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Arabidopsis Cold Stress Tolerance Improvement via *AthHOS1*-targeting *HOS1-amiRNA* Approach

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ABSTRACT

In this study, we examined the efficacy of the artificial microRNAs (amiRNAs) technology in targeting the HOS1 gene for the enhancement of cold stress tolerance in *Arabidopsis thaliana* Ler-0 ecotype. The impact of athHOS1-amiRNA overexpression on the response of transgenic plants to cold stress was assessed using RT-qPCR in 3-week-old seedlings of the T3 generation. Additionally, the response of wild-type plants of the same age to cold stress (4ºC) for various durations (6, 12, 24, 48, and 96 hours) was also evaluated. Comparative analysis revealed that athHOS1-amiRNA downregulated *athHOS1* in transgenic plants after prolonged exposure to low temperature (48 h and 96 h) (Pearson's correlation coefficient of -0.407; P< 0.05). Interestingly, while prolonged cold stress at 96 h led to a significant upregulation of *athHOS1* in wild-type plants, the suppression of athHOS1-amiRNA in transgenic plants disrupted the expected circadian rhythm of *athHOS1* by preventing its upregulation. Furthermore, T3 plants that had been cold-acclimated exhibited a 17% increase in freezing tolerance (-1 to -8°C) compared to wild-type plants, indicating the success of this approach in enhancing Arabidopsis tolerance to low temperatures, at least in the Ler-0 ecotype. In order to gain a deeper understanding of the intricate dynamics of gene/protein networking during cold acclimation and its interaction with the athHOS1-amiRNA approach, further characterization is required. This includes measuring the expression levels and half-life of athHOS1-amiRNA and HOS1 mRNA, as well as evaluating the protein level of HOS1 and its direct targets, such as ICE1, in different Arabidopsis ecotypes and at different time intervals of low temperature exposure.

Keywords: *Arabidopsis thaliana*, Artificial miRNAs**,** Cold acclimation, Freezing tolerance**.**

INTRODUCTION

Staple crop production is globally threatened by abiotic stress magnified by climate change, land degradation, and erosion of natural resources (Arzani and Ashraf, 2016). The response of plants to abiotic stress is mediated by a number of biochemical and molecular pathways and their regulatory mechanisms that determine the ability of distinct plant genotypes to tolerate stress (Saha *et al*., 2016). Low temperatures are one of the major environmental stresses that limit growth, development, productivity and geographical distribution of plants (Zhao *et al*., 2018). Temperatures trigger numerous processes in plants at molecular, biochemical and

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morphological level, which may be represented as alert, acclimation, tolerance, exhaustion and/or recovery phase (Kosová *et al*., 2015). The most studied pathway involved in cold acclimation and freezing tolerance in plants is CBF/DREB pathway (Robison *et al*., 2019). In *Arabidopsis* this pathway involves three groups of CBF/DREB genes (Zhou *et al*., 2017) expressed shortly upon plant exposure to cold stress (Miura and Furumoto, 2013). These genes encode for transcription factors that can bind to C-Repeat elements/Dehydration-Responsive Elements (CRT/DRE), located in the promoters of cold stress responsive genes, inducing their expression. Under low temperatures, the expression of one of these genes, *CBF3/DREB1A* gene, was shown to be induced by *ICE1* gene (Chinnusamy *et al*., 2003; Zuo *et al*., 2019). *ICE1* gene is negatively regulated by *HOS1* gene (high expression of osmotically responsive gene 1), encoding a RING finger protein with E3 ubiquitin ligase activity, that leads to ICE1 ubiquitination and degradation (Ding *et al*., 2015). *HOS1* overexpression in *Arabidopsis* led to cold/freezing sensitivity, while its knock down led to improved cold/freezing tolerance (via its influence on CBFs and downstream genes like RD29A, COR47, COR15A, KIN1, as well as on ICE1) (Chinnusamy *et al*., 2007, 2004; Shi *et al*., 2015). Such *athHOS1* roles in cold signaling qualify this gene as an excellent molecular target for plant cold tolerance improvement studies.

Many reverse genetic approaches based on small RNAs, including Virus-Induced Gene Silencing (VIGS) and RNA-mediated interference (RNAi), have been introduced via gene activity disruption (Gilchrist and Haughn, 2010). Among these methods, the artificial microRNA (amiRNA) technology became one of the preferred gene silencing procedures. This method is based on using 21nt long amiRNA and amiRNA* sequences to replace miRNA and miRNA* sequences in an endogenous miRNA precursor, which eventually knocks down corresponding endogenous target gene. Such approach is more specific, more trans-generation stable, holds multi targeting potential, and displays increased biosafety in comparison to siRNA-mediated gene silencing (Liu and Chen, 2010; Ossowski *et al*., 2008; Schwab *et al*., 2010, 2006). Numerous research groups have reported amiRNA as a tool of choice in genetic and functional studies, like research in plant reproduction, i.e. investigation of the flowering molecular mechanism and underlying genes, as *FT* gene in *Arabidopsis* (Yeoh *et al*., 2011) and *ZCN* gene in corn (Meng *et al*., 2011) in crop improvement studies, like amiRNAs silencing of *Eui1* and *SBE11b* genes or amiRNA *BADH2* suppression that increased seed fragrance in non-fragrant lines, improving quality and economical value of rice (Chen *et al*., 2013). This technology helped also in disclosing the roles of abiotic stress responsive genes, including revealing the stu*CBP80* as the negative regulator of drought tolerance in potato (Pieczynski *et al*., 2013). *Os-amiR393* as a booster of the salinity tolerance in *Arabidopsis* (Gao *et al*., 2010). Furthermore, amiRNA-mediated resistance to viruses has been successfully applied in different crops, i.e. wheat dwarf virus in barley (Kis *et al*., 2016), Brown Streak Disease (CBSD) in Cassava (Wagaba *et al*., 2016), and Sugarcane Mosaic Virus (SCMV) in maize (Xia *et al*., 2018).

Due to the gained knowledge on *HOS1* negative role in *A. thaliana* response to cold stress and the advances that amiRNA technology offers in gene repression approach compared to other silencing methods, in this research, *athHOS1* function was targeted by applying amiRNA method. The aim was to induce cold tolerance in transgenic *Arabidopsis* plants and test its effect during prolonged exposure to low temperatures.

The obtained results are encouraging in regards of continuing the research by conducting more detailed characterization of *athHOS1-amiRNA* transgenic *Arabidopsis* plants at transcriptomic and proteomic level, in different time intervals of exposure to cold stress. Additionally the study closely looks at re-shaping of circadian- and

diurnal-clock related genes expression (including changes in gene splicing patterns) and their influences on the amiRNA approach. These findings may eventually open the new insights for similarly designed researches in different agriculturally important plant species in order to improve their cold/freezing tolerance and yield stabilization.

MATERIALS AND METHODS

Plant Materials

The Ler-0 ecotype seeds of *A.thaliana* L. were surface-sterilized by immersion in 70% ethanol for 1 minute and 1% NaClO3 (Bleach: H2O, 1:4) for 10 minutes. After rinsing the seeds four times using sterilized distilled water, seeds were mixed and suspended into 0.1% agar solution and pipetted onto Petri dishes containing solid ½ MS medium supplemented with 1.5% sucrose. These Petri dishes were firstly incubated at 4°C in dark for 2 to 3 days, then, at 22°C under the light intensity of approximately 100 µmol m^{-2} s^{-1} using 16 hours light/8 hours dark regime in the growth chamber. After two weeks, the seedlings were transplanted into plastic pots containing coco coir and peat moss (in ratio 1:2) (Drake *et al*., 2016) and grown in the growth chamber for 3-4 weeks, until the first inflorescence bolting was observed. In order to remove apical dominance and to obtain synchronized emergence of multiple secondary bolts, subsequently, on the appearance of primary bolts in the majority of plants, the first inflorescence was clipped in each plant. Seven to ten days after clipping, when the majority of the secondary inflorescences had grown to a height of 8-12 cm, the plants were prepared for the floral dip procedure.

Design and Construction of amiRNA

A 21-nucleotide artificial microRNA (amiRNA) sequence targeting the AthHOS1 gene (ID: AT2G39810) was designed using the Web MicroRNA Designer tool (WMD3) developed by Schwab *et al*. (2006). The sequence for HOS1-derived amiR was 5'-UAAAUUCGAAUAUCGAGGCCU-3' (Figure 1-a) and ath-MIR319a precursor was used as a backbone to engineer *HOS1-amiRNA*. The amiRNA containing precursor (Figure 1-b) was generated through site-directed mutagenesis using overlapping PCR with primers shown in the Table S1.

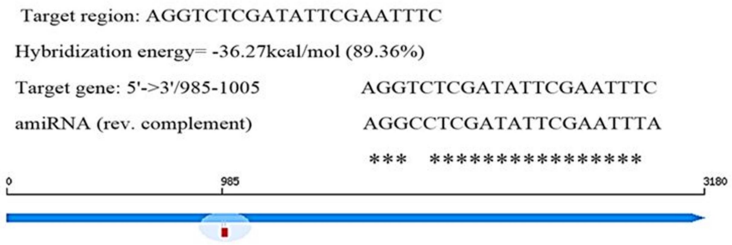
In the first phase, three PCR products of 271bp, 170bp and 298bp in length were amplified separately, using primers A+IV, III+II, and I+B, respectively. Plasmid pRS300, which harbored MIR319a precursor, was employed as a template in these PCR reactions. Amplified PCR products were extracted from the agarose gel, purified and fused in the PCR reaction using primers A+B, delivering 699 bp long recombinant *athHOS1-preamiRNA*.

Cloning and Plant Transformation

Final overlapping of PCR product was ligated into T/A vector pTG19-T (Vivantis). Before ligation, 3'-A overhangs were added to the blunt-ended PCR products using KBC Taq plus DNA Polymerase. Heat shock transformation of *E. coli* strain *DH5α* was performed in water bath at 42°C for 90 seconds. Transformed *E. coli DH5α* cells, containing recombinant vector, were selected by using cultivation on Luria-Bertani (LB) medium with Ampicillin (100 µg mL^{-1}). These were verified by colony PCR and sequencing using forward/reverse PUC/M13 primers (F-5′-GTTTTCCCAGTCACGAC-3′, R- 5′-CAGGAAACAGCTATGAC-3). The PCR amplification conditions were initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94°C for 30 seconds, and 55°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

The binary destination vector, pCAMBIA 1301/2301 and the confirmed carrier vector were digested using *KpnI* and *XbaI*, and the digestion products were separated by agarose

(a)



(b)

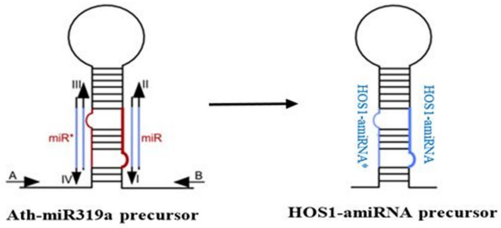


Figure 1. Supplementary information on the miRNA sequence representing the target site position and hybridization energy (**a**) and schematic diagram of *miR319a/miR319a** substitution with *AtHOS1-amiRNA* by using the overlapping PCR approach (**b**).

gel electrophoresis. The 480 bp long fragment released from the carrier vector was sub-cloned into linearized binary vector to obtain the artificial miRNA expression cassette. The secondary structure of sequenced amiRNA precursor 217 nucleotides long was predicted by Mfold web server (Zuker, 2003). The recombinant plasmids were minipreped, and electroporated into the *Agrobacterium tumefaciens* strain *GV3103* and selection of transformed *A. tumefaciens* was done similar to *E. coli* assay.

Plant transformation was performed using floral dip protocol as in Clough and Bent (1998) and Bent (2006). In this method, inflorescences of *Arabidopsis* were dipped for 30 seconds into inoculation medium containing 5% sucrose, 0.05% Silwet L-77, and re-suspended *Agrobacterium,* carrying the genes of interest, and incubated in the dark for 24 hours. Afterwards, plants were placed in the growth chamber at 22°C under 16 hours light/8 hours dark photoperiod regime until seed harvesting.

Transformed Plants Screening

Screening experiments were performed on the first and second generation of transgenic plants (T1 and T2). Sterilized seeds were plated on the solid ½ MS medium supplemented with 1.5% sucrose and 50 µg mL^{-1} kanamycin (Figure S1-a). After 7 to 10 days, green and healthy seedlings were selected and sub-cultured in ½ MS medium without antibiotic (Figure S1-b). Two weeks later, the seedlings were transplanted to the plastic pots containing coco coir: peat moss (in ratio 1:2) (Figure S1-c). After adaptation, young, green and healthy leaves were sampled.

Secondary screening aimed at revealing the presence of T-DNA by isolation of total DNA from 200 mg leaf samples of kanamycin-resistant T1 plants and T2 plants using the method described by Dellaporta *et al*. (1983), followed by PCR identification of *HOS1-amiRNA*. Concordant PCR

reactions were performed on T1 and T2 plants using the primers shown in Table S2.

The test was based on the fact that only transgenic plants could produce eligible amplicons by using different sets of primers: 116bp long PCR product by using primers 1 and 2, 1024 bp long PCR product by using primers 3 and 4, and 323 bp long PCR product by using primers 4 and 5 (Figure S1-d). The presence of contaminating Agrobacterium was investigated through PCR reaction based on the amplification of 730 bp long fragment from the Agrobacterium *Virg* gene using the primers shown in Table S2 (Siritunga and Sayre, 2003). Amplification of Virg gene on DNA genome of the *Arabidopsis* should not be observed.

Cold Stress Treatment

Cold treatments were based on the study of Dong and Pei (2014). T1 and T2 plants were cultivated in trays and mature seeds were collected from transgenic T2 plants, mixed and, as a pooled sample, used for planting T3 generation. Simultaneously, homozygous T3 plants and wild-type seedlings were transplanted on separate halves of the same pot. The 3-week-old seedlings were subjected to the temperature of 4°C in duration of 6, 12, 24, 48 and 96 hours under 16 hours light/8 hours dark photoperiod regime in the growth chamber (the temperature was gradually reduced from 22 to 4°C at 3°C h^{-1} cooling rate). For the control treatment, seedlings were kept at the temperature of 22°C during the same period of time under the same photoperiod regime. In the sampling procedure, whole plants were taken immediately upon the elapsed stress-time, then, frozen in liquid nitrogen and stored at -80ºC.

Freezing Tolerance Assay

Freezing tolerance evaluation was conducted as described in Chinnusamy *et al*. (2003) and Dong *et al*. (2006) with several modifications. Briefly, 10-day-old transgenic and wild-type plants were grown on separate halves of ½ MS media. They were acclimated to cold stress by growing at 4°C under 16 hours light/8 hours dark photoperiod regime for 4 days in the growth chamber (the temperature was gradually reduced from 22to 4°C at 3°C h^{-1} cooling rate). Afterwards, plants were moved on ice and held in the freezing chamber at -1 °C in the dark for 16 hours. Then, the temperature was further lowered to -8°C (at -1°C h^{-1} cooling rate) and plants were kept at this final temperature for 2 hours. Treated plants were then thawed at 4°C overnight and transferred to the growth chamber at 22°C under long-day conditions. After a week, survival rates were scored visually by counting the seedlings that displayed normal growth and green, healthy-looking leaves.

RNA Extraction and cDNA Synthesis

After grinding frozen samples in liquid nitrogen, total RNA was isolated from 100 mg of each sample using TRIzol reagent (Ambion), according to the manufacturer's instruction. Total RNA extracts were treated with DNaseI, RNase Free Enzyme (#EN0521, Thermo Scientific) to eliminate DNA contamination. Afterwards, 1µg of purified total RNA was used for the oligo(dT) synthesis of the first strand of cDNA according to the method of Chen *et al*. (2005) and Varkonyi-Gasic (2007), while miRNA cDNA synthesis was performed using 200 ng of purified RNA and 1 µM of specific stem-loop RT primer (Table S3). Synthesis of both types of cDNAs was carried out using RT Reagent PrimeScript TM (#RR037A, Takara) according to the manufacturer's recommendations.

Expression Analysis

The precise quantification of *HOS1-amiRNA* and *AthHOS1* transcription level

was measured by RT-qPCR, which was performed on a Roter-Gene Q (Qiagen) apparatus using the following thermal cycling profile: denaturation 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. For each reaction, 2 µL of cDNA was added to 10 µL of PCR mixture containing 6 µL of SYBR Premix Ex Taq (Takara) and 0.83 µM of each (forward/reverse) primer. Three technical replicates were carried out for each of three biological replicates and *Arabidopsis* actin 2 (*AthAct2*- At3g18780) was used as an internal control (Egert *et al*., 2013; Kozera and Rapacz, 2013). Primers used for *athAct2, athHOS1-amiRNA* and *athHOS1* RT-qPCR analysis are given in the Table S3.

The relative gene expression was calculated using the $2^{-}\Delta\Delta C_T$ method (Livak Student's t-test was used to analyze differences between treated and non-treated samples (P-value< 0.05) (Goni *et al*., 2009).

RESULTS

Preparation for AthHOS1-miRNA Integration into the *Arabidopsis* Genome

The obtained recombinant *AtHOS1-preamiRNA* (confirmed as a single PCR product of 699 bp, Figure S2) was cloned into pTG19-T vector and confirmed by sequencing. Upon restriction of transformed vector, 480 bp long restriction fragment (confirmed by agarose electrophoresis, Figure S3) was isolated and sub-cloned into the binary vector creating the artificial *athHOS1-miRNA* expression cassette (Figure 2-a), which was also confirmed by sequencing. This cassette encoded for 217 nucleotides long athHOS1 amiRNA precursor. The secondary structure of the construct was predicted as shown in Figure 2-b. Only in selected transformed plants the amplicons of defined lengths were detected: 1024 bp long PCR product, confirming the presence of CaMV35S promoter, Nos terminator sequence and mature *AthHOS1-amiRNA* (Figure 3-a); 116 bp long PCR product, confirming the presence of mature *athHOS1-amiRNA* (Figure 3-b) and 323 bp long PCR product confirming the presence of mature *AthHOS1-amiRNA* and Nos terminator sequence (Figure 3-c), while in the wild type plants no amplification products were obtained. Additionally, PCR results confirmed the absence of Agrobacterium contamination (Figure 3-d).

Results confirmed the presence of mature *AthHOS1-amiRNA* in all examined transgenic plants (Figure S4).

AthHOS1-miRNA Expression in T3 Generation *Arabidopsis* Plants

Temporal profile of *athHOS1* expression showed fluctuations both in transgenic and wild type plants. After 6 h of cold stress exposure, *athHOS1* was 1.24-fold downregulated in the wild type plants, but it was 1.25-fold upregulated in transgenic plants (the difference was not statistically significant). Upon 12 h of the cold stress exposure, *athHOS1* was downregulated in both wild type and transgenic plants, only more prominently in the latter (1.65-fold decrease in the wild type plants vs. 3.22-fold decrease in transgenic plants). Unexpectedly, 24 hours after the cold stress exposure, both types of the plants showed the same response of 1.2-fold upregulation of *athHOS1*, which was followed by *athHOS1* downregulation in both types of plants after 48 hours of cold stress exposure (2-fold decrease in wild type plants vs. 1.78-fold decrease in transgenic plants). Finally, after 96 h of the exposure to cold stress, transgenic and wild type plants showed completely the opposite responses, that included 3.42-fold downregulation of *athHOS1* in transgenic plants and 1.72-fold upregulation of *athHOS1* in wild type plants (Figure 4-a). Hence, while prolonged cold stress led to *athHOS1* upregulation in the wild type plants, it led to prominent downregulation of *athHOS1* in transgenic plants, with approximately 5-fold difference

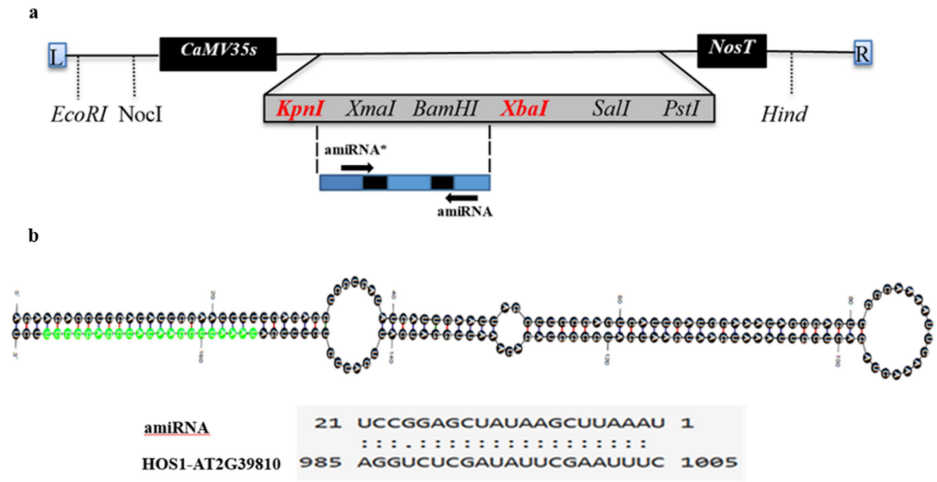


Figure 2. Schematic diagram of amiRNA binary vector generation **(a)**, predicted secondary structure of sequenced amiRNA precursor using Mfold web server and complementarity of the mature amiRNA to the target site in *AthHOS1* gene **(b).**

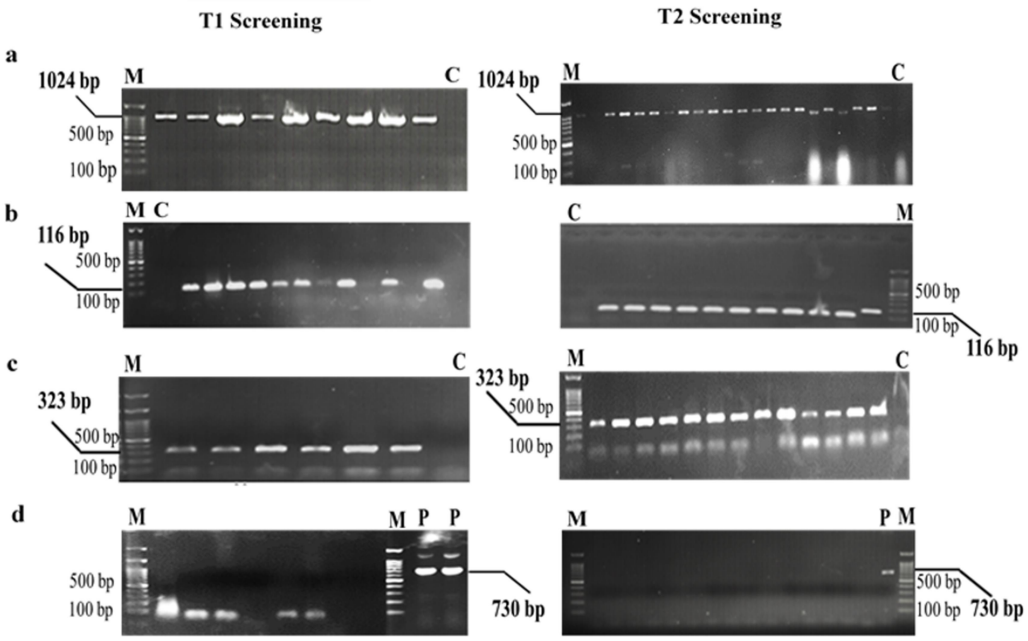


Figure 3. PCR screening of transgenic lines of *A. thaliana* Ler-0 ecotype for the presence of amplicons of the specific lengths: 1024 bp amplicon generated by a primer pair specific to the 35S promoter and the Nos terminator **(a)**, 116 bp amplicon generated by HOS1-amiRNA1-F and HOS1-amiRNA1–R primers **(b)**, 323 bp amplicon generated by HOS1-amiRNA 2-F and Nos terminator sequence **(c)**, 730bp amplicon generated by Agrobacterium *VirG* specific primers; no amplification observed in the tested transgenic plants **(d)**. **M**: 100 bp molecular ladder (#PR901644, CinnaGen); **P**: pCAMBIA2301 plasmid DNA; **C**: Control as a non-transgenic plant; **T1** and **T2** as the first and the second generation of transgene lines progeny.

in the *athHOS1* expression levels between wild type and transgenic plants.

Temporal expression profile of *athHOS1-amiRNA* over 96 hours of transgenic plants exposure to cold stress showed that *athHOS1-amiRNA* was 1.7-fold upregulated at 6 hours, 1.09-fold downregulated at 12 hours, and returned to the level of the reference gene expression at 24 hours (however, the changes of *athHOS1-amiRNA* expression recorded at these time points were not statistically significant). After 24 hours of the cold stress onset, the expression profile of *athHOS1-amiRNA* in transgenic plants showed a continuous increase, recording 5.55-fold upregulation at 48 hours and reaching maximal 9.65-fold upregulation at 96 hours (Figure 4-b) of the cold stress duration.

Comparison of *athHOS1* and *athHOS1-amiRNA* expression profiles in transgenic plants exposed to cold stress revealed that, during the first 24 hours, two expression profiles followed each other, with both being upregulated at 6 h, downregulated at 12 h, and upregulated at 24 h. However, after the first 24 hours of the cold stress exposure, *athHOS1* and *athHOS1-amiRNA* expression profiles in transgenic plants started to oppose each other as *athHOS1* expression was downregulated, while *athHOS1-amiRNA* expression was upregulated at 48 and 96 hours upon the cold stress onset. Even though a negative correlation between *athHOS1-amiRNA* and *athHOS1* was not observed at 6, 12, and 24 hours after transgenic plants exposure to cold stress, with the prolonged cold stress exposure for 48 and 96 hours, a negative regulation of *athHOS1* via *athHOS1-amiRNA* was observed in the transgenic Ler-0 plants (Pearson's correlation coefficient of -0.407;

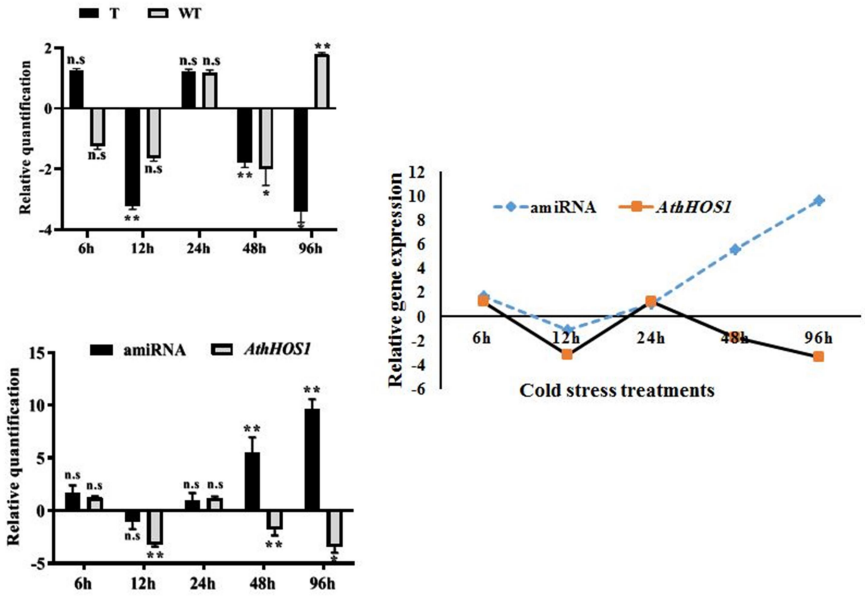


Figure 4. Bar diagram showing the expression profile timelines of *AthHOS1* in transgenic and non-transgenic plants of *A. thaliana* Ler-0 ecotype (**a**), bar diagram showing the expression profile timelines of *AtHOS1-amiRNA* and *AthHOS1* in *A. thaliana* Ler-0 transgenic plants (**b**), graphic representation of the expression pattern of *AtHOS1-amiRNA* and *AthHOS1* response to cold stress (4°C) after 6, 12, 24, 48 and 96 hours in *A. thaliana* Ler-0 transgenic plants (**c**). Mark **n.s** indicate no statistical significance, ***:** Statistical significance at 95% confidence level and ******: Statistical significance at 99% confidence level, according to t-student test. **WT:** Wild Type plants, **T:** Transgene plants, **amiRNA:** *AtHOS1-amiRNA* expression profile/pattern, ***AthHOS1:*** *AthHOS1* expression profile/pattern.

P< 0.05) (Figure 4-c). The detected negative correlation between *athHOS1-amiRNA* and *athHOS1* during prolonged cold stress corresponded to the observed 5-fold difference in *athHOS1* expression levels between transgenic and wild type plants at 96 hours.

Freezing-Tolerance Assay

After 4 days of cold acclimatization, neither transgenic nor the wild type plants showed any obvious signs of chilling injuries (Figure S5). However, the freezing tolerance testing, to which the cold-acclimated transgenic and wild type plants were subsequently subjected, revealed statistically significant differences in their response to freezing temperatures (-1 to -8°C). Results of the freezing test tolerance revealed that transgenic seedlings had the survival rate of 38.5%, while the wild type seedlings had the survival rate of 21.5%. This accounted for a significant increase of 17% of individuals displaying normal growth and healthy-looking green leaves in transgenic plants in comparison to the wild type plants after recovery from freezing test (Figures 5-a and -b).

DISCUSSION

Presented assay was successfully employed to increase *A. thaliana* (Ler-0 ecotype), cold stress tolerance through *athHOS1* suppression mediated by

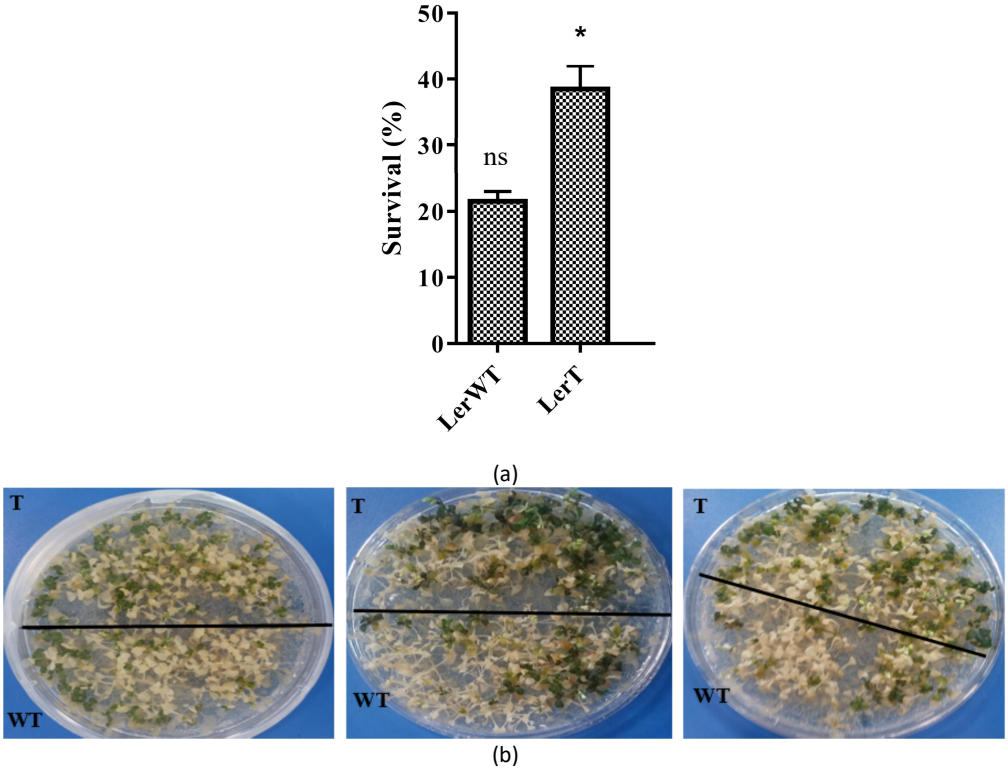


Figure 5. Survival rates of transgenic and wild type *Arabidopsis* plants of Ler-0 ecotype (**a**) and improved survival ratio of *amiRNA-HOS1* overexpressing transgenic Ler-0 plants after a freezing treatment (**b**). **WT:** Wild Type plants, **T:** Transgene plants, **ns:** No statistical significance and *: Statistical significance at 95% confidence level according to Student's *t*-test

athHOS1-amiRNA overexpression. The effect of the assay in transgenic plants was shown to depend on the duration of cold stress, with the highest effect recorded upon prolonged exposure, for a period of 96 hours. This is supported by the finding that negative correlation between *athHOS1-amiRNA* and *athHOS1* target gene was observed only 48 and 96 hours after the cold stress onset. Thus, in order to obtain the significant level of *athHOS1-amiRNA* overexpression, required for affecting the target gene in the desired manner, the duration of cold stress seems to be an important factor. This is not surprising, since it is known that circadian-clock and diurnal-clock harmonize all cell's processes to light and temperature cycles in the environment, including cold acclimation upon longer exposure to low temperatures (Gorpenchenko *et al*., 2023). For instance, there is a record that CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) is associated with cold acclimation in *Arabidopsis*, which self-regulates CCA1 activity via alternative splicing, producing either CCA1α or CCA1β variant, which influence cold acclimation in the opposite manner (35S:CCA1α transgenic plants are tolerant, while 35S:CCA1β transgenic plants are sensitive to freezing) (Seo *et al*., 2012). With such influence of alternative splicing of a single gene and with the ongoing extensive alternative splicing of numerous genes during cold acclimation (Calixto *et al*., 2018; Gallegos, 2018), a complex dynamics of gene/protein networking underlying cold acclimation needs to be carefully investigated. Given the significant function of HOS1 in the process of adapting to low temperatures, it is plausible to infer that athHOS1-amiRNA expression and half-life are also affected by the related factors during cold stress exposure, and that securing athHOS1-amiRNA overexpression is not a straightforward endeavor.

Further supporting this reasoning is the observation that athHOS1-amiRNA was upregulated in transgenic plants at all tested time points, except at 12 hours, when it was downregulated. Moreover, downregulation of its target, *athHOS1*, was observed in both transgenic and wild type plants at the same time. However, the downregulation of *athHOS1* was more pronounced in the transgenic plants. This finding suggested that the expression of athHOS1-amiRNA was influenced by other factors that were active at this time point, thereby impeding the accumulation of athHOS1-amiRNA. Given that HOS1 is crucial for maintaining a normal circadian rhythm and plays a role in regulating mRNA export from the nucleus, it is possible that certain regulatory factors target athHOS1-amiRNA for degradation within the first 12 hours. However, it is also plausible that the amiRNA was able to exert its function to some extent before undergoing degradation, thereby contributing to the inherent downregulation of *athHOS1* at this specific time point. An important observation, underscoring the need for a closer examination of miRNA-mediated gene silencing and the influence of circadian and diurnal networks during cold acclimation, was noted six hours after cold stress exposure. At this early stage, transgenic plants showed upregulation of athHOS1, despite the concurrent upregulation of athHOS1-amiRNA, whereas wild-type plants exhibited downregulation of athHOS1 at the same time point. Since miRNA-mediated gene silencing operates through two concurrent mechanisms—target cleavage and translational inhibition—both influenced by developmental and environmental factors (von Born et al., 2018), our observation at the 6-hour mark could be attributed to the accumulation of athHOS1 transcripts in transgenic plants. This may result from translational inhibition, combined with the lack of transcript cleavage at this specific time point. Consequently, this accumulation could lead to an increase in RT-qPCR detection, while concurrently suppressing the innate mechanisms responsible for the observed downregulation of *athHOS1* in the wild type plants at the same 6 h time interval.

After 24 hours of exposure to cold stress, the expression of *athHOS1* increased in both wild -type and transgenic plants. This increase occurred even though athHOS1-amiRNA was confirmed to be upregulated in the transgenic plants. This suggests that, at this time point, the accumulation of *athHOS1* transcripts in the transgenic plants may result from translation inhibition rather than transcript cleavage, similar to what was observed at the 6h. This inhibition may suppress the innate mechanisms responsible for *athHOS1* upregulation. Alternatively, the 24 hours cold stress might trigger a primal survival mechanism in the plants to protect them from an excessive cold response. This mechanism could prevent the overexpression of athHOS1-amiRNA in transgenic plants by immediately inactivating and/or degrading it, thus allowing undisturbed natural *athHOS1* upregulation at this time. However, as the duration of cold stress increases to 48 h, both types of plants show reduced *athHOS1* expression. This reduction is slightly more pronounced in the wild type plants compared to transgenic ones, despite the confirmed significant upregulation of athHOS1-amiRNA in the transgenic plants. This finding could be attributed to rapid inactivation and/or degradation of amiRNA, which had a slight effect on the innate mechanisms responsible for the downregulation of *athHOS1* at this particular time. After 96 hours of exposure to cold, the transgenic and wild type plants exhibited distinct behaviors. The desired effect of the assay was observed for the first time. At this stage, transgenic plants showed significant upregulation of athHOS1-amiRNA and notable downregulation of *athHOS1*. In contrast, wild type plants showed upregulation of *athHOS1* at the same time point. Therefore, while wild type plants upregulated *athHOS1* at 96 hours to prevent exhaustion from the excessive cold response, the overexpression of AtHOS1-amiRNA in transgenic plants reached a level sufficient to override the primary survival strategy at this time point. This led to significant downregulation of *athHOS1*, allowing for further amplification of the cold signaling in transgenic plants, thereby enhancing their cold and freezing tolerance. This finding is supported by the outcome of the freezing test conducted after 4 days of cold acclimatization, which revealed a 17% higher survival rate for transgenic plants compared to the wild type plants. This ability to tolerate freezing temperatures may be attributed to the production of soluble sugars activated by GIGANTEA (GI) in response to cold stress. Additionally, the influence of *athHOS1* on CBF genes, where *athHOS1* suppression triggers the expression of the CBF-COR regulon and affects the alternative splicing of multiple genes, may also play a role in freezing tolerance (Gil and Park, 2019). Lee *et al*. (2001) reported that during 48 hours of cold stress in the wild type *Arabidopsis* C24 ecotype, *athHOS1* expression was initially undetectable, then, returned to pre-cold treatment levels after 1 hour, and remained consistent until 48 hours when it decreased again. Although this aligns with our findings at 48 hours in Ler-0 wild type plants, there appears to be a contradiction in our results at 6 and 12 hours. However, this discord may be explained by the use of Northern blot for *athHOS1* expression quantification in the reported experiment, while we used RT-qPCR, which is more sensitive in this regard (Dean *et al*., 2002). Another possible explanation could be found in independently reported differences recorded in cold transcriptomes of Ler-0 and C24 ecotypes. Further investigation into the first 24 hours cold treatment in *Arabidopsis* has shown that genes regulated by diurnal and circadian rhythms play a significant role in the variations observed between experiments. Moreover, it was observed that genes responsive to cold are influenced by the time of the day when the experiment was conducted, along with numerous transcription factors, thereby adding to the disparities in the experiments (Bieniawska *et al*. 2008). However, the similar responses of the two different ecotypes after 24 and 48

hours of cold stress exposure could be explained by more noticeable changes detected through both methods, or by the activation of a commonly shared cold survival mechanism that depends on the duration of the cold stress. Exploring the use of the amiRNA approach in different *Arabidopsis* ecotypes is suggested based on these findings. It is advisable to use uniform detection methods and start the experiment at the same time, while also following consistent stress exposure time points. This approach will help gain a thorough understanding of how the genetic background may impact both innate and amiRNA-mediated processes involved in cold response.

Concerning the results obtained in our investigation, additional research is needed to clarify the effect of *athHOS1-amiRNA* approach in different time intervals of the cold (4°C) and freezing (-1 to -8°C) stress exposure. In these experiments, the expression level and half-life of *athHOS1-amiRNA* and *HOS1* mRNA should be followed, along with protein level of HOS1 (and possibly protein levels of some of its direct targets like ICE1), to arrive at definitive conclusions regarding the ongoing processes during specific time of the cold stress exposure. If these forthcoming studies support the safety of such cold tolerance introduction, then this method could be extended for studying in other plant species, like crops that benefit from developing cold tolerance (i.e. almond, grapevine, and tea) (Karimi *et al*., 2016; Sun *et al*., 2015; Zhang *et al*., 2014).

CONCLUSIONS

This study presented encouraging findings regarding the improvement of cold and freezing stress through the application of exogenous amiRNAs. Nevertheless, additional research is necessary to address the inquiries raised by this investigation, specifically concerning the impact of diurnal and circadian genes on the amiRNA method. Subsequent studies should concentrate on examining the effects of athHOS1-amiRNA during various time intervals of low temperature exposure and in diverse *Arabidopsis* ecotypes, before considering its application in agriculturally important plant species for cold tolerance enhancement.

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بهبود تحمل استرس سرما آرابیدوپسیس از طریق رویکرد HOS1-amiRNA با هدف AthHOS1

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چکیده

در این مطالعه، کارایی فناوری microRNA های مصنوعی (amiRNAs) را در هدف قرار دادن ژن HOS1برای افزایش تحمل تنش سرما در اکوتیپ Arabidopsis thaliana Ler-0 بررسی شد. تأثیر بیان بیش از حد athHOS1- amiRNA بر پاسخ گیاهان تراریخته به تنش سرما با استفاده از RT-qPCR در نهال‌های ۳ هفته‌ای نسل T3 ارزیابی شد. علاوه بر این، واکنش گیاهان نوع وحشی هم سن به تنش سرما (٤ درجه سانتیگراد) برای مدت‌های مختلف (٦ ساعت، ۱۲ ساعت، ۲٤ ساعت، ٤٨ ساعت و ۹٦ ساعت) نیز مورد ارزیابی قرار گرفت. تجزیه و تحلیل مقایسه ای نشان داد که athHOS1-amiRNA باعث کاهش athHOS1در گیاهان تراریخته پس از قرار گرفتن طولانی مدت در دمای پایین (٤٨ ساعت و ۹٦ ساعت) (ضریب همبستگی پیرسون ۰.٤۰۷-؛ P< 0.05) شد. جالب توجه است، در حالی که تنش سرمای طولانی در ۹٦ ساعت منجر به تنظیم مثبت athHOS1 در گیاهان نوع وحشی شد، سرکوب athHOS1-amiRNA در

گیاهان تراریخته با جلوگیری از تنظیم دخیل، ریتم شبانه روزی مورد انتظار athHOS1 را مختل کرد. علاوه بر این، گیاهان T3 که به سرما سازگار شده بودند، ۱۷ درصد افزایش در تحمل یخ زدگی (۱- تا ۸- درجه سانتیگراد) در مقایسه با گیاهان نوع وحشی نشان دادند که نشان دهنده موفقیت این رویکرد در افزایش تحمل Arabidopsisبه دماهای پایین است. حداقل در اکوتیپ .Ler-0 به منظور به دست آوردن درک عمیق تر از پویایی پیچیده شبکه ژن / پروتئین در طول سازگاری با سرما و تعامل آن با رویکردathHOS1-amiRNA ، توصیف بیشتر مورد نیاز است. این شامل اندازه گیری سطوح بیان و نیمه عمر athHOS1-amiRNA و HOS1 mRNA، و همچنین ارزیابی سطح پروتئین HOS1 و اهداف مستقیم آن، مانندICE1 ، در اکوتیپ های مختلف Arabidopsis و در فواصل زمانی مختلف قرار گرفتن در معرض دمای پایین است.