A Study of Arabidopsis Cold Stress Tolerance Improvement via AthHOS1-Targeting HOS1-AmiRNA Approach

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Abstract

In this study the artificial microRNAs (amiRNAs) technology targeting \textit{HOS1} gene was tested for its applicability for the improvement of cold stress tolerance in Landsberg-0 (Ler-0) ecotype of \textit{Arabidopsis thaliana}. The chosen approach was designed to suppress \textit{AtHOS1} gene expression through the overexpression of \textit{amiRNA-HOS1}. The effect of \textit{AtHOS1-amiRNA} overexpression to transgenic plants' response to cold stress was determined by Real Time PCR. The expression levels of \textit{amiRNA} and its target, \textit{AtHOS1} gene, were observed in 3-week old seedlings of T3 generation and in wild-type plants after 6h, 12h, 24h, 48h and 96h of their exposure to cold stress (4ºC). Comparative analysis revealed that \textit{AtHOS1-amiRNA} negatively regulated \textit{AtHOS1} in transgenic plants upon plants lengthen exposure (for 48h and 96h) to low temperature (Pearson's correlation coefficient of -0.407; $P < 0.05$). Even though prolonged cold stress caused extended up regulation of \textit{AtHOS1} in wild type plants, in transgenic plants \textit{AtHOS1-amiRNA} suppression disturbed expected \textit{AtHOS1} circadian rhythm by preventing further \textit{AtHOS1} up regulation. Moreover, transgenic plants showed \textit{AtHOS1} down regulation 96h after the cold stress onset, due to sufficient overexpression of \textit{AtHOS1-amiRNA}, which allowed cold signaling amplification in transgenic plants. As a result of that, cold-acclimated transformed plants displayed 17% higher freezing tolerance (-1°C to -8°C) in comparison to wild type plants, demonstrating the success of chosen approach in improving Arabidopsis tolerance to low temperatures, at least in Ler-0 ecotype.

Introduction

Low temperatures are one of the major environmental stresses that limit growth, development, productivity and geographical distribution of plants (Ahanger et al. 2017; Zhao et al. 2018). Based on the intensity of low temperatures, induced stress can be classified as cold (temperatures within the range 0–20°C) or freezing stress (temperatures below 0°C) (Ma et al. 2018). Low temperatures trigger numerous processes in plants at molecular, biochemical and morphological level, which may be represented as alert, acclimation, tolerance, exhaustion (under prolonged and intense stresses) and/or recovery phase (after stress factor removal; may lead to a new homeostasis maintenance) (Kosová et al. 2015). For discovering metabolic pathways involved in cold signaling in plants, various forward and reverse genetic approaches were used (Dong and Pei 2014). Among the most studied pathways involved in cold acclimation and frost tolerance in plants is CBF/DREB pathway (Jia et al. 2016; 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, \textit{CBF3/DREB1A} gene, was shown to be induced by \textit{ICE1} gene (inducer of \textit{CBF} expression 1) encoding \textit{ICE1} transcription factor, overexpression of which enhances cold tolerance in transgenic Arabidopsis plants (Chinnuasmy et al. 2003; Zuo et al. 2019). This gene is negatively regulated by \textit{HOS1} gene (high expression of osmotically responsive gene 1), encoding a RING finger protein with E3 ubiquitin ligase activity, that leads to \textit{ICE1} ubiquitination and degradation (interfering with \textit{CBF1} and
CBF2 expression as well) (Ding et al. 2015). In transgenic Arabidopsis plants HOS1 overexpression led to cold/freezing sensitivity, while its knock out led to improved cold/freezing tolerance (via its influence on CBFs and downstream genes such as RD29A, COR47, COR15A, KIN1, as well as on ICE1) (Chinnusamy et al. 2007; Chinnusamy et al. 2010; Barrero-Gil; Shi et al. 2015). Further research showed that AtHOS1 has even wider role, acting as a major integrator of temperature information and as a part of the plant clock system (MacGregor and Penfield 2015; Gil and Park 2018). Moreover, hos1 mutants display defect of the circadian clock through altered nucleo-cytoplasmic mRNA export that causes huge transcriptome changes (MacGregor et al. 2013) and affects cold signaling. All of discovered AtHOS1 roles, involving its direct (via CBF-COR regulon) and indirect effect (by disrupting the circadian clock) on cold signaling, make it an excellent molecular target for plant cold tolerance improvement studies.

Many reverse genetic approaches, like insertional, fast-neutron or chemical mutagenesis, TILLING, virus-induced gene silencing (VIGS) and RNA-mediated interference (RNAi) have been introduced via gene activity disruption (Gilchrist and Haughn 2010). Among methods based on small RNAs, 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 leads to the knock down of endogenous target gene. Asterisk (*) marks antisense sequence of mature amiRNA or miRNA, which is determined according to the thermodynamic stability and nucleotide bias at the 5'-end and generally degraded) (Schwab et al. 2006; Sablok et al. 2011). Such approach is more specific, more trans-generation stable, which possess multitargeting potential and displays increased biosafety in comparison to siRNA-mediated gene silencing (Parizotto et al. 2004; Duan et al. 2008; Ossowski et al. 2008; Zhao et al. 2008; Lin et al. 2009; Molnar et al. 2009; Park et al. 2009; Liu and Chen 2010; Schwab et al. 2010). Even with recent development of novel technologies for gaining insights into gene functions, like CRISPR-Cas9 technology that enables genome editing, amiRNA technology remains preferable method in functional analysis projects based on gene silencing, as currently more feasible and reliable strategy (Zhang et al., 2018). 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 (Schwartz et al. 2009; Yeo et al. 2011) and ZCN gene in corn (Meng et al. 2011), revealing the role of AGP proteins in pollen and ovary development in Arabidopsis (Coimbra et al. 2009) or revealing the role of sm TAF10 gene in male sterility induction in eggplant (Toppino et al. 2011). This technology was also used in crop improvement studies, like amiRNAs silencing of Eui1 and SBE11b genes or amiRNA BADH2 suppression that increased seed fragrance in non-fragrant lines, which improved quality and economical value of rice (Chen et al. 2008; Chen et al. 2013b). In another study, the roles of biotic and abiotic stress responsive genes have been revealed by amiRNAs-mediated gene silencing including disclosing a negative correlation between AthMYB14 and AthCBF in Arabidopsis cold stress tolerance (Chen et al. 2013a) or stuCBP80 being negative regulator of drought tolerance in potato (Pieczynski et al. 2013) or Os-amiR393 increasing the salinity tolerance in Arabidopsis (Gao et al. 2011), etc. 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 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, with aim to induce cold tolerance in transgenic Arabidopsis plants and test its effect during prolonged exposure to low temperatures. Obtained results are encouraging in regards of continuing the research by conducting more detailed characterization of AtHOS1-amiRNA transgenic Arabidopsis plants at RNA, protein, metabolite and phenotype levels and in different time intervals. These findings will open the door 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**

In this study Landsberg-0 (Ler-0) ecotype of Arabidopsis thaliana L. was used. The seeds of Arabidopsis plants were surface-sterilized by immersion in 70% ethanol for 1 min followed by immersion in 1% NaClO$_3$ (Bleach: H$_2$O, 1:4) for 10 min. After rinsing the seeds four times using sterilized distilled water, seeds were mixed in melted 0.1% water agar and plated on the solid ½ MS medium supplemented with 1.5% sucrose in Petri dishes. These Petri dishes were firstly incubated at 4°C in dark for 2 to 3 days to enable growth/development synchronization, following by another incubation step at 22°C under the light intensity of approximately 100 µM/cm2/s using 16h light/8h 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) and grown in the growth chamber for 3-4 weeks (under the same conditions as described above) until the first inflorescences bolting were 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 inflorescences were clipped. Upon 7-10 days on clipping, when the most of secondary inflorescences reached 8-12cm of height, plants were prepared for floral dipping.

**Design and construction of amiRNA**

21mer amiRNA sequence for AthHOS1 gene (ID: AT2G39810) was designed by the Web MicroRNA Designer tool (WMD3) (Schwab et al. 2006). The sequence for HOS1-derived amiR was 5'-UAAAUUGCAGAGGCCC-3' (Fig. 1a). Ath-MIR319a precursor was used as a backbone to engineer HOS1-amiRNA. The amiRNA containing precursor (Fig. 1b) was generated through site-directed mutagenesis using overlapping PCR and the following primers:

1. I 5'-gaTAAATTCGAATATCGAGGCTtctctcttttgtattcc-3'
2. II 5'-gaAGGCTCGATATTCGAAATGAtcaagagaatcaatga-3'
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 RS300, which harbored the 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 699bp long recombinant \textit{AthHOS1-preamiRNA}.

**Cloning and plant transformation**

Final overlapping PCR product was ligated into T/A vector (pTG19-T, Vivantis). Before ligation, 3'-A overhangs (A-Tailing) were added to the blunt-ended PCR products. Transformed \textit{Escherichia coli} (\textit{E.coli}) strain \textit{DH5α}, containing recombinant vector, were selected by using cultivation on Luria-Bertani (LB) media containing Ampicillin (100\,\mu g/ml) and afterwards verified by colony PCR and sequencing. For colony PCR, selected bacterial colonies were mixed with 20\,\mu l of PCR reaction mixture containing 10X Taq Plus buffer, 1.5U Taq Plus DNA polymerase (#K1116-500, Kawsar Biotech Company), 2mM MgCl₂, 200\,\mu M of each of dNTPs and 0.25\,\mu M of each of forward/reverse PUC/M13 primer. The PCR amplification conditions were as follows: initial denaturation at 94°C for 3 min and 30 cycles of denaturation at 94°C for 15 sec, followed by annealing at 55°C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 5 min. PCR products were analyzed by 1% agarose electrophoresis. Recombinant plasmids were extracted from transformed bacterial colonies and sent to Macrogen Company (Korea) for sequencing with forward PUC/M13 primer.

The binary destination vector, pCAMBIA 1301/2301 and confirmed carrier vector were digested using \textit{KpnI} and \textit{XbaI} and obtained digestion products were separated by agarose gel electrophoresis. The 480bp long fragment released from the carrier vector was sub-cloned into linearized binary vector to get the artificial miRNA expression cassette. The secondary structure of the sequenced amiRNA precursor 217 nucleotides long was predicted by Mfold web server (Zuker 2003). The recombinant plasmids were electroporated into the \textit{Agrobacterium tumefaciens} strain \textit{GV3103}. The selection of transformed \textit{A. tumefaciens} was done similarly as the assay performed on \textit{E.coli}.

Plant transformation was accomplished by floral dip protocol (Clough and Bent 1998; Bent 2006). In this method, inflorescences of Arabidopsis were dipped in duration of 30 second into inoculum media containing 5% sucrose, 0.05% Silwet L-77 and resuspended Agrobacterium carrying the genes of interest and incubated in the dark for 24 hours. Afterwards, plants were placed in the growth chamber under 16h light/8h dark photoperiod regime at 22 °C to complete their development until seed harvesting.

**Transformed plants screening**
Screening experiments were performed on first and second transgene plants generations (T1 and T2 generations). Sterilized seeds of Arabidopsis transgenic lines were plated on the solid ½ MS medium supplemented with 1.5% sucrose and 50μg/ml kanamycin, since only transgenic seeds were able to germinate and grow normally in this medium (Fig. S1a). After 7 to 10 days, green and healthy seedlings were selected and sub-cultured in ½ MS medium without antibiotic (Fig. S1b). Two weeks later, the seedlings were transplanted to the plastic pots containing coco coir: peat moss (in ratio 1:2) (Fig. S1c). After transferring healthy plants to the pot and adapting them, 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 (1983) followed by the identification of HOS1-amiRNA using PCRs. Corroborant PCR reactions were performed on T1 and T2 plants using the following primers:

1. HOS1-amiRNA1-F  5’-GATGCGGTTAGACAAATTGGATCA-3’
2. HOS1-amiRNA1-R  5’-TCAAGCATGTTTTTGTCAGGA-3’
3. CaMV35S promoter-F 5’-GACCTAACAGAACTCGCGT-3’
4. Nos terminator-R 5’-TGCCAATGTTTTGAACGCTG-3’
5. HOS1-amiRNA 2-F 5’-AGCTTCCGACTCATCCA-3’

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, 1024bp long PCR product by using primers 3 and 4, and 323bp long PCR product by using primers 4 and 5 (Fig. S1d).

The presence of contaminating Agrobacterium was investigated through PCR reaction based on the amplification of 730bp long fragment from the Agrobacterium Virg gene using primers Virg-F: 5’-CCTTACGATCCACGCTTCA-3’ and Virg-R: 5’-GGCACATCGCTTACTCCTCA-3’) (Siritunga and Sayre 2003).

**Cold stress treatment**

Cold treatments were based on the study of Dong and Pei (2014). T1 and T2 plants were cultivated in trays filled with 1.5-2.5 cm of standing water. T3 mature seeds were collected from the transgenic T2 plants and cultivated in drained trays which were alternately irrigated and nourished with fertilizer solution (20-20-20) every 10 days. Simultaneously, homozygous T3 plants and wild-type seedlings were transplanted on separate halves of the same pot and 3-week old seedlings were subjected to the temperature of 4° C in duration of 6h, 12h, 24h, 48h and 96h under 16h light/8h dark photoperiod regime in the growth chamber (the temperature was gradually reduced from 22°C to 4°C using the chamber cooling rate of 3°C/h). 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 (containing new and old leaves) were taken immediately upon elapsed stress-time and after freezing in liquid nitrogen stored at -80ºC.
Freezing tolerance assay

The evaluation of freezing tolerance was conducted as described in Chinnusamy et al. (2003) and Dong et al. (2006), with several modifications. Briefly, 10-day-old transgenic Arabidopsis plants with HOS-amiRNA-overexpression 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 16h light/8h dark photoperiod regime for 4 days in the growth chamber (the temperature was gradually reduced from 22°C to 4 °C using the chamber cooling rate of 3°C/h). Afterwards, plants were moved on ice and held in a freezing chamber at -1°C in the dark for 16 h. Upon this time the temperature was further lowered to -8°C (at chamber cooling rate of -1°C/h) and plants were held at this final temperature for 2h. After freezing treatment, Petri dishes with plants were thawed at 4°C overnight and transferred to a growth chamber at 22°C under long-day condition. After a week, survival rates of seedlings were scored visually based on counting the ones displaying normal growth and green, healthy looking leaves.

RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg of grinded samples using TRIzol reagent (Ambion) according to the manufacturer’s instruction and treated with the DNasel, RNase Free Enzyme (#EN0521, Thermo Scientific) to eliminate DNA contamination. Afterwards 1 µg of total RNA was used for the oligo (dT) cDNA first strand synthesis according to the method of Chen et al. (2005) and Varkonyi-Gasic et al. (2007), while miRNA cDNA synthesis was performed using 200 ng of purified RNA and 1 µM of specific stem-loop RT primer (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCACGGCCT-3'). Synthesis of both types of cDNA 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 min, followed by 40 cycles of amplification (95°C for 5 s and 60°C for 30 s). For each reaction, 2µl of cDNA was added to 10µl of PCR mixture containing 6 µl of SYBR Premix Ex Taq (Takara) and 0.83µM forward/reverse primers. 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). Gene-specific primers used for AthAct2 amplification were Act2-F: 5’- TCCGCTCTTTTTTCTCAAGGT-3’ and Act2-R: 5’- AGTGGAGGTTCACAGATCCAA-3’. The other gene-specific primers, used to assess HOS1-amiRNA and AthHOS1 expression, were as follows: amiRNA-F: 5’-ATCCAGTGACGGGTCCGAGG-3’, amiRNA-R: 5’-GC GGCGTAAATTCGAATATCG-3’, HOS1-F: 5’-TGCCAATGTTGAACGCTG-3’ and HOS1-R: 5’-AGCTTCCGACTTCTCATCCA-3’.

The relative gene expression level was calculated using the 2−ΔΔCT method (Livak and Schmittgen 2001). Student’s t-test was used to analyze differences between treated and non-treated samples (for a
statistically significant difference $P$-value was required to be less than 0.05) (Goni et al. 2009).

**Results**

Obtained recombinant $AtHOS1$-$preamiRNA$ (confirmed as a single PCR product of 699 bp, Fig. S2) was cloned into pTG19-T vector, which was confirmed by sequencing. Upon restriction of transformed vector, 480 bp long restriction fragment (confirmed by agarose electrophoresis, Fig. S3) was sub-cloned into the binary vector creating the artificial $AtHOS1$-$miRNA$ expression cassette (schematically presented in the Fig. 2a), which was also confirmed by sequencing. This cassette encoded for 217 nucleotides long $AtHOS1$ amiRNA precursor and the prediction of its secondary structure is shown in Fig. 2b. 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 $AtHOS1$-$amiRNA$ (Fig. 3a); 116 bp long PCR product, confirming the presence of mature $AtHOS1$-$amiRNA$ (Fig. 3b) and 323 bp long PCR product confirming the presence of mature $AtHOS1$-$amiRNA$ and Nos terminator sequence (Fig. 3c), while in non-transgenic plants no amplification products were obtained. Also, PCR confirmed there were no contaminations with Agrobacterium (Fig. 3d).

Molecular analysis results revealed the presence of mature $AtHOS1$-$amiRNA$ in all examined transgenic plants (Fig. S4). Considering the time profile of $AtHOS1$ expression it was fluctuating both in transgenic and wild type plants. Obtained results showed that after 6h of the exposure to cold stress $AtHOS1$ was 1.24-fold down regulated in wild type plants, but, surprisingly, it was 1.25-fold up regulated in transgenic plants (only the observed differences were not statistically significant). Upon 12h of the exposure to cold stress $AtHOS1$ was down regulated in both non-transformed and transformed plants, only more prominently in the later (1.65-fold decrease in wild type plants vs. 3.22-fold decrease in transgenic plants). Unexpectedly, 24h after the cold stress exposure, the both type of plants showed the same response of 1.2-fold up regulation of $AtHOS1$, which was followed by $AtHOS1$ down regulation in both type of plants at 48h of cold stress (2-fold decrease in wild type plants vs. 1.78-fold decrease in transgenic plants). Finally, at 96h of the exposure to cold stress, transgenic and wild type plants showed completely opposite responses, that included 3.42-fold down regulation of $AtHOS1$ in transgenic plants and 1.72-fold up regulation of $AtHOS1$ in wild type plants (Fig. 4a). Hence, while prolonged cold stress led to $AtHOS1$ up regulation in wild type plants, it led to prominent down regulation of $AtHOS1$ in transgene plants, with approximately 5-fold difference in the $AtHOS1$ expression levels between non-transgenic and transgenic plants.

Considering expression profile of $AtHOS1$-$amiRNA$ over 96h of transgenic plants exposure to cold stress, it was 1.7-fold up regulated at 6h, 1.09-fold down regulated at 12h and then returning to the level of the reference gene expression at 24h (however, the changes of $AtHOS1$-$amiRNA$ expression recorded at 6h, 12h and 24h were not statistically significant). After 24h of the cold stress onset the expression profile of $AtHOS1$-$amiRNA$ in transgenic plants showed a continuous increase, recording 5.55-fold up regulation at 48h and reaching maximal 9.65-fold up regulation at 96h (Fig. 4b) of the cold stress duration. Comparison of $AtHOS1$ and $AtHOS1$-$amiRNA$ expression profiles in transgenic plants exposed to cold
stress revealed that during the first 24h the two expression profiles followed each other and that both were up regulated at 6h, down regulated at 12h and up regulated at 24h. However, after the first 24h of the exposure to cold stress AtHOS1 and AtHOS1-amiRNA expression profiles in transgenic plants started to oppose each other as the recorded AtHOS1 expression was down regulated, while AtHOS1-amiRNA expression was up regulated at 48h and 96h upon the cold stress onset. Even though the negative correlation between AtHOS1-amiRNA and AthHOS1 was not observed at 6, 12 and 24 hours after the transgene plants were exposed to cold stress, with the prolongation of cold stress exposure to 48h and 96h, negative regulation of AthHOS1 via AtHOS1-amiRNA was observed in the transgenic Ler-0 plants (Pearson's correlation coefficient of -0.407; P < 0.05) (Fig. 4c). Detected negative correlation between AtHOS1-amiRNA and AthHOS1 during extended cold stress corresponded with the observed 5-fold difference in AthHOS1 expression levels between transformed and non-transformed plants at 96h.

After 4 days of the acclimatization to cold, both transformed and non-transformed plants did not show any obvious signs of chilling injuries (Fig S5). However, the freezing-tolerance testing, to which cold-acclimated transformed and non-transformed plants were subsequently subjected to, revealed statistically significant differences in their response to freezing temperatures (-1°C to -8°C). Results of the freezing test tolerance revealed that transgenic seedlings had the survival rate of 38.5%, while wild type plantlets had the survival rate of 21.5%. Thus, transgenic plants showed 17% higher number of plants displaying normal growth and healthy looking green leaves in comparison to wild type plants after recovery from freezing test. These results showed that approach using overexpressed AtHOS1-amiRNA to target AtHOS1 was successful in increasing the cold and freezing tolerance in Arabidopsis transgenic plants, leading to 17% higher survival rate in transgenic plants, at least in Ler-0 ecotype (Fig. 5a, 5b).

**Discussion**

Presented assay was successfully employed to increase A. thaliana Ler-0 plants cold stress tolerance through AtHOS1 suppression mediated by AtHOS1-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 96h, to low temperatures. This is supported by the finding that negative correlation between AtHOS1-amiRNA and AtHOS1 target gene was observed only 48h and 96h after the cold stress onset. Thus, in order to obtain the higher level of the AtHOS1-amiRNA overexpression, required to exert desired effect on the target gene, the duration of cold stress seems to be an important factor. Interestingly, while at all other tested time intervals AtHOS1-amiRNA was up regulated, it was down regulated only at 12h, which may imply that other players took action at this time point, influencing AtHOS1-amiRNA expression/accumulation under tested experimental conditions. Being that the accumulation of (artificial) miRNA is under the influence of the expression profile and dynamics of the main players involved in (artificial) miRNA biosynthesis and degradation, it is needed to further investigate how AtHOS1-amiRNA expression/accumulation is timely influenced under tested conditions and via which molecular factors/pathways.
Another interesting observation was recorded 6h upon exposure to cold stress, when \textit{AtHOS1} in transgenic plants was up regulated despite recorded \textit{AtHOS1-amiRNA} up regulation and observed \textit{AtHOS1} down regulation in wild type plants at the same time point. This may suggest that the presence of \textit{AtHOS1-amiRNA} at this particular time point included additional players and/or their post-translational modifications that impacted the expression of \textit{AtHOS1} as the target gene (since each (artificial) miRNA family specifically regulates its targets and the ones involved in cold signaling are time- and temperature-sensitive) (von Born et al. 2018). Also, 24h upon exposure to cold stress, despite the confirmed \textit{AtHOS1-amiRNA} up regulation, \textit{AtHOS1} in transgenic plants was up regulated and as high as \textit{AtHOS1} in wild type plants at the same time point. So, 24h of the cold stress onset the ongoing mechanisms in transformed and non-transformed plants led to the same final effect, which is \textit{AtHOS1} prevention of cold signaling amplification. This may imply that at 24h the entire survival mechanism aims to preserve both transformed and non-transformed plants from the excessive cold response, while acquired overexpression of \textit{AtHOS1-amiRNA} in transgenic plants at this time point is still not sufficient enough to overcome the primary survival response. However, survival strategy changes with further cold stress prolongation to 48h in which both type of plants allowed cold signaling amplification by down regulating \textit{AtHOS1}. Finally, further elongation of cold stress to 96h led to another change in the survival strategy toward another prevention of the cold signaling amplification, only at this time point it resulted in divergent response in transformed and non-transformed plants (because of significantly increased level of \textit{AtHOS1-amiRNA} overexpression in transgenic plants). While wild type plants at 96h reacted by \textit{AtHOS1} up regulation to prevent plants exhaustion due to excessive cold response, overexpression of \textit{AtHOS1-amiRNA} in transgenic plants reached a sufficient level at this time point to overrule the primary survival strategy, leading to significant down regulation of \textit{AtHOS1} and allowing further cold signaling amplification in transgenic plants. Additional research is needed to clarify the effect of \textit{AtHOS1-amiRNA} approach in different time intervals of the cold (4°C) and freezing (-1°C to -8°C) stress exposure (i.e. RACE technique may be suitable for accurately determining the target gene).

Considering down regulation of \textit{AtHOS1} in wild type Arabidopsis Ler-0 plants observed 12h, 48h and 96 h and up regulation at 6h and 24h upon cold stress onset, such finding is partially in concordance with the report of Lee et al. (2001) in wild type Arabidopsis C24 ecotype. Their report recorded temporary down regulation of \textit{AtHOS1} gene at the beginning of cold stress (after 30 min), followed by up regulation within first hour, which was kept ongoing over the first 24h and then down regulated 48h after the cold stress onset in C24 wild type plants. However, our finding in Ler-0 wild type plants at 12h was discordant from this previous report, which could possibly be explained by later reported differences in cold transcriptomes between Ler-0 and C24 ecotype (Barah et al. 2013). Nonetheless, the similar response of wild type plants of two ecotypes observed at 24h and 48h of cold stress exposure may be explained by the activation of the general time-dependent cold survival mechanism with the prolongation of the cold stress, independently of ecotype. Our finding that wild type Ler-0 plants showed up regulated \textit{AtHOS1} after 96h implied that the extended cold stress caused its increase in order to prevent further cold response that could be exhausting to a plant. Oppositely, at the same time point amiRNA approach changed biorhythm and circadian clock linked to \textit{AtHOS1} expression in transgenic plants through \textit{AtHOS1}
down regulation, enabling amplification of cold signaling and increasing transgene plants tolerance to low temperatures. Concordantly, after 4 days of cold acclimatization the freezing test showed 17% increase in the survival rate of transgenic Ler-0 plants in comparison to wild type plants. Obtained freezing tolerance is provided, on one hand side, through soluble sugar production activated via GIGANTEA (GI) induced by cold stress, and on the other hand side, through CBF genes that are under AtHOS1 influence (AtHOS1 suppression induces CBF-COR regulon, which also influences differential alternative splicing of genes under the regulon control) (Gil and Park 2018). Nonetheless, since AtHOS1 gene displays complex pleiotropic role (i.e. it impacts flowering time via chromatin remodeling of Flowering Locus C, facilitates nucleocytioplasmatic mRNA transport important for maintaining overall circadian periodicity via associating with nuclear pore complex, etc.), its expression regulation during prolonged cold stress will consequently affect all its roles. In order to reach firm conclusion on the different effects of performed transformation further experimental investigations are needed at transcriptomic and proteomic level. Future transcriptomic research should investigate to a greater detail to what extent and in which way AtHOS1 down regulation affects mRNA export and circadian clock in transgenic plants as well as temperature (within temperature interval from 22°C to -8°C) and developmental stage influence on the AtHOS1-amiRNA approach. 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 will benefit from developing cold tolerance (i.e. almond, grapevine and tea (Karimi et al., 2016; Sun et al., 2015; Zhang et al., 2014)).

Conclusion

This study investigated amiRNA application in improving cold/freezing stress tolerance in Arabidopsis Ler-0 ecotype through AthHOS1 knock out mediated by AtHOS1-amiRNA over expression. This approach resulted in a statistically significant negative regulation of AthHOS1 expression via AtHOS1-amiRNA which was recorded with the prolongation of the cold stress (4°C) exposure to 48h and 96h. Finally, transgenic Ler-0 plants displayed improved tolerance to low temperatures, which was confirmed by 17% higher survival rate of cold acclimatized transgenic plants in comparison to cold acclimatized wild type plants when exposed to freezing temperatures (-1°C to -8°C). Transgene Ler-0 plants became more resilient to freezing stress after 4 days cold acclimatization, displaying greater number of plants with normal growth and green leaves in comparison to wild type plants under the same conditions. Even though the obtained results look promising, further characterization of AtHOS1-amiRNA approach effects in different time intervals of low temperatures exposure and in different Arabidopsis ecotypes is needed (especially due to complex pleiotropic role of AtHOS1 gene), before it can be considered to be extended for use for cold tolerance introduction in some of agriculturally important plant species.

Abbreviations

Artificial microRNAs (amiRNAs)

C-repeat responsive element binding factor/dehydration-responsive element binding factor) (CBF/DREB)
C-repeat elements/dehydration-responsive elements (CRT/DRE)

Inducer of CBF expression 1 (ICE 1)

High expression of osmotically responsive (HOS1)

Targeting Induced Local Lesions IN Genomes (TILLING)

Virus-induced gene silencing (VIGS)

RNA-mediated interference (RNAi)

Web MicroRNA Designer tool (WMD3)

Landsberg-0 (Ler-0)

Base pair (bp)

Luria-Bertani (LB)

Declarations

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Code availability: Not applicable

Ethical Procedure:

- The research meets all applicable standards with regard to the ethics of experimentation and research integrity, and the following is being certified/declared true.
- As an expert scientist and along with co-authors of concerned field, the paper has been submitted with full responsibility, following due ethical procedure, and there is no duplicate publication, fraud, plagiarism, or concerns about animal or human experimentation.

Author contribution statement
Behrouz Shiran (BS) conceived and designed the research. Marzieh Karimi (MK), BS and Mohammad Rabei (MR) conducted experiment. MK and Hossein Fallahi (HF) carried out experiment and analyzed data. MK, BS and Bojana Banović Đeri (BBD) wrote the manuscript. All authors read and approved the manuscript.

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**Figures**

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)
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-amRNA1-F and HOS1-amRNA1–R primers (b), 323 bp amplicon generated by HOS1-amRNA 2-F and Nos terminator sequence (c), 730bp amplicon generated by Agrobacterium VirG specific primers (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
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
Figure 5

Survival rates of transgenic and wild type Arabidopsis plants of Ler-0 ecotype (a) and improved survival ratio of amiRNA-HOST overexpressing transgenic Ler-0 plants after a freezing treatment (b) WT- wild type plants, T – transgene plants, n.s. - no statistical significance and * - statistical significance at 95% confidence level according to t-student test

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