



Comparative analysis of MIR168 promoters in three plant species

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ABSTRACT. MicroRNAs (miRNAs) play important roles in the regulation of gene expression by post-transcriptionally targeting mRNAs for cleavage or translational repression. miR168 is a key miRNA because it regulates the expression of the slicer protein ARGONAUTE1 (AGO1), which catalyzes mRNA cleavage. Interestingly, plant miR168s are highly evolutionarily conserved; however, it is unclear whether MIR168 promoter elements and expression patterns are also conserved. Here, we isolated MIR168 promoters from monocot rice and dicot grape genomes. To determine the expression pattern, different promoters were fused to a beta-glucuronidase reporter gene and the resulting constructs were then transformed in *Arabidopsis*. The results revealed clear differences in the MIR168 promoter sequence of monocot and dicot plant species. Moreover, the pattern of MIR168 promoter expression differed between monocots and dicots. These

results suggest that, unlike that of miR168, the MIR168 promoter is not conserved in monocots and dicots.

Key words: MIR168; Promoter; Rice; Grape

INTRODUCTION

MicroRNAs (miRNAs) are a large class of short non-coding RNAs (19-25 nt long), which have emerged as important post-transcriptional regulators of protein-coding genes and target mRNAs for cleavage or translational repression (Sunkar and Zhu, 2004; Bartel, 2004; Voinnet, 2009). miRNAs have important regulatory roles in multiple fundamental processes in plant growth and development, and in the regulation of plant gene silencing (Sunkar and Zhu, 2004; Mallory and Vaucheret, 2006). Moreover, miRNAs are involved in various responses to biotic and abiotic stresses, such as oxidative, drought, mineral nutrient deficiency, and fungal invasion stress (Navarro et al., 2006; Lu and Huang, 2008; Khraiwesh et al., 2012; Gupta et al., 2014).

Among the miRNAs, miR168 represents a group of plant-specific miRNAs, whose target gene, ARGONAUTE1 protein (AGO1), is the basic component of RNA-induced silencing complex (RISC), which is involved in the cleavage of target mRNAs. miR168 plays a pivotal role as it regulates the expression of AGO1, a mechanism through which all microRNAs can down-regulate their targets (Vaucheret et al., 2006; Gazzani et al., 2009). In addition, miR168 is reportedly involved in the regulation of plant growth, fruit development, and response to different stresses, such as UV-B and aluminum, and is also up-regulated during viral infection in plants, during which it is ubiquitous in plant-virus interactions (Várallyay et al., 2010; Lima et al., 2011; Wang et al., 2013; Xian et al., 2014).

Plant miRNAs are evolutionarily conserved in a wide variety of species, ranging from mosses and ferns to dicots (Arazi et al., 2005; Jones-Rhoades et al., 2006; Fattash et al., 2007). Interestingly, miR168a are also highly conserved in different plants, from rice to *Arabidopsis* (Table 1). Despite an increasing number of reports predicting miRNAs and demonstrating their roles in the development and morphogenesis of plants, relatively little is known about the conservation of miR168 regulatory elements in various plant species (Han et al., 2014). It is generally accepted that the ACGT core element (ACE) is functionally important in a variety of promoters that respond to different stresses, such as light (Donald and Cashmore, 1990), jasmonic acid (Mason et al., 1993), and hormones stresses (Shen et al., 1993). The ACGT core sequences are found in *Arabidopsis thaliana* and also in rice (Mundy et al., 1990), wheat (Marcotte et al., 1989), and barley (Shen et al., 1996), suggesting that ACEs are conserved across plant species. Moreover, the copy number of ACGT elements in a promoter can significantly affect gene expression (Mehrotra et al., 2005). In this study, we compared regulatory differences (transcription factor binding sites and expression patterns) controlling the expression of miR168 in different plant families. These findings could lead to a better understanding of miR168 biogenesis and regulatory mechanisms for use in research in different plant species, from monocots to dicots.

MATERIAL AND METHODS

Plant material

Leaves of *Oryza sativa* were collected in Trentino Alto Adige (Italy). Tissue was

collected from *Vitis vinifera*, which were grown in a greenhouse from leaves collected in Trentino Alto Adige (Italy). *A. thaliana*, ecotype Columbia, was used as the wild-type control in this study and grown under long photoperiod conditions (16 h light/8 h dark) at 25°C in a greenhouse. Transgenic *Arabidopsis* seeds were surface sterilized in 5% hypochlorite solution, treated with 70% ethanol, and rinsed in distilled water. Sterilized seeds were plated on solidified Murashige-Skoog (MS) medium with kanamycin, supplemented with 1% sucrose. T1 seeds were germinated and grown in a controlled chamber at 20°C and 40% relative humidity under continuous illumination.

Table 1. *MIR168* sequences of different plant species.

Species	<i>MIR168</i> sequence
<i>OrySat</i>	Ucgcuuuggucagaucgggac (osa-MIR168a)
<i>VitVin</i>	Ucgcuuuggucaggucgggaa (vvi-MIR168)
<i>PopTri</i>	Ucgcuuuggucaggucgggaa (ptc-MIR168a)
<i>SolLyc</i>	Ucgcuuuggucaggucgggac (sly-MIR168a)
<i>AraTha</i>	Ucgcuuuggucaggucgggaa (ath-MIR168a)

Isolation of MIR168 promoter in different species

Genomic DNA of *O. sativa* and *V. vinifera* was extracted from leaves using the Hexadecyltrimethyl Ammonium Bromide (CTAB) method (Doyle, 1987). The upstream fragments of MIR168 were amplified from genomic DNA using the primers detailed in Table 2. Primers were designed on conserved upstream sequences of MIR168 or on the highly conserved sequences of the mature miR168 and miR168*. Amplification products were cloned in pGEM-T (Promega) vectors. At least eight clones corresponding to each product were sequenced bi-directionally to confirm their identity. Multiple sequence alignments were performed with manual editing in Bioedit. PLANTCARE was used to analyze promoter core elements (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Table 2. PCR primer sequences.

Species	Primers
<i>OrySat</i>	F: 5'-CACCTTTGAAACGAGGGATTTTATAGG-3' R: 5'-CGGTACCGGCGCCAAGAGACGACGAGACG-3'
<i>VitVin</i>	F: 5'-GACACTTCTAGCATCTTCAATACA-3' R: 5'-CGATTCAGTTGATGCAAGGCGGGA-3'

Expression analysis of pMIR168 from different species

The determined *pMIR168* regions from three species were cloned into the Gateway pENTR/D-TOPO vector (Life Technologies) according to the manufacturer instructions. The resulting plasmids were recombined with PKGWFS7 to drive the expression of an enhanced green fluorescent protein-beta glucuronidase (eGFP-uidA) fusion reporter construct. The three constructs were pOrySatMIR168a:GUS, pVitVinMIR168:GUS, and pAraThaMIR168a:GUS. Each plasmid was used to transform 4-week-old *A. thaliana* plants by the floral dipping method. A total of 50-60 primary transgenic plants were screened per construct by histochemical β -Glucuronidase (GUS) staining.

Histochemical GUS staining

T1 7-day-old seedlings were harvested. Histochemical GUS staining was performed following the standard procedure (Jefferson et al., 1987) with a modified buffer containing 1 mg/mL X-GLUC (5-bromo-4-chloro-3-indolyl- β -D-glucuronic cyclohexylamine salt), 50 mM sodium phosphate (pH 7.0), 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 10 mM Ethylenediaminetetraacetic acid (EDTA). Plant seedlings were incubated in buffer overnight at 37°C in the dark. Prior to observation, tissue samples were cleared with a graded ethanol dehydration series to 70% ethanol. For GUS quantification, at least 50 independent transgenic lines for each construct were assessed. At least 12 repeats were carried out per line. Microscopic inspection was carried out on a Leica microscope (Leica MZ16F).

RESULTS AND DISCUSSION

DNA sequence variation in MIR168 promoter regions

miR168 sequences are conserved between monocotyledonous and dicotyledonous plants (Table 1). However, the most interesting results relate to the copy number of the G-box (CACGTG) of MIR168 promoters from different species were different. G-box (CACGTG) is the most prevalent G-box in all ACGT classes (Figure 1 and [S1 Figure](#)) (Mehrotra et al., 2013). The MIR168 promoter of *A. thaliana* contains three G-box motifs (CACGTG) (Gazzani et al., 2009). However, no G-box motif was found in the MIR168 promoter of monocot species like rice, and two were found in the MIR168 promoter of dicot species such as grape. Therefore, the evolution of the MIR168 promoter sequence does not correspond closely with that of MIR168 sequences. It is reported that these G-boxes are differentially regulated by the distance between two copies of the motif (Mehrotra and Mehrotra, 2010). Moreover, the G-box copy number in a promoter, and the distance from the transcriptional start site, also result in drastically altered gene expression, causing changes in the pattern of expression and even in resistance (Mehrotra et al., 2005, 2013). Thus, the degree of conservation of different regulatory elements may decrease depending on the motif in monocots and dicots. Of note, no G-box motif was present in the promoter of monocot species, such as rice. Given the variable copy number of the ACGT core sequence, attempts should be made to analyze the expression patterns of the ACGT core element in MIR168 promoters of different plant species.

Differential expression of MIR168 promoters in transgenic plants

To further investigate whether differences in the MIR168 promoters of monocots and dicots determined the similarities and differences in expression, promoters from different species were transformed in *A. thaliana*. We generated a reporter line in which the beta-GUS coding region was placed under the control of MIR168 promoter fragments (pOrySatMIR168a:GUS, pVitVinMIR168:GUS). After GUS staining was performed on single copy transgenic lines, the results showed there were obvious differences in expression patterns among angiosperm, suggesting that the expression patterns of miR168 genes are not conserved (Figure 2, 3, and 4). Two patterns can be observed in pOrySatMIR168a: GUS transgenic plants (Figure 3), and GUS staining can only be detected in the blade tip (Figure 3A) and emerging leaf

primordia (Figure 3B). However, more plant organs of GUS staining can be observed in the seedlings of pVitVinMIR168: GUS and pAraThaMIR168a: GUS transgenic plants, compared with those of pOrySatMIR168a: GUS transgenic plants (Figure 2 and 4). pVitVinMIR168: GUS and pAraThaMIR168a: GUS transgenic plants displayed the same pattern of staining as pOrySatMIR168a: GUS plants. Moreover, the vasculature of the cotyledons was also stained, and GUS expression was observed in the lateral and primary root tips of pVitVinMIR168: GUS transformed plants (Figure 4).

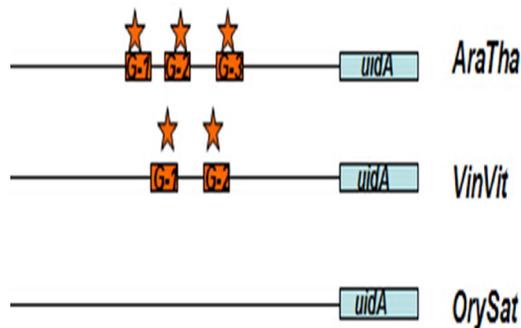


Figure 1. pAraThaMIR168a, pVitVinMIR168, and pOrySatMIR168a:GUS constructs are represented. G-box copy numbers in MIR168 promoters of *Arabidopsis*, grape, and rice are shown. Orange box: G-box.

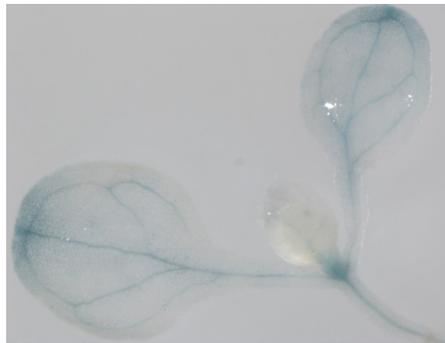


Figure 2. GUS-staining of transformed *Arabidopsis* lines carrying the pAraThaMIR168a:GUS constructs. GUS staining can be observed in the blade tip, vasculature of the cotyledons, and emerging leaf primordia.

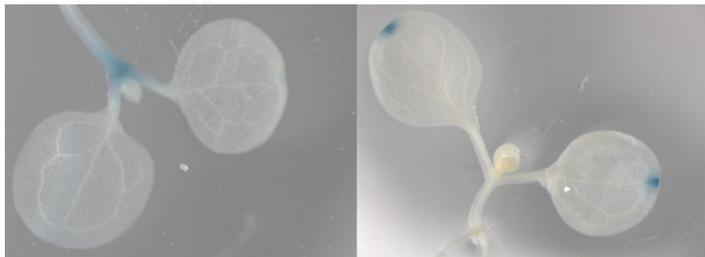


Figure 3. Histochemical analysis of pOrySatMIR168a:GUS overexpression. **A.** and **B.** Two patterns of GUS expression. GUS staining can be observed in the emerging leaf primordia and blade tip.



Figure 4. Histochemical analysis of pVitVinMIR168:GUS overexpression. Three patterns were observed. **A.** GUS expression in emerging leaf, primordia vasculature of the cotyledons and the blade tip. **B.** GUS staining in the lateral root tip, and **C** shows GUS staining in the primary root tip.

The relative position of the ACGT core motif, and its copy number upstream of the promoter, affects the recognition of transcription factors and therefore, affects development and the response to different environments, such as flower development and pathogen defense (Chuang et al., 1999; Zhou et al., 2000; Mehrotra et al., 2005). Multiple ACGT motifs, which bind synergistically to transcription factors, can form enhancer elements (Mehrotra et al., 2013). Our data suggest that there are no G-box motifs in the rice MIR168 promoter, whereas two and three G-boxes can be found in the grape and *Arabidopsis* MIR168 promoters, respectively. Additionally, there were significant differences in the expression patterns of G-boxes as controlled by promoters. We suggest that the apparent preference for a particular copy number shows that the MIR168 promoter of different species might be implicated in different physiological functions across monocots and dicots. In addition, variations in the correlation patterns between monocots and dicots might be implicated in the functional difference in transcriptional regulation. Therefore, further investigation of functional analysis would be of interest.

In conclusion, this is the first study that has attempted to analyze MIR168 promoters in monocots and dicots. Our results demonstrate that obvious differences exist within the MIR168 promoter sequences, although MIR168 sequences are highly conserved in monocots and dicots. We further show that the pattern of miR168 expression in different plant species differed in transformed plants due to the regulation of their MIR168 promoters. Therefore, it may be suggested that MIR168 promoters are not conserved between monocots and dicots. Further studies are required to clarify the regulatory relevance of these MIR168 promoters in monocot and dicots.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

S1 Figure. G-box is highlighted in the MIR168 promoter of grape (two G-box) and rice (no G-box). **A.** MIR168 promoter sequence in rice (no G-box). **B.** MIR168 promoter sequence in grape (two G-boxes; highlighted).