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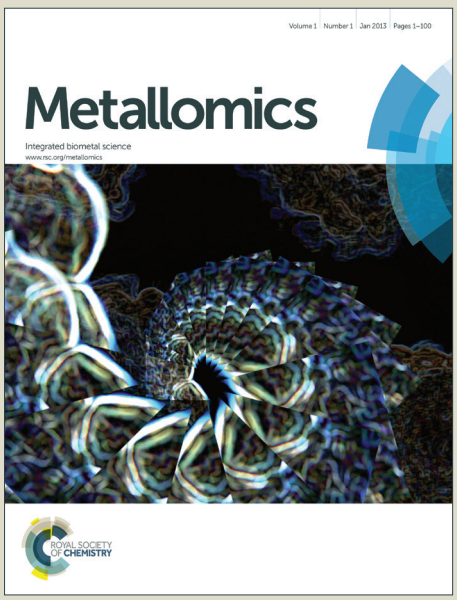

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miRNA plays role on antagonistic effect of selenium on arsenic stress in rice seedlings

Chandana Pandey[1], Badmi Raghuram[2], Alok Krishna Sinha[2] and Meetu Gupta[1*]

[1]Department of Biotechnology, Jamia Millia Islamia, New Delhi, India

[2]National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India

***Corresponding Author**

Dr. Meetu Gupta
Department of Biotechnology
Jamia Millia Islamia
Jamia Nagar
New Delhi 110025
India
Email: meetu_gpt@yahoo.com
Phone: +91 9818911750

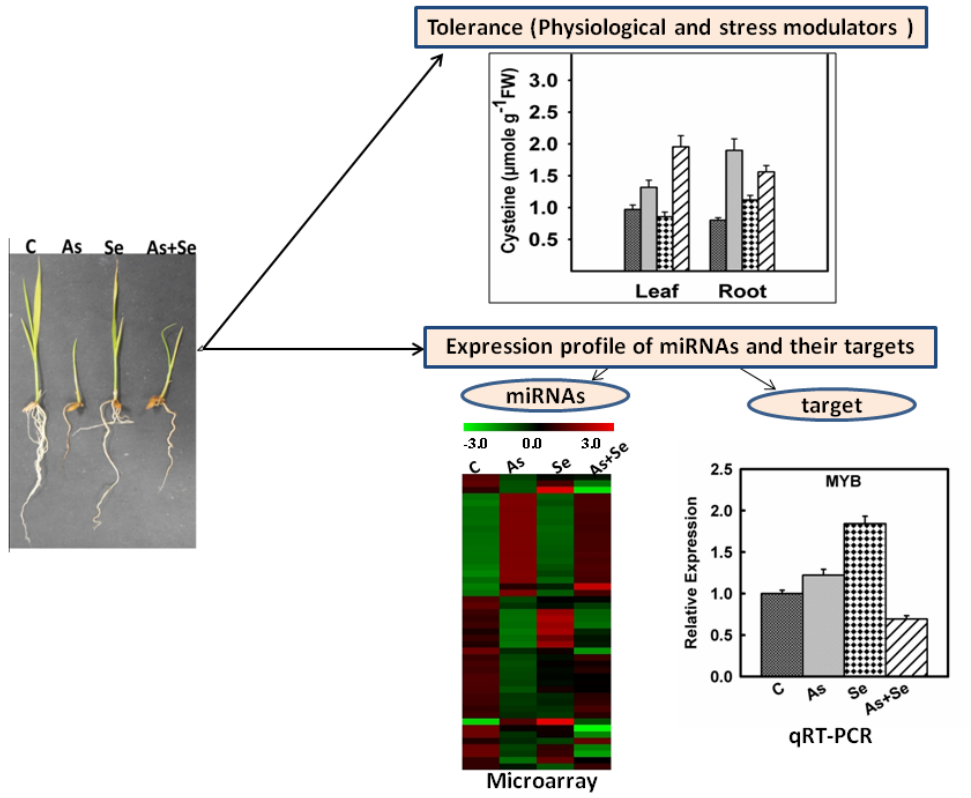
Running Title: miRNA during arsenic and selenium antagonism in rice

ABSTRACT

MicroRNAs (miRNA), the small non coding RNA has been implicated in various biological processes including adaptation during environmental stress. The present work explores the involvement of miRNA during arsenic (As) and selenium (Se) treatment in rice seedlings. Arsenic is a heavy metalloid causing severe adverse effect on growth and development of plants while Se is another metalloid and an essential micro-nutrient when present in appropriate amount. It was observed that presence of Se along with As mitigated the adverse effect of As on seedling germination, root-shoot growth, total chlorophyll and protein contents. Measurement of stress indicators such as proline, cysteine and MDA also indicated similar effects. Analysis of miRNA profile by microarray under As, Se and As+Se treatments exhibited differential regulation of at least 46 miRNAs in rice seedlings compared to untreated control. 18 of these miRNAs showed differential regulation among different treatments. Further the microarray data was validated using real time PCR. The target genes of a few of these miRNAs showed inverse transcript accumulation. The possible role of miR395 and miR398 in antagonistic effect on adverse response of As in the presence of Se in rice seedlings is discussed.

Keywords: Arsenic, Heavy metal stress, microarray, miRNA, Rice, Selenium

Graphical abstract



INTRODUCTION

Arsenic (As) is a non-essential metalloid in the environment and potentially toxic to plants, animals and human beings.[1,2] Mainly two forms of inorganic As exist in nature, such as arsenate (AsV) and arsenite (AsIII). Under aerobic conditions Arsenate, As (V) is a stable and competes with phosphate, while As (III) is the dominant form in an anaerobic condition which reacts with –SH groups of the enzyme and inhibits several cellular processes.[3,4] The cellular toxicity caused by As on the physiological and biochemical processes in plants, involving damage of chloroplast membrane,[5] stimulation of free radicals and formation of reactive oxygen species,[6-8] peroxidation of membrane lipid,[9] and cross-linking with thiol compound.[10] The potential of As tolerance, based on response of the various pathways involved in tolerance and detoxification processes depends also on the early perception of As induced stress. To know about the molecular mechanism of response to As stress in plants it is very important to understand the pathways that play role to counteracting As stress and to identify the genes responsible in toxicity and tolerance. Selenium (Se), on the other hand is an essential micronutrient for humans and animals,[11] mainly due to its antioxidative properties and role in hormonal balance. Essentiality of Se in plants remains controversial, although its role has been considered to be beneficial in plants capable of accumulating large amount of the element.[12] Depending on the concentration, the effect of Se in plant changes from beneficial to toxic.[11] Selenium at low concentration can enhance the growth in non-accumulating plants by acting as an antioxidant to increase enzyme glutathione peroxidase (GSH-Px) activity and decrease lipid peroxidation.[13] For instance enhanced antioxidative ability was observed in white clover (*Trifolium repens* L.) shoot with Se concentration lower than 200μg Kg^{-1}. Selenium is found to promote the growth and resistance in plants under

certain abiotic stresses such as drought,[14,15] salinity,[16] chilling,[17] UV radiations[18] and metals.[19-21] Moreover, wheat grown in seleniferous soil accumulate Se-Met, one of the main dietary sources for Se.[22]

Arsenic and selenium are both metalloids with similar chemical properties, coexist in contaminated soil and may have antagonistic or synergistic effect on plants. Therefore, biological interactions between As and Se depend on their respective chemical forms. In prokaryotes, both show similar roles in metabolic function such as assimilation, methylation and detoxification.[23] Oxidative damage caused by As in plants is reduced with Se application. Recently, Malik et al.[21] reported that As (10 μM) supplemented with Se (5 μM) showed improved growth by causing less damage to membrane, chlorophyll and cellular viability induced by arsenic. However, contrasting observations on As-Se interactions in higher plants is also reported, for example, application of Se increased the toxicity of As in *Thunbergia alata*,[24] while in barley As application increased the uptake of Se.[25] Hu et al.[26] also reported that presence of Se restricts the translocation of As in rice roots. It shows that variations might exist among higher plants under the influence of these metals. Hence, the present study may shed light for better understanding of the molecular mechanisms of plant response to As-Se stress. The molecular mechanisms in response to heavy metals/mettaloids involved in displaying the observed effects are still elusive. At the transcriptional level, microRNAs (miRNAs) have emerged as the key factors in transcriptional regulation and are involved in a variety of processes from plant development to various biotic and abiotic stresses. miRNAs are 21 nucleotide single stranded noncoding RNA molecules, extensively involved in regulation of gene expression.[27,28] The heavy metals pose much concern when they affect the major staple crop plants during production. Studies on heavy metal toxicity of crop plants,

especially rice are focused on variety of heavy metals. Previous studies showed that miRNAs implicate in essential physiological, developmental and signalling processes.[29,30] Evidence suggested that miRNAs in plants responded to heavy metal stress.[31,32] Huang et al.[33] identified 13 miRNAs involved in sulphate deficiency in *Brassica napus* under Cd stress. Through PCR based analysis, Zhou et al.[34] reported response of miR393 and miR171 in *Medicago truncatula* under heavy metal stress. Additionally, 19 potential novel miRNA responsive to Cd were identified by using conventional sequencing approaches.[35] Recently, high-throughput gene expression profiles with microarray technology, and their application in comparative studies, helped in revealing the role of different gene regulation caused due to metal toxicity and tolerance. Ding et al.[31] reported that miR166, miR171, miR390, miR156 and miR168 responded to Cd stress in rice and miR396, miR397, miR398, miR408 were related to Cd exposure in *Brassica*.[36] Furthermore, Yu et al.[37] identified 396 new As (III) responsive miRNAs, out of which 14 were involved in regulating gene expression in transcriptional signalling and metabolism in rice seedlings. Sharma et al.[38] using a miRNA microarray identified several differentially regulated miRNA in contrasting As accumulating rice varieties exposed to As (III) and As (V).

Arsenite [As (III)] and selenate [Se (VI)] were selected as metalloid in the present study. Although, As (III) and selenite (Se IV) are the dominant forms in rice grown paddy fields, Se (VI) was preferred over Se (IV) mainly for two reasons, first inorganic forms of Se differ in terms of absorption and mobility within plants. Inside the plant, Se (VI) is more easily transported to shoots, while Se (IV) tends to accumulate in plant roots causing reduction in nutrient elements in rice grains.[26] This indicates that Se (VI) might help in mitigating As contamination and improving Se nutrition in rice. Benefits of Se (VI) over Se (IV) was reported in rice[26] and lettuce

plants.[39] Secondly, As (III) is transported into rice by aquaporins, such as Si transport, hence competition between As (III) and Se (IV) uptake is possible through the transporters.[3] On the other hand, Se (VI) helps in mitigation of As by protective mechanisms as an antioxidant.[26] Experiments have also been carried out previously with the combination of As (III) and Se (VI) in *Chlorophytum comosum*[40] and in cereal grains, wheat and rice using NanoSIMS analysis.[41]

Rice (*Oryza sativa* L.), one of the major staple crops worldwide, accumulates more As because of its higher bioavailability in the flooded paddy soil. For this reason, rice is the major source of inorganic As in a rice-based diet.[42] Several rice miRNAs have been identified that play role in response to abiotic stress[43] however, comprehensive work involving morphology, physiology and transcriptional studies including the role of miRNAs in response to As and its interaction with other metals are still lacking. Therefore, we investigated the effect of As, Se and their combinatorial effect on model crop plant rice. Various physiological and biochemical variables were analysed followed by miRNA transcript profiling of rice seedlings. In this study 46 As-Se responsive miRNAs were identified out of which 8 were further validated by using real time PCR. Identification of As-Se responsive miRNA and their targets could provide more insights into understanding the molecular mechanism of response to these metal induced toxicity or tolerance in plants.

EXPERIMENTAL

Plant material and growth condition

Rice seeds (*Oryza sativa* L. var. IR64) were obtained from Indian Agriculture Research Institute, Pusa, New Delhi. Seeds were surface sterilized with 70% (v/v) ethanol for 15 min followed by thoroughly washing twice with double distilled water.

Equal number of seeds (25) were allowed to germinate on moist cotton bed in dark and watered with 5% Hoagland nutrient solution for 2 days. Seedlings were removed from dark and divided into four groups, group I to IV. Three groups were treated with metal salts ($NaAsO_2$, Na_2SeO_4) using different combinations, C-control (without metal), As(III)-150µM, Se (20µM), As(III)+Se (150+20µM). Seedlings were transferred to light (a 16h photoperiod) with day/night temperature of 25 ± 2^0C for 12 days in a controlled environmental growth chamber with 70% relative humidity. All nutrient solutions were changed twice per week. After harvesting, each plant was separated into leaves and roots, washed thoroughly with distilled water, frozen in liquid nitrogen and stored in -80^0C for further analysis. Plants not subjected to any of these treatments served as the experimental control.

Analysis of physiological and biochemical variables

Twelve days old treated and untreated plants were measured for their shoot length (SL) and root length (RL) using meter scale. Seed germination test was performed in seven day old seedlings.

Total Chlorophyll content was estimated following the method of Arnon.[44] Protein estimation was carried out following Bradford[45] using bovine serum albumin (BSA) as a standard.

Malondialdehyde (MDA) content was estimated following Heath and Packer[46] by reaction with thiobarbituric acid (TBA). Level of proline was measured following Bates.[47] Method of Gaitonde[48] was followed for the estimation of cysteine.

RNA isolation

Total RNA from each sample was isolated using Tri reagent (Sigma). Briefly, 100 mg of tissue was ground in liquid nitrogen followed by chloroform extraction and isopropanol precipitation of total RNA. For small RNA enrichment, isopropanol precipitation was carried out in -20˚C for overnight followed by two 70% ethanol washes before dissolving in DEPC treated ddH_2O. DNaseI treatment was performed before proceeding to cDNA synthesis.

MicroRNA expression profiling by microarray

The small RNA enriched total RNA was used to perform miRNA microarray analysis. The isolated RNA was assessed for quality and integrity using Bioanalyzer (Agilent 2100). Poly-A tailing and biotinilysation was performed using Flashtag HSR biotin labelling kit (Affymetrix) according to manufacturer's instructions. Biotin labelled RNA was then hybridized on Gene chip^R miRNA 3.0 array (Affymetrix) in hybridization oven followed by washing and staining according to manufacturer's protocols. The slides were scanned for fluorescence using GenePix 4000B Scanner to obtain CEL files, which were later normalised using Expression console software and analysed using Gene Spring 12.0. Fold change cut-off of ≥1.5 was applied and the resulting differential expression was considered significant.

Reverse transcription and Real Time PCR

Oligo $(dT)_{18}$ primed first strand cDNA was synthesised using Superscript ^{TM} Double-stranded cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. Briefly, 2µg of DNase free total RNA was incubated with 5x buffer, oligo $(dT)_{18}$ primer, dNTPs and reverse transcriptase at 42˚C for 60 min followed by 85 ˚C for 5 min to inactivate the reverse transcriptase. For miRNAs, stem-loop primers for selected mature miRNAs were designed as described by Ding et al.[31] Reverse

transcription using total RNA was carried out in 20µl reaction in presence of specific stem-loop primers for individual miRNAs. The reaction mix was incubated in a thermocycler for 30 min at 16 ˚C, followed by pulsed reversed transcription of 60 cycles at 30 ˚C for 30 s, 42 ˚C for 30 s and 50 ˚C for 1s then following incubation at 85 ˚C for 5 min to inactivate the reverse transcriptase. Actin was used as the internal control.

Real-time PCR was carried out on ViiA7 platform (Applied Biosystems) using Power SYBR® Green PCR Master Mix following 40 cycles of denaturation, annealing and extension. Each sample was analyzed in triplicates and calculations were performed using $^{\Delta\Delta}$CT method[49].

Target gene prediction for heavy metal-induced miRNAs

The target transcripts of heavy metal induced miRNAs were predicted using psRNA Target server (http://plantgrn.noble.org/psRNATarget/) using default parameters for *Oryza sativa* transcript database. User-submitted small RNAs with preloaded transcripts option was chosen for prediction of targets for miRNAs showing differential expression under heavy metal stress. Among the list of predicted targets, a single target having lower expectation values and biologically relevant ones were chosen for each miRNA.

MicroRNA promoter selection and *cis*-acting element analysis

Pre-miRNA sequences of osa-miRNAs were downloaded from miRBase, miRBase Release21.0 (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=osa). Further, the promoters of all the rice miRNAs were obtained from PMRD database. These obtained sequences were then analyzed by using Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[50] a database for the *in*

silico analysis of promoter sequences and analysis of plant *cis*-acting regulatory elements.

RESULTS AND DISCUSSION

Physiological and biochemical evidences for As and Se interaction on rice seedling growth

To study the response of rice seedlings to As and Se, plants were grown in Hoagland media supplemented with either As (150 μM), Se (20 μM) or both in combinations. Seven and twelve days old rice seedlings were used for estimating seed germination, shoot length (SL) and root length (RL), respectively, grown under the above mentioned treatments. Arsenic treatment caused a negative effect on seed germination displaying only 72% of the germinated seeds, while As in combination with Se showed 82% germination as compared to their control depicting the antagonistic effect of Se towards As (Table S1, ESI). Further, seed germination was 98% upon Se treatment which was similar to that of control suggesting that Se alone had no effect on seed germination. Shoot length (SL) was similarly affected by As treatment displaying inhibition in growth (46%) which was rescued in the presence of Se (28%) over the control value. Root growth inhibition was observed in presence of either As (60%), Se (12%) or in their combination (35%) as compared to control, while the effect of As alone was more followed by As+Se and Se alone (Figure 1A, Table S1, ESI).

Estimation of chlorophyll and total protein content were determined to reveal the physiological status of the plants upon treatment with As and Se. Chlorophyll content decreased under As exposure and addition of Se did not show much improvement in the chlorophyll content. However, Se alone showed 74% increase as

compared to only As treatment (Figure 1B). Total protein content in both shoot (38%) and root (36%) was inhibited by As treatment while Se in combination rescued the effect of As. Interestingly, it was observed that the presence of only Se also decreased the total protein content in root (25%) over the control values (Figure 1C). These observations point out to the fact that Se acts antagonistically to As stress in maintaining the normal physiology and morphology of the plant. Inhibitory effect due to As and enhancement in the morphology of plant in the presence of Se, is in agreement with the previous study on mungbean seedlings.[51]

It is reported that As toxicity is mediated mainly by the oxidative stress and the damage caused due to As was lowered with Se application, which could be related to elevated levels of stress indicators and modulators.[51-53] The accumulation of MDA (stress indicator) and proline and cysteine (stress modulators) were similarly affected under different treatments. The degradation of poly-unsaturated lipids by reactive oxygen species gives rise to Malondialdehyde (MDA) content. The estimation of MDA indirectly reflects the level of oxidative stress in the plant. At least two fold increase in MDA content was observed in As treated shoots which was only little relieved in combination with Se (Figure 1D). Only 10% increase in MDA levels were observed in root length with Se alone treatment. These results clearly suggest that As can induce oxidative stress in the plant and Se to some extent can alleviate the toxic effects of As. Previous study on rice also indicated significant impact on MDA level under As stress,[54] which could result in altered membrane permeability and, consequently, increased ion leakage.

The increase of proline and cysteine in As treated leaves and roots were significant but the synergistic effect with Se decreased their accumulation considerably (Figure 1E, F). The additive effect of As+Se increased proline and

cysteine content significantly, 54% & 101% in leaves and 92% & 95% in roots, respectively, over the control seedlings. Cysteine, a thiol containing amino acid, is known to play a role in antioxidant defence and detoxify excess metal ions through GSH-PCs synthesis. Increase in cysteine content may be attributed to the involvement of sulphur assimilation pathway or stimulation of sulphate transporters involved in glutathione and PC. In case of proline, which acts as radical scavenger and cellular redox potential buffer, co-application of Se and As has more prominent effects than their individual applications. Our findings are in concordance with the observations reported previously under single or mixed metal conditions.[51-53] The overall results showed that the application of Se effectively improved the growth of seedlings as compared to the individual application of As. Improvement in the level of stress indicators and modulators shows that Se acts antagonistically to As adverse effect on rice plant.

Expression profiling of miRNAs in response to As, Se and As+Se stress in rice seedlings

After establishing that Se antagonistically affects the adverse effect of As on rice seedlings, the effect of these two metals was studied, independently as well as in combinations on miRNA profiling. miRNA microarray analysis was performed using GeneChip® miRNA 3.0 arrays. Small RNA enriched total RNA was poly-A tailed and biotinylated as described in methods section and hybridized on miRNA 3.0 array chips. After hybridization, the chips were scanned and the CEL files obtained were normalized using Expression console and analyzed using GeneSpring 12.1 software. Fourty-four miRNAs were found to be differentially regulated in the treatments with respect to control with the $\geq$ 1.5 fold change cut-off (Figure 2A). Among them 26 miRNAs displayed the same pattern of regulation in all the different treatments (As,

Se and in combination) as compared to control, whereas 18 miRNAs displayed differential transcript accumulation between As and Se treated samples (Table S2, ESI). The pattern of regulation for all the miRNAs remained similar across the As and Se treated samples. The miRNAs were oppositely regulated in As and Se samples whereas the treatment of As+Se resulted in the similar regulation as As itself. For example, almost all members of miR395 family were upregulated in arsenic and As+Se treatments but downregulated in Se treatment. Also, miR399d was downregulated in As and As+Se treatments but upregulated in Se treatment. The data indicate that Se when present in combination with As had very little or no effect on miRNA levels.

The construction of Venn-diagrams revealed that two miRNAs were common in the up-regulated category in all the expression ratios (As vs control (As/C), Se vs control (Se/C), As+Se vs control (As+Se/C), whereas fifteen miRNA was common in As/C and As+Se/C. In the down-regulated category, twenty five miRNAs were common in all the expression ratios (Figure 2B). This observation suggests that As and Se independently give rise to the up-regulation of different miRNAs. However, when the up and down regulation of miRNA were compared among the different treatments a different picture of miRNA regulation was revealed. For example 25 miRNA was found to be upregulated and 17 down regulated when control samples were compared with As treated samples. While 23 and 26 miRNA were upregulated when Se and As+Se samples were compared with As treated samples, respectively, while 19 and 16 miRNAs were down regulated when the same comparison was made (Table S2, ESI). The other details of number of miRNAs getting up and down regulated when comparisons are made among different treatments are mentioned in Table S2 (ESI). The details of miRNA involved in these regulation are given in Table

S3 (ESI) (all treatments versus Control), Table S4 (ESI) (all treatments versus As treatment), Table S5 (ESI) (all treatments versus Se treatment), and Table S6 (ESI) (all treatments versus As+Se treatment). The numbers of miRNA getting up- or down-regulated when different treatments were compared among each other are represented in Venn diagram in Figures S1, S2 and S3 (ESI).

Expression patterns of heavy metal responsive miRNAs obtained by microarray analysis were validated using stem-loop RT-PCR analysis. The expression patterns obtained by stem-loop RT-PCR were very much in accordance with the microarray results. The miRNAs like miR159, miR171, miR396, miR398, miR399 and miR415 displayed downregulation in As treated plants whereas their levels were restored when As was treated in combination with Se (Figure 3). However, it was found that the expression of a few miRNAs like miR811, miR812, miR3980, and miR5082 was slightly deviated from microarray results only in the samples that included Se (Figure 3). Recent studies have identified a set of miRNAs from *B. napus*,[33,36,55,56] rice,[31,35] *Medicago truncatula*,[34,57] Arabidopsis[58] and found to be regulated by different heavy metals. However, only a few reports are available under As stress.[38,59,60]

In the previous study, Liu and Zhang[60] analysed the miRNA expression pattern and identified 67 As(III)-responsive miRNAs belonging to 26 miRNA families from rice and found a total of 54 and 13 miRNAs to be significantly down and up-regulated, respectively. Among these, miR159, miR171, miR396, miR399, miR812, and miR815 are also identified to be As responsive in the present study. Recently, Sharma et al.[38] identified 114 As(III)-responsive miRNAs belonging to 30 miRNA families from High As accumulating Rice Germplasm (HARG) rice cultivars and 166 As(III)-responsive miRNAs belonging to 62 miRNA families from Low As

accumulating Rice Germplasm (LARG) rice cultivars. Out of these, expression of seven [miR396, miR399, miR408, miR528, miR1861, miR2102, miR2907] miRNAs families were up regulated. In addition, members of the miR164, miR171, miR395, miR529, miR820, miR1432 and miR1846 were down-regulated. In the present study, a similar pattern was observed in miR396, miR399, miR171, miR395, and miR1846 families under As stress, whereas remaining members of these miRNA families showed variable expression pattern. However, we have identified a set of additional As-stress responsive miRNAs. Several As-responsive miRNAs families are common in two plant species and expression pattern of their individual members is different. The effect of As stress in miRNA regulation is also reported in *Brassica juncea* by Srivastava et al.[61] However, the regulation of miRNA by combatorial effect of two elements are rather absent in the literature. The findings of the present work on regulation of miRNA by As and Se, independently and in combinations gives a new insight in the complex regulatory mechanism by miRNA under metal induced stress.

In this study, miRNA microarray data revealed identification of 46 metal (As, Se and As+Se) responsive miRNAs in rice. For As treatment, 150 μM concentration was chosen, as it caused damage to rice without resulting in death, while, 20 μM Se was considered non-toxic for the plant. Members of miR395 and miR1433 showed up-regulation under As stress (Table S3, ESI). Unlike, As stress, Se treatment individually showed upregulation of three miRNAs, miR171, miR399 and miR1433. However, up and downregulated miRNAs were same in As+Se treatment when compared with only As induced miRNAs. There is no report available about the role of miRNA under Se stress in plants. However, induction of miR171 (involved in hormone signalling and developmental processes) and miR399 family has been reported in *Medicago truncatula* under Hg stress.[57] Targets of these miRNA are

detailed in Table 1. Presented results showed better response in plant growth morphology and stress modulator parameters under the treatment of Se alone as compared to As or As+Se treatments. These results are in accordance of the previous results observed in rice variety PB1 under As, Se and As+Se treatment.[62] In addition, it was observed that the physiological response of metal treatments can be partly due to the transcriptional reprogramming induced by the toxicity of metals. MicroRNA expression profiling of all the metal treated seedlings provided better insights into the overall effect imposed by the metals on rice seedlings. The miR395 family is upregulated upon As and As+Se treatment whereas downregulated upon Se treatment. Targets of miR395 involves mainly two families of sulphate assimilation pathway, namely ATP sulfurylase (APS) and sulphate transporter (SULTR2;1).[63] Zhang et al.[56] observed transgenic rapeseed (*Brassica napus*) overexpressing miR395 under Cd stress and further correlated with the growth response. Results showed higher expression of miR395 along with higher content of chlorophyll, glutathione and non-protein thiols in the transformants than the wild type. Similarly, in the present study plants showed better growth response, role of stress indicators, modulators and induction of miR395 under As+Se treatment over to As or Se treatment alone. These data indicate that As induced miR395 might regulate the sulphate assimilation pathway thereby indirectly regulating glutathione and phytochelatin biosynthesis, which has a role in complexation of metal ions as a defense strategy against metal stress. Furthermore, this observation indicates that miR395 family is responsive to both As and Se and that As and Se impart different responses in the plant even at transcriptional level. Clearly, the differential accumulation of miR395 family is not because of oxidative stress, as both As and to a little extent Se induced oxidative stress observed from physiological and biochemical variables. Other miRNAs like

miR159, miR171, miR396, miR398, miR399, miR415, miR811, miR812, miR815, miR821, miR1875, miR3980, miR5076 and miR5082 were all downregulated in all the treatments studied. But, a closer analysis revealed that the effect of As and Se were antagonistic on the levels of miRNAs as revealed by their fold change (FC) values (Table S3, ESI). Expression of miR159 is always repressed under the metal exposure, for instance, miR159a is downregulated in both As and Se treatment but its fold change values are -0.841 and -0.326 respectively.

Target gene prediction and validation

The differential transcript accumulation of miRNAs due to As, Se and their combination would lead to the transcript variation of their respective target genes. To identify the putative target genes of the differentially accumulated miRNAs, psRNA target server was used. The details of the miRNAs, target genes and their functions are listed in Table 1. Among several predicted target genes, only the ones with low E-score were considered and proceeded for further investigations.

In principle, the miRNAs and their respective targets display inverse transcript co-relations. Considering this fact, the inverse transcript co-relations were analyzed for the miRNA:target pairs using qRT-PCR. The expression levels of the miRNAs and their corresponding transcripts were analysed in all the four samples – control, As, Se and As+Se treated rice seedlings. Interestingly, five out of eight miRNAs – miR159, miR171, miR395, miR398 and miR415 displayed inverse transcript correlation with their predicted target genes indicating the validity of the regulation involved (Figure 4). Correlation coefficients calculated for the expression patterns of all the miRNA:target pairs were negative for four of the miRNA:target pairs, miR159:MYB, miR395:CYTB, miR398:ZSD and miR415:RP further establishing the biological relevance of their regulation (Table 1). The relevance of the results

obtained can be very well seen in miR398:ZSD target pair analysis. The expression of copper/zinc superoxide dismutase is very high in As treated samples, which is known to function in antioxidant defense and might be the reason for down regulation of miR398. Our analysis establishes the effect and relevance of As, Se and their combination on rice seedlings, however further work is necessary to reveal the underlying regulatory mechanisms of miRNA by combinatorial effect of As and Se.

Identification of the metal stress-responsive *cis*-elements in the miRNA promoters

To get an insight into the regulation of selected miRNAs, the potential stress responsive *cis*-acting elements in the metal responsive miRNA promoters were searched by PlantCARE.[50] First, the promoters of the selected eight rice miRNAs were analyzed. Furthermore, our extensive motif analysis of these putative promoters identified many *cis*-elements that were essential for the initiation of gene transcription and might play role in transcriptional regulation of gene expression in response to heavy metals. *Cis*-acting elements in the promoters of the eight validated metal stress responsive miRNAs included: ARE (anaerobic-responsive element); ABRE (ABA-responsive element); GARE (gibberellins-responsive element); HSE (heat stress-responsive element); O_2 site (oxidative stress-responsive element), etc. (Table S7, ESI). All eight validated miRNAs had AREs in their promoter region, which responded to low oxygen stress, low temperature, dehydration stress, and submergence conditions.[52,53] TATA box and CAAT elements were distributed more than fifteen regions in promoter of all miRNAs. The presence of heat stress responsive element (HSE, miR159, miR171, miR396 and miR398) and abscisic acid (ABA) responsive elements (ABRE, miR159, miR399 and miR415) indicates their stress responsive expression to temperature, drought and high salt. Also, no metal-

responsive elements (MREs) were found in the *cis*-acting elements of the miRNAs which indicates that the differential expression observed upon As treatment is due to the primary effects (ROS burst, peroxidation of membrane lipids and etc.) and secondary effects (temperature, hypoxia, dehydration etc.). The finding of these stress-responsive *cis*-elements clearly suggested that these miRNAs might play diverse functions in oxidative stress, growth and developmental processes, environmental signals under heavy metal stress.

Conclusions

MicroRNA has emerged as major player regulating variety of biological processes in living organisms. There are several reports of miRNA playing role under abiotic and biotic stress including heavy metal stresses. In the present work, an attempt has been made to study the regulation of miRNA profile by a combination of As and Se in rice seedlings. The adverse effect of As on growth of rice plant is ameliorated in the presence of Se. Selenium inhibited the adverse effect of As when present together in seedling germination, growth and other related physiological and biochemical parameters. Analysis of miRNA profile indicated that some of the members are playing major role in this fine adjustment of Se and As together in rice system. Out of 46miRNA found to be differentially regulated in Se, As and As+Se treatment compared to control, 18 showed differential regulation among the treatments. miR395 exhibited up-regulation in the presence of As and As+Se while down regulation when only Se was present. While, there were other 25 miRNA genes that showed down regulation in all the treatments. These observations point towards distinct role of miR395 in the adverse effect of Se on As stress. The targets of the differentially regulated miRNA genes showed inverse correlation. The target of miR398, copper/zinc super oxide dismutase showed up-regulation while miR398

itself was down regulated suggested the importance of ROS scavenging enzymes during this As-Se interaction.

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Electronic Supporting Information:

Venn diagram showing the number of miRNA up- or down regulated of all treatments versus As treatment (Figure S1), versus Se treatment (Figure S2) and versus As+Se treatment (Figure S3). Germination percentage of seeds and root and shoot length under different treatments (Table S1), number of miRNA regulated by different treatments (Table S2), list of differentially regulated miRNAs of all treatments versus control (Table S3), versus As treatment(Table S4), versus Se treatment (Table S5) and versus As+Se treatment (Table S6), distribution pattern of *cis*-acting element in the promoter of the eight identified metal stress miRNAs (Table S7) and list of primer sequences used for qRT-PCR (Table S8).

REFERENCES

1. P. Tripathi, A. Mishra, S. Dwivedi, D. Chakrabarty, P. K. Trivedi, R. P. Singh and R. D. Tripathi, Differential response ofoxidative stress and thiol metabolism in contrasting rice genotypes for arsenic tolerance, *Ecotoxol. and Environ. Safety.,* 2012, **79,** 189-198.

2. M. A. Ahmad, R. Gaur and M. Gupta, Comparative biochemical and RAPD analysis in two varieties of rice (*Oryza sativa*) under arsenic stress by using various biomarkers, *J. Hazard. Mater.,* 2012, **217–218,** 141-148.
3. F.-J. Zhao, Y. Ago, N. Mitani, R.-Y. Li, Y.-H. Su, N. Yamaji, S.P. McGrath and J. F. Ma, The role of the rice aquaporin Lsi1 in arsenite efflux from roots, *New Phytol.,* 2010, **186,** 392-399.
4. S.-F. Fu, P.-Y. Chen, Q. T. T. Nguyen, L.-Y. Huang, G.-R. Zeng, T.-L. Huang, C.-Y. Lin and H.-J. Huang, Transcriptome profiling of genes and pathways associated with arsenic toxicity and tolerance in *Arabidopsis, BMC Plant Biol.,* 2014, **14,** 94.
5. N. Stoeva and T. Bineva, Oxidative changes and photosynthesis in oat plants grown in As-contaminated soil, *Bulg. J. Plant Physiol*., 2003, **29,** 87–95.
6. J. Hartley-Whitaker, G. Ainsworthand A. A. Meharg, Copper- and arsenate inducedoxidative stress in *Holcus lanatus* L. clones with differential sensitivity. *Plant Cell Environ.,* 2001, **24,** 713–722.
7. R. Requejo and M. Tena, Proteome analysis of maize roots reveals that oxidative stress is a main contributing factor to plant arsenic toxicity, *Phytochemistry,* 2005, **66,** 1519-1528.
8. S. Mallick, G. Sinam and S. Sinha, Study on arsenate tolerant and sensitive cultivars of *Zea mays* L. Differential detoxification mechanism and effect on nutrients status, *Ecotoxicol. Environ. Safety,* 2011, **74,** 1316-1324.
9. M. Moller, P. E. Jensen and A. Hansson, Oxidative modifications to cellular components in plants, *Annu. Rev. Plant Biol.,* 2007, **58,** 459-481.

10. K. T. Kitchin and K. Wallace, Dissociation of arsenite-peptidecomplexes: triphasic nature; rate constants, half-lives, and biological importance, *J. Biochem. Mol. Toxicol.,* 2006, **20,** 48–56.
11. M. Tamaoki, J. L. Freeman and E. A. H. Pilon-Smits, Cooperative Ethylene and Jasmonic Acid Signaling Regulates Selenite Resistance in Arabidopsis, *Plant Physiol.,* 2008, **146,** 1219–1230.
12. K. Shanker, Countering UV-B stress in plants: Does selenium have a role?, *Plant Soil,* 2006, **282,** 21-26.
13. M. Djanaguiraman, A. K. Shanker, J. A. Sheeba, D. D. Devi and U. Bangarusamy, Selenium-an antioxidative protectant in soybean during senescence, *PlantSoil,* 2005, **272,** 77-86.
14. V. V. Kuznetsov, V. P. Kholodova, V. V. Kuznetsov and B. A. Yagodin, Selenium regulates the water status of plants exposed to drought, *Dokl. Biolog. Sci.,* 2003, **390,** 266–268.
15. X. Q. Yao, J. Z. Chu and C. J. Ba, Antioxidant responses of wheat seedlings to exogenous selenium supply under enhanced ultraviolet-B, *Biol. Trace Elem. Res.,* 2010, **136,**96–105.
16. B. Hawrylak-Nowak, Beneficial effects of exogenous selenium in cucumber seedlings subjected to salt stress, *Biol. Trace Elem. Res.,* 2009, **132,** 259-269.
17. J. Chu, X. Yao and Z. Zhang, Responses of Wheat seedlings to exogenous selenium supply under cold stress, *Biol. Trace Elem. Res.,* 2010, **136,** 355–363.
18. X. Q. Yao, J. Z. Chu and G.Y. Wang, Effects of selenium on wheat seedlings under drought stress. *Biol. Trace Elem. Res.,* 2009, **130**, 283–290.

19. Z. Pedrero, Y. Madrid, H. Hartikainen and C. Camara, Protective effect of seleniumin broccoli (*Brassica oleracea*) plants subjected to cadmium exposure, *J. Agri Food Chem.,* 2008, **56,** 266–271.

20. P. Cartes, A. Jara, L. Pinilla, A. Rosas and M. Mora, Selenium improves the antioxidant ability against aluminium-induced oxidative stress in ryegrass roots, *Annals Appl. Biol.,* 2010, **156,** 297-307.

21. J.A. Malik, S. Kumar, P. Thakur, S. Sharma, N. Kaur, R. Kaur, D. Pathania, K. Bhandhari, N. Kaushal, K. Singh, A. Srivastava and H. Nayyar, Promotion of growth in mungbean (*Phaseolus aureus* Roxb.) by selenium is associated with stimulation of carbohydrate metabolism, *Biol. Trace Elem. Res.,* 2011, **143,** 530–539.

22. G. Lyons, I. Ortiz-Monasterio, J. Stangoulis and R. Graham, Selenium concentration in wheat grain: Is there sufficient genotypic variation to use in breeding?, *Plant Soil,* 2005, **269,** 369–380.

23. J. E. Stolz, P. Basu, J. M. Santini and R.S. Oremland, Arsenic and selenium in microbial metabolism, *Annu. Rev. Microbiol.,* 2006, **60,** 107-130.

24. K. Bluemlein, E. Klimm, A. Raab and J. Feldmann, Selenite enhances arsenate toxicity in *Thunb. alata*, *Environ. Chem.,* 2009, **6,** 486–494.

25. S. Ebbs and W. Leonard, Alteration of selenium transport and volatilization in barley (*Hordeum vulgare*) by arsenic, *J. Plant Physiol.,* 2001, **158,** 1231–1233.

26. Y. Hu, G. L. Duan, Y. Z. Huang, Y. X. Liu and G. X. Sun, Interactive effects of different inorganic As and Se species on their uptake and translocation by rice (*Oryza sativa* L.) seedlings, *Environ. Sci. Pollut. Res.,* 2013, **21,** 3955–3962.

27. R. Sunkar and J. K. Zhu, Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis, *Plant Cell,* 2004, **16,** 2001–2019.

28. S. Lu, Y. H. Sun, R. Shi, C. Clark, L. Li andV. L. Chiang, Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from Arabidopsis, *Plant Cell,* 2005, **17,** 2186–2203.

29. A. C. Mallory and H. Vaucheret, Functions of microRNAs and related small RNAs in plants, *Nat. Genet.,* 2006, **38,** 850.

30. R.-F. Virginia and O. Voinnet, Roles of Plant Small RNAs in Biotic Stress Responses, *Annu. Rev. Plant Biol.,* 2009, **60,** 485-510.

31. Y. Ding, Z. Chen and C. Zhu, Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*), *J. Exp. Bot.,* 2011, **62,** 3563–3573.

32. Y. Fang, K. Xie and L. Xiong, Conserved miR164-targeted NAC genes negatively regulate drought resistance in rice, *J. Exp. Bot.,* 2014, **65,** 2119–2135.

33. S. Q. Huang, A. L. Xiang, L. L. Che, S. Chen, H. Li, J. B. Song and Z. M. Yang, A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress, *Plant Biotech. J.,* 2010, **8,** 887–899.

34. Z. S. Zhou, S. Q. Huang and Z. M. Yang, Bioinformatic identification and expression analysis of new microRNAs from *Medicago truncatula*, *Biochem. Biophy. Res. Commu.,* 2008, **374,** 538–542.

35. S. Q. Huang, J. Peng, C. X. Qiu and Z. M. Yang, Heavy metal-regulated new microRNAs from rice, *J. Inorg. Bioch.,* 2009, **103,** 282–287.

36. Z. S. Zhou, J. B. Song and Z. M. Yang, Genome-wide identification of *Brassica napus* microRNAs and their targets in response to cadmium, *J. Exp. Bot.* 2012, **63,** 4597-4613.

37. L.-J. Yu, Y.-F. Luo, B. Liao, L.-J. Xie, L. Chen, S. Xiao, J.-T. Li, S.-N. Hu, and W.-S. Shu, Comparative transcriptome analysis of transporters, phytohormone and lipid metabolism pathways in response to arsenic stress in rice (*Oryza sativa*), *New Phytol.,* 2012, **195,** 97–112.

38. D. Sharma, M. Tiwari, D. Lakwani, R.D. Tripathi and P.K. Trivedi, Differential expression of microRNAs by arsenate and arsenite stress in natural accessions of rice, *Metallomics*, 2015, **7**, 174.

39. S. J. Ramos, V. Faquin, L. R. G. Guilherme, E. M. Castro, F. W. Ávila, G. S. Carvalho, C. E. A. Bastos and C. Oliveira, Selenium biofortification and antioxidant activity in lettuce plants fed with selenate and selenite, *Plant Soil Environ*., 2010, **56**, 584–588.

40. S. E. Afton, B. Catron and J. A. Caruso, Elucidating the selenium and arsenic metabolic pathways following exposure to the non-hyperaccumulating Chlorophytum comosum, spider plant, J. Exp. Bot., 2009, **60**, 1289–1297.

41. K. L. Moore, M. Schro¨der, E. Lombi, F.-J. Zhao, S. P. McGrath, M. J. Hawkesford, P. R. Shewry and C. R. M. Grovenor, NanoSIMS analysis of arsenic and selenium in cereal grain, *New Phytol*., 2010, **185**, 434–445.

42. P. N. Williams, A. Villada, A. Raab, J. Figuerola, A. J. Green, J. Feldmann andA. A.Meharg, Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared towheat and barley, *Environ. Sci. Techn.,* 2007, **41,** 6854–6859.

43. B. Zhao, L. Ruqiang, L. Ge, W. Li, H. Xiao, H. Lin, K. Ruan and Y. Jin, Identification of drought-induced microRNAs in rice, *Biochem. Biophy. Res. Commu.,* 2007, **354,** 585–590.

44. D. E. Arnon, Copper enzymes in isolated chloroplast, polyphenol oxidase in *Beta vulgaris*, *Plant Physiol.,* 1949, **24,** 1–15.

45. M. M. A. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analyt. Biochem.,* 1976, **72,** 248–254.

46. R. L. Heath and L. Packer, Photoperoxidation in isolated chloroplasts kinetics and stoichiometry of fatty acid peroxidation, *Arch. Biochem. Biophy.,* 1968, **125,** 189–198.

47. L. S. Bates, R. P. Waldren and I. D. Teare, Rapid determination of free proline for water stress studies, *Plant Soil,* 1973, **39,** 205–207.

48. M. K. A. Gaitonde, spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids, *Biochem. J.,* 1967, **l104,** 627-633.

49. K. J. Livak and T. D. Schmittgen, Analusis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ Method, *METHODS,* 2001, **25,** 402-408.

50. M. Lescot, P. Déhais, G. Thijs, K. Marchal, Y. Moreau, Y. V. D. Peer, P. Rouzé and S. Rombauts, PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *insilico* analysis of promoter sequences, Nucl. Acids Res., 2002, **30,** 325-327.

51. J. A. Malik, S. Goel, N. Kaur, S. Sharma, I. Singh and H. Nayyar, Selenium antagonises the toxic effects of arsenic on mungbean (*Phaseolus aureus*

Roxb.) plants by restricting its uptake and enhancing the antioxidative and detoxification mechanisms, *Environ. Exp. Bot.,* 2012, **77,** 242-248.

52. S. Mishra, A. B. Jha andR. S. Dubey, Arsenite treatment induces oxidative stress, up regulates antioxidant system, and causes phytochelatin synthesis in rice seedlings, *Protoplasma,* 2011, **248,** 565-577.
53. M. A. Ahmad and M. Gupta, Exposure of *Brassica juncea* (L.) to arsenic species in hydroponic medium: comparative analysis in accumulation and biochemical and transcriptional alterations, *Environ. Sci. Pollut. Res.,* 2013, **20,** 8141–8150.
54. M. Shri, S. Kumar, D. Chakrabarty, P. K. Trivedi, S. Mallick, P. Misra, D. Shukla, S. Mishra, S. Srivastava, R. D. Tripathi and R. Tuli, Effect of arsenic on growth, oxidative stress, and antioxidant system in rice seedlings, *Ecot. Environ. Safety,* 2009, **72,** 1102–1110.
55. F. L. Xie, S. Q. Huang, K. Guo, Y. Y. Zhu, L. Nie and Z. M. Yang, Computational identification of novel microRNAs and targets in *Brassica napus*, FEBS Lett., 2007, 581, 1464–1473.
56. L. W. Zhang, J. B. Song, X. X. Shu, Y. Zhang, Z. M. Yang, miR395 is involved in detoxification of cadmium in *Brassica napus, J. of Hazard. Mater.,* 2013, **250– 251**, 204– 211.
57. Z. S. ZHOU, H Q ZENG , Z P LIU and Z. M. YANG, Genome-wide identification of *Medicago truncatula* microRNAs and their targets reveals their differential regulation by heavy metal, Plant, Cell and Environ., 2012, **35**, 86–99

58. H. H. Liu, X. Tian, Y. J. Li, C. A. Wu and C. C. Zheng, Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*, *RNA*, 2008, **14**, 836–843.

59. R. Tuli , D. Chakrabarty, P. K. Trivedi and R. D. Tripathi, Recent advances in arsenic accumulation and metabolism in rice. Mol. Breeding., 2010, **26**, 307-323.

60. Q. Liu and H. Zhang, Molecular identification and analysis of arsenite stress-responsive miRNAs in rice, *J. Agri.Food Chem.,* 2012, **60,** 6524–6536.

61. S. Srivastava, A. K. Srivastava, P. Suprasanna and S. F. D'Souza, Identification and profiling of arsenic stress-induced microRNAs in *Brassica juncea, J. Exp. Bot.,* 2013, **63,** 695–709.

62. C. Pandey and M. Gupta, Selenium and auxin mitigates arsenic stress in rice (*Oryza sativa* L.) by combining the role of stress indicators, modulators and genotoxicity assay, *J. Hazard. Materl.,* 2015, **287**, 384-391.

63. Z. M. Yang and J. Chen, A potential role of microRNAs in plant response to metal toxicity, *Metallomics*, 2013, **5**, 1184-90.

Table 1: List of predicted target genes of differentially expressed miRNAs in arsenic, selenium and arsenic+selenium treatments along with the function of target genes and correlation coefficient of their regulation with miRNAs.

Sl. No.	MicroRNA	Target genes	Target functions	Correlation coefficients
1	osa-miR159a	MYB transcription factor	Synthesis of anthocyanins, primary and secondary metabolite response to biotic and abiotic stress	-0.045001191
2	osa-miR171a	GRAS domain transcription factors (SCARECROW-like)	Pyridoxin biosynthesis protein ER1, phase transition and floral meristem determination, glycosyl hydrolase	0.046446
3	osa-miR395a	Cytochrome b5-like heme/steroid binding domain containing protein	Environmental stress response, controls lateral roots formation, response to sulphate uptake and nutrition stress in plant	-0.891437887
4	osa-miR396e-3p	Growth-regulating factor 1	Cell division and differentiation during leaf development	0.953946
5	osa-miR398b	Copper/zinc superoxide dismutase	Antioxidant defense	-0.369504376
6	osa-miR399a	Ubiquitin conjugating enzyme protein	Phosphate-starvation conditions	0.346962
7	osa-miR415	40S ribosomal protein S10	Positive regulator of cell proliferation, profiling under	-0.775870021

			oxidative stress	
8	osa-miR1875	CDP-diacylglycerol-inositol 3-phosphatidyltransferase1, putative, expressed	Signal transducer, GPCR and tyrosine kinase activity.	0.603373

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Figure legends:

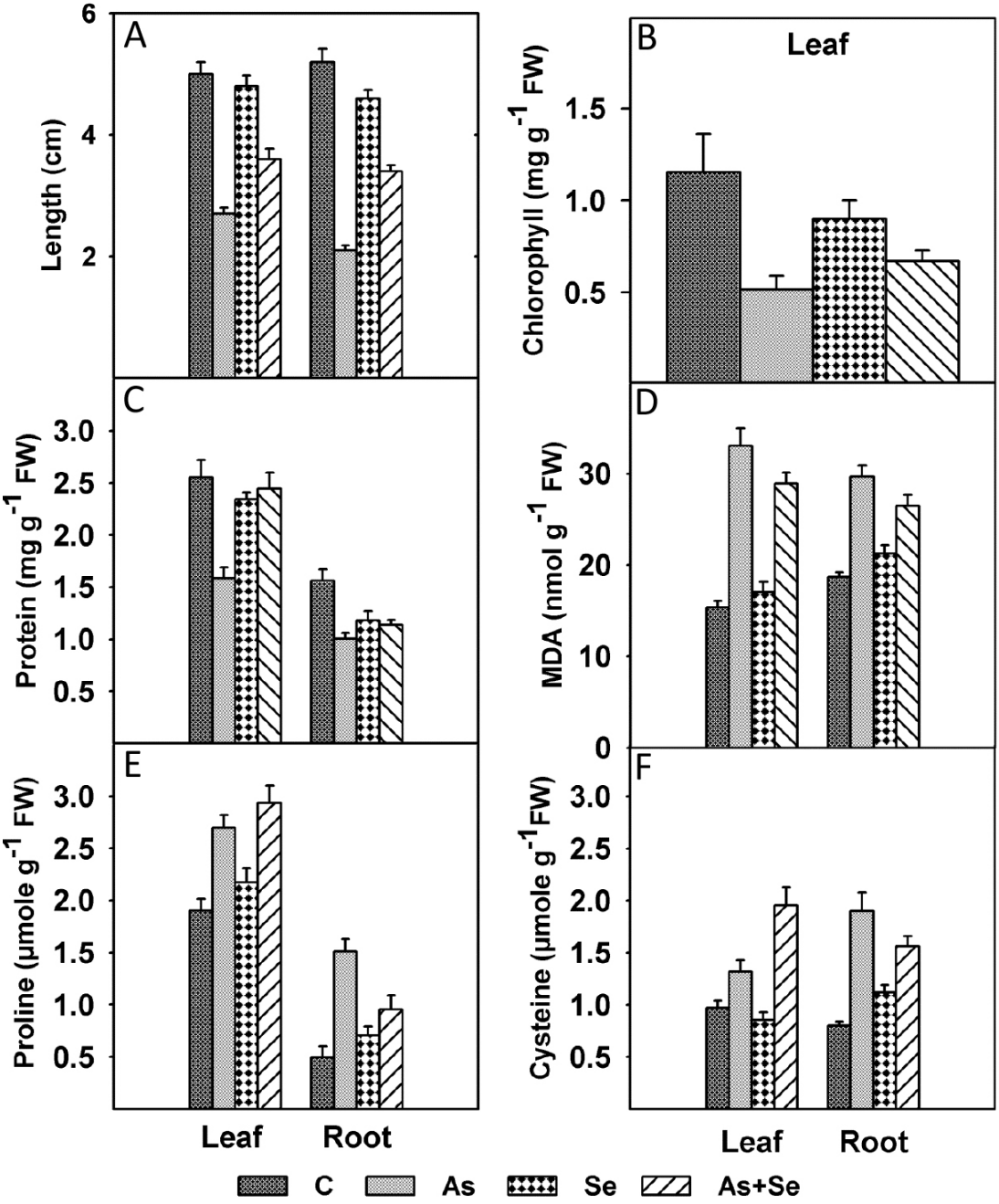
Fig. 1: Effect of arsenic and selenium on the plant morphology and stress indicator parameters. Rice seedlings growth is inhibited and display variations in (A) shoot and root length upon treatment with As, Se and As+Se. The metal treated seedlings accumulated different levels of (B) chlorophyll and (C) total protein. Oxidative stress signature components – (D) MDA, (E) proline and (F) cysteine also were differentially accumulated upon treatment with heavy metals. All the experiments were carried out in three biological replicates.

Fig.2: Differential expressions of miRNAs indicate their role in arsenic and selenium response. (A) Heat map of 46 differentially regulated miRNAs in arsenic, selenium and arsenic+selenium treated rice seedlings. Heat map was generated using signal intensities obtained after normalisation of the microarray data by Multi experiment Viewer (MeV). (B) Venn-diagrams representing the number of unique and common miRNAs up- and down- regulated in As, Se and As+As treated rice seedlings with respect to untreated control.

Fig.3: Validation of the expression levels of miRNAs obtained by microarray. Stem-loop qRT-PCR was used to validate the accumulation of few differentially regulated miRNAs in heavy metal treated rice seedlings. All qRT-PCR was carried out in three biological replicates with three technical replicate each. Rice actin gene was taken as internal control.

Fig.4: Inverse transcript correlation of the miRNAs and their predicted targets. qRT-PCR analysis of four miRNA:target pairs display inverse transcript correlation which suggest their biological significance. All qRT-PCR was carried out in three

biological replicates with three technical replicate each. Rice actin gene was taken as internal control.



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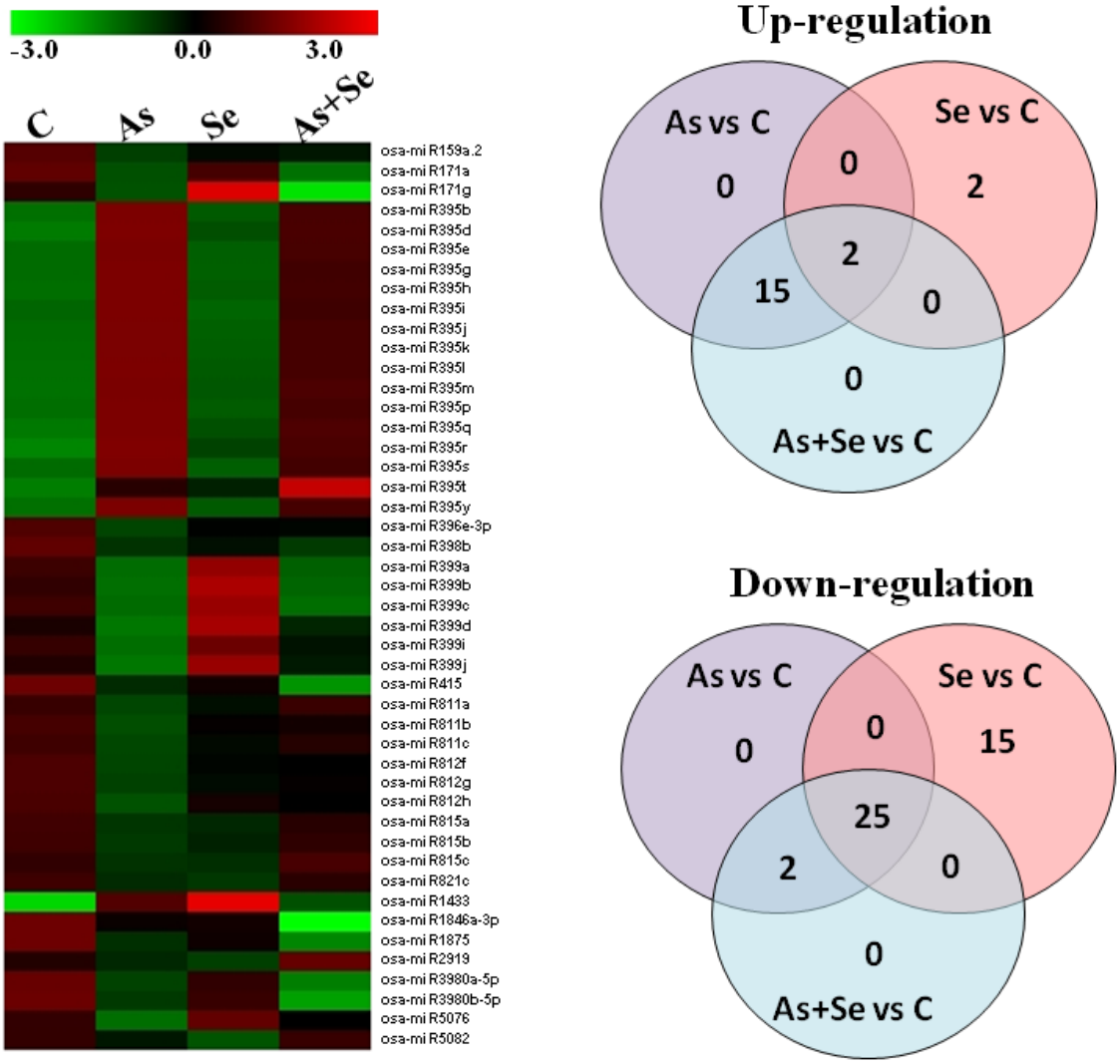

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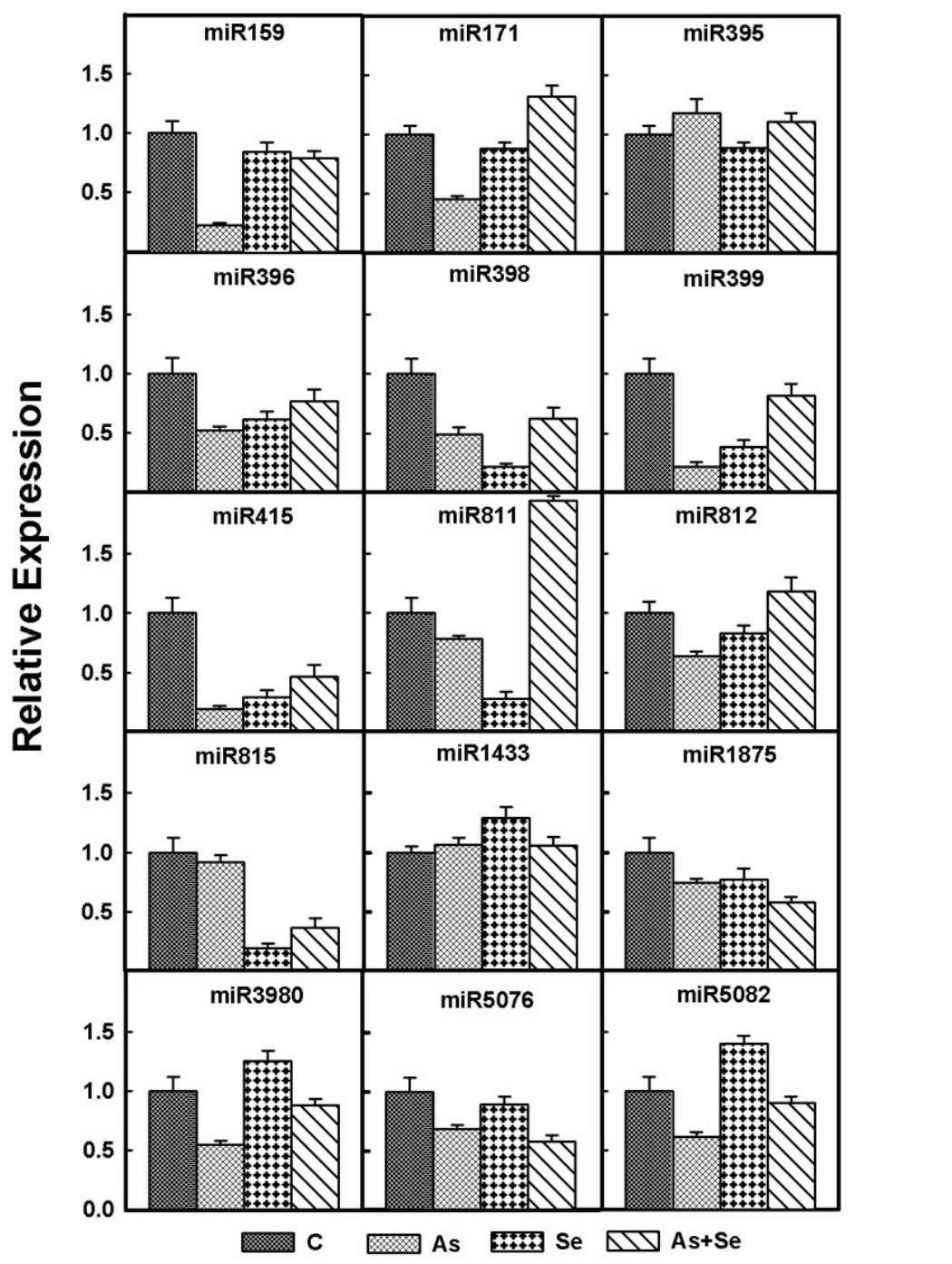

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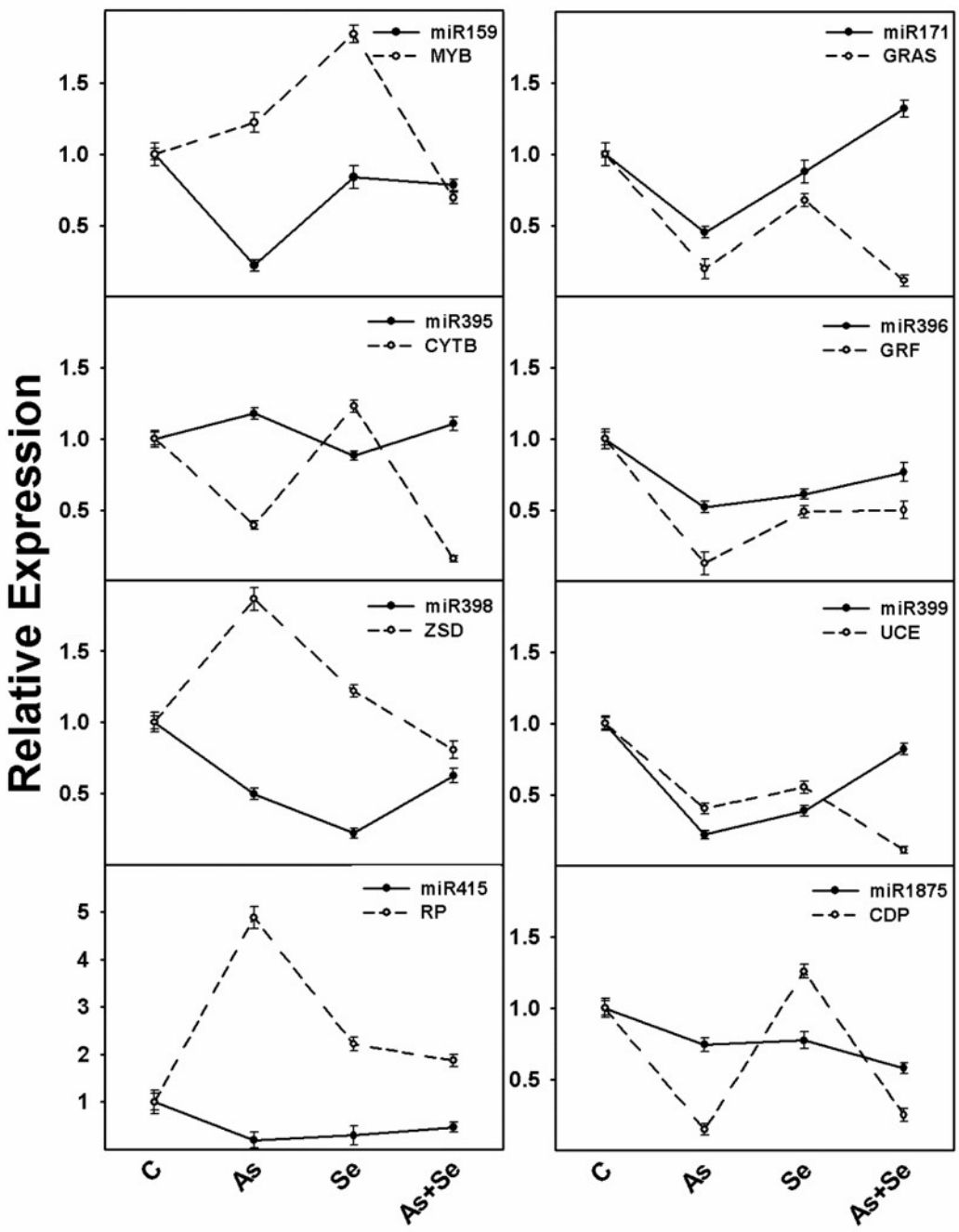

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