

# Differentially Expressed MicroRNAs Link Cellular Physiology to Phenotypic Changes in Rice Under Stress Conditions

Rumdeep K. Grewal<sup>1,2,3</sup>, Shradha Saraf<sup>1,3</sup>, Arindam Deb<sup>1</sup> and Sudip Kundu<sup>1,\*</sup>

<sup>1</sup>Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, Kolkata 700009, India

<sup>2</sup>Department of Botany, Bhairab Ganguly College, Kolkata 700056, India

<sup>3</sup>These authors contributed equally to this work

\*Corresponding author: E-mail, [skbmbg@caluniv.ac.in](mailto:skbmbg@caluniv.ac.in); Fax, 91-33-23519755.

(Received August 9, 2017; Revised July 9, 2018)

**Plant microRNAs (miRNAs) and their target genes have important functional roles in nutrition deficiency and stress response. However, the underlying mechanisms relating relative expression of miRNAs and target mRNAs to morphological adjustments are not well defined. By combining miRNA expression profiles, corresponding target genes and transcription factors that bind to computationally identified over-represented cis-regulatory elements (CREs) common in miRNAs and target gene promoters, we implement a strategy that identifies a set of differentially expressed regulatory interactions which, in turn, relate underlying cellular mechanisms to some of the phenotypic changes observed. Integration of experimentally reported individual interactions with identified regulatory interactions explains how (i) during mineral deficiency *osa-miR167* inhibits shoot growth but activates adventitious root growth by influencing free auxin content; (ii) during sulfur deficiency *osa-miR394* is involved in adventitious root growth inhibition, sulfur and iron homeostasis, and auxin-mediated regulation of sulfur homeostasis; (iii) *osa-miR399* contributes to cross-talk between cytokinin and phosphorus deficiency signaling; and (iv) a feed-forward loop involving the *osa-miR166*, trihelix and HD-ZIP III transcription factors may regulate leaf senescence during drought. This strategy not only identifies various regulatory interactions connecting phenotypic changes with cellular or molecular events triggered by stress, but also provides a framework to deepen our understanding of stress cellular physiology.**

**Keywords:** Abiotic stress • MicroRNA • Mineral deficiency • Next-generation sequencing • Regulatory cascade • Transcription factor.

**Abbreviations:** ARF, auxin response factor; CRE, *cis*-regulatory element; DCL1, Dicer-Like 1; DEM, differentially expressed miRNA; GEO, Gene Expression Omnibus; HYL1, HYPONASTIC LEAVES; miRNA, microRNA; NGS, next-generation sequencing; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; sRNA, small RNA.

## Introduction

Plants have evolved sophisticated strategies at morphological, biochemical and molecular levels to withstand stress

conditions. Numerous studies have shown that plant genes are regulated under stress conditions such as drought, salinity, cold, oxidative stress and mineral deficiency (Zhu 2002, Paul et al. 2015). Studies in *Arabidopsis thaliana* under stress have shown that stress-induced regulation operates at various levels, and regulatory molecules and small RNAs (sRNAs) are integral to this regulation (Sunkar et al. 2007). Indications of the importance of sRNAs in stress response came from in silico analysis of microRNAs (miRNAs) and their targets, and cloning of stress-induced miRNAs from *Arabidopsis* (Jones-Rhoades and Bartel 2004, Sunkar and Zhu 2004). Further investigations revealed that small regulatory RNAs play an important role in the post-transcriptional regulation of gene expression in all biosystems studied so far, including rice (He and Hannon 2004). Depending on their origin, structure, associated effector proteins and biological roles, small regulatory RNAs have been classified into several groups (Ketting 2011). miRNAs are associated with post-transcriptional regulation through cleavage and translation inhibition (Bartel 2004, Chen 2005). miRNAs are approximately 19–24 nucleotides in length, with a nucleotide overhang at the 3' end in the duplex conformation (Chen 2005). miRNA genes encode primary miRNAs (pri-miRNAs), containing a long sequence of several hundred nucleotides (Chen 2005). The pri-miRNAs are then converted to precursor miRNAs (pre-miRNAs) by RNase III-like enzymes i.e. Dicer-Like (DCL1), HYPONASTIC LEAVES (HYL1) and SERRATE (SE) proteins (Park et al. 2005). The pre-miRNA is converted into an miRNA duplex structure with methylation at the 3' terminus and is exported to the cytoplasm (Park et al. 2005). While entering the cytoplasm, one of the duplex strands of miRNA is directed to the exosome for degradation. The other strand (mature miRNA) is exposed to the RNA-induced silencing complex (RISC) and is incorporated into an ARGONAUTE (AGO) complex. It then binds to target transcripts on the basis of sequence complementarity, and the target transcript is cleaved by the AGO complex.

Plant miRNAs have been primarily associated with development, nutrient uptake and responses to biotic and abiotic stress (Jones-Rhoades and Bartel 2004, Sunkar and Zhu 2004). miRNA target genes may include transcription factors or functional enzymes having important roles in stress response. The role of miRNAs in the abiotic stress response has been shown in *Arabidopsis* using miRNA metabolism mutants. An *A. thaliana*

mutant with a defect in HYL1 has increased sensitivity to ABA (Lu and Fedoroff 2000), DCL1 and HUA ENHANCER 1 (HEN1) mutants are more susceptible to salt and osmotic stress (Zhang et al. 2008). Differential expression of miRNAs under various stress conditions such as cold, drought, salt, UV-B radiation, oxidative stress or mechanical stress (Sunkar and Zhu 2004, Sunkar et al. 2007, Jeong et al. 2011, Paul et al. 2015) is well documented. In Arabidopsis, Sunkar and Zhu (2004) found miR393 to be up-regulated by cold, dehydration, salinity and ABA treatments, while miR319c was induced by cold but not by the other treatments; miR389a, however, was down-regulated by all stress treatments applied. Previous studies have also reported that several miRNAs are associated with the uptake and transport of nutrient minerals in plants (Kehr 2013). In rice, miR827 is associated with phosphorus (P) mobilization in old leaves (Wang et al. 2012). The expression of miR398a has also been found to be modulated by P, carbon and nitrogen (N) limitation (Kuo and Chiou 2011).

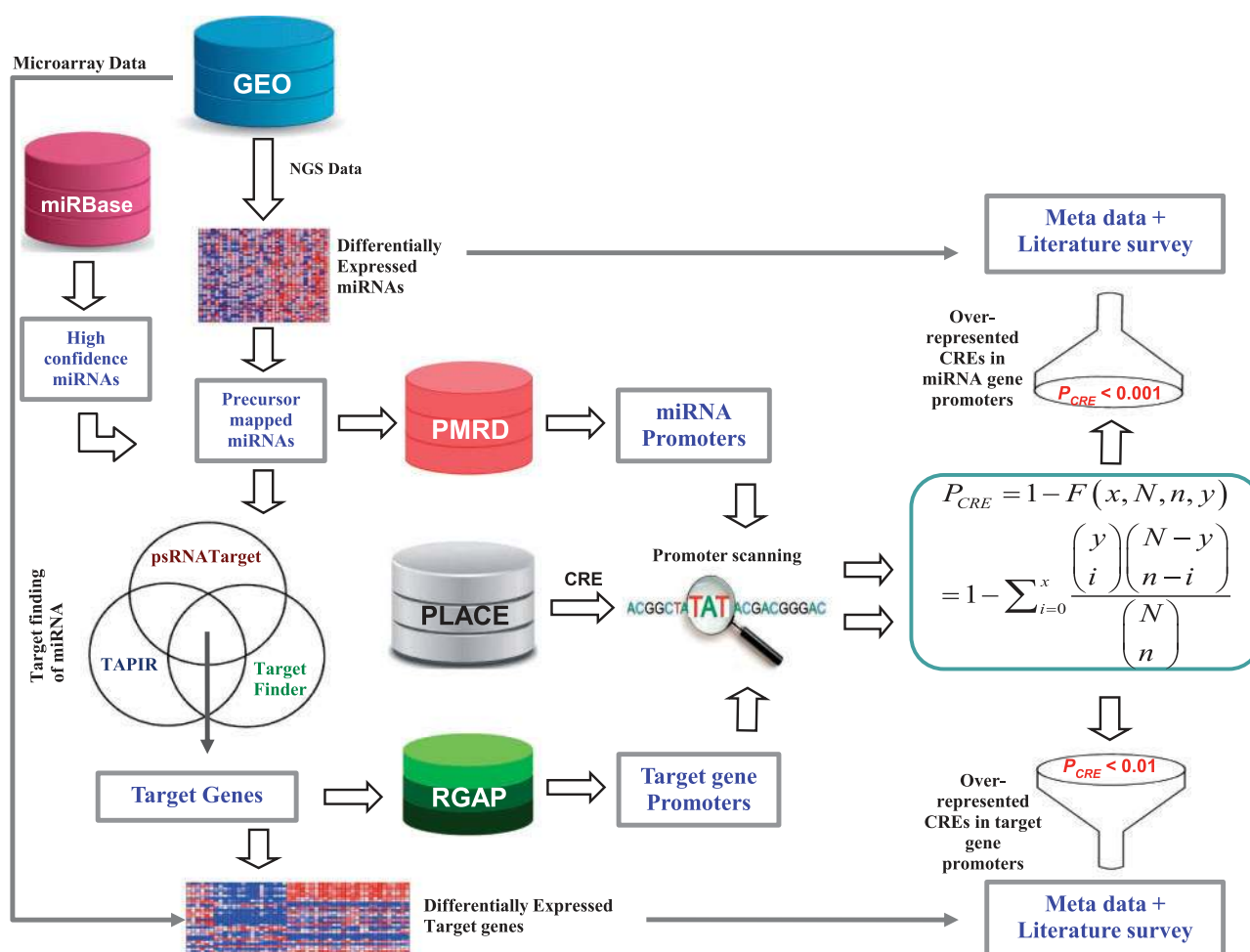
Differential expression of miRNAs indicates that miRNA genes themselves are regulated at the transcriptional level. A previous study has reported that the expression of miR399 is regulated by the MYB transcription factor PHR1 (Franco-Zorrilla et al. 2004). Transcriptional control of genes may be brought about by *cis*-regulatory elements (CREs), located in promoters of miRNA genes, and their corresponding transcription factors. Similar to promoters of mRNA-encoding genes, promoters of miRNA genes harbor multiple CREs. miRNA gene expression depends on binding of the corresponding transcription factors to CREs and the specific combinatorial logic among them. Transcription factor-mediated regulation may result in condition-specific miRNA accumulation. Several computational approaches aimed at understanding the CRE-mediated combinatorial regulation at the promoter regions of genes (Pilpel et al. 2001, Kato et al. 2004, Deb et al. 2016) are available. The existing computational approaches may be adapted to study possible CRE-mediated regulation of differentially expressed miRNA (DEM) genes, but only a few studies have attempted to do so (Meng et al. 2009, Zhao and Li 2013). The analysis of CRE elements in DEM promoters may be used to reveal the diverse set of causal factors that possibly regulate the expression of miRNAs.

In the present study, our aims were (i) to identify the DEMs and their differentially expressed target genes; (ii) to predict the over-represented CREs in promoters of both miRNAs and targets; and (iii) by integrating these results with previously documented molecular interactions, to relate these interactions with the observed phenotype under stress. To meet these goals, we analyzed miRNA expression data sets that included abiotic stress (cold, drought and salt) in panicles and seedlings, and mineral deficiency [potassium (K), P, N and sulfur (S)] in root and shoot tissues of *Oryza sativa*. Analysis of the expression profile of miRNAs, corresponding target genes and transcription factors that bind to over-represented CREs found to be common in miRNAs and target gene promoters (Fig. 1) allowed us to identify various regulatory interactions that may connect phenotypic changes observed under stress conditions to the cellular and molecular events triggered by the same stress.

## Results

### Tissue-specific differential expression of miRNAs in abiotic stress and mineral-deficient conditions

A filtered set of 62 validated rice miRNAs was used for this study. A set of 182 miRNAs collected from miRBase v21 (<http://www.mirbase.org/>) were mapped exactly on precursor sequences obtained from MSU Rice Genome Annotation Project Version 7, resulting in 106 precursor sequences corresponding to 155 miRNAs. Out of these 155 miRNAs, we chose only those miRNAs for which all three software tools (psRNATarget, Tapir and Target Finder) predicted at least one common target. The 112 miRNAs so obtained included both 5p and 3p forms, and hence shared the same promoter. Again as mature miRNAs encoded by different *MIR* genes may have the same mature sequence, our selected set of miRNAs included 62 validated rice miRNAs corresponding to 79 promoters (Fig. 1). These 62 unique mature miRNA sequences were mapped to 14 next-generation sequencing (NGS) libraries (Supplementary Table S1). Normalized expression values for miRNAs were obtained for each data set (Supplementary Table S2). The data sets include abiotic (cold, drought and salt) stress conditions in two rice tissues, namely the panicle and seedling, and mineral deficiency (K, P, N and S) conditions in rice root and shoot (Supplementary Table S2). Fold change values of miRNAs were calculated for every data set with respect to the respective controls to find DEMs. If the fold change value was  $\geq 5$ , the miRNA was considered to be differentially up-regulated; conversely if the fold change was  $\leq 0.2$ , then the miRNA was considered to be differentially down-regulated under that particular stress condition in this study. Out of 62 mature unique miRNAs, 47 miRNAs were found to be differentially expressed (DEM) in at least one stress condition considered in the present study. Out of these 47 DEMs, three DEMs were down-regulated in the panicle (osa-miR-167d-3p, osa-miR167e-3p, i-3p and osa-miR396c-5p) and one was down-regulated in the seedling (osa-miR166i-5p) under all conditions (Fig. 2A; Supplementary Fig. S4). Based on tissue-specific differential expression, we observed that one DEM (osa-miR162a) was exclusively up-regulated whereas one DEM (osa-miR528-5p) was exclusively down-regulated in the root (Fig. 2). Members of osa-miR167 (osa-miR167d-3p, osa-miR167e-3p, i-3p and osa-miR167h-3p) were found to be differentially regulated in rice shoot (fold change 0.03–0.17) and root (fold change 1.2–3.33) under all the mineral-deficient conditions considered (Supplementary Table S2). Along with osa-miR167, expression of osa-miR394 was also induced in the case of S deficiency in rice roots. However, fold change for osa-miR394 was higher (2.15) as compared with osa-miR167 (1.55). Again in rice roots, we found osa-miR399, osa-miR162 and osa-miR1846d-3p to be up-regulated during P deficiency. Osa-miR162 was found to target DCL-1 (LOC\_Os03g02970) protein, which is a vital enzyme in the miRNA biogenesis pathway, and osa-miR1846 was found to target Metallo-beta-lactamase (LOC\_Os09g26760), an exonuclease.



**Fig. 1** Overview of the work flow. High confidence miRNAs were downloaded from miRBase (182 miRNAs). miRNAs were mapped to precursor sequences (106 miRNAs). miRNA promoter sequences were obtained from PMRD (155 promoters). miRNA targets were predicted by using three software tools, psRNA Target, Tapir and Target. Those miRNAs which have at least one target commonly predicted by all three tools were selected (112 miRNAs). The CRE information was retrieved from the PLACE database and using an in-house Perl program as well as the Signal Scan program. miRNA promoter sequences were scanned to determine occurrences of CREs. Over-represented CREs were computed in the promoters of miRNA and its target genes using statistical analysis. CREs with  $P$ -values  $< 0.001$  (FDR confidence level 99.75%) were considered as over-represented in a promoter. Small RNA, next-generation sequencing data were obtained from the GEO database for different rice tissues under abiotic and mineral deficiency stress conditions. Multiple microarray data sets for the above-mentioned conditions were obtained from the GEO database. These microarray data sets were used to compute expression of the target genes and transcription factors (TFs) associated with over-represented CREs found in promoters of both differentially expressed miRNAs and their respective targets under a particular condition. The GEO2R tool was used to find differential expression of the desired loci under different conditions, and only those target genes or TF(s) with adjusted  $P$ -values  $< 0.05$  were considered.

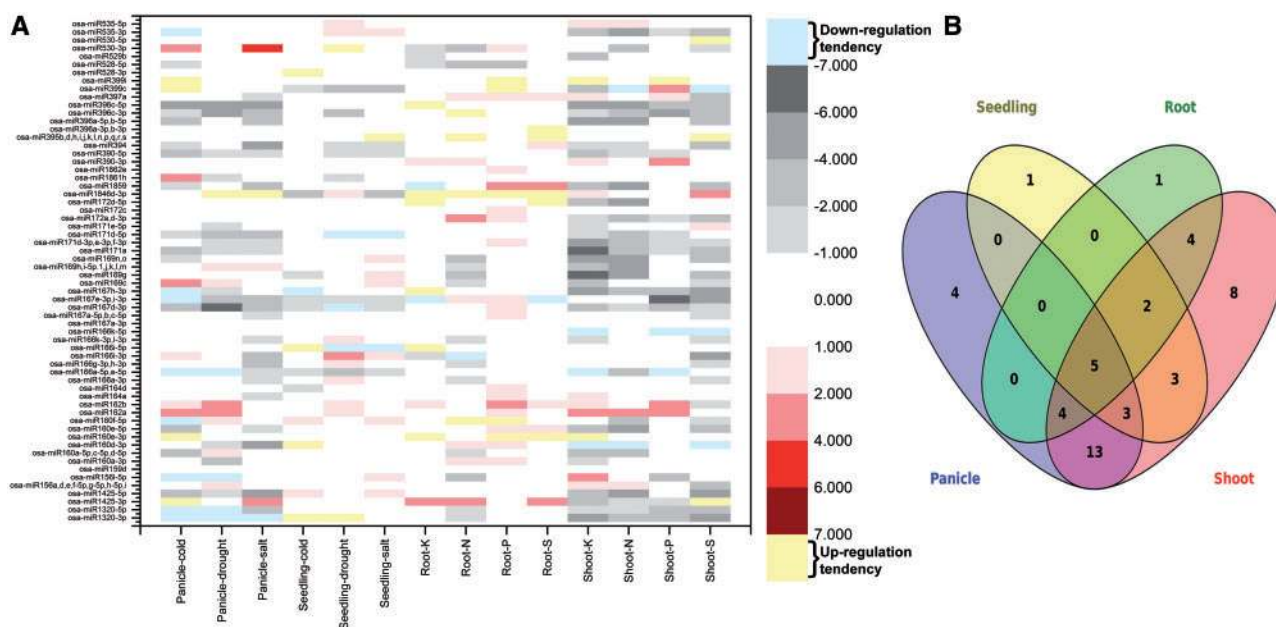
## Downstream effect of DEMs

To understand the downstream effect of miRNA differential expression, we predicted targets for DEMs using three different miRNA target identifier tools. Specifically, we considered only those targets which were predicted by all three tools (see the Materials and methods: miRNA sequence data). The list of DEMs and their corresponding commonly predicted targets is given in Supplementary Table S3; for 47 DEMs, we obtained 219 unique targets. Targets predicted by our analysis matched with validated miRNA targets listed in DIANA-TarBase v7.0 (Vlachos et al. 2015), e.g. LOC\_Os05g40700 is a validated target of osa-miR1425-5p (Li et al. 2010) and was also predicted by our analysis to be so. On average, each DEM targets six genes;

osa-miR171d-5p was found to have the highest number of targets, 25 in total; whereas 12 DEMs were found to have a single target each (Supplementary Table S3). We observed that few miRNA targets were transcription factors; for example, miR169g targets the nuclear transcription factor Y subunit (LOC\_Os12g42400).

## Over-represented CREs in stress-responsive miRNAs

As more than one miRNA precursor may give rise to the same mature miRNA, we obtained 62 miRNA precursors corresponding to 47 DEMs. We applied cumulative hypergeometric statistics (Deb and Kundu 2015) to predict over-represented CREs



**Fig. 2** Distribution of differentially expressed miRNAs (DEMs). (A) Distribution of DEMs under three different abiotic stresses (cold, drought and salt) and four mineral deficiencies (K, N, P and S) in four rice tissues (panicle, seedling, root and shoot). The red and gray-black colors represents up-regulated and down-regulated DEMs, respectively (except light blue and yellow). The intensity of the color is directly proportional to the log-transformed fold change value for miRNA. The light blue color indicates a tendency towards down-regulation; as the treated sample value is zero, a quantitative value of fold change has not been calculated. The yellow color indicates a tendency towards up-regulation; as the control value, is zero a quantitative value of fold change has not been calculated (see Supplementary Table S2; Fig. S4). (B) Venn diagram shows the number of DEMs in four rice tissues (panicle, seedling, root and shoot). Five DEMs are commonly found in all four tissues, whereas tissue-exclusive DEMs are indicated in the diagram.

in individual promoters of all 62 miRNA precursors (Supplementary Fig. S1). The CREs with a  $P$ -value  $< 0.001$  [false discovery rate (FDR) confidence level = 99.75%] were considered to be over-represented. The number of over-represented CREs predicted ranged between 21 and 38; on average, approximately 30 CREs were over-represented in each promoter. The lowest number of CREs was found in osa-miR162b and the highest was found in osa-miR162a.

Out of 170 over-represented CREs predicted in 47 DEMs promoters, 63.3% (109 CREs) were found to be common in all tissue types (panicle, seedling, root and shoot) whereas 50.5% (87 CREs) were common in both abiotic stress and mineral-deficient conditions (cold, drought, salt, K, N, P and, S) considered in the present study (Supplementary Fig. S2). A subset of common CREs may arise due to common DEMs under different conditions and/or tissue types. Interestingly, when we compared CREs of those DEMs which are not common in all four tissues under study, we still observed that 35 CREs were common (**Fig. 2B**). This indicates that a set of transcription factors may regulate different DEMs in different tissues (Supplementary Fig. S3).

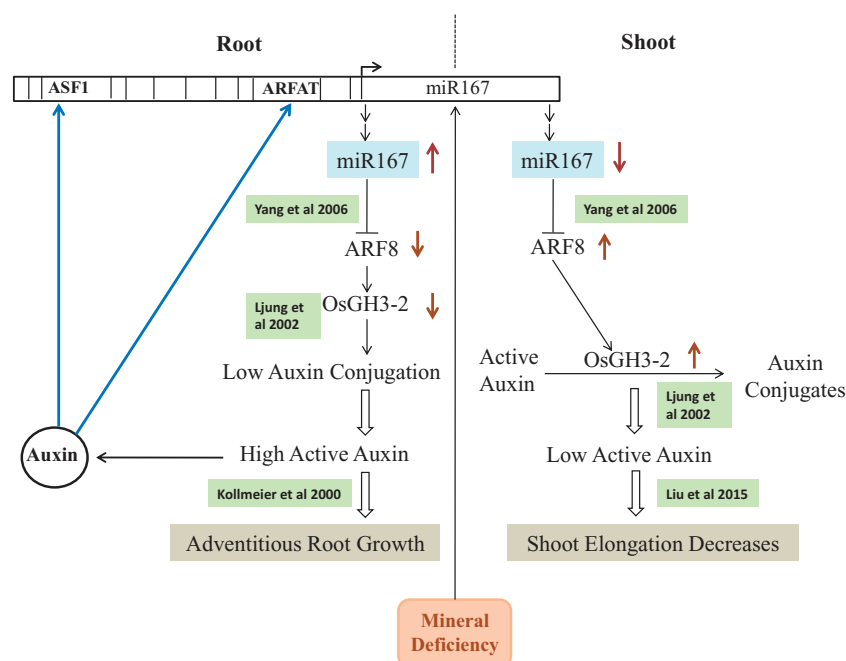
**The same transcription factors regulate expression of the miRNAs and corresponding targets under study**

Over-represented CREs were estimated in miRNAs and their target gene promoters. We observed that all miRNAs shared at

least one CRE with their corresponding target gene promoters (Supplementary Table S4); for instance, the promoter of osa-miR530-5p and the promoter of its target, a C3HC4-type domain-containing protein (LOC\_Os02g14990.1), share five common CREs (Supplementary Table S4). These results imply that transcription factors associated with these common CREs regulate the expression of both miRNAs and their corresponding targets (Wang et al. 2011). The transcription factors corresponding to over-represented CREs were identified using the PLACE database and data mining. We could identify transcription factors (for 63 CREs) belonging to 18 different transcription factor families (Supplementary Table S5).

In order to understand how transcription factors associated with common CREs influence expression of miRNAs (DEMs) and target genes under the same stress, we used publicly available microarray data under abiotic (cold, drought and salt) and mineral deficiency (K, P, N and S) stress conditions in rice panicle, seedlings, root and shoot (Supplementary Table S1). GEO2R (Barrett et al. 2013) was used to find differential expression of the desired loci under different conditions, and only those target genes or transcription factor(s) with an adjusted *P*-value <0.05 were considered for further analysis.

We analyzed the expression of transcription factors along with the expression of DEMs and their target genes using the above-mentioned data sets (Supplementary Table S6). This allowed us to identify several molecular interactions comprising transcription factors, miRNAs and their target genes. For instance, we found



**Fig. 3** Schematic representation of regulation of auxin content in shoots and roots by *osa*-miR167 under mineral deficiency. In root, *osa*-miR167 is up-regulated, resulting in degradation of ARF8 mRNA. Hence ARF8 is not available for activating OsGH3-2, leading to a decrease in auxin conjugation. Free auxin is available for adventitious root growth. Free auxin also augments *osa*-miR167 expression through ASF1 and ARFAT, auxin-responsive CREs. In the shoot, *osa*-miR167 is down-regulated, resulting in accumulation of ARF8; ARF8 activates OsGH3-2, which conjugates auxin. Lack of free auxin leads to a decrease in shoot elongation. Black arrows indicate interactions known from previous experimental studies; references are mentioned in green boxes beside each interaction. Blue and red arrows indicate findings of the present analysis; the direction of red arrows (up or down) indicates the expression level of the gene product.

that the expression of *osa*-miR399c is down-regulated during salt stress in seedlings, whereas its target LOC\_Os05g45350.2 (DnaJ domain-containing protein) is up-regulated and the common transcription factor MYB is also up-regulated (Rabbani et al. 2003, Yang et al. 2012, Xiong et al. 2014).

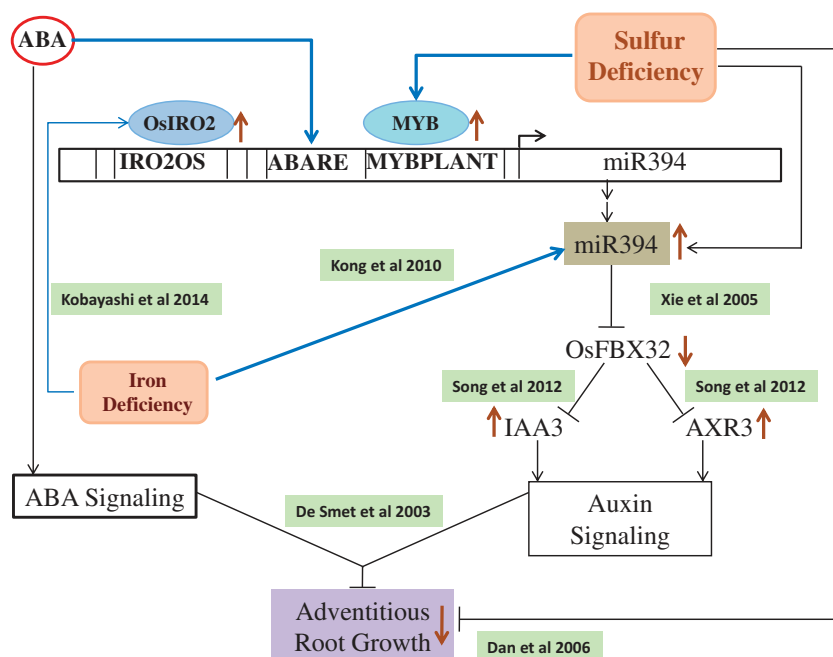
### Towards stress-responsive regulatory subnetworks

We were able to reconstruct interaction pathways relating miRNA to phenotypes by combining our findings with previous knowledge. As mentioned above ('Tissue-specific differential expression of miRNAs in abiotic stress and mineral-deficient conditions') we were able to identify some DEMs that show tissue- and condition-specific expression. Members of *osa*-miR167 were found to be differentially expressed in rice shoot and root under all the mineral-deficient conditions considered. miRNA167 expression is reported to be controlled by auxin, and it is known to target the auxin response factor, ARF8, involved in auxin conjugation (Yang et al. 2006). When we analyzed promoters of these miRNAs, interestingly we found auxin-responsive CREs (ARFAT, ASF1MOTIFCAMV, CATATGGMSAUR, NTBBF1ARROLB and SURECOREATSULTR11) to be over-represented (Fig. 3).

Besides *osa*-miR167, expression of *osa*-miR394 was also found to be induced in rice root under S deficiency. Moreover, we found that *osa*-miR394 family members target S transporters and bi-functional 3-phosphoadenosine 5-phosphosulfate synthase in rice; both of these observations are in agreement with

previous findings (Huang et al. 2010, Jeong et al. 2011). When we analyzed the CREs of the *osa*-miR394 promoter, we found the iron (Fe) starvation-related CRE IRO2OS, MYB-transcription factor binding (MYBPLANT) and ABA-responsive CREs (ABREATRD22, ABRELATERD1, ABREOSRAB21, ABRERATCAL, ABREZMRAB28, ACGTABREMOTIFA2OSEM, DPBFCORED CDC3 and LTRECOREATCOR15) to be over-represented, indicating that S deficiency may be related to Fe starvation and ABA response (Fig. 4).

During P deficiency, we found *osa*-miR399i, *osa*-miR162 and *osa*-miR1846d-3p to be up-regulated in rice root. On target prediction, we found that *osa*-miR399i targets ubiquitin-conjugating enzyme (LTN1), *osa*-miR162 targets DCL1, and *osa*-miR1846d-3p targets Metallo-beta-lactamase, an endonuclease. Previous studies have reported differential expression of miR399 and LTN1 during P deficiency (Hu et al. 2011). However, the roles of *osa*-miR162 and *osa*-miR1846d-3p remain to be explored. The MYB transcription factor OsPHR-binding CREs were found to be over-represented in promoters of both *osa*-miR399i (OsPHR1-binding CRE PIBS) and *osa*-miR162 (OsPHR2-binding CRE PIBS-like CREs). OsPHR transcription factors are known to influence P starvation signaling (Zhou et al. 2008). We also found eight CREs (DOFCOREZM:DOF, ARR1AT:MYB, CACTFPPCA1, GTGANTG10, MYCCONSUS USAT:MYC, SORLIP1AT, ARR1AT:ARR1, CURECORECR:SPL and CAATBOX1:HAP) common to all of above three miRNAs. Amongst these, ARR1AT, a cytokinin response



**Fig. 4** Schematic representation of integration of S and Fe deficiency response by *osa-miR394*. During S deficiency, *osa-miR394* is up-regulated by MYB transcription factor and ABA; *osa-miR394* is also up-regulated by OsIRO2 during Fe deficiency. *osa-miR394* targets OsFBX32, an auxin response gene repressor, resulting in an increase in expression of IAA3 and AXR3. Auxin and ABA interaction result in inhibition of adventitious root growth. Black arrows indicate interactions known from previous experimental studies; references are mentioned in green boxes beside each interaction. Blue and red arrows indicate the findings of the present analysis; the direction of red arrows (up or down) indicates the expression level of the gene product.

regulator, is interesting as cytokinin is known to inhibit P transport and signalling (Shen et al. 2014) (Fig. 5).

## Discussion

We analyzed DEMs in rice panicle and seedling under several abiotic (cold, drought and salt) and mineral deficiency (K, P, N and S) stress conditions in rice root and shoot. We predicted the CREs in promoters of the miRNAs that show condition-specific expression and identified corresponding transcription factors. To understand the biological significance of transcriptional regulation of these miRNAs, we explored their corresponding target genes and CRE-mediated transcriptional regulation of target genes. For both analysis of expression of genes and CRE predictions, statistical tests were performed, and results that passed stringent statistical cut-offs were taken for further analysis. We validated our results through a literature review. Our results, supported by previous experimental evidence, were used to formulate regulatory signaling interactions in the present study. This strategy allowed us to detect various regulatory signaling interactions that may connect phenotypic changes observed under stress conditions to the cellular and molecular events triggered by stress conditions.

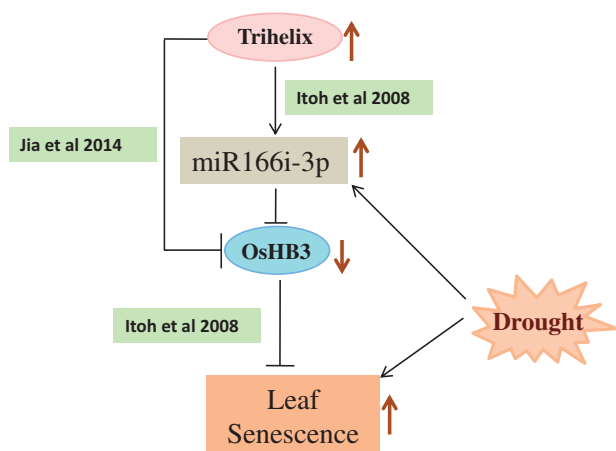
## During mineral deficiency *osa-miR167* inhibits shoot growth but activates adventitious root growth

We analyzed DEMs in both rice root and shoot data sets under mineral deficiency. We found that some members of *osa-*

*miR167* were down-regulated in the shoot but tended towards up-regulation in the root under all the mineral-deficient conditions considered. Previous studies have reported that *osa-miR167* expression is controlled by auxin (Yang et al. 2006). Our analysis of *osa-miR167* members also found that auxin-responsive CREs (ARFAT, ASF1MOTIFCAMV, CATATGG MSAUR, NTBBF1ARROLB and SURECOREATSULTR11) are over-represented in all of the members of the *osa-miR167* family. Auxin and its interaction with other plant hormones are known to play a pivotal role in shaping plant architecture (Vandenbussche and Van Der Straeten 2004, Gallavotti 2013). The patterning of lateral root formation under various stresses is directly related to auxin distribution (Kollmeier et al. 2000, López-Bucio et al. 2002). *miRNA167* is known to target ARF8 (Yang et al. 2006); ARF8, in turn, activates OsGH3-2, a protein that catalyzes the conjugation of auxin to different compounds and controls the cellular concentration of free auxin (Ljung et al. 2002, Staswick et al. 2005) (Fig. 3). ARF8 overexpression is also known to inhibit lateral root formation (Tian et al. 2004). Our results indicate that during mineral deficiency, *osa-miR167* expression is down-regulated in the shoot; this facilitates the accumulation of ARF8 and activation of OsGH3-2, leading to conjugation of auxin. Hence auxin is not available in the free state in the shoot, which results in stunting. On the other hand, *osa-miR167* expression is up-regulated in the root to inhibit ARF8, which in turn lowers OsGH3-2 activity and increases free auxin in the cell. This increase in cellular auxin on the one hand further augments *osa-miR167* expression by a loop, and on the other hand leads to the formation of adventitious



IRO2OS is an Fe deficiency response CRE; it is a binding site of the transcription factor OsIRO2, which is a positive transcriptional regulator of Fe deficiency-responsive genes (Kobayashi et al. 2014). As biosynthesis of Fe–S clusters requires both Fe and S in a definite stoichiometric ratio, Fe and S homeostasis are known to be related (Forieri et al. 2013). A previous report states that release of phytosiderophores, used to chelate Fe<sup>3+</sup>, is reduced under S deficiency in barley; on re-supply of sulfate, phytosiderophore secretion is increased (Astolfi et al. 2010). This indicates that Fe and S absorption by roots are related. Conversely, Fe deficiency is known to induce changes in expression of S transporters and several genes of the S metabolic pathway in durum wheat (Ciaffi et al. 2013). Moreover,



**Fig. 6** Schematic representation of regulation of leaf senescence during drought by trihelix transcription factors. Trihelix transcription factors induce expression of osa-miR166i-3p during drought. osa-miR166i-3p targets OsHB3, a HD-ZIP III transcription factor involved in leaf senescence, resulting in an increase in leaf senescence. Trihelix transcription factors also repress OsHB3 directly. Black arrows indicate interactions known from previous experimental studies; references are mentioned in green boxes beside each interaction. Blue and red arrows indicate the findings of the present analysis; the direction of red arrows (up or down) indicates the expression level of the gene product.

miR394 is known to be up-regulated during Fe deficiency in Arabidopsis (Kong and Yang 2010). These findings led us to hypothesize that the osa-miR394 expression level, which controls the expression of S transporters, may be influenced by Fe deficiency through the interaction of the Fe deficiency response transcription factor OsIRO2. Thus osa-miR394 may be involved in integrating S and Fe stress responses (Fig. 4).

### Osa-miR399 contributes to cross-talk between cytokinin and P deficiency signaling

P deficiency is known to induce adventitious root growth in rice (Niu et al. 2013). The DEMs during P deficiency in root tissue were analyzed and we found osa-miR399 to be up-regulated. Osa-miR399 is known to be induced during P deficiency in rice shoot and root tissues, and its target ubiquitin-conjugating enzyme (LTN1) is known to be down-regulated (Hu et al. 2011) (Fig. 5). In rice, two MYB transcription factor OsPHR genes (homologous to AtPHR1), OsPHR1 and OsPHR2, were shown to influence the P starvation signaling pathway by regulating the expression of P starvation-induced genes (Zhou et al. 2008). It is also known that OsPHR2 positively regulates the expression of miR399 in rice (Wu and Wang 2008, Zhou et al. 2008) and that miR399 targets LTN1, a ubiquitin-conjugating enzyme involved in degradation of rice high-affinity phosphate transporters (PHTs), OsPT2. The rice *ltn1* mutant shows enhanced P uptake and root growth under P deficiency (Hu et al. 2011). Thus down-regulation of LTN1 by osa-miR399 leads to root growth and P uptake.

In addition to osa-miR399, osa-miR162 and osa-miR1846d-3p were also found to be up-regulated during P deficiency in

root tissue. The MYB transcription factor OsPHR1 binds to P1BS (GNATATNC), whereas a combination of P1BS and P1BS-like is essential for stable binding by OsPHR2 (Ruan et al. 2015). Our CRE analysis of up-regulated DEMs in rice root tissue suggests that P1BS (GCATATCC) is present in the osa-miR399 promoter, and P1BS-like motifs (GAATATAC and GAATATTC) are present on the miR162 promoter. These findings indicate that osa-miR162 is involved as the putative regulator of P deficiency response in root and shoot tissue of rice along with osa-miR399. It may be regulating the levels of osa-miR399 by inhibiting Dicer protein, which regulates the biogenesis of miRNAs in a cell.

When we analyzed the CRE over-represented in the promoters of these miRNAs (osa-miR162, osa-miR1846d-3p and osa-miR399), we found eight common CREs (DOFCOREZM:DOF, ARR1AT:MYB, CACTFPPCA1, GTGANTG10, MYCCONSEN SUSAT:MYC, SORLIP1AT, ARR1AT:ARR1, CURECORECR:SPL, CAATBOX1:HAP). Amongst these, ARR1AT is a binding site of ARR1, a cytokinin response regulator (Sakai et al. 2000, Oka et al. 2002). Cytokinin is known to suppress phosphate deficiency responses (Martín et al. 2000); conversely, P deficiency decreases cytokinin content (Martín et al. 2000). Cytokinin inhibits both P transport and signaling (Shen et al. 2014), by regulating expression of transcription factors (OsARF16) (Shen et al. 2014) and transporters (OsPT) (Hatorangan et al. 2009) at the transcriptional level. Our analyses suggest that cross-talk between cytokinin and P starvation signaling may also involve post-transcriptional regulation through miRNAs.

### Trihelix transcription factors influence leaf senescence during drought

Transcription factors and miRNAs are the two most important families of *trans*-acting gene expression-regulating factors in multicellular organisms (Hobert 2008). We found that miRNAs and their respective target gene promoters shared over-represented CREs. This indicated that a common transcription factor might regulate both miRNA and the corresponding target gene. On analysis of these motifs, we found that different regulatory interactions are present during different stress conditions.

Drought stress is known to induce leaf senescence; here we describe miRNA- and transcription factor-mediated regulatory interactions that may be involved in this process (Fig. 6). Trihelix transcription factors (LOC4347833, LOC4330441, LOC4336540, LOC4335704 and LOC4328005) were found to be up-regulated during drought conditions in rice seedling. Trihelix proteins are known to induce expression of osa-miR166i-3p, which represses the target protein OsHB3 (LOC\_Os12g41860.1) (Itoh et al. 2008). OsHB3 is an HD-ZIP III transcription factor; it is involved in leaf initiation and inhibits leaf senescence (Itoh et al. 2008). HB3 is known to be repressed by a trihelix transcription factor in Arabidopsis (Jia et al. 2014). Leaf senescence is a common response of plants to drought as it reduces the surface area open for evaporation and conserves moisture. Our observation indicates that during drought trihelix transcription factors are up-regulated, which

on one hand down-regulates OsHB3 and on the other hand induces osa-miR166i-3p, which in turn targets OsHB3, leading to leaf senescence.

Regulatory signaling interactions proposed by the present study were derived through our analysis of expression of data sets, CRE predictions and literature review. Our results challenge experimental plant biologists to access these hypotheses by direct experimental testing (see Supplementary File S4 for further discussion). Under a particular condition/stress, RT-PCR may be used for analysis of gene expression. Transcription factor–CRE interactions may be studied using chromatin immunoprecipitation assays, DNA footprinting and expression of a reporter gene fused with the promoter with the mutated CRE of interest.

Phenotypic alterations are an outcome of dynamic interactions among many regulatory components involving multiple genes. Regulatory interactions discussed above contribute to phenotypic changes observed under stress conditions used for the present study, but other components may also be involved.

## Conclusion

We predicted CREs to be over-represented in promoters of miRNA genes and their targets; we then related our predictions to expression profiles of the same miRNAs, their targets and transcription factors that were likely to interact with both miRNAs and their corresponding target promoters. This strategy allowed us to identify regulatory molecular interactions that connect molecular events to phenotypic changes during stress. During mineral deficiency, osa-miR167 inhibits shoot growth but activates adventitious root growth by influencing the free auxin content. During mineral deficiency, osa-miR167 expression is down-regulated in the shoot; this facilitates the accumulation of ARF8 and activation of OsGH3-2, leading to conjugation of auxin. On the other hand, osa-miR167 expression in root inhibits ARF8, an auxin response factor, which in turn lowers OsGH3-2 activity and increases free auxin in the root (Fig. 3). Previous studies have demonstrated that during S deficiency lateral root growth is inhibited, S and Fe homeostasis are related, and auxin influences S homeostasis. We found that the activity of osa-miR394 is able to explain and integrate all of the above observations into a single regulatory process. Our results indicate that during S deficiency, ABA-responsive bZIP transcription factors or, during Fe starvation, OsIRO2 bind to the miR394 promoter and increase its expression. osa-miRNA394 then degrades OsFBX32; this activates auxin response genes, i.e. IAA3 and AXR3, leading to adventitious root growth inhibition (Fig. 4). Cytokinin is known to inhibit both P transport and signaling at the transcriptional level; our results indicated that this cross-talk may also involve miRNAs (osa-miR399 and osa-miR162) and the ubiquitination pathway (LTN1) (Fig. 5). According to our findings, leaf senescence during drought might be regulated by a feed-forward interaction involving osa-miR166 and trihelix and HD-ZIP III transcription factors (Fig. 6). Altogether our findings indicate that study of interactions at the molecular level may help to explain,

at least partially, how regulation of the cellular–molecular events leads to phenotypic changes.

## Materials and Methods

### miRNA sequence data

We collected rice miRNA sequences from miRBase v21 (<http://www.mirbase.org/>). Only those miRNAs that were ranked as high confidence miRNAs in miRBase were selected to ensure that the miRNA data set would be of high quality (Kozomara and Griffiths-Jones 2014); the data set consisted of 182 miRNAs. These 182 selected miRNAs were then again filtered based on the exact mapping of miRNAs on precursor sequences. The precursor sequences for selected miRNAs were obtained from rice MSU Rice Genome Annotation Project Version 7; a total of 121 sequences were retrieved. The obtained sequences were fitted to the MSU Rice Genome Annotation Project Version 6.1; this process reduced the miRNA precursor number to 106 sequences. These 106 precursor sequences correspond to 155 miRNA sequences. These sequences were further filtered based on whether three different softwares, namely psRNATarget (Dai and Zhao 2011), Tapir (Bonnet et al. 2010) and Target Finder v1.6 (<https://github.com/carringtonlab/TargetFinder>) (Srivastava et al. 2014), predicted at least one common target. The default cut-off scores used for target prediction were 3, 4 and 4 for psRNATarget, TAPIR and TargetFinder, respectively. Finally, the number of miRNA precursor sequences was reduced to 92, corresponding to 112 miRNA sequences.

### Promoter sequences

Some of the miRNAs, out of the 112 that we considered for analysis, included both 5p and 3p forms and hence shared the same promoter. Moreover, as mature miRNAs encoded by different miRNA genes may have the same mature sequence, we ended up with 79 promoters which correspond to 62 unique mature miRNA sequences. We collected 1 kb upstream promoter sequences of rice miRNAs from the Plant miRNA Database, PMRD (<http://bioinformatics.cau.edu.cn/PMRD/>). Promoter sequences (1 kb upstream) of the rice target loci were collected from the Rice Genome Annotation Project, version 6.1 (Ouyang et al. 2007). RepeatMasker version open-4.0.2 (Smit et al. 2015) was used to mask interspersed and simple repeats in the promoter sequences.

### sRNA expression data set

The processed sRNA data set (GSE32973) was obtained from the Gene Expression Omnibus (GEO) (Edgar et al. 2002) for rice under abiotic (cold, drought and salt) and mineral deficiency (K, P, N and S) stress conditions in four tissues, namely panicle, seedlings, root and shoot (Supplementary Table S1).

### Target gene expression microarray data set

The multiple microarray data set was obtained from the GEO database (Edgar et al. 2002) for rice seedling and panicle tissue under cold, drought and salinity stress, and rice root and shoot tissues under N and P deficiency (GSE67373, GSE6901, GSE26280, GSE38102, GSE52159, GSE6187, GSE4438 and GSE66935) (Supplementary Table S1).

### Identification of DEMs across various stress samples

Processed sRNA data sets (GSE32973) were obtained from the GEO and used for analysis. sRNA reads that were 20–25 nucleotides long were mapped to the MSU Rice Genome Annotation Project Version 6.1 using the tool Bowtie (Langmead et al. 2009). Each mapped miRNA read was normalized to  $1 \times 10^{-7}$  by the total number of sRNA reads in each library (excluding the reads which do not map to the rice genome and other non-coding RNAs [tRNA, rRNA, snRNA and snoRNA]) (Saraf et al. 2015). We then looked for the presence of the 62 selected unique mature miRNA sequences in each sRNA data set (Supplementary Table S2), and their expression values were retrieved across different samples using an in-house-developed Perl script (Saraf et al.

2015). To identify DEMs, the fold change value of selected miRNA sequence was calculated for every library (stress condition with respect to control condition). If the fold change value is  $\geq 5$  or  $\leq 0.2$ , then the miRNA is considered to be differentially up- or down-regulated, respectively, in that particular stress condition in this study (Fig. 2A; Supplementary Fig. S4) (Chang et al. 2010). After determining such miRNAs, common over-represented CREs were identified in them. The process was repeated with all sRNA libraries; the pipeline is given in Fig. 1.

## Identification of CREs in miRNA and target gene promoters

The information on CREs was retrieved from the PLACE (Plant cis-acting regulatory element) database (Higo et al. 1999). The 1 kb upstream sequences of miRNA genes and their target genes were scanned to obtain the occurrences of the CREs using an in-house Perl program as well as the Signal Scan program (Prestridge 1991). Previous studies have applied different statistical enrichment tests (Roider et al. 2009, Deb and Kundu 2015) to identify the over-represented CREs in respect of their occurrences in the promoters of the whole genome. We applied cumulative hypergeometric statistics (Deb et al. 2016) (Equation 1) to retrieve the over-represented CREs in each promoter of miRNA genes and their target genes.

$$P_{CRE} = 1 - F(x, N, n, y)$$

$$= 1 - \sum_{i=0}^x \frac{\binom{y}{i} \binom{N-y}{n-i}}{\binom{N}{n}} \quad (1)$$

Where  $N$  is the total number of occurrences of all CREs in the rice genome,  $y$  is the total number of occurrences of a considered CRE in the genome,  $x$  is the number of occurrences of that CRE in an individual promoter of a gene,  $n$  is the total number of occurrences of all CREs in that promoter and  $i$  is the summation index. Thus,  $P_{CRE}$  is the probability of occurrence of a considered CRE in a particular promoter.

The significance level has been defined in terms of a 'P-value' for the CREs present in miRNAs and their target promoters. Correction for the FDR was performed using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). The actual  $P$ -values were compared with Benjamini–Hochberg critical values and found to be significant at a 99.75% confidence level (García-Arenzana et al. 2014). The CREs with  $P$ -values  $< 0.01$  (FDR confidence level = 99.75%) were considered as over-represented in the target promoters. The targets of the miRNAs are protein-coding genes of the rice genome which have been widely studied for promoter analysis (Mohanty et al. 2012, Deb et al. 2016). On the other hand, inadequate analyses have been done on the promoter elements of the miRNAs. Therefore, for further analysis of CREs in rice miRNA promoters, a higher stringent level of filtering has been considered to obtain high confidence data. Here, in miRNA promoters, the CREs with  $P$ -values  $< 0.001$  (FDR confidence level = 99.75%) were considered as highly over-represented and were taken for further analysis.

## Target gene and transcription factor expression analysis from microarray data

Microarray data sets from rice seedling and panicle under cold, drought and salinity stress and from rice root and shoot under N and P deficiency (GSE6901, GSE26280, GSE38102, GSE52159, GSE6187 and GSE66935) were used to compute expression of the target genes and transcription factors associated with over-represented CREs found in promoters of both DEMs and their respective targets under a particular condition (Supplementary Table S1). Each microarray data set contained three replicates. GEO2R (Barrett et al. 2013) was used to find differential expression of the desired loci under different conditions, and only those target genes or transcription factor(s) with an adjusted  $P$ -value  $< 0.05$  were considered for further analysis.

## Supplementary Data

Supplementary data are available at PCP online.

## Funding

This work was supported by the Centre of Advanced Study (CAS) by the University Grants Committee (UGC) [No. F5.1/2015/CASI(SAP-II)]; the Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta; Department of Biotechnology, Government of India [a Bioinformatics National Certification (BINC) fellowship to S.S.]; and UGC, Government of India [a Research Fellowship in Sciences for meritorious students (RFSMS) to A.D.].

## Disclosure

The authors have no conflicts of interest to declare.

## References

- Astolfi, S., Zuchi, S., Hubberten, H.-M., Pinton, R. and Hoefgen, R. (2010) Supply of sulphur to S-deficient young barley seedlings restores their capability to cope with iron shortage. *J. Exp. Bot.* 61: 799–806.
- Barrett, T., Wilhite, S.E., Ledoux, P., Evangelista, C., Kim, I.F., Tomashevsky, M., et al. (2013) NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Res.* 41: D991–D995.
- Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281–297.
- Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 57: 289–300.
- Bonnet, E., He, Y., Billiau, K. and Van de Peer, Y. (2010) TAPIR, a web server for the prediction of plant microRNA targets, including target mimics. *Bioinformatics* 26: 1566–1568.
- Chang, K.H., Mestdagh, P., Vandesompele, J., Kerin, M.J. and Miller, N. (2010) MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. *BMC Cancer* 10: 173.
- Chen, X. (2005) MicroRNA biogenesis and function in plants. *FEBS Lett.* 579: 5923–5931.
- Ciaffi, M., Paolacci, A.R., Celletti, S., Catarcione, G., Kopriva, S. and Astolfi, S. (2013) Transcriptional and physiological changes in the S assimilation pathway due to single or combined S and Fe deprivation in durum wheat (*Triticum durum* L.) seedlings. *J. Exp. Bot.* 64: 1663–1675.
- Dai, X. and Zhao, P.X. (2011) psRNATarget: a plant small RNA target analysis server. *Nucleic Acids Res.* 39: W155–W159.
- Dan, H., Yang, G. and Zheng, Z.-L. (2006) A negative regulatory role for auxin in sulphate deficiency response in *Arabidopsis thaliana*. *Plant Mol. Biol.* 63: 221–235.
- Deb, A., Grewal, R.K. and Kundu, S. (2016) Regulatory cross-talks and cascades in rice hormone biosynthesis pathways contribute to stress signaling. *Front. Plant Sci.* 7.
- Deb, A. and Kundu, S. (2015) Deciphering cis-regulatory element mediated combinatorial regulation in rice under blast infected condition. *Plos One*. 10: e0137295.
- De Smet, I., Signora, L., Beeckman, T., Inzé, D., Foyer, C.H. and Zhang, H. (2003) An abscisic acid-sensitive checkpoint in lateral root development of *Arabidopsis*. *Plant J.* 33: 543–555.

- Edgar, R., Domrachev, M. and Lash, A.E. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30: 207–210.
- Falkenberg, B., Witt, I., Zanor, M.I., Steinhauser, D., Mueller-Roeber, B., Hesse, H., et al. (2008) Transcription factors relevant to auxin signalling coordinate broad-spectrum metabolic shifts including sulphur metabolism. *J. Exp. Bot.* 59: 2831–2846.
- Fioreri, I., Wirtz, M. and Hell, R. (2013) Toward new perspectives on the interaction of iron and sulfur metabolism in plants. *Front. Plant Sci.* 4: 357.
- Franco-Zorrilla, J.M., González, E., Bustos, R., Linhares, F., Leyva, A., et al. (2004) The transcriptional control of plant responses to phosphate limitation. *J. Exp. Bot.* 55: 285–293.
- Gallavotti, A. (2013) The role of auxin in shaping shoot architecture. *J. Exp. Bot.* 64: 2593–2608.
- García-Arenzana, N., Navarrete-Muñoz, E.M., Lope, V., Moreo, P., Vidal, C., Laso-Pablos, S., et al. (2014) Calorie intake, olive oil consumption and mammographic density among Spanish women. *Int. J. Cancer* 134: 1916–1925.
- Hatorangan, M.R., Sentausa, E. and Wijaya, G.Y. (2009) In silico identification of cis-regulatory elements of phosphate transporter genes in rice (*Oryza sativa* L.). *J. Crop Sci. Biotechnol.* 12: 25–30.
- He, L. and Hannon, G.J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* 5: 522–531.
- Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* 27: 297–300.
- Hobert, O. (2008) Gene regulation by transcription factors and microRNAs. *Science* 319: 1785–1786.
- Hu, B., Zhu, C., Li, F., Tang, J., Wang, Y., Lin, A., et al. (2011) LEAF TIP NECROSIS1 plays a pivotal role in the regulation of multiple phosphate starvation responses in rice. *Plant Physiol.* 156: 1101–1115.
- Huang, S.Q., Xiang, A.L., Che, L.L., Chen, S., Li, H., Song, J.B., et al. (2010) A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress. *Plant Biotechnol. J.* 8: 887–899.
- Itoh, J.-I., Hibara, K.-I., Sato, Y. and Nagato, Y. (2008) Developmental role and auxin responsiveness of class III homeodomain leucine zipper gene family members in rice. *Plant Physiol.* 147: 1960–1975.
- Jeong, D.-H., Park, S., Zhai, J., Gurazada, S.G.R., De Paoli, E., Meyers, B.C., et al. (2011) Massive analysis of rice small RNAs: mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage. *Plant Cell* 23: 4185–4207.
- Jia, H., Suzuki, M. and McCarty, D.R. (2014) Regulation of the seed to seedling developmental phase transition by the LAFL and VAL transcription factor networks. *Wiley Interdiscip. Rev. Dev. Biol.* 3: 135–145.
- Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* 14: 787–799.
- Kato, M., Hata, N., Banerjee, N., Fletcher, B. and Zhang, M.Q. (2004) Identifying combinatorial regulation of transcription factors and binding motifs. *Genome Biol.* 5: R56.
- Kehr, J. (2013) Systemic regulation of mineral homeostasis by micro RNAs. *Front. Plant Sci.* 4: 145.
- Ketting, R.F. (2011) The many faces of RNAi. *Dev. Cell* 20: 148–161.
- Kobayashi, T., Itai, R.N. and Nishizawa, N.K. (2014) Iron deficiency responses in rice roots. *Rice (N Y)* 7: 27.
- Kollmeier, M., Felle, H.H. and Horst, W.J. (2000) Genotypical differences in aluminum resistance of maize are expressed in the distal part of the transition zone. Is reduced basipetal auxin flow involved in inhibition of root elongation by aluminum? *Plant Physiol.* 122: 945–956.
- Kong, W.W. and Yang, Z.M. (2010) Identification of iron-deficiency responsive microRNA genes and cis-elements in *Arabidopsis*. *Plant Physiol. Biochem.* 48: 153–159.
- Kozomara, A. and Griffiths-Jones, S. (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42: D68–D73.
- Kuo, H.-F. and Chiou, T.-J. (2011) The role of microRNAs in phosphorus deficiency signaling. *Plant Physiol.* 156: 1016–1024.
- Langmead, B., Trapnell, C., Pop, M. and Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10: R25.
- Li, Y.-F., Zheng, Y., Addo-Quaye, C., Zhang, L., Saini, A., Jagadeeswaran, G., et al. (2010) Transcriptome-wide identification of microRNA targets in rice. *Plant J.* 62: 742–759.
- Ljung, K., Hull, A.K., Kowalczyk, M., Marchant, A., Celenza, J., Cohen, J.D., et al. (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol. Biol.* 49: 249–272.
- López-Bucio, J., Hernández-Abreu, E., Sánchez-Calderón, L., Nieto-Jacobo, M.F., Simpson, J. and Herrera-Estrella, L. (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the *Arabidopsis* root system. *Plant Physiol.* 129: 244–256.
- Lu, C. and Fedoroff, N. (2000) A mutation in the *Arabidopsis* HYL1 gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. *Plant Cell* 12: 2351–2366.
- Martín, A.C., Del Pozo, J.C., Iglesias, J., Rubio, V., Solano, R., De La Peña, A., et al. (2000) Influence of cytokinins on the expression of phosphate starvation responsive genes in *Arabidopsis*. *Plant J.* 24: 559–567.
- Meng, Y., Huang, F., Shi, Q., Cao, J., Chen, D., Zhang, J., et al. (2009) Genome-wide survey of rice microRNAs and microRNA–target pairs in the root of a novel auxin-resistant mutant. *Planta* 230: 883–898.
- Mohanty, B., Herath, V., Wijaya, E., Yeo, H.C., de los Reyes, B.G. and Lee, D.-Y. (2012) Patterns of cis-element enrichment reveal potential regulatory modules involved in the transcriptional regulation of anoxia response of japonica rice. *Gene* 511: 235–242.
- Nikiforova, V.J., Daub, C.O., Hesse, H., Willmitzer, L. and Hoefgen, R. (2005) Integrative gene-metabolite network with implemented causality decipherers informational fluxes of sulphur stress response. *J. Exp. Bot.* 56: 1887–1896.
- Niu, Y.F., Chai, R.S., Jin, G.L., Wang, H., Tang, C.X. and Zhang, Y.S. (2013) Responses of root architecture development to low phosphorus availability: a review. *Ann. Bot.* 112: 391–408.
- Oka, A., Hiroe, S. and Shintaro, I. (2002) His-Asp phosphorelay signal transduction in higher plants: receptors and response regulators for cytokinin signaling in *Arabidopsis thaliana*. *Genes & genetic systems* 77: 383–391.
- Ouyang, S., Zhu, W., Hamilton, J., Lin, H., Campbell, M., Childs, K., et al. (2007) The TIGR Rice Genome Annotation Resource: improvements and new features. *Nucleic Acids Res.* 35: D883–D887.
- Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H. and Poethig, R.S. (2005) Nuclear processing and export of microRNAs in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 102: 3691–3696.
- Paul, S., Datta, S.K. and Datta, K. (2015) miRNA regulation of nutrient homeostasis in plants. *Front. Plant Sci.* 06: 232.
- Pilpel, Y., Sudarsanam, P. and Church, G.M. (2001) Identifying regulatory networks by combinatorial analysis of promoter elements. *Nat. Genet.* 29: 153–159.
- Potters, G., Pasternak, T.P., Guisez, Y., Palme, K.J. and Jansen, M.A.K. (2007) Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci.* 12: 98–105.
- Prestridge, D.S. (1991) SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. *Bioinformatics* 7: 203–206.
- Rabbani, M.A., Maruyama, K., Abe, H., Khan, M.A., Katsura, K., Ito, Y., et al. (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiol.* 133: 1755–1767.
- Roider, H.G., Lenhard, B., Kanhere, A., Haas, S.A. and Vingron, M. (2009) CpG-depleted promoters harbor tissue-specific transcription factor binding signals—implications for motif overrepresentation analyses. *Nucleic Acids Res.* 37: 6305–6315.

- Ruan, W., Guo, M., Cai, L., Hu, H., Li, C., Liu, Y., et al. (2015) Genetic manipulation of a high-affinity PHR1 target cis-element to improve phosphorous uptake in *Oryza sativa* L. *Plant Mol. Biol.* 87: 429–440.
- Sakai, H., Takashi, A. and Atsuhiko, O. (2000) Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. *The Plant Journal* 24: 703–711.
- Saraf, S., Sanan-Mishra, N., Gursansky, N.R., Carroll, B.J., Gupta, D. and Mukherjee, S.K. (2015) 3' and 5' microRNA-end post-biogenesis modifications in plant transcriptomes: evidences from small RNA next generation sequencing data analysis. *Biochem. Biophys. Res. Commun.* 467: 892–899.
- Shen, C., Yue, R., Yang, Y., Zhang, L., Sun, T., Tie, S., et al. (2014) OsARF16 is involved in cytokinin-mediated inhibition of phosphate transport and phosphate signaling in rice (*Oryza sativa* L.). *PLoS One* 9: e112906.
- Smit, A.F., Hubley, R. and Green, P. (2015) RepeatMasker Open-4.0. 2013–2015. *Institute for Systems Biology*. <http://repeatmasker.org>.
- Song, J.B., Huang, S.Q., Dalmay, T. and Yang, Z.M. (2012) Regulation of leaf morphology by microRNA394 and its target LEAF CURLING RESPONSIVENESS. *Plant Cell Physiol.* 53: 1283–1294.
- Srivastava, P.K., Moturu, T.R., Pandey, P., Baldwin, I.T. and Pandey, S.P. (2014) A comparison of performance of plant miRNA target prediction tools and the characterization of features for genome-wide target prediction. *BMC Genomics* 15: 348.
- Staswick, P.E., Serban, B., Rowe, M., Tiriyaki, I., Maldonado, M.T., Maldonado, M.C., et al. (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. *Plant Cell* 17: 616–627.
- Sunkar, R., Chinnusamy, V., Zhu, J. and Zhu, J.-K. (2007) Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci.* 12: 301–309.
- Sunkar, R. and Zhu, J.-K. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* 16: 2001–2019.
- Tian, C., Muto, H., Higuchi, K., Matamura, T., Tatematsu, K., Koshiba, T., et al. (2004) Disruption and overexpression of auxin response factor 8 gene of Arabidopsis affect hypocotyl elongation and root growth habit, indicating its possible involvement in auxin homeostasis in light condition. *Plant J.* 40: 333–343.
- Vandenbussche, F. and Van Der Straeten, D. (2004) Shaping the shoot: a circuitry that integrates multiple signals. *Trends Plant Sci.* 9: 499–506.
- Vlachos, I.S., Paraskevopoulou, M.D., Karagkouni, D., Georgakilas, G., Vergoulis, T., Kanellos, I., et al. (2015) DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res.* 43: D153–D159.
- Wang, C., Huang, W., Ying, Y., Li, S., Secco, D., Tyerman, S., et al. (2012) Functional characterization of the rice SPX-MFS family reveals a key role of OsSPX-MFS1 in controlling phosphate homeostasis in leaves. *New Phytol.* 196: 139–148.
- Wang, Y., Li, X. and Hu, H. (2011) Transcriptional regulation of co-expressed microRNA target genes. *Genomics* 98: 445–452.
- Wu, P. and Wang, X.M. (2008) Role of OsPHR2 on phosphorus homeostasis and root hairs development in rice (*Oryza sativa* L.). *Plant Signal. Behav.* 3: 674–675.
- Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A. and Carrington, J.C. (2005) Expression of Arabidopsis MIRNA genes. *Plant Physiol.* 138: 2145–2154.
- Xiong, H., Li, J., Liu, P., Duan, J., Zhao, Y., Guo, X., et al. (2014) Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. *PLoS One* 9: e92913.
- Yang, A., Dai, X. and Zhang, W.-H. (2012) A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice. *J. Exp. Bot.* 63: 2541–2556.
- Yang, J.H., Han, S.J., Yoon, E.K. and Lee, W.S. (2006) Evidence of an auxin signal pathway, microRNA167–ARF8–GH3, and its response to exogenous auxin in cultured rice cells. *Nucleic Acids Res.* 34: 1892–1899.
- Zhang, J.-F., Yuan, L.-J., Shao, Y., Du, W., Yan, D.-W. and Lu, Y.-T. (2008) The disturbance of small RNA pathways enhanced abscisic acid response and multiple stress responses in Arabidopsis. *Plant. Cell Environ.* 31: 562–574.
- Zhao, X. and Li, L. (2013) Comparative analysis of microRNA promoters in Arabidopsis and rice. *Genomics Proteomics Bioinformatics* 11: 56–60.
- Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., et al. (2008) OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiol.* 146: 1673–1686.
- Zhu, J.-K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53: 247–273.