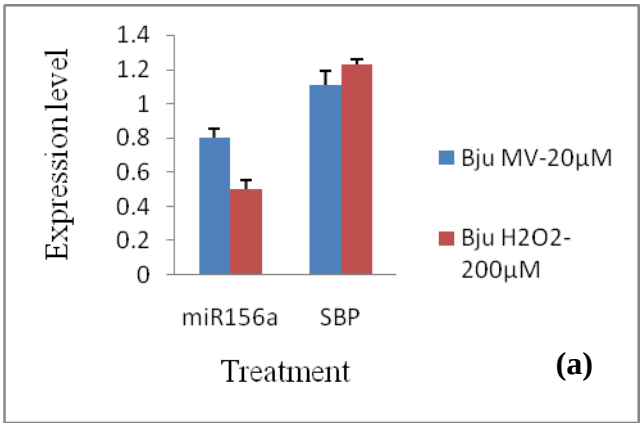
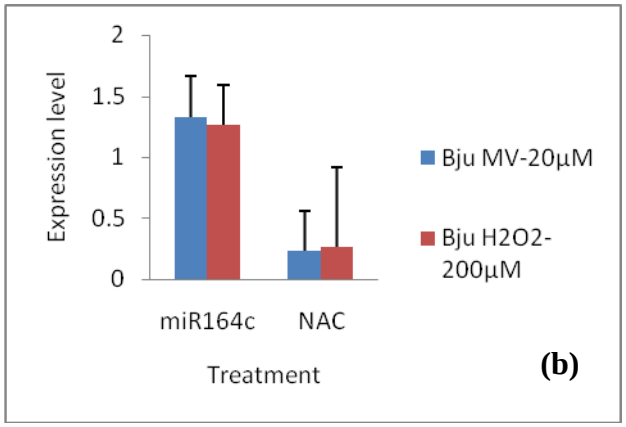


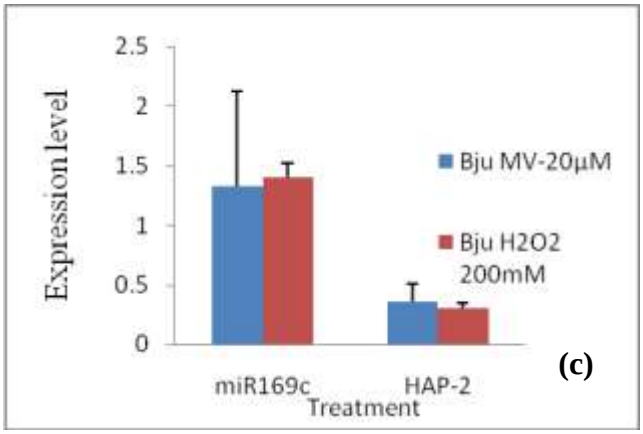
Figure 1: Hybridisation blots for miRNAs in response to oxidative stress and control plants in *Brassica juncea*

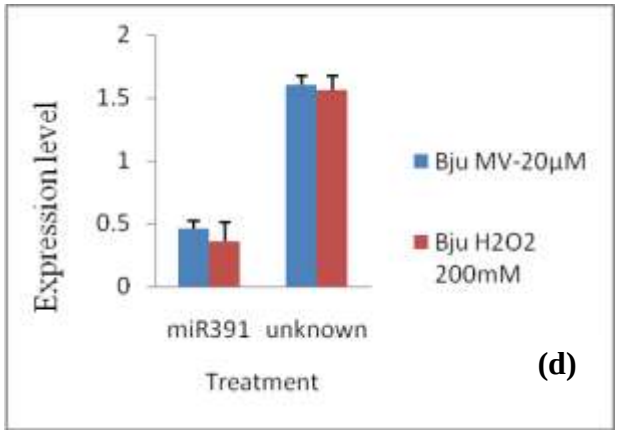
3.3 Evaluation of miRNA target gene expression under oxidative stress

As previously described the role of microRNAs has been well established in regulating the expression of their respective target genes [16], so we proceeded to correlate the differential expression of miRNAs with their respective target transcripts. Expression analysis of target genes (Bra004363, Bra022685, Bra011583, Bra013888 and Bra036649) was carried by using a quantitative RT-PCR. As previously described we employed the relative fold change method to evaluate the expression of target genes in stress-treated plants in comparison to controls. The expression profiles of miRNAs and their predicted targets are depicted in **Figure 2 (a-e)**. We observed a significant change in the expression of target genes under stress conditions when compared to the respective control samples. *HAP-2* was slightly down-regulated while its associated miR164c showed corresponding minor up-regulation predicting mRNA cleavage activity of the miR 164c.









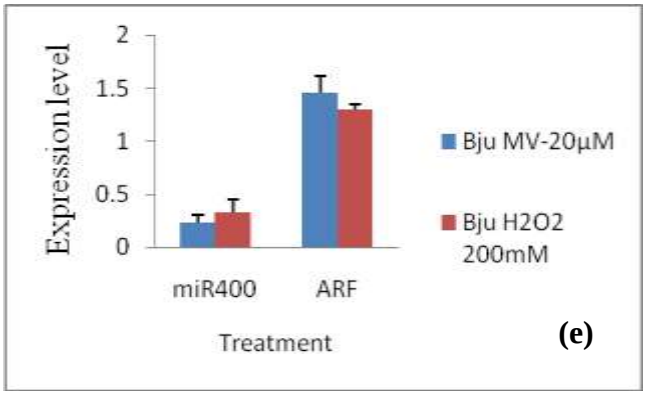


Fig. 2 (a-e): The relative expression of miR156a, miR164c, miR169c, miR 391 and miR400 and their respective target accessions was measured under oxidative stress conditions using qPCR. Plants were subjected for different time durations to either MV (20µM) stress or H_2O_2 (200mM) stress. Values are averaged for three biological replicates (N=3±SD).

4. DISCUSSION

Oxidative stresses like other environmental stresses lead to loss of crop productivity due to accumulation of reactive oxygen species [17]. These reactive oxygen species have been seen leading to lipid peroxidation by causing accumulation of malondialdehyde (MDA) apart from causing damages to several fundamental biosynthetic processes of the cell [18]. Oxidative stresses lead to reduction in photosynthetic efficiency of plants fundamentally by leading to decrease in the pigment concentrations such as chlorophyll. However plants have employed several defence mechanisms to counteract ROS. One such mechanism which is fundamentally important is the production

of antioxidant enzymes. In this work we have been able to evaluate the responses of plants in terms of pigment degradation and lipid peroxidation.The estimation of chlorophyll content has been used by previous workers to correlate it with abiotic stress responses [19]. Similar influence of lipid peroxidation on the survival of plants under stress is well documented. A number of recent reports have been able to link various oxidative stresses in *Arabidopsis* and Tobacco [20]. Hence changes in lipid peroxidation levels in plants can be used as an essential component to evaluate stress response in plants. Lipid peroxidation which can be measured by estimating the MDA content of plants is indicative of oxidative damage. Several studies have revealed a positive correlation between lipid peroxidation and abiotic stresses [21]. We have also been able to evaluate the expression of several conserved miRNAs in the genome of *Brassica juncea*, on the basis of homology with the genome of *Brassica napus*. All the five miRNAs analysed showed differential expression under oxidative stress relative to control samples.

5. CONCLUSION

MicroRNAs have pacified, to a large extent, the ongoing debates on the regulation of gene expression under various kinds of abiotic stresses. This work highlights the role of some of the conserved miRNAs involved in the regulation of plant responses to oxidative stresses. Deep sequencing approaches which are preferred at present due to their data generation and accuracy can be used to construct miRNA libraries for plants that are at present out of human contravention. Apart from their role in plant development, it is being inferred that bigger problems of environmental stresses can be dealt in with much ease by employing regulators such as miRNAs to develop transgenic lines which shall culminate into providing better adaptability to plants in general.

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