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HOS1 Regulates Argonaute1 by Promoting the Transcription of the MicroRNA Gene *MIR168b* in *Arabidopsis*

Bangshing Wang[1], **Cheng-Guo Duan**[1], **Xingang Wang**[1], **Yueh-Ju Hou**[1], **Jun Yan**[1], **Caiqiu Gao**[1,2], **Jin-Hong Kim**[1,3], **Huiming Zhang**[1], and **Jian-Kang Zhu**[1,4,*]

[1]Department of Horticulture and landscape Architecture, Purdue University, West Lafayette, IN 47907, USA

[2]State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, 26 Hexing Road, Harbin 150040, China

[3]Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, 29 Genmgu-gil, Jeongeup-si, Jeollabuk-do 580-185, Republic of Korea

[4]Shanghai Center for Plant Stress Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

Summary

Proper accumulation and function of miRNAs is essential for plant growth and development. While core components of the miRNA biogenesis pathway and miRNA-induced silencing complex have been well characterized, cellular regulators of miRNAs remain to be fully explored. Here we report that HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) is a regulator of an important miRNA, mi168a/b, that targets the *ARGONAUTE 1* (*AGO1*) gene in *Arabidopsis*. HOS1 functions to regulate plant cold stress responses as an ubiquitin E3 ligase, associate with the nuclear pores to regulate mRNA export and circadian clock and flowering time by binding to chromatin of the flowering regulator *FLC*. We isolated a loss-of-function *Arabidopsis* mutant of *HOS1* in a genetic screen for enhancers of *sic-1*, which is defective in miRNA biogenesis. Like other *hos1* mutant alleles, the new *hos1-7* mutant allele flowered early and was smaller in stature than the wild type. Dysfunction in HOS1 reduced the abundance of miR168a/b but not of other miRNAs. In *hos1* mutants, *pri-MIR168b* and *pre-MIR168b* levels were decreased, and RNA polymerase II occupancy was reduced at the promoter of *MIR168b* but not *MIR168a*. Chromatin immunoprecipitation assays revealed HOS1 protein is enriched at the chromatin of the *MIR168b* promoter. The reduced miR168a/b level in *hos1* mutants results in an increase in the mRNA and protein levels of its target gene, *AGO1*. Our results reveal that HOS1 regulates miR168a/b and AGO1 levels in *Arabidopsis* by maintaining proper transcription of *MIR168b*.

*Corresponding author: jkzhu@purdue.edu.

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Keywords

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Introduction

MicroRNAs (miRNAs) are 21-nucleotide (nt)-long small RNAs that induce post-transcriptional gene silencing through mRNA cleavage and/or translational repression. The functional importance of miRNAs in plant growth and development and stress responses has been extensively documented (Jones-Rhoades et al., 2004; Sunkar et al., 2007; Chen 2008; Zhu 2008; Chuck et al., 2009; Poethig 2009; Rubio-Somoza et al., 2009; Voinnet et al., 2009). In *Arabidopsis*, miRNA biogenesis and the core components involved in this process are well understood. First, RNA polymerase II transcribes the *MIR* genes, and then 5′ cap and 3′ poly A tails are added to produce *pri-MIRNA* transcripts. These *pri-MIRNA* transcripts fold into imperfect stem-loop secondary structures by base pairing within the transcripts. The stem-loop structure of *pri-MIRNA* is processed by DICER-LIKE1 (DCL1), an RNase III enzyme, to remove the 5′ and 3′ ends to produce *pre-MIRNA*, which is further processed by DCL1 into 21-nt-long miRNA/miRNA* duplexes.

Other components required for the proper functioning of DCL1 are HYPONNASTIC LEAVES1 (HYL1) (Dong et al., 2008), a dsRNA-binding protein, and SERRATE (SE), a C2H2 zine-finger protein (Dong et al., 2008). HUA ENHANCER1 (HEN1), a methyltransferase that catalyzes the 2′-O-methylation of the ribose sugar at the 3′ end of miRNA, which helps stabilize the miRNA (Yu et al., 2005). HASTY (HST), a homolog of mammalian EXPORTIN 5, guides the export of the methylated miRNA/miRNA* duplex from nucleus to cytosol (Park et al., 2005). The mature miRNAs exported to the cytosol are incorporated into the ARGONAUTE1 (AGO1) protein, which is a core component of the RNA-induced silencing complex (RISC). The RISC with a specific miRNA scans for complementary mRNA transcripts and directs the cleavage or translational repression at the target mRNAs (Jones-Rhoades et al., 2004; Baumberger and Baulcombe, 2005). Many other components play roles in the production of mature miRNAs, such as ABA HYPERSENSITIVE 1/CAP-BINDING PROTEIN 80 (ABH1/CBP80) and CBP20. Mutations in these genes cause dysfunction during the processing of *pri-MIRNA* transcripts into mature miRNA, which leads to reduced abundance of mature miRNAs (Laubinger et al., 2008). ABH1 may protect the capped miRNA from RNA decay and may function to bring *pri-MIRNA* to DCL1/HYL1/SE for processing of mature miRNA (Chen 2008). The hnRNP-like glycine-rich RNA-binding protein GRP7 showed its role in regulating pre-mRNA splicing (Köster et al., 2014). Recently, additional components involved in miRNA biogenesis have been identified. These include Erecta mRNA Under-expressed (EMU) (Furumizu et al., 2010), TOUGH (TGH) (Ren et al., 2012), STABILIZED1 (STA1) (Chaabane et al., 2013), SICKLE (SIC) (Zhan et al., 2012), and MODIFIER of SNC1 2 (MOS2) (Wu et al., 2013). However, the precise roles of these new components in miRNA biogenesis remain unclear.

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Arabidopsis has 10 AGO proteins (Fagard et al., 2000; Carmell et al., 2002), among which AGO1 is the main protein that mediates miRNA-dependent silencing. Unlike its paralogs, the AGO1 transcript has a sequence complementary to miR168a/b, and *AGO1* mRNA is cleaved at the site of miR168a/b complementarity (Vazquez et al., 2004). Furthermore, a decrease in mature miR168a/b in flowers of *hen1-1* results in an increase in the *AGO1* mRNA level (Vazquez et al., 2004).

The *Arabidopsis* HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) functions as an ubiquitin E3 ligase (Dong et al., 2006). HOS1 is a negative regulator of cold-responsive genes like *CBFs* and of their downstream cold-regulated target genes such as *RD29A* and *COR15A* (Ishitani et al., 1998; Lee et al., 2001; Dong et al., 2006). HOS1 negatively regulates the cold response pathway at least in part by targeting the INDUCER OF CBF EXPRESSION1 (ICE1), which is a MYC transcription factor. ICE1 is important for induction of *CBF* genes under cold conditions (Chinnusamy et al., 2003; Lee et al., 2005), and it is marked by HOS1-mediated ubiquitination for protein degradation (Dong et al., 2006). HOS1 is also involved in regulating flowering time. Two different mechanisms by which HOS1 regulates the flowering pathway have been recently reported. First, HOS1 regulates the abundance of CONSTANS (CO), a photoperiod sensor (Jung et al., 2012; Lazaro et al., 2012). Previous report shows that CO is targeted by CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), a CUL4 E3 ligase, for degradation during dark photoperiods (Jang et al., 2008). Under cold stress conditions, CO is tagged by HOS1 for degradation (Jung et al., 2012). It has also been speculated that HOS1 may be the E3 ligase that targets CO for degradation during light photoperiods (Lazaro et al., 2013). With respect to the second mechanism, HOS1 regulates the transcription of *FLOWERING LOCUS C* (*FLC*) under cold stress by interacting with FVE and HDA6 (Jung et al., 2013). Transcriptional regulation of *FLC* by HOS1 does not involve the degradation of FVE or HDA6 (Jung et al., 2013). In addition, HOS1 associates with the nuclear pore, and is important for circadian clock that has a critical role in gating the cold response (MacGregor et al., 2013).

Here, we report the isolation of a new *hos1* mutant allele, *hos1-7*, from an enhancer screen in the *sic-1* mutant background. We discovered that HOS1 specifically regulates the level of miR168a/b. HOS1 modulates the level of miR168a/b by regulating the transcription of the *MIR168b* gene. We show that HOS1 is important for AGO1 mRNA and protein levels, and suggest that this helps explain the broad function of HOS1 in plant growth, development and stress tolerance.

Results

Identification of the *hos1-7* mutant allele from a *sic-1* enhancer screen

Previously, we found that a loss-of-function mutation in the *SICKLE* (*SIC*) gene resulted in an increase in *proRD29A-LUC* transgene expression under abiotic stresses such as cold, NaCl, and ABA (Zhan et al., 2012). SIC is a proline-rich protein involved in the biogenesis of some miRNAs as well as the degradation of some spliced introns (Zhan et al., 2012). To identify additional cellular factors that may regulate miRNA accumulation, we carried out a forward genetic screen in the *sic-1* background. An EMS mutant population was generated

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in the *sic-1* background for screening for putative enhancers of the *sic-1* mutant. One such mutant was isolated based on its enhanced cold stress-induced LUC expression phenotype. Through map-based cloning, the enhancer mutation was found in the *At2g39810/HOS1* gene (Figure 1A, and S1), which was previously identified as an ubiquitin E3 ligase that functions as a negative regulator of cold stress-responsive gene expression in *Arabidopsis* (Ishitani et al., 1998; Lee et al., 2001; Dong et al., 2006). The *hos1-7sic-1* mutant displayed an enhanced LUC phenotype under cold conditions compared to *sic-1* and the wild type (Col-0 ecotype with *gl-1* mutation harboring *proRD29A-LUC* transgene) (Figure 1B). Based on Northern and quantitative real-time PCR (qRT-PCR) analyses, the *hos1-7sic-1* had higher *LUC* transcript levels than *sic-1* or the wild type under cold treatment (Figure 1C and 1D). Slight increases in endogenous *RD29A* (Figure 1D) and *COR15A* (Figure 1C) were also found in *hos1-7sic-1* compared to *sic-1* and the wild type under the same conditions. The *hos1-7sic-1* mutant had smaller leaves and plant size than *sic-1* or the wild type (Figure S2A). Furthermore, multiple siliques emerged from the same node in *hos1-7sic-1* and in *sic-1* (Figure S2B), and the mature plant was shorter for *hos1-7sic-1* than for *sic-1* or the wild type (Figure S2C and S2D). To confirm that these developmental phenotypes were due to mutations in the *HOS1* gene, we isolated the single *hos1-7* mutant from a backcross to the original wild type; we then compared the developmental phenotype of the *hos1-7* single mutant with that of the previously identified *hos1-3* mutant (SALK_069312, Col-0 background). Consistent with previous reports, *hos1-7* displayed similar developmental phenotypes as *hos1-3*. The leaves were smaller for both mutants than for the wild type (Figure S2E). The *hos1-7* also displayed an early flowering phenotype as reported in other *hos1* mutants (Figure S2F, Ishitani et al., 1998; Lazaro et al., 2012). In addition, the mature *hos1-7* mutant plant was shorter than the wild type (Figure S2G). All of the phenotypes were similar for the *hos1-7* single and the *hos1-7sic-1* double mutant except for the emergence of multiple siliques from one node in the *hos1-7sic-1* double mutant, which may be caused by the *sic-1* mutation. Our observations of the *hos1-7* mutant are consistent with the notion that HOS1 is important for plant growth, development and cold stress responses (Ishitani et al., 1998).

HOS1 is required for proper accumulation of mature miR168a/b in *Arabidopsis*

Because *sic-1* is involved in miRNA biogenesis, we examined whether the mutation in *HOS1*, may also affect the accumulation of miRNA. Accumulation of miR159a and miR165 did not differ between *hos1-7sic-1* and *sic-1*, but miR168a/b was less abundant in *hos1-7sic-1* than in *sic-1* (Figure S3A). We measured compared miRNA accumulation in *hos1-1* (Ishitani et al., 1998) and *hos1-3* (Figure 1A) and in their respective wild types. Accumulation of most of the examined miRNAs did not substantially differ between the wild types and *hos1* single mutants except for miR168a/b (Figure 2A). Accumulation of mature miR168a/b was reduced in both the *hos1* single mutants (Figure 2A). To further confirm the reduction of miR168a/b, we measured the accumulation of miR168a/b in the *hos1-7* single mutant. Consistent with the results obtained with the other *hos1* alleles, a clear reduction in the mature miR168a/b was evident in the *hos1-7* single mutant (Figure S3B). Reduction in mature miR168a/b was compared between *hos1-3* and *mir168a-2* mutant, mature miR168a/b was significantly lower in *mir168a-2* than in *hos1-3* also when compared

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to WT (Figure S3C). Furthermore, increase in *AGO1* transcript level was higher in *mir168a-2* than *hos1-3* (Figure S3D) consistent with reduction in mature miR168a/b.

To determine whether a mutation in *HOS1* is responsible for the reduction in miR168a/b, level, we used complementation lines harboring native *proHOS1::HOS1-4xmyc* in the *hos1-3* mutant allele. The complemented lines had a restored *hos1-3* developmental phenotype (Figure S2H). According to Northern analysis, accumulation of mature miR168a/b was similar in the complemented lines and the wild type but was reduced in *hos1-3* (Figure 2B). Because HOS1 was previously determined to function as an ubiquitin E3 ligase, we next asked whether the reduction in miR168a/b might be due to a dysfunction of HOS1 as an ubiquitin E3 ligase. To help answer this question, we used previously reported transgenic lines, the wild type, and *hos1* harboring over-expressed *ICE1* tagged with *GFP* (*WT/OE GFP-ICE1* and *hos1/OE GFP-ICE1*) (Dong et al., 2006). ICE1 was previously reported as a direct target of the ubiquitin E3 ligase activity of HOS1, i.e., HOS1 functions as an ubiquitin E3 ligase to target ICE1, which leads to ICE1 degradation through a proteasome degradation pathway (Dong et al., 2006). The wild type and *hos1* transgenic lines with *GFP-ICE1* were treated with 50 μM MG132 for 24 hours, which inhibits the proteasome degradation pathway. Treated samples were divided into two subsamples: miRNA levels were determined by Northern blot analysis in one subsample, and protein levels were determined by Western blot analysis in the other subsample. Consistent with Dong et al. (2006), the GFP-ICE1 protein level was higher in *hos1* than in the wild type under the control DMSO treatment (Figure 2C), and GFP-ICE protein level was higher in both *hos1* and the wild type under MG132 treatment than under the control DMSO treatment (Figure 2C), indicating the inhibition of proteasome degradation pathway by MG132 treatment. The reduction of miR168a/b was not recovered by MG132 treatment (Figure 2D), suggesting that the regulation of miR168a/b by HOS1 did not result from a loss of function for HOS1 to cause degradation of ICE1. These results indicated that HOS1 is needed for proper accumulation of mature miR168a/b and that the reduction in miR168a/b may be associated with a malfunction in an unidentified role of HOS1 that differs from its previously reported role as an E3 ligase.

HOS1 functions at the transcriptional level to regulate *MIR168b*

We detected a reduction in mature miR168a/b levels in *hos1* mutant alleles (Figure 2A and S3). To further investigate whether the reduction is due to malfunction at the transcriptional or post-transcriptional level, we measured the levels of miR168a/b precursors in several experiments. Using qRT-PCR, we examined the expression level of *pri-MIR159a*, *pri-MIR168a*, and *pri-MIR168b*. The *pri-MIR168b* level was lower in *hos1* mutants than in the respective wild types (Figure 3A), while *pri-MIR168a* and *pri-MIR159a* levels were similar between the *hos1* mutants and their wild type controls (Figure 3A). To further confirm the qRT-PCR results, we used Northern blot analysis to measure the transcript levels of *pre-MIR168a* and *pre-MIR168b*. Consistent with the qRT-PCR results, the *pre-MIR168b* but not *pre-MIR168a* transcript level was lower in *hos1* mutants than in the wild type controls (Figure 3B). The decrease in *pri-* and *pre-MIR168b* suggests in the *hos1* mutants that HOS1 may affect the accumulation of mature miR168a/b at the transcriptional level and that HOS1 specifically affects *MIR168b* (Figure 3A and 3B).

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The qRT-PCR and Northern blot analysis results indicated that loss of *HOS1* may reduce the transcription of *MIR168b* but not of *MIR168a* (Figure 3A and 3B). To further confirm these results, we performed chromatin immunoprecipitation (ChIP) using an RNA polymerase II-specific antibody to examine the enrichment of pol II at the *MIR168a* and *MIR168b* promoter regions. We detected a decrease in pol II occupancy at the promoter region of *MIR168b* in *hos1-1* compared to the wild type (Figure 3C, right panel), while pol II occupancy was similar in *hos1-1* and the wild type at the *MIR168a* region (Figure 3C, left panel). From these results, it is clear that loss-of-function *hos1* mutants had a deficiency during the transcription of *MIR168b* but not *MIR168a* (Figure 3). This indicates that *MIR168b* is a target for transcriptional regulation by HOS1.

Enrichment of HOS1 protein at the promoter region of *MIR168b*

The specific regulation of *MIR168b* transcription by HOS1 indicates that HOS1 may bind to the *MIR168b* promoter region and regulate its transcription. To test this hypothesis, we conducted a ChIP assay using native *proHOS1::HOS1-4xmyc* transgenic lines to determine whether HOS1 enrichment can be found at the promoter region of *MIR168b in vivo*. HOS1 was previously reported to regulate the transcription of *FLC* (Jung et al., 2013). We used two regions near or at the *FLC* region as positive controls for the ChIP assay based on Jung et al. (2013). Consistent with the previous report, enrichment of HOS1 was detected at the *FLC* locus in our ChIP assay (Figure 4). Furthermore, we detected the enrichment of HOS1 protein at the *MIR168b* region but not at the *MIR168a* region or in the wild type without the myc-tagged HOS1 transgene (Figure 4). To test whether HOS1 protein may bind to the *MIR168b* promoter DNA directly, MBP-tagged HOS1 protein was expressed in *E. coli*, and DNA binding was examined *in vitro* by electrophoretic mobility shift assay (EMSA). Our repeated EMSA experiments failed to detect any HOS1 binding to the promoter region of *MIR168b* or *MIR168a in vitro* (Figure S4). Together, the EMSA and ChIP assay results suggested that HOS1 does not directly bind to the promoter DNA of *MIR168b* but associates with the chromatin of the *MIR168b* promoter to regulate transcription of *MIR168b*.

Mutation to HOS1 affects the proper balance of AGO1 due to reduced miR168a/b

AGO1 is a core component of the RISC complex that represses miRNA targets (Vaucheret et al., 2006; Vaucheret 2009; Va'rallyay et al., 2010). Because the mutations in HOS1 results in a reduction of mature miR168a/b and because miR168a/b directs the cleavage of *AGO1* mRNA to maintain the proper balance and production of AGO1 protein levels (Vaucheret et al., 2004), we suspected that the *hos1* mutations may alter the proper balance and maintenance of *AGO1* mRNA transcript level and AGO1 protein level. qRT-PCR and Northern blot analyses revealed an increase in *AGO1* mRNA transcript level in the *hos1* mutants compared to the wild type controls (Figure 5A). Similarly, the use of an AGO1 protein-specific antibody revealed an increase in AGO1 protein level in the *hos1* mutants (Figure 5B). These results demonstrate that HOS1, by maintaining the proper transcript level of *MIR168b*, plays an important role in maintaining the proper balance of both the *AGO1* transcript level and the AGO1 protein level.

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Discussion

HOS1 is an ubiquitin E3 ligase in *Arabidopsis* (Dong et al., 2006), and it regulates the cold response pathway by targeting ICE1, a transcription factor, which activates downstream cold-responsive genes (Lee et al., 2001; Dong et al., 2006). HOS1 can also regulate the flowering pathway by targeting CO for degradation (Jung et al., 2012; Lazaro et al., 2012). Mutants of miRNA biogenesis are known to accumulate reduced amounts of mature miRNAs, while the *pri-* and *pre-MIRNA* levels are increased compared to the wild type (Han et al., 2004; Vazquez et al., 2004; Lobbes et al., 2006; Yang et al., 2006; Dong et al., 2008; Kim et al., 2008; Laubinger et al., 2008; Zhan et al., 2012; and Chaabane et al., 2013). The *hos1-7sic-1* double mutant showed decreases in miR168a/b compared to its background *sic-1*. Furthermore, other *hos1* single mutant alleles also showed reduced accumulation of mature miR168a/b, while accumulation of other miRNAs tested was not affected by the *hos1* mutations. We found that *pri-* and *pre-MIR168b* transcript levels were reduced in the *hos1* mutants, while *pri-* and *pre-MIR168a* transcript levels were not affected. These results were further supported by ChIP results showing that the enrichment of RNA polymerase II was decreased at the promoter region of *MIR168b* but not of *MIR168a*. These results support the idea that HOS1 promotes proper transcription of *MIR168b* but not *MIR168a*. A similar case of regulation was previously reported in which a mutation of *POWERDRESS* (*PWR*) resulted in the reduction of *MIR172a-c* but not of *MIR172d* or *MIR172e*, in which transcription of *MIR172a-c* was affected by a mutation to PWR (Yumul et al., 2013). Also, the promoter region of *MIR168a* contains an *ARBE* motif where ABFs bind and positively regulate the transcription of *MIR168a* (Li et al., 2012). The overexpression of *ABFs* results in an increase in miR168a/b, and the increase is enhanced by treatment with abiotic stresses such as cold or ABA because of an increase in transcriptional activity (Li et al., 2012). Abiotic stress can also induce miR168a/b by increasing the transcription of *MIR168a* (Jia et al., 2009; Liu et al., 2008; Li et al., 2012). According to our ChIP results, HOS1 was enriched at the promoter region of *MIR168b* but not *MIR168a*, which provides further evidence that HOS1 regulates the transcription of *MIR168b* to maintain the proper accumulation of mature miR168a/b. The transcriptional regulatory role of HOS1 is also supported by a recent study where HOS1 was found to bind to *FLC* chromatin in the presence of FVE to prevent the binding of HDA6, which resulted in activation of *FLC* and a delay in flowering (Jung et al., 2013). The latter research also showed that enrichment of HOS1 at the *FLC* chromatin promoted the transcription of *FLC* (Jung et al., 2013). HOS1 interacts with FVE and HDA6, and the binding of HOS1 to *FLC* chromatin is dependent on FVE (Jung et al., 2013). At the present time, we do not know how HOS1 associates with the chromatin of the *MIR168b* promoter. Since HOS1 does not seem to bind directly to the promoter DNA, it is possible that HOS1 may associate with the promoter chromatin by interacting with other chromatin regulators.

The proper maintenance of AGO1 is essential for plant growth and development because excessive amounts of AGO1 can result in growth defects (Vaucheret et al., 2004). AGO1 homeostasis is mainly controlled by the presence of miR168a/b and the interaction between AGO1 and miR168a/b (Vaucheret et al., 2006; Vaucheret 2009; Va'rallyay et al., 2010). A mutated form of *AGO1* mRNA in the miR168-binding region results in an increase in *AGO1*

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mRNA due to the failure of miR168a/b to bind to the mutated form of *AGO1* mRNA; this causes developmental defects, because a large excess of AGO1 protein interferes with RISC function (Vaucheret et al., 2004). The *hos1* mutants showed reduced mature miR168a/b due to reduction in transcription of *MIR168b*. Consistent with reports in the literature, we detected an increase in *AGO1* mRNA in the *hos1* mutants, and this increase was translated into an increase in the AGO1 protein level. These results are also consistent with previous finding in which a *mir168a-2* mutant resulted in a decrease in mature miR168a/b and an increase in *AGO1* mRNA level (Vaucheret et al., 2009). Previously it was noted that *MIR168a* predominantly produces a 21-nt miR168 while *MIR168b* produces similar amounts of 21-nt and 22-nt miR168 (Rajagopalan et al., 2006). Some researchers have suggested that *miR168b* contributes less than *miR168a*, our result further support this idea, reduction of miR168a/b were lower in *mir168a-2* than *hos1-3* compared to WT. Accumulation of mature miR168a/b was significantly lower in *mir168a-2* mutant compared to *hos1-3*, which indicates *miR168a* is represented more than the *mir168b* in mature miR168a/b. However, *miR168b* has been shown to rescue the developmental defects in 4m-AGO1 (mutated form of *AGO1* mRNA) (Vaucheret et al., 2009). It has also been suggested that both 21-nt and 22-nt miR168a/b may be required for the proper maintenance of levels of AGO1 transcript and protein.

Mutation to the *HOS1* gene results in developmental defects (Ishitani et al., 1998; Lazaro et al., 2012). Like other *hos1* mutant alleles, the newly isolated *hos1-7* allele displayed early flowering and a reduced plant size. Like the *hos1* mutants, plants expressing miR168a/b-resistant forms of *AGO1* mRNAs, *2m-AGO1* and *4m-AGO1*, also over-accumulate *AGO1* mRNAs and produce abnormally small leaves and small plants (Vaucheret et al., 2004). It is possible that the role of HOS1 in regulating miR168a/b and AGO1 levels contributes to the function of HOS1 in plant development. HOS1 also has an important function in cold stress tolerance (Ishitani et al., 1998). The function of HOS1 in plant cold tolerance could not be fully explained by its role as an ubiquitin E3 ligase to cause degradation of ICE1 (Dong et al., 2006). In *hos1* mutant plants, the positive regulator of cold stress responsive genes, ICE1, is more stable; however, the *hos1* mutant plants are more sensitive to freezing without cold acclimation (Ishitani et al., 1998; Dong et al., 2006). HOS1 regulates miR168a/b that in turn regulates AGO1 mRNA and protein levels. AGO1 is important for the function of all miRNAs and tasiRNAs (Jones-Rhoades et al., 2004). Therefore, by regulating miR168a/b and AGO1, HOS1 may affect the function of all miRNAs and tasiRNAs, some of which may be important for plant freezing tolerance in the absence of cold acclimation.

Experimental procedures

Plant materials and growth conditions

To screen for putative mutants affecting the accumulation of miRNAs, we introduced the miRNA biogenesis component *sic-1* mutant (Zhan et al., 2012) to the EMS mutagen and then screened for putative *sic-1* enhancer mutants, *sic-1* was originally isolated in mutant screening in Col-0 ecotype (in *gl-1* mutant) with *proRD29A-LUC* transgene refer to as wild type in this study (Zhan et al., 2012). One such mutant, *hos1-7sic-1,* showed an enhanced LUC phenotype under cold conditions and was identified as a *sic-1* enhancer. See *sic-1* map

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based cloning details in Supporting Methods S1. Two other *Arabidopsis hos1* mutant alleles were used in this study: *hos1-1* was isolated from the EMS pool from C24 ecotype harboring the same *proRD29a-LUC* transgene (Lee et al., 2001, Dong et al., 2006); and *hos1-3* is a T-DNA insertion mutant allele in Col-0 background and was obtained from *TAIR* (SALK_069312c).

A 2314-bp sequence upstream of the *HOS1* initiation start codon with *HOS1* genomic sequence (without termination stop sequence and *3′UTR* sequence) was PCR amplified using Phusion high-fidelity DNA polymerase (M0530S, New England Biolab). The PCR product was then inserted into pENTR/D-Topo according to the manufacturer's instructions (K2400-20, Invitrogen) and named *pENTR/proHOS1::HOS1*. Inserted sequences were confirmed by sequencing, and *pENTR/proHOS1::HOS1* was used as a template for the LR Reaction using Gateway LR Clonase II (11791-100, Invitrogen) as instructed by the manual into a gateway destination vector *pGWB-16* (-4xmyc). The construct carrying *proHOS1::HOS1-4xmyc* was transformed into the *hos1-3* mutant via the traditional floral dip method (Clough and Bent, 1998).

Luciferase imaging

Twelve-day-old seedlings (wild type, *sic-1*, and *hos1-7sic-1*) were kept at 4 °C for 24 h to induce the *proRD29A-LUC* transgene. The LUC images were obtained using a low-light video imaging system (Princeton Instruments) with WinView software (Princeton Instruments; Chinnusamy et al., 2002).

RNA analysis

Accumulations of small RNAs were detected by Northern blot analysis. To obtain small RNAs, the total RNA from 12-day-old seedlings was extracted using Trizol (15596-026, Ambion) according to the manufacturer's instructions. Low molecular weight RNAs were purified using PEG enrichment method, and small RNA Northern blot analysis was carried out as described previously (Zheng et al., 2007). The end labeling method was used to label the probes used in miRNA detection, and the random priming method was used to label the probe used to detect mRNA transcripts by Northern blot analysis. The probes used for Northern blot analysis are listed in Table S1. qRT-PCR was used to check the transcript levels. Total RNAs were extracted using Trizol (15596-026, Ambion) and were treated with Turbo DNase (AM1907, Ambion) to remove DNA contamination. DNase-treated RNAs were used to perform first-strand cDNA synthesis using the qScript Flex cDNA kit (95049-100, Quanta). qPR-PCR was performed as described previously (Zhan et al., 2012) with primers listed in Table S1.

Western blot analysis

Tissue from 12- to 14-day-old seedlings was ground in liquid N_2 and then dissolved in protein extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 10% glycerol (v/v), 1 mM DTT, 1 mM petablock, and protein inhibitor cocktail (11836153001, Roche)). The preparation was briefly mixed with a vortex apparatus and then centrifuged at 4 °C at 15000 g for 30 minutes. The supernatant containing total proteins was collected for protein normalization and western analysis. Anti-GFP tag antibody (11814460001, Roche)

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was used to determine the GFP-ICE protein level, and anti-AGO1 antibody (AS09 527, Agrisera) was used to determine the AGO1 protein level in *hos1* mutants and the wild type.

Chromatin Immunoprecipitation (ChIP)

Twelve-day-old seedlings were collected for ChIP assay. Sample preparation and overall ChIP procedures were as previously described (Wierzbicki et al., 2008). Plants of the transgenic line harboring *proHOS1::HOS1-4xmyc* were first kept at 4 °C for 48 h to induce *in vivo* HOS1 protein activity so as to obtain a strong ChIP signal. The anti-myc antibody (05-724, Milipore) was used to check for the enrichment of HOS1 at *MIR168a, MIR168b*, and *FLC*. An anti-Pol II C-terminal domain (CTD) repeat antibody (ab817, Abcam) was used to check the enrichment of RNA polymerase II at *MIR168a* and *MIR168b* in the *hos1-1* mutant and the wild type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance Statement

Proper accumulation and function of miRNAs is essential for plant growth and development. Here we show a new role of previously identified HOS1 in regulating miR168a/b. We discovered HOS1 regulate *MIR168b* gene during transcription and it is required for proper expression of *MIR168b*. This role of HOS1 helps explain its broad function in plant growth, development and stress tolerance.

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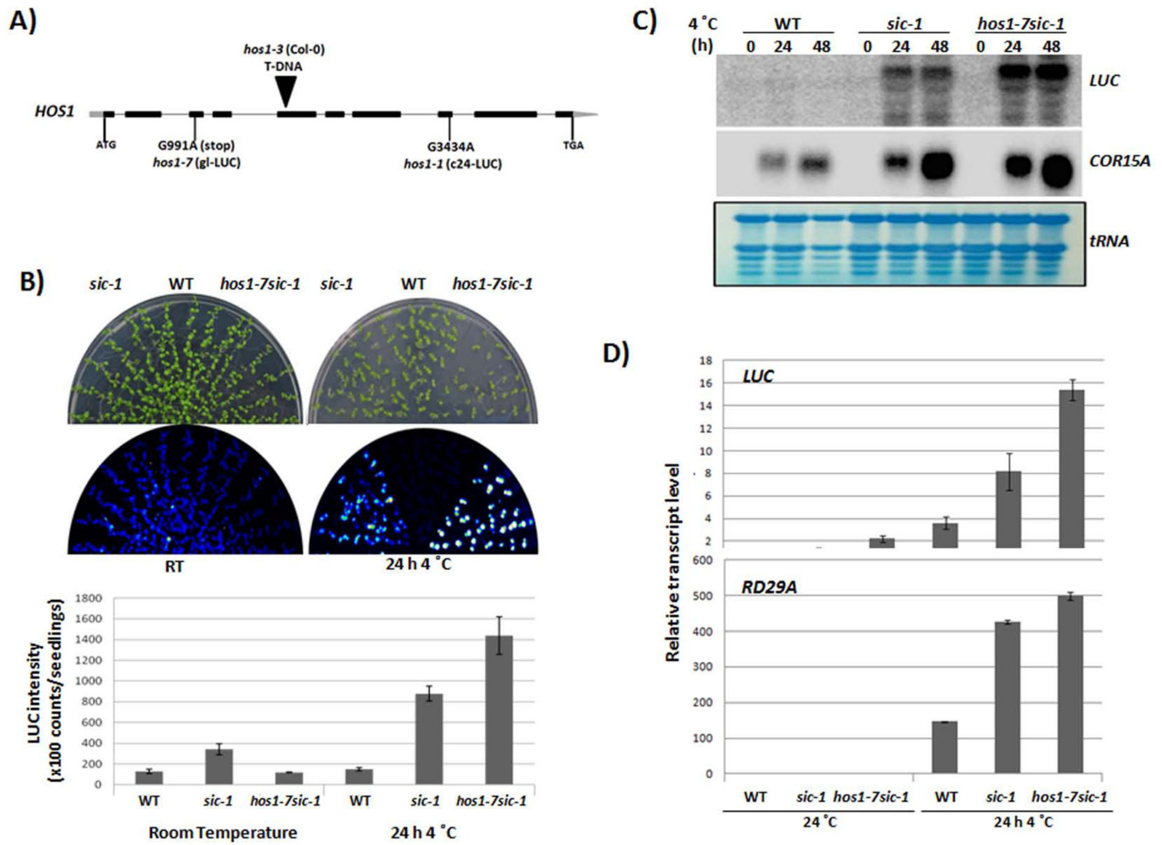

Figure 1.

Identification and characterization of the *hos1-7sic-1* mutant. (A) Diagram of the HOS1 gene and the *hos1* mutant alleles used in this study. (B) Bioluminescence images of wild type (Col-0 ecotype (with *gl-1* mutantion) harboring *proRD29A-LUC* transgene), *sic-1*, and *hos1-7sic-1* under control and cold conditions, and the quantification of LUC intensity in wild type, *sic-1*, and *hos1-7sic-1* DATA are average of 100 seedlings intensity, error bars represent s.d.'s (n = 100). (C) Northern analysis of transcript levels of transgene *LUC* and *COR15A* in gl-LUC, *sic-1*, and *hos1-7sic-1*. (D) qRT-PCR of the relative expression of *LUC* and *RD29A* in *gl-LUC*, *sic-1*, and *hos1-7sic-1*. *Actin2* was used as internal control and error bars represent s.d.'s (n = 4).

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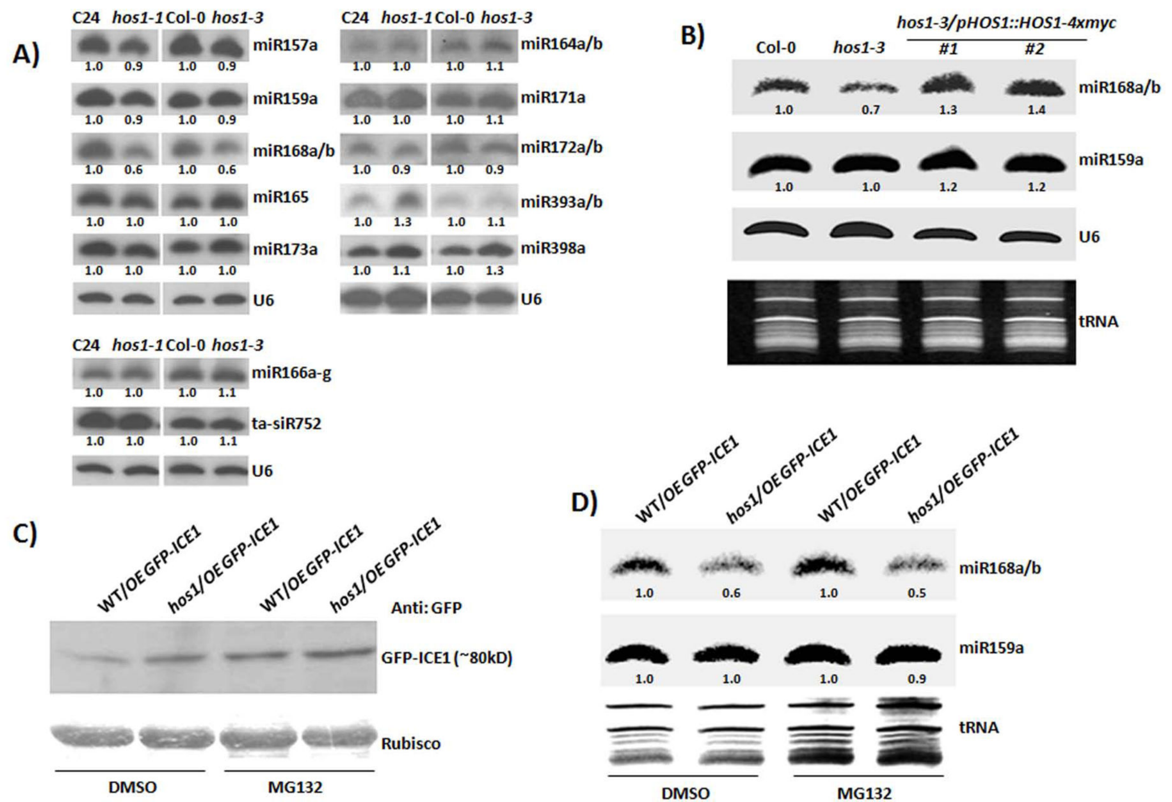

Figure 2.

HOS1 is required for proper accumulation of mature miR168a/b. (A) miRNA accumulation in *hos1* mutants. Two *hos1* mutant alleles (*hos1-1* and *hos1-3*) showed a reduction in mature miR168a/b, while other miRNAs were not affected by the *hos1* mutations. *hos1-1* in C24 ecotype harboring *proRD29A-LUC* transgene and *hos1-3* is T-DNA insertion mutant (Salk_069312c). U6 indicates RNA loading control. Signal intensity was measured with ImageJ and normalized to loading control U6. Relative expression was normalized to wild type. (B) Restored mature miR168a/b in complemented transgenic lines. The genomic DNA sequence of *HOS1* with its native promoter was transformed into the *hos1-3* mutant. miR168a/b was restored to wild type levels in the complemented transgenic lines. U6 indicates RNA loading control. Signal intensity was measured with ImageJ and normalized to loading control U6. Relative expression was normalized to wild type. (C) GFP-ICE protein detection by Western blot analysis. Wild type and *hos1* mutant harboring over-expression of *GFP-ICE1* were treated with 50 μM MG132 for 24 h to inhibit the proteasome mediated protein degradation. DMSO was used as the treatment control. The increase in GFP-ICE1 protein level in the wild type under MG132 treatment compared to DMSO control treatment indicates the expected inhibition of the proteasome degradation pathway. Coomassie blue staining of Rubisco was used as loading control. (D) Accumulation of miR168a/b after MG132 treatment. MG132 treatment did not affect the accumulation of mature miR168a/b, indicating that the accumulation of miR168a/b was not affected by HOS1-mediated ubiquitination and degradation of ICE1. Signal intensity was measured with ImageJ and normalized to loading control tRNA. Relative expression normalized to wild type.

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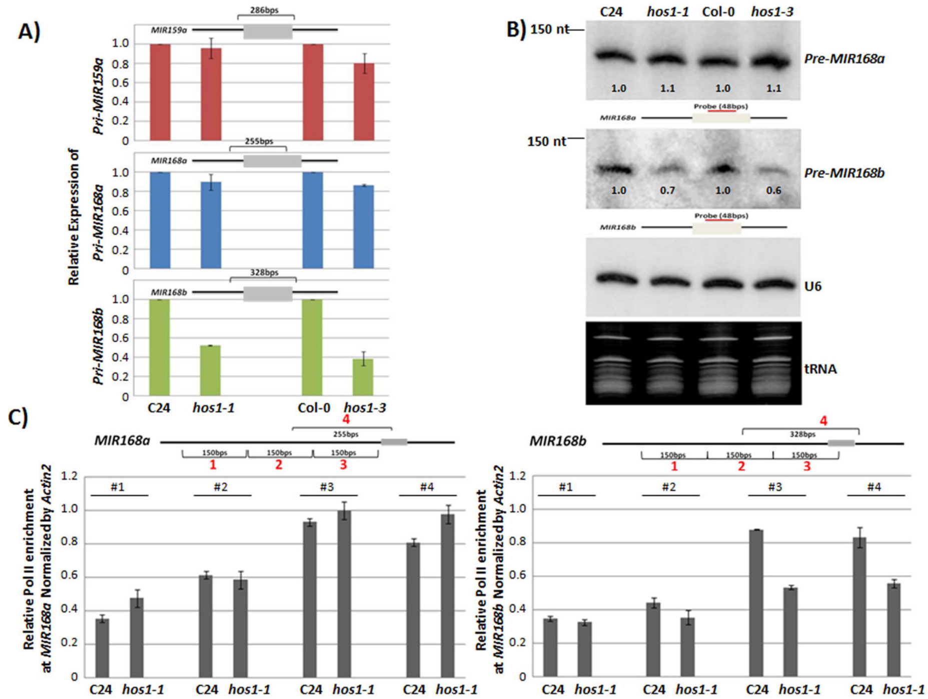

Figure 3.

HOS1 regulates the transcription of *MIR168b*. (A) Reduction of *pri-MIR168b* in *hos1* mutants. *pri-MIR59a*, *pri-MIR168a*, and *pri-MIR168b* levels were examined in *hos1-1* and *hos1-3* mutants. *Actin2* was used as internal control and error bar represents s.d.'s (n = 4). (B) Reduction of *pre-MIR168b* in *hos1* mutants as detected by Northern analysis. U6 was used as an internal control. Signal intensity was measured with ImageJ and normalized to loading control U6. Relative expression was normalized to wild type. (C) ChIP analysis showing the decrease in wild type and *hos1-1* at *MIR168a* (left panel). The ChIP signal was normalized by *actin2* and error bars represent s.d.'s (n = 4).

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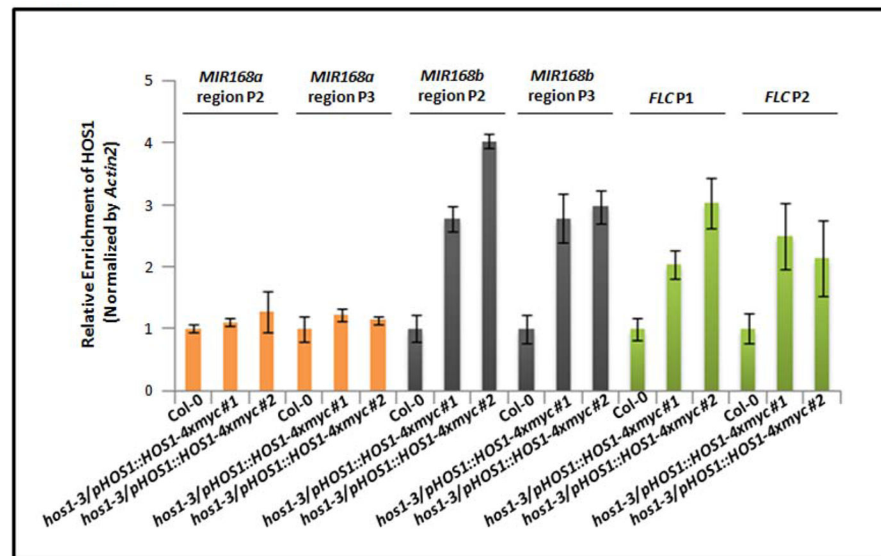

Figure 4.

HOS1 associates with the chromatin at the promoter region of *MIR168b.* ChIP assays using *proHOS1::HOS1-4xmyc* transgenic plants. ChIP assays were carried out using an anti-MYC antibody. *FLC P1* and *P2* were used as positive controls as reported previously (Jung et al., 2013). ChIP signal was normalized by *actin2* and error bars represent s.d.'s (n = 4)

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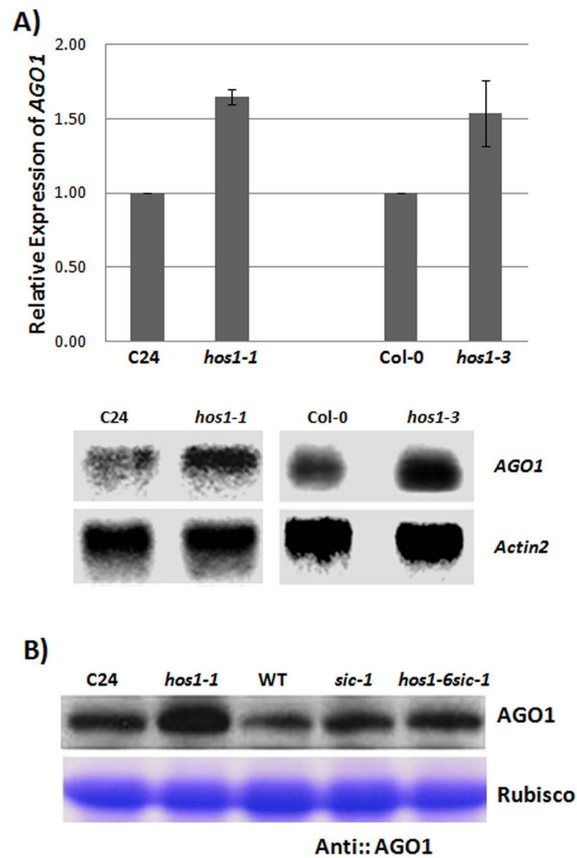

Figure 5.

HOS1 is required for proper maintenance of AGO1. (A) Increase in *AGO1* transcript in *hos1* mutants. qRT-PCR (upper panel) and Northern analysis (lower panel) revealed an increase in *AGO1* mRNA in *hos1-1* and *hos1-3* alleles compared to their respective wild types. *Actin2* was used as internal control and error bars represents s.d.'s (n = 4) for qRT-PCR. Northern signal was measured with imageJ and normalized to loading control actin2. Relative expression was normalized to wild type. (B) Increase in AGO1 protein level in *hos1* mutants. A Coomassie blue-stained band corresponding to the Rubisco largest subunit is shown as a loading control.