HOS1 regulates Argonaute1 by promoting transcription of the microRNA gene MIR168b in Arabidopsis

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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 ARGONAUTE1 (AGO1) gene in Arabidopsis. HOS1 functions as an ubiquitin E3 ligase to regulate plant cold-stress responses, associates with the nuclear pores to regulate mRNA export, and regulates the circadian clock and flowering time by binding to chromatin of the flowering regulator gene FLOWERING LOCUS C (FLC). In a genetic screen for enhancers of sic–1, we isolated a loss-of-function Arabidopsis mutant of HOS1 that is defective in miRNA biogenesis. HOS1 functions as an ubiquitin E3 ligase to regulate plant cold-stress responses, associates with the nuclear pores to regulate mRNA export, and regulates the circadian clock and flowering time by binding to chromatin of the flowering regulator gene FLOWERING LOCUS C (FLC). In a genetic screen for enhancers of sic–1, we isolated a loss-of-function Arabidopsis mutant of HOS1 that is defective in miRNA biogenesis. Like other hos1 mutant alleles, the hos1–7 mutant 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 that of MIR168a. Chromatin immunoprecipitation assays revealed that 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.

Keywords: gene expression, transcriptional regulation, microRNA, MIR168b, HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1, Arabidopsis thaliana.

INTRODUCTION
microRNAs (miRNAs) are 21 nt 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 and Bartel, 2004; Sunkar et al., 2007; Chen, 2008; Zhu, 2008; Chuck et al., 2008; Poethig, 2009; Rubio-Somoza et al., 2009; Voinnet, 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 recognized by Dicer-Like1 (DCL1), an RNase III enzyme, to remove the 5’ and 3’ ends, producing pre-miRNA, which is further processed by DCL1 into 21 nt miRNA/miRNA* duplexes.

Other components required for the proper functioning of DCL1 are HYponastic LEAVES1 (HYL1) (Dong et al., 2008), a dsRNA-binding protein, and SERRATE (SE), a C3H2 zinc-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 stabi-
lize the miRNA (Yu et al., 2005). HASTY (HST), a homolog of mammalian EXPORTIN5, guides the export of the methylated miRNA/miRNA* duplex from the nucleus to the 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). RISCs containing specific miRNAs scan for complementary mRNA transcripts and direct the cleavage or translational repression at the target mRNAs (Jones-Rhoades and Bartel, 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 CAP-BINDING PROTEIN 20 (CBP20). Mutations in these genes cause dysfunction during the processing of pri-miRNA transcripts into mature miRNAs, which leads to reduced abundance of mature miRNAs (Laubinger et al., 2008). ABH1 may protect the capped miRNA from RNA decay, and allow DCL1/HYL1/SE complex to process pri-miRNA into mature miRNA (Chen, 2008). The heterogeneous nuclear ribonucleoprotein-like glycine-rich RNA-binding protein GRP7 has been shown to play a role in regulating pre-miRNA 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.

Arabidopsis has ten AGO proteins (Fagard et al., 2000; Carmelli 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., 2004a,b). Furthermore, a decrease in mature miR168a/b in the flowers of the hen1–1 mutant resulted in an increase in the AGO1 mRNA level (Vazquez et al., 2004a,b).

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 such as those encoding C-REPEATED/DRE BINDING FACTORS (CBFs), and 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 is marked by HOS1-mediated ubiquitination for protein degradation (Dong et al., 2006). HOS1 is also involved in regulating flowering time. Two mechanisms by which HOS1 regulates the flowering pathway have recently been reported. First, HOS1 regulates the abundance of CONSTANS (CO), a photoperiod sensor (Jung et al., 2012; Lazaro et al., 2012). A previous report showed 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., 2012). With respect to the second mechanism, HOS1 regulates the transcription of FLOWERING LOCUS C (FLC) under cold stress by interacting with FVE and HISTONE DEACETYLASE 6 (HDA6) however, 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 the circadian clock that plays a critical role in gating the cold response (MacGregor et al., 2013).

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 expression of the proRD29A-LUC transgene under abiotic stresses such as cold, NaCl and abscisic acid (Zhan et al., 2012). SIC is a proline-rich protein that is 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 performed a forward genetic 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 transcription of the MIR168b gene. We show that HOS1 is important for regulating AGO1 mRNA and protein levels, and suggest that this may explain the broad function of HOS1 in plant growth, development and stress tolerance.
with the gl-1 mutation and harboring the proRD29A-LUC transgene) (Figure 1b). Based on Northern and quantitative real-time PCR analyses, hos1–7 sic–1 had higher LUC transcript levels than sic–1 or the wild-type under cold treatment (Figure 1c,d). Slight increases in endogenous RD29A (Figure 1d) and COR15A (Figure 1c) were also found in hos1–7 sic–1 compared to sic–1 and the wild-type under the same conditions. The hos1–7 sic–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 hos1–7 sic–1 and in sic–1 (Figure S2b), and the mature plant was shorter for hos1–7 sic–1 than for sic–1 or the wild-type (Figure S2c,d). To confirm that these developmental phenotypes were due to mutations in the HOS1 gene, we isolated the single hos1–7 mutant by a backcross to the original wild-type, and 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 to hos1–3. The leaves were smaller for both mutants than for the wild-type (Figure S2e). The hos1–7 mutant also displayed an early-flowering phenotype as reported for 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 mutant and the hos1–7 sic–1 double mutant except for the emergence of multiple siliques from one node in the hos1–7 sic–1 double mutant, which may be caused by the sic–1 mutation. Our observations of the hos1–7 mutant are consistent with HOS1 being 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 miR168 did not differ between hos1–7 sic-1 and sic-1, but mir168a/b was less abundant in hos1–7 sic-1 than in sic-1 (Figure S3a). We compared miRNA accumulation in hos1–1 (C24 ecotype with proRD29a-LUC transgene), (Ishitani et al., 1998) and hos1–3 (Figure 1a) and their respective wild-types. Accumulation of most of the examined miRNAs did not substantially differ between the wild-types and the 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 mutants, a clear reduction in mature mir168a/b was evident in the hos1–7 single mutant (Figure S3b). The reduction in mature mir168a/b was compared between hos1–3 and the mir168a–2 mutant with respective WT, and the level of mature mir168a/b was significantly lower in mir168a–2 than in hos1–3 compared to its respective WT (Figure S3c). Furthermore, the increase in the AGO1 transcript level was higher in mir168a–2 than in hos1–3 (Figure S3d), consistent with the 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. The complemented lines had a restored hos1–3 developmental phenotype back to WT (Figure S2h). Northern analysis showed that 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 shown to function as a ubiquitin E3 ligase, we next determined whether the reduction in mir168a/b is due to a dysfunction of HOS1 as an ubiquitin E3 ligase. To help answer this question, we used the previously reported transgenic lines comprising 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 to be a direct target of the ubiquitin E3 ligase activity of HOS1. 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 quantitative real-time PCR, we examined the expression level of pre-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 quantitative real-time PCR results, we used Northern blot analysis to measure the transcript levels of pre-MIR168a and pre-MIR168b. Consistent with the PCR results, the pre-MIR168b transcript level but not the pre-MIR168a transcript level was lower in hos1 mutants than in the wild-type controls (Figure 3b). The decrease in pri- and pre-MIR168b in the hos1 mutants suggests that HOS1 may affect the accumulation of mature mir168a/b at the transcriptional level, and that HOS1 specifically affects MIR168b (Figure 3a,b).

The quantitative real-time PCR and Northern blot analysis results indicated that loss of HOS1 may reduce the transcription of MIR168b but not of MIR168a compared to WT (Figure 3a,b). To further confirm these results, we performed chromatin immunoprecipitation (ChIP) using an RNA polymerase II-specific antibody to examine the enrichment of RNA polymerase II at the MIR168a and MIR168b promoter regions. We detected a decrease in RNA polymerase II occupancy at the promoter region of MIR168b in hos1–1 compared to the wild-type (Figure 3c, right), while RNA polymerase II occupancy was similar in hos1–1 and the wild-type at the MIR168a region (Figure 3c, left). From these results, it is clear that loss-of-function hos1 mutants show a deficiency during transcription of the other sub-sample. Consistent with the results obtained by Dong et al. (2006), the GFP-ICE1 protein level was higher in hos1–1 than in the wild-type under the control treatment (dimethylsulfoxide) (Figure 2c), indicating inhibition of the proteasome degradation pathway by MG132 treatment. The reduction of mir168a/b was not restored to WT level by MG132 treatment (Figure 2d), suggesting that the regulation of mir168a/b by HOS1 did not result from a loss of function for HOS1 as an E3 ligase mediated-degradation similar to ICE1. These results indicated that HOS1 is required 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 quantitative real-time PCR, we examined the expression level of pre-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 quantitative real-time PCR results, we used Northern blot analysis to measure the transcript levels of pre-MIR168a and pre-MIR168b. Consistent with the PCR results, the pre-MIR168b transcript level but not the pre-MIR168a transcript level was lower in hos1 mutants than in the wild-type controls (Figure 3b). The decrease in pri- and pre-MIR168b in the hos1 mutants suggests that HOS1 may affect the accumulation of mature mir168a/b at the transcriptional level, and that HOS1 specifically affects MIR168b (Figure 3a,b).
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 performed a ChIP assay using native proHOS1::HOS1-4xmyc transgenic lines to determine whether HOS1 enrichment is 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 based on Jung et al. (2013) at or near the FLC region as positive controls for the ChIP assay. Consistent with the previous report, enrichment of HOS1 was detected at the FLC locus in our ChIP assay (Figure 4). Furthermore, we detected enrichment of HOS1 protein at the MIR168b promoter region in our ChIP assay (Figure 4).
region but not at the MIR168a region or in the negative control col-0 without the myc-tagged HOS1 transgene (Figure 4). To test whether HOS1 protein binds directly to the MIR168b promoter DNA, maltose binding protein-tagged HOS1 protein was expressed in Escherichia coli, and DNA binding was examined in vitro by electrophoretic mobility shift assay (EMSA). Our repeated EMSA experiments did not detect any HOS1 binding to the promoter region of MIR168b or MIR168a in vitro (Figure S4). Together, the EMSA and ChIP assay results suggest 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 of HOS1 affects the proper balance of AGO1 due to reduced miR168a/b levels

AGO1 is a core component of the RISC complex that represses miRNA targets (Vaucheret et al., 2006; Vaucheret, 2009; Várallyay et al., 2010). Because mutations in HOS1 result in a reduction of mature miR168a/b, and because miR168a/b directs the cleavage of AGO1 mRNA to maintain the proper balance of AGO1 protein levels (Vaucheret et al., 2004), we suspected that the hos1 mutations may alter the proper balance and maintenance of the AGO1 mRNA transcript level and the AGO1 protein level. Quantitative real-time PCR and Northern blot analyses revealed an increase in the AGO1 mRNA transcript level in the hos1 mutants compared to the wild-type controls (Figure 5a). Similarly, use of an AGO1 protein-specific antibody revealed an increase in the 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.

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 an internal control. Values are means ± SD (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 using ImageJ, and normalized to that of the loading control. Relative expression was normalized to wild-type.
(c) ChIP analysis showing the decrease in RNA polymerase II occupancy at MIR168b (right) and no significant difference between wild-type and hos1–1 at MIR168a (left). The ChIP signal was normalized against actin2. Values are means ± SD (n = 4).
HOS1 is an ubiquitin E3 ligase in Arabidopsis (Dong et al., 2006), and 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 may 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., 2004a,b; 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 hos1–7 sic–1 double mutant showed decreases in miR168a/b compared to 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 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 (Yumul et al., 2013). Also, the promoter region of MIR168a contains an ARBE motif to which ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTORS (ABFs) bind and positively regulate the transcription of MIR168a (Li et al., 2012). The over-expression of ABF genes results in an increase in miR168a/b, and the increase is enhanced by treatment with abiotic stresses such as cold or abscisic acid because of an
increase in transcriptional activity (Li et al., 2012). Abiotic stress may also induce miR168a/b by increasing the transcription of MIR168a (Liu et al., 2008; Jia et al., 2010; 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. As HOS1 does not appear 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 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; Várályi et al., 2010). A mutated form of AGO1 mRNA in the miR168-binding region results in an increase in AGO1 mRNA due to 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 levels of mature miR168a/b due to a 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 a previous study in which a mir168a–2 mutant showed in a decrease in mature miR168a/b and an increase in the AGO1 mRNA level (Vaucheret, 2009). Previously, it was noted that MIR168a predominantly produces a 21 nt miR168, while MIR168b produces similar amounts of 21 and 22 nt miR168 (Rajagopal et al., 2006). Some researchers have suggested that MIR168b produces less mature miR168a/b than MIR168a, and our results further support this idea, as the reduction of miR168a/b was lower in mir168a–2 than hos1–3 compared to wild-type. Accumulation of mature miR168a/b was significantly lower in the mir168a–2 mutant compared to hos1–3, which confirms that MIR168a produces more mature miR168a/b than MIR168b. However, miR168b has been shown to rescue the developmental defects in 4 m-AGO1 (a mutated form of AGO1 mRNA) (Vaucheret, 2009). It has also been suggested that both 21 and 22 nt miR168a/b may be required for the proper maintenance of the levels of AGO1 transcript and protein.

Mutation of 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, 2 m-AGO1 and 4 m-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 causing 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, which in turn regulates AGO1 mRNA and protein levels. AGO1 is important for the function of all miRNAs and tasiRNAs (Jones-Rhoades and Bartel, 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 performed EMS mutagenesis on the miRNA biogenesis component mutant sic–1 (Zhan et al., 2012), and then screened for putative sic–1 enhancer mutants, sic–1 was originally isolated in a mutant screening of the Col-0 ecotype with the gl-1 mutation and harboring the profD294-LUC transgene (Zhan et al., 2012), referred to as one of three (ecotypes) wild-type in this study. One such mutant, hos1–7 sic–1, showed an enhanced LUC phenotype under cold conditions, and was identified as a sic–1 enhancer. The cloning details are provided in Methods S1. Two other Arabidopsis hos1 mutant alleles were used in this study: hos1–1 was isolated from the EMS pool from the C24 ecotype harboring the profD294-LUC transgene (Lee et al., 2001; Dong et al., 2006), and hos1–3 is a T-DNA insertion mutant in the Col-0 background and was obtained from the Arabidopsis Information Resource (SALK_069312c). A 2314 bp sequence upstream of the HOS1 initiation start codon together with the HOS1 genomic sequence (without the termination stop sequence and 3' UTR sequence) was PCR-amplified using Phusion high-fidelity DNA polymerase (M0530S, New England Biolabs; http://www.neb.com). The PCR product was then inserted into pENTR/D–Topo (Invitrogen; http://www.invitrogen.com) according to the manufacturer’s instructions, and named pENTR/
proHOS1::HOS1. Inserted sequences were confirmed by sequencing, and by a LR reaction, the genomic sequence was cloned into Gateway destination vector pGW-B-16 (with 4xmyc at the C terminal region) for Agrobacterium-mediated plant transformation. The construct carrying proHOS1::HOS1-4xmyc was transformed into the hos1-3 mutant via the floral-dip method (Clough and Bent, 1998).

Luciferase imaging

Twelve-day-old seedlings (wild-type, sic-1 and hos1-7 sic-1) were kept at 4°C for 24 h to induce expression of the proRD29A-LUC transgene. The LUC images were obtained using a low-light video imaging system (Princeton Instruments; http://www.princetoninstrument.com) with WinView software (Princeton Instruments) (Chinnusamy et al., 2003).

RNA analysis

Accumulation of small RNAs was detected by Northern blot analysis. To obtain small RNAs, the total RNA from 12-day-old seedlings was extracted using Trizol (Ambion; http://www.lifetechnologies.com) according to the manufacturer’s instructions. Low-molecular-weight RNAs were purified using the poly(ethylene glycol) enrichment method (Zheng et al., 2007), and small RNA Northern blot analysis was performed 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. Quantitative real-time PCR was used to check the transcript levels. Total RNAs were extracted using Trizol (Ambion) and were treated with Turbo DNase (Ambion) to remove DNA contamination. DNase-treated RNAs were used to perform first-strand cDNA synthesis using the qScript Flex cDNA kit (Quanta; http://www.quantabio.com). Quantitative real-time PCR was performed as described previously (Zhan et al., 2012) using the primers listed in Table S1.

Western blot analysis

Tissues from 12–14-day-old seedlings were ground in liquid N2 and then dissolved in protein extraction buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 10% v/v glycerol, 1 mM dithiothreitol, 1 mM phenethyl alcohol (Boehringer Mannheim, Roche; http://www.lifescience.roche.com) and protein inhibitor cocktail (Roche)]. The preparation was briefly mixed with a vortex apparatus, and then centrifuged at 4°C at 15 000 g for 30 min. The supernatant containing total proteins was collected for protein normalization and Western analysis. Anti-GFP tag antibody (Roche) was used to determine the GFP-ICE protein level, and anti-AGO1 antibody (Agrisera; http://www.agrisera.com) 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 the ChIP assay. Sample preparation and overall ChIP procedures were performed as previously described (Wierzbiicki 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 to obtain a strong ChIP signal. Anti-myc antibody (Millipore; http://www.milmipore.com) was used to check for enrichment of HOS1 at MIR168a, MIR168b and FLC. An anti-RNA polymerase II C-terminal domain repeat antibody (Abcam; http://www.abcam.com) was used to check the enrichment of RNA polymerase II at MIR168a and MIR168b in the hos1-1 mutant and the wild-type.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Identification of the hos1-7 mutant by map-based cloning.

Figure S2. Developmental phenotypes of hos1-7 sic-1 and hos1-7.

Figure S3. Accumulation of miRNA in hos1-7 sic-1 and hos1-7 mutants.

Figure S4. Hos1 does not directly bind to the MIR168a and MIR168b promoters.

Table S1. Primer information.


REFERENCES


