Delineating the tissue-mediated drought stress governed tuning of conserved miR408 and its targets in rice

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Abstract
Engineering drought tolerance in rice needs to focus on regulators that enhance tolerance while boosting plant growth and vigor. The present study delineated the concealed function and tissue-mediated interplay of the miR408/target module in imparting drought stress tolerance in rice. The plant miR408 family comprises three dominant mature forms (21 nt), including a distinct monocot variant (F-7 with 5′ C) and is divided into six groups. miR408 majorly cleaves genes belonging to the blue copper protein in addition to several other species-specific targets in plants. Comparative sequence analysis in 4726 rice accessions identified 22 sequence variants (SNP and InDELS) in its promoter (15) and pre-miR408 region. Haplotype analysis of the sequence variants indicated eight haplotypes (three: Japonica-specific and five: Indica-specific) of the miR408 promoter. In drought-tolerant Nagina 22, miR408 follows flag leaf preferential expression. Under drought conditions, its levels are upregulated in flag leaf and roots which seems to be regulated by a differential fraction of methylated cytosines (mCs) in the precursor region. The active pool of miR408 regulated targets under control and drought conditions is impacted by the tissue type. Comparative expression analysis of the miR408/target module under different sets of conditions features 83 targets exhibiting antagonistic expression in rice, out of which 12 genes, including four PLANTACYANINS (OsUCL6, 7, 9 and 30), PIRIN, OsLPR1, OsCHUP1, OsDOF12, OsBGLU1, glycin-rich cell wall gene, OsDUT, and OsERF7, are among the high confidence targets. Further, overexpression of MIR408 in drought-sensitive rice cultivar (PB1) leads to the massive enhancement of vegetative growth in rice with improved ETR and Y(II) and enhanced dehydration stress tolerance. The above results suggest that miR408 is likely to act as a positive regulator of growth and vigor, as well as dehydration stress, making it a potential candidate for engineering drought tolerance in rice.

Keywords miR408 · Conserved · Drought · N22 · miRNA target

Introduction
Global climatic fluctuations are the ultimate threat to the aim of achieving future food security for the constantly increasing population. Drought is one of the leading adversities responsible for global yield losses of major cereals, specifically rice. To bypass these hurdles, it is important to comprehensively delineate the complex multi-level molecular regulatory mechanisms that are activated and functioning in response to drought stress, especially in wild-tolerant cultivars. MicroRNAs (miRNAs) have recently emerged as crucial key regulators of molecular stress-responsive strategies. miRNAs are tiny (20-22 nt), indispensable, post-transcriptional regulators of the core to sophisticated molecular gene circuits impacting growth, development, and biotic, as well as abiotic, stress responses in plants (Chithung et al.,
In rice, several miRNA target modules were shown to regulate key developmental processes including plant architecture (Jiao et al. 2010), reproduction (Yang et al. 2019), and grain yield (Wang et al. 2021a). However, limited miRNAs were studied in detail for their role in rice stress response. For instance, miR1871 and miR1432 act as negative regulators of rice blast disease tolerance (Li et al. 2021a, b), while miR528 and miR172 positively regulate the salt stress tolerance in rice (Wang et al. 2021b; Cheng et al. 2021). Differential regulation of several miRNAs in response to drought was reported by previous studies (Nadarajah et al. 2012). In contrast, miR408 acts as a positive regulator of drought tolerance in rice, tomato (Zhang et al. 2010), and Arabidopsis (Jiang et al. 2021). The miR408 gene module plays a critical role in conferring drought stress in plants (Öztürk Gökçe et al. 2021; Bhogireddy et al. 2021). The miR408/F-box gene module plays a critical role in conferring drought stress in Arabidopsis (Ni et al. 2012). miR169o acts as a positive regulator of drought tolerance in poplar by regulating its target, Nuclear transcription factor Y (NF-YA) 6A (Jiao et al. 2021). In contrast, miR169n functions as a negative regulator of drought tolerance by targeting NF-YA8 in Brassica napus (Li et al. 2021a, b). Moreover, several other miRNAs/target nodes are reported as the critical regulator of drought stress tolerance, e.g., miR827/Rab17 in barley (Ferdous et al. 2016), miR169/NF-YA in tomato (Zhang et al. 2010), and miR166-OsHB4 (HOMEO-DOMAIN CONTAINING PROTEIN4) in rice (Zhang et al. 2018a). With the advancement and ease in the accessibility of small RNA sequencing technologies, we have witnessed a surge in the identification and annotation of a large repertoire of miRNA families in diverse plant species. More recently several comprehensive databases such as PmiREN (Plant miRNA Encyclopedia; https://pmiren.com; Guo et al. 2022) and sRNAanno (http://www.plantsrnas.org; Chen et al. 2021) in addition to miRBase (the microRNA database; https://www.mirbase.org; Kozomara et al. 2019) have cataloged miRNA discovery, annotation, and functional analysis in diverse plant species. This has enabled comparative genomics to explore the evolution of miRNAs across diverse groups of plant lineage. While several of the miRNA families exhibited lineage or species-specific occurrences, most are conserved along with the evolution of plants (Cupe-rus et al. 2011).

MIR408 is one of the most conserved regulators of plant growth, development, and stress response (Zhang et al. 2017; Pan et al. 2018; Zhang et al. 2018b; Feng et al. 2013; Balyan et al. 2017). miR408 is a critical node in the copper economy mode conserved in different plant species (Zhang and Li 2013; Yamasaki et al. 2009). In rice, the miR408-UCL8 (Uclacyanin-like protein 8) module is implicated in the control of grain yield and photosynthesis (Zhang et al. 2018a). Recently, PHOTOCYCLE INTERACTING FACTOR 1 (PIF1)-mediated direct transcriptional repression of miR408 modulated the post-transcriptional regulation of blue copper protein, PLANTACYANIN (PCY), during the early germination in Arabidopsis (Jiang et al. 2021). In response to copper and light, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7) and ELONGATED HYPOCHOTYL 5 (HY5) coordinate the transcriptional activation of miR408 resulting in repression of targets to allocate copper to plastocyanin (Zhang et al. 2014). MIR408 overexpression in Arabidopsis, rice, and tobacco showed increased chloroplastic copper, plastocyanin abundance, and photosynthesis in addition to higher rates of vegetative growth and enhanced seed size and weight (Pan et al. 2018; Song et al. 2018). Furthermore, miR408-TaTOC1s (Triticum aestivum TIMING OF CAB EXPRESSION-A1) is involved in the regulation of heading time in wheat (Zhao et al. 2016). MIR408-mediated enhanced biomass and yield traits were primarily due to cell expansion (Song et al. 2018). In addition to being the conserved positive regulator of plant growth and development, miR408 is also reported to be modulated in response to several environmental cues. MIR408 overexpressing cowpea lines displayed enhanced drought and salinity tolerance (Mishra et al. 2021). Moreover, the improved drought tolerance due to overexpression of MIR408 was also reported in ryegrass (Hang et al. 2020) and chickpea (Hajyzadeh et al. 2015). In addition, MIR408 overexpression leads to enhanced heat stress tolerance in ryegrass (Taier et al. 2021).

Previously, our group demonstrated the cultivar-specific drought stress-mediated response of miR408 in rice where its levels were upregulated in the flag leaf of tolerant cultivar but reduced in sensitive cultivar (Balyan et al. 2017; Mutum et al. 2013). In rice, the predominant targets of miR408 are several members of the plantacyanin family (Mutum et al. 2013). In addition, several other genes from diverse families are also reported as targets of miR408 as evidenced from prediction as well as parallel analysis of RNA ends (PARE) data analysis. However, in rice, only a few targets were discussed in detail. Further, how the spatiotemporal and environment-specific abundance and regulation of miR408 could tailor the cleavage and abundance of its target transcripts still needs to be clarified. In addition, besides having few reports on miR408-mediated drought tolerance in plants, direct evidence of it in rice is still lacking.

Here in the present study, we map the detailed conservation and diversification of miR408 and its targets in plants. Complementing the miR408 levels with RNA-seq profiles of putative targets in flag leaf, inflorescence, and root under control and drought-stressed conditions of N22 rice identified several high-confidence targets. Considering the drought-regulated tolerant cultivar-specific upregulation of
miR408, here, the overexpression (OE) of MIR408 construct was generated in drought-sensitive rice cultivar. MIR408-OE leads to the massive enhancement of vegetative growth in rice with an improved electron transport rate (ETR) and effective photosynthetic efficiency Y(II) and ROS levels. At transcriptome-wide scale, drought-responsive miR408-regulon contributes towards the drought response majorly by photosynthetic light harvesting in photosystem I, starch and sucrose catabolism, zinc ion transport, phytocyanin, and cupredoxins. Further, MIR408-OE lines perform better than wild type (WT), suggesting miR408 as a positive regulator of growth, vigor, and dehydration response in rice. Therefore, miR408 could be an important regulator for engineering and achieving crops with enhanced vigor, yield, and drought tolerance.

Materials and methods

Plant material and stress conditions

During the onset of summer, seedlings of N22 and PB1 were raised under culture room conditions (temperature = 28 °C ±2 with a photocycle of 14 h light and 10 h dark; 60–75% relative humidity) and nourished with rice growth medium (RGM; Yoshida et al., 1976). One-month-old seedlings were transplanted to the beds prepared specially for control and drought stress application at university of Delhi South Campus (UDSC) fields. Special care has been taken to avoid any water flow towards stress beds and for that plastic shelters were designed to protect from rain and boundaries were covered with plastic sheets. As the duration of each cultivar is different so the experiment was planned in such a way that equal stress was given to each cultivar. For drought stress, water supply was stopped 10 days before the expected date of 50% flowering of each cultivar. Stress was measured by estimating the soil moisture content (SMC) using the Hydra Probe® Soil Sensor (Stevens® Water Monitoring System, Inc.) and by observing rolling of leaves. Flag leaf and spikelets from control and stressed plants at heading stage of both PB1 and N22 were collected and immediately frozen in liquid nitrogen and stored at −80 °C until use.

Multiple sequence alignment and phylogenetic analysis of miR408 family

The precursor and mature miR408 sequences from plant species belonging to different families were extracted from PmiREN2.0 (Plant miRNA Encyclopedia; https://www.pmiREN2.0 (Plant miRNA Encyclopedia; https://www.pmiREN2.0 (Plant miRNA Encyclopedia; https://www.pmiREN.com; Guo et al. 2022). Multiple sequence alignment followed by phylogenetic tree construction of precursor miR408 was performed using the alignment tool of CLC genomics (version: 9, Qiagen). The evolutionary tree was constructed using the maximum likelihood method and Tamura-Nel model (Tamura et al. 2021).

miR408 target identification and their conservation analysis

Degradome-identified targets of miR408 were extracted from PmiREN2.0 (Plant miRNA Encyclopedia; https://www.pmiREN.com) for 13 plant species available in the database. Targets belonging to category ≤ 2 and cleavage evidence from the ≥ 1 degradome library were considered. For studying the conservation of targets of miR408 in plants, the degradome-identified targets from different plant species were extracted from PmiREN2.0 (Plant miRNA Encyclopedia; https://www.pmiREN.com). The degradome analyzed targets were used to extract their full-length complementary DNA (cDNA) sequences from Phytozome (version: 13, https://phytozome-next.jgi.doe.gov) followed by their phylogenetic analysis. The miR408 target genes from 16 different species with degradome evidence were used for conservation analysis. A phylogenetic tree was built using the NGPhylogeny.fr (https://ngphylogeny.fr) tool following the PhyML 3.0 (Guindon et al. 2010) workflow. The tree was converted into a circular format and visualized using the MEGA11 software (Tamura et al. 2021).

miRNA and mRNA expression analysis

The total RNA was extracted using TRI-reagent (Sigma-Aldrich) followed by DNase I treatment (Thermo Scientific) as per the manufacturer’s instructions. The quality and quantity of RNA were analyzed on Nanovue (GE Healthcare Life Sciences) followed by verification of RNA (1 μg) on formaldehyde agarose gel (1.2 %). The high-quality total RNA (A260/280 = 1.8–2.0 and A260/230 = ≥ 2.0) was used to enrich the small RNA using equal volumes of 4M LiCl followed by polyadenylation using a Poly(A) Tailing Kit (Ambion). An amount of 2 μg of polyadenylated small RNA and total RNA was reverse transcribed using miR_oligoT_RTQ (100 bp) (Ro et al. 2006) and oligodT (25 bp), respectively, followed by cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen) as per instructions. All the primers for the real-time PCR analysis were designed using the “Primer Express 2.0” software (expected product size = 100–120 bp) (Life Technologies) followed by the individual pair confirmation using the BLAST program in the RGAP database (rice.plantbiology.msu.edu; Kawahara et al. 2013) against genomic and cDNA sequences.
To analyze the expression of miRNAs, qRT-PCR was performed using a reaction mixture (7 μl) containing cDNA (2 fold diluted; 1 μl), 2X Taqman Fast Universal PCR master mix (3.5 μl, Applied Biosystems) with 10 mM miR408 specific forward primer (0.6 μl), 10 mM universal fluorogenic probe (0.16 μl) specific to miR_oligodT_RTO, and 10 mM RTQ universal reverse primer (0.6 μl) in Rotor Gene Q (Qiagen) according to the manufacturer’s protocol. For target gene expression and pre-miR408, Fast SYBR Green master mix (3.5 μl) was used with gene-specific primers (0.3 μl each), and 1 μl of the diluted cDNA template to make the final reaction of 7 μl was run on Rotor Gene Q (Qiagen) as per manufacturer’s protocol. Rice SS and Actin were used as endogenous control for miRNA and mRNA expression, respectively. The ΔΔCt method was followed to calculate the relative fold change (2−ΔΔCt) in expression. The sequence of primers is listed in Table S1. For all qRT-PCR-based expression analysis, at least three biological and three technical replicate were analyzed.

Gene ontology analysis and pathway analysis

The Gene Ontology (GO) enrichment analysis was executed using the ShinyGO tool (v 0.7.41 http://bioinformatics.sdstate.edu/go/; Ge et al. 2020) following a P-value cutoff (FDR) of ≤ 0.05.

RNA-seq profiles of miR408-3p targets in rice

The expression of miR408-3p targets was extracted from the flag leaf, inflorescence, and root of N22 under control as well as drought as conditions from an in-house generated transcriptome of N22 (NCBI accession: PRJEB47431: Gour et al. 2021). Transcriptome data of the wild type and MIR408 transgenic seedlings were obtained from NCBI-SRA (Accession no. PRJNA412295; Zhang et al. 2017). The raw RNA-seq files were analyzed using the RNA seq tool of the CLC genomics workbench (version: 9, Qiagen) to obtain the expression values of genes.

Determining mCs

Methylation levels in different tissues under control and drought conditions were determined by analyzing the whole genome bisulfiite datasets using the bisulfite sequencing plugin of the CLC Genomics Workbench (version: 9, Qiagen) following default parameters. Briefly, in order to call mCs, we deployed a bidirectional protocol to map to the reference genome. The mapped reads were used as an input for calling the methylation level based on a Fisher exact test (p-value ≤ 0.05) after removing non-specific matches, duplicate matches, and broken pairs reads. The error rate was determined by aligning reads to rice chloroplast and found to be ~1.44% in both the control and drought stress samples. After incorporating the error rate, binomial distribution was calculated and the mCs were filtered based on a p-value ≤ 0.005 with at least 5 reads in both the replicates. The mCs passing all these criteria were used for further analyses. Transcript information from RGAP (Rice Genome Annotation Project; V7.0: http://rice.uga.edu; Kawahara et al. 2013) was used to annotate the methylated sites through R Bioconductor packages of GenomicFeatures (v 1.46.1; Lawrence et al. 2013) and ChIPSeeker (v1.30; Yu et al. 2015). All the analyses were performed using custom R scripts built in-house. All the relevant information regarding methylated cytosines in the promoter and precursor region was extracted for flag leaf, roots and inflorescence under drought conditions.

Identification of sequence variation and haplotype evaluation in 4726 rice accessions

The DNA sequence variations (SNPs and Indels) in the miR408 precursor and its 1kb upstream regulatory regions were detected in 4726 accessions using the “variations by region tool” of RiceVarMap v2.0 (http://ricevarmap.ncpgr.cn; Zhao et al. 2021). The variation IDs were then used as input for haplotype analysis using the “Haplotype Network analysis” tool of RiceVarMap2.

Cloning of miR408 precursor into binary vector and plant transformation

To generate the Ubi::MIR408 construct, the 213 bp [Chr1:12301661-12301873 (+)] pre-miR408 was PCR amplified using Phusion (Thermo) Taq DNA Polymerase from genomic DNA isolated from N22 seedlings using 5′-AGCGGTACCAGGTATTCAGATTGG-3′ and 5′-AGAGCTCACAGAGAGAAGAGAGAAG-3′ primers. The PCR product was cloned into the pB4NU overexpression binary vector between the KpnI and SacI restriction sites. The construct was cloned in E. coli and confirmed by digestion and sequencing and then mobilized into Agrobacterium for plant transformation. The transgenic rice plants overexpressing MIR408 under drought-sensitive PB1 background were generated as per the protocol (Toki et al. 2006). To obtain the homozygous transgenic plants, mature seeds from the T1 transgenic plants were germinated on fresh 1/2 × MS medium containing 50 mg/L Hygromycin. Homozygous transgenic plants, heterozygous plants, and negative plants were differentiated by their germination ratios (100, 75, and 0%, respectively). Four homozygous lines were used for morphometric analysis at the 15-day-old seedling stage. Then, the detailed morphophysiological (40-day-old) and dehydration stress tolerance assay (60-day-old) were
Morphometric and dehydration stress analysis of the over-expression transgenic plants

The wild type (WT) and MIR408-OE seeds were sterilized with 70% ethanol and then with 0.1% HgCl₂, having a few drops of Tween-20 with shaking for 15 min. They were then washed with reverse osmosis (RO) water several times and soaked in RO water overnight in the dark. The next day, the seeds were spread on a cotton bed in small plastic trays evenly moistened with RO water. Trays were covered with a clean film and allowed to grow at 28 °C ± 2 in culture room conditions with a photocycle of 14 h light and 10 h dark for 1 week in rice growth medium (RGM; Yoshida et al., 1976). One-week seedlings were transferred to test tubes and grown hydroponically under culture room conditions (temperature = 28 °C ± 2 with a photocycle of 14 h light and 10 h dark; 60–75 % relative humidity). The transgenic lines were analyzed for their morphometric differences from the wild-type plants. The shoot length, root length, root density, 3rd leaf length and width (from the midpoint), and whole plant weight at different stages (15-day-old; 40-day-old) were recorded for both transgenic lines and wild-type plants. For most of the lines, more than 10 plants were analyzed for each parameter. Measurements of relative water content (RWC) were determined as described previously (Dansana et al. 2014). Leaf segments measuring 5 cm were cut with the help of scissors and weighed immediately to determine the fresh weight (FW). Then, the segments were submerged in water for an overnight period and their turgor weight (TW) was recorded. To determine the dry weight (DW), the segments were dried in the oven for 24 h and weighed. The RWC was calculated using the following equation: \((FW-DW)/(TW-DW)\) ×100.

Sixty-day-old WT and MIR408-OE plants were given polyethylene glycol (20% PEG-8000)-induced dehydration stress (Mahreen et al., 2022; Yang et al., 2012) in nutrient solution (Yoshida et al., 1976) in glass tubes for 1 and 7 h followed by 2 days of recovery in a nutrient solution without PEG-8000. The control plants were maintained in normal nutrient solution only.

Chlorophyll content and fluorescence measurement

Chlorophyll was extracted from the wild type (WT) and transgenic leaves with dimethyl sulfoxide (DMSO). An amount of 50 mg of finely chopped leaves was incubated with 1 ml of DMSO at 65 °C for about 1 h. A volume of 50 µl of the extract was diluted to 200 µl, and the absorbance was measured at 645 nm and 663 nm on infinite® M200 PRO (Tecan). The concentration of total chlorophyll and chlorophyll a/b was calculated by the equation proposed by Arnon (1956). The chlorophyll fluorescence parameters were measured using a pulse-amplitude modulation fluorometer (Junior-PAM, H. Waltz, Germany). Plants were dark-adapted for 30 min before measuring the maximum photosynthetic efficiency (Fv/Fm). The electron transport rate (ETR) and effective photosynthetic efficiency (YII) were recorded in light-adapted plants. More than 10 plants were used for each reading.

Results

Conservation and diversification of miR408 across plant kingdom

To extensively track the sequence diversity and conservation of miR408 in the plant kingdom, we have extracted the mature (miR408) and precursor (pre-miR408) sequences from 97 plant species annotated in the PmiREN database covering bryophytes, spermatophytes, lycophytes, magnoliophyta, and dicotyledons (Fig. S1a). miR408 was not annotated in Chlorophyceae (green algae) in any of the databases. In the majority of the plant species (n = 63), the miR408 family comprises a single precursor (Fig. 1a). However, several plant species do show expansion of the miR408 family and exhibited two (eighteen species), three (nine species), four (six species), and six (one species) precursors (Fig. 1a). The highest number of miR408 family members, i.e., six precursors (MI408a-f) were annotated in Triticum aestivum (Fig. 1a). The size of miR408 ranges from 20 to 22 bp, but most of the miR408 precursors (n = 142) generate 21 bp mature miR408 (Fig. 1b; Table S2). Next, we investigated the sequence conservation of the MIR408 family at the level of mature and precursor levels in the plant kingdom. As expected, the mature miR408 showed a high degree of conservation across 97 plants species wherein the core miR408 is represented by “UGCACUGCCCUUCCCGGCU” (20 bp) except Han-miR408, Can-miR408, Sha-miR408, and Syl-miR408, wherein one SNP in the core sequence was detected (Fig. S1b). The variations were observed at the 5’ and 3’ end, respectively. The plant miR408 family is represented by 14 mature sequence forms (F) annotated across diverse plant groups (Fig. 1c). The miR408-F3 (UGCACUGCC UCUUCCCGGCU) is the most predominant, abundant, and universal mature form arising from the processing of 67 miR408 precursors annotated in diverse plant species ranging from simpler bryophytes (Physcomitrella patens), spermatophytes (Ginkgo biloba and Picea abies) to Liliopsida (Araceae, Asparagaceae, Musaceae and Poaceae) to eudicots (Actinidiaceae, Asteraceae, Brassicaceae, Caricaceae, Convolvulaceae, Euphorbiaceae, Fabaceae,
### c

| miR408 Sequence Variant | No. of Precursors | Lignans | Phenylpropanoids | Sterols | Terpenoids | Triterpenoids | Alkaloids | Cinnamic acid derivatives | Indole alkaloids |
|-------------------------|------------------|--------|------------------|---------|------------|--------------|-----------|---------------------------|-----------------|
| F1                      | USGACAGGCUUUUCUGCCU | 16     | 1                | 2       | 1          | 3            | 1         | 1                         | 1               |
| F2                      | USGACAGGCUUUUCUGCCU | 12     | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F3                      | USGACAGGCUUUUCUGCCU | 6      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F4                      | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F5                      | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F6                      | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F7                      | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F8                      | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F9                      | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F10                     | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F11                     | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F12                     | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F13                     | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| F14                     | USGACAGGCUUUUCUGCCU | 5      | 1                | 2       | 1          | 2            | 1         | 1                         | 1               |
| Total                   |                  | 185    |                  |         |            |              | 1         |                           |                 |

### d

- miR408 precursors > 1
- Node colors indicate the number of miR408 precursors.
Malvaceae, Nelumboonaceae, Oleaceae, Rosaceae, Rutaceae, Solanaceae, Amaranthaceae, Araliaceae, Apiaceae, Cleomaceae and Rhamnaceae) (Fig. 1c). In addition, the other prominent miR408 mature form, i.e., miR408-F-10 (AUGCACUGCCUCCUCCUGGC) was mostly present in Fabaceae (n = 23) followed by other eudicots. Interestingly, miR408-F-7 (CUGCAGUCCUCUUCCUGGC) was present only in Poaceae (n = 16). Other sequence variants were confined to only a few species (Fig. 1c). As the different miR408 forms have a variable 5’ base, the role of different ARGONAUTs (AGOs) in addition to AGO1 in miR408 loading in a species-specific context is quite a possibility. The extensive conservation of mature miR408 across diverse plant groups suggests the indispensable function of miR408 in shaping key processes of plant development.

To assess the evolutionary conservation and diversification of the miR408 family in the plant kingdom, 156 miR408 precursor sequences from 97 plant species were used as the input for alignment followed by phylogenetic analysis using the maximum likelihood method and Tamura-Nel model in MEGA11 (Table S3). The miR408 precursor alignment demonstrated the peak conservation in and around the miRNA/miRNA* regions (Fig. S2). The plant miR408 family can be divided into six groups represented by lycophytes (G-I), bryophytes (G-II), gymnosperms (G-III), Malvales (G-IV), Poales (G-V), and eudicots (G-VI) (Fig. 1d). To our surprise, the Tae-MIR408b was clustered together with Smo-MIR408s (lycophytes). The Atr-MIR408 (Amborella trichopoda) was distinct from other lycophytes and placed alone in the tree. Meanwhile, all the pre-miR408s from different species of Malvaceae clustered together (G-IV) (Fig. 1d).

Most of the species of this family have four miR408 precursors. The miR408 precursors from all species belonging to Poaceae were clustered together with Nelumboonaceae and Amaranthaceae in G-V. All other eudicots were represented in a giant cluster (G-VI) constituting several sub-clusters (VI-A to I) (Fig. 1d). The sub-cluster VI-A constitutes the pre-miR408 of the Solanaceae family, represented by eight species. Another family-specific sub-cluster was seen for Brassicaceae (VI-F). Several species belonging to different dicot families were clubbed together in sub-cluster VI-I, wherein Fabaceae predominates the most (Fig. 1d).

### Comparative analysis of DNA sequence variations and haplotype evaluation in rice

To delineate the extent of sequence variation in upstream regulatory and precursor pre-miR408 in available sequenced genomes of rice, the precursor and 1 Kb upstream cis-regulatory region (Ch01: 12300661 to 12301873) was analyzed for sequence variations (SNP and Indels) across 4726 rice accessions using the RiceVarMap2 (http://ricevarmap.ucr.ac.cn; Zhao et al. 2021) database. As a result, a total of 22 sequence variants were detected, out of which 15 lie within the promoter while seven lies in the putative precursor region downstream of mature (Fig. 2a–b). Four variants (vg0112300662, vg0112300679, vg0112300683, and vg01123001149) that differ from the reference represent the primary allele with a frequency ranging from 0.647 to 0.678 (Fig. 2a–b). In addition, the other three variants (vg0112300734, vg0112300737, vg0112300779) were also very abundant in rice accessions. Eight variants represent low frequency and are present in small subsets of rice accessions (vg0112300685, vg0112300704, vg0112301017, vg0112301021, vg0112301030, vg0112301098, vg0112301361, and vg0112301478) (Fig. 2a–b).

Next, the overlap and intersection of the above variants were studied with the presence of cis-regulatory motifs in the promoter region to see possible disruption or modification of cis-regulatory motifs. Therefore, the promoter region was screened for different cis-regulatory motifs using the New Place database (https://www.dna.affrc.go.jp/PLACE/?action=newplace, Higo et al. 1999) and the resulting motifs were mapped on the promoter region using CLC genomics (version: 9, Qiagen) (Fig. S3). Five variants, i.e., vg-5, 6, 7, 9, and 10, intersect with several cis-regulatory motifs. Variant vg-5 overlaps in the SEF4 binding site (SEFMOTIFGM5S), while vg-6 interferes with the binding site of WRKY71 (WRKY71OS) and GTGA motif (GTGANTG10). Adjacently, vg-7 overlaps with the binding sites of DOF proteins (DOFCOREZM). Interestingly, vg-9 and 10 lie in the region with TATABOX5 and MARTBOX (Fig. S3). The variants that lie in the promoter region were used to evaluate the haplotype using the Haplotype Network Analysis tool of RiceVarMap2. As a result, eight haplotypes were calculated using the following variants: vg0112300662, vg0112300679, vg0112300683, vg0112300685, vg0112300734, vg0112300779, vg0112301030 and vg0112301361 (Fig. 2c–d). Haplotype I, IV and VIII were dominated by japonica accessions while hap-II, III, V, VI, and VII were indica-specific (Fig. 2c, d). The variations in the miR408 precursor and promoter region between N22 and PB1 were already revalidated in our previous studies (Mutum et al.)
The comparison of the precursor sequences in the above varieties revealed identical sequences, except for a substitution at position 146 bp in N22. Thus, the differential expression of miR408 in these rice varieties in response to drought conditions cannot be accounted for by differences in their precursor or promoter sequences.

Fig. 2 Sequence variants and haplotype analysis of miR408 and its promoter region in 4726 rice accessions. a The plots show the position and frequency of the primary allelic variants identified in the region of pre-miR408 and its promoter region (1 kb) using the Rice-VarMap2 database. The coordinates marked with green and red lines represent the promoter and precursor of miR408, respectively. b The detailed list of variants with primary and secondary alleles. c The plot shows the result of the haplotype network analysis generated by Rice-VarMap2. Only haplotypes found in ≥10 rice accessions were used to construct the haplotype network. d The details of eight haplotypes were detected on the basis of variants in the promoter region.

2013 and Balyan et al. 2017). The comparison of the precursor sequences in the above varieties revealed identical sequences, except for a substitution at position 146 bp in N22. Thus, the differential expression of miR408 in these

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Transcriptional and epigenetic regulation of miR408 in N22

To investigate the impact of different tissue types on the regulation of the miR408-target module under control as well as in drought conditions in tolerant rice cultivar, N22, the expression of miR408-3p was extracted from the small RNA sequencing data available for flag leaf, inflorescence and roots of N22 under control and drought conditions (PRJNA294727; Zhang et al. 2017). Under control conditions, miR408-3p was highly expressed in flag leaf (Fig. 3a) as compared to roots and inflorescence. In flag leaf and roots of N22, miR408-3p was upregulated while it showed slight downregulation (non-differential) in inflorescence under drought stress (Fig. 3b). Then, we examined the pre-miR408 levels in roots and flag leaf of N22 under drought conditions to see whether the differential regulation was also seen in the precursor or not. Similar to the mature, the precursor was also upregulated in flag leaf and roots (Fig. 3c). In summary, miR408-3p exhibited differential expression in tissue as well as in drought in tolerant N22 rice.

Furthermore, to understand the role of DNA methylation in deriving the drought response of miR408-3p, the single-base resolution of methylation levels was examined in all three tissues under control and drought conditions. For the above analysis, the pre-miR408 along with 1 kb cis-regulatory region was used, and the differential methylation levels in a different context (CpG, CHH, and CHG) was extracted from in-house generated whole-genome bisulfite sequencing (WGBS) data available for flag leaf, inflorescence and root of N22 under drought stress. No methylated cytosine (mCs) was observed in the promoter region while all the observed mCs lie in the precursor region (Fig. 3d-e). Under control conditions, a higher fraction of mCs representing all three contexts were observed in inflorescence. In addition, mCs in the CHG context were only observed in inflorescence and their fraction further increased under drought conditions (Fig. 3d-e). While under drought conditions, the fraction of mCs in the CpG and CHH context was increased in flag leaf as well as in roots. The enhanced fraction of mCs in the CpG and CHH context in the precursor in flag leaf and root might be contributing towards the upregulation of miR408-3p under drought (Fig. 3d, e).

Comparative expression analysis of miR408-3p/ target module

To understand the diversity and conservation of genes targeted by miR408 in plants, the targets having evidence from the degradome were obtained from the PmiREN database for Oryza sativa, Arabidopsis thaliana, Brachypodium distachyon, Glycine max, Zea mays, Medicago truncatula, Physcomitrella patens, Solanum lycopersicum, Solanum tuberosum, Triticum aestivum, Brassica napus, Selaginella moellendorffii, and Vitis vinifera. Phylogenetic analysis of all the above targets clearly showed the enrichment of the blue copper family as the dominant target class including plantacyanins. In addition, laccases were also regulated by miR408 in Glycine max and Solanum lycopersicum (Fig. S4). A cluster of copper-transporting ATPases as miR408 targets was also observed in Zea mays, Solanum lycopersicum, and Arabidopsis thaliana (Fig. S4). Several genes encoding for glycin-rich proteins were also cleaved by miR408 in Brassica napus, Oryza sativa, Arabidopsis thaliana, and Solanum lycopersicum (Fig. S4). The above data suggest that the pool of miR408 targets is diverse with a preference for genes encoding for the blue copper family.

In rice, miR408-3p predominantly targets the plantacyanin family members, while several other targets belonging to diverse protein classes were predicted as well as identified through degradome analysis from diverse tissue types and cultivars. However, in literature-reported studies, only a few targets have been associated with miR408-mediated regulation (Singroha et al. 2021). Furthermore, it is critical to understand how miR408-3p derives the differential regulation of its target transcripts in different tissue types and stress conditions. It is possible that miR408 may result in complete suppression of some targets while at the same time acting as a rheostat for others to slightly dim their expression or even have no effect. The above relationship could also be impacted by basal expression of the miR408-target node in different tissue types vis-à-vis environmental conditions. Therefore, here in the present study, efforts were directed to delineate the regulatory activity of miR408-3p on modulating its target transcripts by utilizing the comparative miRNome vs. transcriptome analysis in different tissues of N22 under drought stress. The miRNome and RNA-seq resources generated by our group were used to extract the miRNA-target expression in flag leaf, inflorescence, and root under drought stress at the heading stage of development. The targets with evidence of cleavage by miR408-3p identified through degradome analysis were retrieved from the PmiREN database (category ≤ 2 and cleavage evidence from more than one degradome dataset). In total, 119 genes were targeted by miR408-3p including its bona fide target gene family consisting of 11 plastocyanin-like domain-containing proteins (Table S4). In addition, several other types of genes encoding different types of protein classes were also identified from the degradome data. Out of the 119 targets, 9 belong to the “0” category, while 9 and 94 were among categories 1 and 2, respectively. Seven targets, LOC_Os03g49820 (score: 2, expressed protein), LOC_Os01g02110 (score: 2, helix-loop-helix DNA-binding domain-containing protein), LOC_Os01g03530 (score: 1.5, multicopper oxidase domain-containing protein), LOC_Os07g43540 (score: 2, ORC6-Putative origin recognition
Fig. 3 Transcriptional regulation of miR408 in different tissues of N22 under control and drought stress conditions. a miR408-3p expression (transcript per million values: TPM) in flag leaf (FL), inflorescence (INF), and roots (RT) of N22 under control conditions. b Drought-mediated expression of miR408-3p in the root (RT), flag leaf (FL), and inflorescence (INF) of N22 under drought conditions. The relative fold change in expression (TPM values) of miR408-3p under drought in comparison to control conditions was plotted in the graph. c The qRT-PCR mediated expression analysis of pre-miR408 in flag leaf (FL) and roots (RT) of N22 in response to drought. Rice actin was used as an internal control. For each condition, three technical and three biological replicates were analyzed. The error bars in graphs denote standard error. d–e The comparison of methylation levels (single base level) of different contexts, i.e., CpG (orange triangle), CHG (green square), and CHH (blue circle) in the promoter (1 kb upstream of pre-MiR408) and precursor region (Ch01: 12301661-12301873) of MiR408 in different tissues of N22 under control and drought conditions. Only the methylation levels following the p-value criterion of ≤ 0.05 were shown in the Figure.
Expression analysis of miR408-3p targets in miR408-OE plants

To further study the impact of pre-miR408 overexpression on its target transcripts, the RNA-seq data of seedlings of MIR408 overexpression and WT rice were downloaded from NCBI (BioProject PRJNA412295, Zhang et al. 2017) followed by RNA-seq analysis using CLC genomics (version 9, Qiagen). The differential expression of pre-miR408 and its targets were extracted and the relative fold expression in MIR408-OE with respect to WT was calculated. In the MIR408-OE line, the pre-miR408 was eight times upregulated confirming the optimum level of overexpression in lines. Out of 119 targets, 11 genes (LOC_Os04g46130, LOC_Os02g52180, LOC_Os08g37670, LOC_Os06g11490, LOC_Os02g06530, LOC_Os03g50160, LOC_Os03g51340, LOC_Os03g09940, LOC_Os03g01930, LOC_Os10g36580, and LOC_Os03g07590) exhibited significant downregulation (fold change (FC) ≤ 0.5) in MIR408-OE (Fig. 4d). Twenty-one targets were reduced to an FC range from 0.80 to 0.50
Fig. 4 Comparison of miR408-3p and its target gene expression in rice. a The expression comparison of miR408-3p and its target genes in roots (RT) with respect to flag leaf (FL) under control conditions. The drought-mediated comparative expression analysis of miR408-3p target module in roots (b) and flag leaf (c) of N22. d qRT-PCR validation of root-enriched drought-responsive miR408-3p targets and pre-miR408 in roots of N22. The expression was the mean of three biological replicates, and three technical repeats were used. Rice actin gene was used as the endogenous control. e The relative expression levels of different miR408-3p targets and pre-MiR408 in RNA-seq of transgenic rice seedlings overexpressing miR408 were demonstrated by box plot (BioProject PRJNA412295; Zhang et al. 2017). Each dot represents one target gene. f The upset plot showing the intersection and overlap of miR408-3p targets showing opposite expression to miR408-3p in different conditions in rice.
in MIR408-OE. A large number of targets, i.e., 61 showed a non-differential expression pattern in MIR408-OE. Moreover, 17 targets were observed to be slightly upregulated upon MIR408 overexpression (Fig. 4e; Fig. S6).

To summarize, the miR408-target regulatory relationship across different tissues, overexpression lines and drought, and the comparative intersection of targets following the inverse expression to miR408 levels were studied. The data suggest the coherent co-expression of the miR408-target module in all tissues but both miR408 and some targets do exhibit tissue preference (Fig. S6). Furthermore, the dynamic selection and miR408-mediated regulation or the degree of anticorrelation in the expression of target nodes were modulated by the tissue types and stress conditions. Out of 119 targets, 83 genes showed antagonistic expression to miR408-3p in either of the studied domains (Fig. 4f). One target gene encoding for PIRIN (LOC_Os08g27720) exhibited antagonistic expression with miR408-3p in all four contexts. Another set of 11 genes including four PLANTACYANINS (OsUC16: LOC_Os02g52180, 7: LOC_Os03g15340, 9: LOC_Os03g50160 and 30: LOC_Os08g37670), OsLPR1 (LOC_Os01g03530), OsCHUP1 (LOC_Os03g07590), OsDOF12 (LOC_Os03g07360), OsBGLU1 (LOC_Os03g53800), glycine-rich cell wall gene (LOC_Os10g31660), OsDUT (LOC_Os03g46640), and OsERF7 (LOC_Os06g07030) are among the high confidence targets as they followed anticorrelation under three conditions including few root-specific targets (Fig. 4f). Twenty-five targets are significantly modulated by the tissue-mediated (flag leaf to root) as well drought-mediated dynamic shift in the expression of miR408-3p in roots. The above observation highlights the critical role of miR408-3p in shaping the root’s target pool at the tissue as well as at the stress tolerance level. Twenty-seven showed anticorrelation in any two conditions (Fig. 4f). While a large number of targets were only observed to be regulated in an opposite manner in one condition, e.g., 10 in FL (drought), 11 in MIR408-OE, 9 in the root (drought) and 14 in a tissue biased manner (root/flag leaf) (Fig. S6). To understand the exact role of miR408 targets, the trait ontology (TO) terms associated with the genes were extracted from the rice Oryzabase database (https://shigen.nig.ac.jp/rice/oryzabase, Fig. S6b). Several targets were observed to be associated with salt tolerance (eight genes), drought tolerance (five genes), bacterial blight (four genes), plant height (four), jasmonic acid sensitivity (three), heat tolerance (three), and cold tolerance (three) suggesting the central possible role of miR408-3p in multiple stress tolerance (Fig. S6b). The above data demonstrated the impact of tissue and stress on the mode of miR408-directed regulation of target transcripts suggesting that at a particular set of conditions, miR408 derives significant clearance of some targets while acting as a buffering agent for others to eliminate the noise.

High levels of miR408 enhances the vegetative vigor and dehydration stress tolerance of the drought sensitive rice

Our present and previous data (Balyan et al. 2017; Mutum et al., 2013) clearly demonstrated the important role of the miR408-target module in the drought stress response of rice cultivars. Considering the drought-tolerant cultivar-specific expression of miR408-3p and lack of functional evidence of miR408-3p-mediated drought tolerance, over-expression MIR408 rice lines were generated in the drought-sensitive cultivar, PB1 background (Wild type: WT) (Fig. S7). Four homozygous transgenic lines were obtained and subjected to an in-depth phenotypic analysis in 15-day-old plants in T2 generation (Fig. S7). The overexpression lines exhibited variable magnitude of miR408-3p upregulation (Fig. S7b), and the highest overexpression was observed for MIR408-OE-16 (~16-fold; Fig. S7b). All four MIR408-OE lines exhibited significant increments in shoot length, root length, and seedling weight, suggesting miR408 as a positive regulator of rice growth and vigor (Fig. S7). In comparison to other MIR408-OE lines, line 16 showed the highest growth enhancement and miR408-3p expression; therefore, MIR408-OE-16 was selected for further investigation at T2 generation. Comprehensive morphometric analysis of 40-day old WT and MIR408-OE-16 (referred to as MIR408-OE in upcoming sections) plants clearly showed the role of miR408-3p in enhancing vegetative growth and development of rice plants (Fig. 5). Plants show vigorous growth and reach the 4-leaf-stage, whereas control WT plants reach only the 3-leaf-stage (Fig. 5a, b). There is a significant increase in shoot length, fresh weight, root length, and root density (Fig. 5c–f; n = 10). In addition to an increase in the leaf blade length, there is a significant increase in the leaf blade width as well (Fig. 5g–i; n = 10). The chlorophyll content (chlorophyll “a” and “b”) increased by as much as 20–25% (Fig. 5j; n = 10). While the maximum photosynthetic efficiency (Fv/Fm) is similar, there was a significant increase in the effective photosynthetic efficiency Y(II) and electron transfer rate (ETR) in the overexpression lines even under normal growth conditions (Fig. 5k and l).

At the molecular level, the expression of seven degradome identified targets were analyzed in MIR408-OE-16 plants. As expected, five (LOC_Os03g15340, LOC_Os08g37670, LOC_Os02g43660, LOC_Os06g15600, LOC_Os03g50160, LOC_Os06g11490, LOC_Os09g29390) targets were downregulated while LOC_Os08g37670 remained non-differential (Fig. 5m). Surprisingly, LOC_Os03g50160 was upregulated in MIR408-OE plants (Fig. 5m).

As miR408-3p followed cultivar-biased drought regulation, it showed upregulated expression in tolerant cultivars and downregulated expression in sensitive ones (Balyan et al. 2017; Mutum et al., 2013). To understand the impact
of miR408 on dehydration stress tolerance in sensitive rice cultivar, the 60-day-old WT and MIR408-OE plants were screened for dehydration stress tolerance (Fig. 6) by growing in rice growth medium (Yoshida et al., 1976) along with 20% PEG-8000 (Mahreen et al., 2022; Yang et al., 2012). After 1 h of PEG-induced dehydration stress, clear leaf rolling with a needle-like appearance was visible in WT plants (Fig. 6a–c). In contrast, the leaves of MIR408-OE had just
started to roll from the tips and no needle-like leaf was seen (Fig. 6a–c). Even after 7 h, MIR408-OE plants were in better condition with some expanded leaves (Fig. 6d). Further, transgenic plants performed better than WT plants during recovery (Fig. 6e). The impact of drought stress was estimated on basis of the expression of the drought-induced stress marker gene, bZIP transcription factor (OsbZIP23; Xiang et al., 2008; Balyan et al., 2017). Here also, dehydration stress leads to the upregulation of OsbZIP23 in WT and MIR408-OE plants (Fig. 6f) indicating that plants experienced sufficient drought stress. Comparative physiological evaluation of the WT and transgenic lines showed slightly higher relative water content (RWC) (Fig. 5g; n = 3) and increased Fv/Fm values (Fig. 6h; n = 5) after 7 h of PEG treatment in MIR408-OE plants. Although there is an overall decrease in the Y(II) and ETR in both WT and MIR408-OE, but MIR408-OE plants still maintain higher Y(II) and ETR than the WT plants (Fig. 6i–j; n = 5). Similarly, the plant weight also decreased in stress conditions in both WT and MIR408-OE, still, the MIR408-OE maintained a much higher mass as compared to WT in stress conditions (Fig. 6k; n = 5). The histochemical staining of leaf segments for the detection of hydrogen peroxide (H$_2$O$_2$) using 3,3′-diaminobenzidine (DAB) showed a slight increase in the H$_2$O$_2$ levels in MIR408-OE plants under control conditions (Fig. 7a). As reported previously also in WT plants the levels of H$_2$O$_2$ falls upon dehydration (Fig. 7a). While MIR408-OE plants maintained higher H$_2$O$_2$ levels in both conditions (Fig. 7a). Furthermore, the regulation of pre-miR408 was checked in WT and transgenics under dehydration. As reported previously also (Mutum et al. 2013), pre-miR408 was decreased in WT (PB1) upon dehydration stress and the as-expected increased levels are observed in MIR408-OE under control as well as under dehydration stress (Fig. 7b). To enquire further into the reason of high H$_2$O$_2$ levels, the expression analysis of key genes involved in the reactive oxygen species (ROS) pathway has also been done (Fig. 7b). Results showed comparatively higher expression of copper-zinc superoxide dismutase (CzSOD; LOC_Os07g46990), catalase-A (CAT-A; LOC_Os02g24000), catalase B (CAT-B; LOC_Os06g51150), and ascorbate peroxidases (APX1- LOC_Os03g17690 and APX2- LOC_Os07g49400) were observed in MIR408-OE under control as well as in dehydration stress (Fig. 7b). As the canonical targets of miR408 belonging to the phytocyanin family were already analyzed in MIR408-OE plants and mostly followed downregulation due to miR408 (Fig. 5m). Here, the qRT-PCR expression analysis of some high-confidence targets identified in the study other than the canonical phytocyanin targets was performed under dehydration in WT and MIR408-OE plants. All four targets [OsLPR1 (LOC_Os01g03530), Ubiquitin family protein (LOC_Os01g68950), OsDOF12 (LOC_Os03g07360), and expressed protein (LOC_Os02g49870)] were upregulated in dehydrated WT plants opposite to miR408 expression (Fig. 7b and c). While low levels of the above targets were observed in MIR408-OE in control and dehydration except for LOC_Os02g49870. The above molecular characterization suggests that the modulation of miR408 levels upon overexpression and dehydration was also reflected in the regulation of its targets. In the previous sections, the regulation of miR408 targets has been shown in different tissues and MIR408-OE through comparative transcriptomics (Fig. 4). The transcriptome data of miR408-OE (PRJNA412295; Zhang et al. 2017) showed differential regulation of a large number of genes belonging to different classes in addition to its targets. Therefore, to see the drought-response of global miR408-regulon (genes differentially regulated in MIR408-OE transcriptome), the miR408-mediated up (FC ≥ 3) and downregulated (FC ≤ −3) genes were extracted and their drought responsive expression dynamics were compared in different tissue (flag leaf, inflorescence and root) under drought stress in N22 (Fig. 7d). Further, the miR408-regulated and dehydration-responsive genes were characterized through gene ontology enrichment analysis (Fig. 7e–f). The miR408-upregulated and dehydration-responsive gene clusters were involved in photosynthesis, light harvesting, starch, and sucrose metabolism; zinc-ion transport; isoprenoid biosynthesis, and lipid biosynthesis (Fig. 7e). Further as expected, the miR408-downregulated and dehydration-responsive gene cluster demonstrates enrichment of plantacyanin, cuperodoxin, polysaccharide catabolism, and lipid transfer processes. The above data suggest miR408 may act as a positive regulator of growth, development and water stress tolerance in rice.

**Discussion**

Several miRNA families in plants are conserved across diverse lineages, while the majority are confined to some specific group or species (Cuperus et al. 2011). miR156, miR159, miR160, miR166, miR171, miR390, miR408, and...
miR395 are present in all plant lineages, while several others displayed restricted taxonomic conservation, e.g., miR528 (monocots), miR472 (rosids) and miR857 (eurosids) (Cuperus et al. 2011). miR408 was one such conserved miRNA family that was reported to have a key role in mechanisms regulating growth, development, stress response, and...
Fig. 7 Impact of miR408 on drought stress-regulated transcriptome in rice. 

a. Histochemical staining by DAB for the detection of H$_2$O$_2$ accumulation in WT and MIR408-OE leaf segments under control and dehydration stress. 
b. qRT-PCR analysis of key genes involved in the maintenance of ROS homeostasis in the WT and MIR408-OE plants under control (C) and dehydration stress (Deh). Data represents the average value of at least two biological and three technical replicates. 
c. The expression analysis of miR408 targets in WT and MIR408-OE plants under control (C) and dehydration stress (Deh). 
d. Comparative drought regulation of differentially regulated genes (up: FC ≥ 3; down: FC ≤ 0.4) reported in miR408-OE transcriptome in flag leaf (FL), inflorescence (Inf), and roots (RT) of N22. 
e–f The Gene Ontology enrichment-based characterization if miR408-mediated drought stress-regulated genes using ShinyGO (version 0.77). Data represents the average value of at least two biological and three technical replicates. Rice actin gene was used as the endogenous control, and ddCt method was used to calculate the relative fold expression.
miR408 was reported to be associated with both AGO1 and AGO2 in Arabidopsis (Maunoury and Vaucheret 2011). The origin and activity of isomiRs processed from several miRNAs including miR408 were reported in rice (Balyan et al. 2020). At the level of precursor, the regions around the miR408 and miR408* exhibited extensive conservation. The course of evolution through phylogenetic analysis suggested the origin of miR408 in bryophytes followed by gymnosperms. The miR408 from basal eudicot Nelumbo nucifera shared homology with the Poaceae-specific clade. The Malvaceae clade seems to be more ancient than other eudicots. The giant dicot clade was further divided into sub-clades.

miR408-target modules exhibited differential tissue/drought-mediated regulation in rice

In our previous study, we have reported the drought-tolerant cultivar-specific upregulation of miR408-3p in flag leaf (Balyan et al. 2017; Mutum et al. 2013) of rice. Here, the comparative expression profile of miR408-3p in flag leaf, roots, and inflorescence of N22 showed that it was expressed in higher amounts in flag leaf and low levels were maintained in roots. Furthermore, miR408-3p followed upregulated expression in roots and flag leaf under drought due to the transcriptional upregulation of its precursor in both the tissues. Furthermore, OsSPL9 was known to regulate the transcriptional activation of pre-miR408 under copper starvation conditions induced by drought stress in N22 (Balyan et al. 2017). Other than that, the transcriptional regulation of miR408 is yet to be uncovered in rice. Dynamic DNA methylation is critical in shaping the transcriptome of rice under natural as well as under specific environmental conditions including drought (Rajkumar et al. 2020; Wang et al. 2016). However, only a handful of reports demonstrated the role and impact of DNA methylation on the transcriptional regulation of MIR genes in plants (Ci et al. 2015; Rambani et al. 2020). Here, the comparative single base methylation levels, mCs in the promoter and MIIR408 gene body in flag leaf, root and inflorescence under control and stress conditions suggest the possible role of epigenetic regulation of MIIR408 loci in N22 which needs to be proved further. The dynamic methylation sites were only identified in the miR408 gene body and not in the promoter. mCs in the CpG and CHH context are prevalent in all three tissues but the CHG context was specific to inflorescence only. Moreover, the significant increase in the fraction of mCs in the CpG and CHH context under drought in both roots and flag leaf followed a positive correlation with the miR408-3p expression. The positive correlation between the expression and the methylation levels in the gene body was also observed in previous reports (Liang et al. 2019; Wang et al. 2016).

Importantly, the above observed tissue-mediated drought-responsive differential expression of miR408-3p mediates nutritional requirements (Zhang et al. 2017; Pan et al. 2018; Song et al. 2018; Ma et al. 2015). The present study investigated the miR408 taxonomic conservation across the plant kingdom followed by in-depth characterization of expression dynamics of miR408-target modules in drought stress in rice. Moreover, overexpression of MIR408 enhanced the vigor, photosynthesis, and dehydration stress tolerance of drought-sensitive rice suggesting the role of miR408 as a positive key regulator of both rice growth and dehydration response.

miR408 is highly conserved across plants

miR408 was shown to be taxonomically conserved across plants (Pan et al. 2018; Hang et al. 2020) but the support was from a limited number of species. Recently, the extensive annotation of miR genes in plants was performed by several groups and cataloged in different databases, providing an excellent opportunity to study the evolutionary conservation and diversification of miRNAs in plants. We utilized the 156 miR408 precursors from 97 plant species representing different taxonomic groups of plants from bryophytes to eudicots, for detailed comparative sequence analysis at mature and precursor levels. miR408 was absent from chlorophytes and bryophytes as suggested by its presence in Marchantia polymorpha and Physcomitrella patens (sRNAnno and PmiREN). Unlike multigene conserved families such as miR156 and miR172, miR408 is represented by only a single precursor in a majority of species (n = 63), while in several species, expansions did happen as evidenced from multiple miR408 precursors, e.g., wheat where six miR408 precursors have been annotated. The plant miRNA family members derived from the same family are mostly similar at mature as well as at precursor levels suggesting their recent and still progressing expansion (Li and Mao 2006). Here, the different precursors of miR408 and mature sequence share high homology within a species suggesting their origin due to tandem duplications. Further, ~92% of miR408 precursors processed into 21 bp mature miR408, while 20 and 22 bp mature were also found in some species. The mature sequence is fairly preserved and the divergence was only restricted to 5′ and 3′ ends. Furthermore, the plant miR408 can be divided into 14 mature sequence variants, out of which three forms, i.e., miR408-F3, 7 and 10 dominate over other forms. miR408-F-7 (CUG CAC UGC CUC UUC CCU GGC) was observed to be confined to only species belonging to Poaceae, while miR408-F-10 (AUG CAC UUC CCU GGC) was mostly seen in Fabaceae. The most abundant miR408-F3 (UGCCUCUCCCCUGGC) showed ubiquitous distribution across different plant groups. As the above three forms differ in 5′ base, it is interesting to investigate the involvement of different AGO proteins in miR408 loading in a species-dependent manner.
the post-transcriptional regulation of their target pool differentially depending on stress, tissue and the stage of development. The differential pools of targets are regulated by miR408-3p at the level of tissue (root vs. flag leaf), drought stress (in roots and flag leaf), and MIR408 overexpression. Several miR408 targets followed tissue preferential expression including some of the top category targets which exhibited root-specific expression in N22. The tissue-restricted expression could be one of the reasons for the non-identification of targets in a degradome with very low alignment scores. This suggests that the cellular context plays an important role in regulating the miR408-3p target pool in N22. A recent report showed that the patterns and levels of miRNAs in maize are largely determined at the level of transcription and finely tuned post-transcriptionally in a tissue-dependent manner (Ma et al. 2021). Based on the condition/tissue-specific target expression versatility, 83 targets have been identified with inverse expression to miR408 in at least one condition. Furthermore, 12 targets are regulated inversely in most conditions suggesting the critical role of miR408-3p in post-transcriptional regulation of these targets. Root-specific, LOC_Os10g31660 encode glycine-rich cell wall structural protein 2, followed opposite expression in roots under drought as well as in flag leaf vs roots. It was regulated in response to drought in rice (Zhang et al. 2012) and exhibited a root cap restricted expression pattern (Wang et al. 2021c). OsBGLU1 is probably involved in cell wall modification by modulating sucrose metabolism (Wang et al. 2014a). Another high-confidence root-specific target OsERF7 was reported to be upregulated under excess copper treatment (Sudo et al. 2008) and drought stress (Sircar et al. 2015) in rice. OsDOF12 also exhibited inverse expression to miR408-3p in most conditions in N22. In rice, it was involved in flowering under long day conditions (Li et al. 2009). Recently, its role in rice architecture has been delineated by suppressing brassinosteroid signaling (Wu et al. 2015). The above candidates include novel miR408 targets that were unreported in previous reports. A total of 50 miR408-3p/target nodes were responsive to drought, while 49 showed modulations even change in a cellular context.

miR408 likely to act as a positive regulator of drought and vegetative growth in rice

Further insight into the functionality of miR408 was gained by overexpressing miR408 in the drought-sensitive rice cultivar (PB1) to explore the impact of elevated levels of miR408 on rice growth and stress tolerance. In PB1, miR408 was downregulated under drought stress (Mutum et al., 2013; Balyan et al., 2017). Indeed, the transgenic plants were more vigorous than WT plants with enhanced root length, the number of lateral roots, shoot length, leaf width, and seedling weight along with increased chlorophyll. Interestingly, there was no noticeable effect on the Fv/Fm in the transgenic plants but there was a significant increase in the ETR and Y (II) values as compared to the WT PB1 plants. In Arabidopsis, MIR408 overexpressing plants showed higher chlorophyll and plastocyanin levels due to increased copper delivery (Zhang et al. 2014). Plastocyanin role is indispensable for plant photosynthesis and its protein levels and ETR increases with the increase in copper levels (Abdel Ghany and Pilon 2008). Plastocyanin levels are known to affect plant vegetative growth and photosynthesis (Weigel et al. 2003; Pesaresi et al. 2009). The above-observed increase in ETR and Y(II) is due to the post-transcriptional down-regulation of phytocyanin family members which are the canonical targets of miR408 in plants. The levels of several phytocyanins (LOC_Os03g15340, LOC_Os08g37670, LOC_Os02g43660, LOC_Os06g15600, LOC_Os03g50160, LOC_Os06g11490, LOC_Os09g29390) were downregulated. Due to the degradation of phytocyanins, the released copper is diverted to plastocyanin and leads to enhance ETR and plastoquinone and pigments (Shikanai et al., 2003) In rice, the miR408-UCL8 (LOC_Os03g50140) was involved in enhancing grain yield, panicle branching, grain number, and photosynthesis (Zhang et al. 2017). UCL8 showed high enrichment in the pistil, young panicles, seeds, and inflorescence meristem (Zhang et al. 2018a). In the present study, UCL8 was inflorescence enriched but it did not follow drought-mediated inverse expression in N22. Still most of the phytocyanin family remained uncharacterized in rice. Other miR408 high confidence targets other than phytocyanins also downregulated in MIR408-OE and also regulated under drought opposite to miR408. The enhanced growth parameters were in concordance with the results obtained for MIR408 overexpression in rice, Arabidopsis, and tobacco (Song et al. 2018; Pan et al. 2018). As miR408 was downregulated in drought-sensitive cultivar reported by our previous report, we want to see whether the overexpression lines performed better than the WT when challenged with dehydration stress by PEG. Drought tolerant wheat genotypes maintain better ETR levels than the sensitive ones under drought (Subrahmanya et al. 2006). Moreover, miR408 overexpression displayed improved thermotolerance in ryegrass, drought tolerance in ryegrass, drought, and salinity tolerance in cowpea and are important in wounding response in sweet potato. However, the direct role of miR408 in improving drought tolerance in high-yielding, drought-sensitive rice backgrounds is still lacking. The drought-responsive miR408-mediated transcriptome-wide regulon again highlight the role of photosynthesis, light harvesting, sucrose and sugar metabolism, phytocyanins, zinc-ion transport, and copperoxidents. Here, the slight increase in H2O2 levels were observed in MIR408-OE but this was not consistent with the expression of key genes involved in ROS homeostasis. CzSOD, catalases, and APX were upregulated
in MIR408-OE under both control as well as stress conditions. Thus, tolerance of miR408-overexpression transgenic plants could be attributed to the enhanced growth and higher basal levels of ETR and Y(II) even under stress. Enhanced ETR is associated with higher photosynthesis, plant growth, tolerance to oxidative stress (Chida et al. 2007; Takahara et al. 2010), and drought tolerance (Wang et al. 2014b), as was observed in our study. Here, we provide evidence for a novel pathway where the miR408-mediated decrease in the copper containing protein targets provide more copper supply to the plastocyanin which increases the plant ETR, biomass, and ROS and ultimately makes them more tolerant to drought stress.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10142-023-01111-2.

**Author contribution** SR conceived the research and supervised the experiments; SB executed all the experiments and analyzed the data; SK and RJ analyzed the methylation status of the miR408 region; PRB performed the phylogenetic analysis of targets; RC complemented the expression analysis; SR and SB wrote the manuscript.

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**Data Availability** All the data related to the study has been provided within the manuscript as main figures or supplementary material.

**Declarations**

**Ethics approval** Not applicable

**Competing interests** The authors declare no competing interests.

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