



Efficient use of artificial micro-RNA to downregulate the expression of genes at the post-transcriptional level in *Arabidopsis thaliana*

A. Ud-Din^{1,4}, M. Rauf⁴, S. Ghafoor¹, M.N.K. Khattak², M.W. Hameed³, H. Shah⁵, S. Jan¹, K. Muhammad¹, A. Rehman² and Inamullah¹

¹Department of Genetics, Garden Campus, Hazara University, Mansehra, Pakistan

²Department of Zoology, Garden Campus, Hazara University, Mansehra, Pakistan

³Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Karachi, Pakistan

⁴Institute of Biochemistry and Biology, University of Potsdam, Potsdam, Germany

⁵National Agricultural Research Centre, Islamabad, Pakistan

Corresponding author: A. Ud-Din

E-mail: azizkhan91@yahoo.com / azizkhan@hu.edu.pk

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ABSTRACT. Micro-RNAs are cellular components regulating gene expression at the post-transcription level. In the present study, artificial micro-RNAs were used to decrease the transcript level of two genes, *AtExpA8* (encoding an expansin) and *AHL25* (encoding an AT-hook motif nuclear localized protein) in *Arabidopsis thaliana*. The backbone of the *Arabidopsis* endogenous *MIR319a* micro-RNA was used in a site-directed mutagenesis approach for the generation of artificial micro-RNAs targeting two genes. The recombinant cassettes were expressed under the control of the CaMV 35S promoter in individual *A. thaliana* plants. Transgenic lines of the third generation were tested by isolating total RNA and by subsequent cDNA synthesis using oligo-dT18 primers and mRNAs as templates. The

expression of the two target genes was checked through quantitative real-time polymerase chain reaction to confirm reduced transcript levels for *AtExpA8* and *AHL25*. Downregulation of *AtExpA8* resulted in the formation of short hypocotyls compared with those of the wild-type control in response to low pH and high salt concentration. This technology could be used to prevent the expression of exogenous and invading genes posing a threat to the normal cellular physiology of the host plant.

Key words: Artificial micro-RNA; *Arabidopsis thaliana*; qRT-PCR; *AtExpA8*; *AHL25*

INTRODUCTION

Gene expression can be affected by two classes of small RNAs in animals and plants: small interfering RNA and micro-RNA (miRNA). Normal gene function can be interfered on several pre- and post-transcriptional levels by these small RNAs. To inhibit the translation of messenger RNA (mRNA) into a functional protein, cells use protein machinery called RNA-induced silencing complex (RISC). RISC uses small RNAs to recognize and target a very specific complementary motif in the nucleic acids (Bartel, 2004; Filipowicz, 2005). miRNAs are widely distributed, usually 18 to 25 nucleotides noncoding small RNAs. They play important roles in plant development and responses to different kinds of biotic and abiotic stresses by regulating gene expression (Lagos-Quintana et al., 2003; Bartel, 2004). A number of putative miRNAs have been predicted with the help of high throughput sequencing and computational biology (Sunkar and Jagadeeswaran, 2008; Chandrasekaran et al., 2013). Recently, in animals and plants, endogenous miRNA precursors have been used to develop artificial miRNA technology. This has led to the generation of target-specific miRNA for gene silencing (Schwab et al., 2006; Molnar et al., 2009; Zhou et al., 2013).

Plant cells secrete different types of proteins and many other enzymes that modify cellulose and other polysaccharides in the cell wall (Fry, 1995). One of these proteins is expressed by expansins, which trigger changes in the cell wall by loosening it (Cosgrove, 2000). Expansins are involved in many processes, mainly in the modification of cell walls including wall dissolution, pollen-tube invasion, cell separation, and control of plant cell growth (Rose and Bennett, 1999; Lee et al., 2001; Cosgrove et al., 2002; Li et al., 2003). Expansin-mediated cell wall enlargement is required for the complex and highly coordinated pattern of growth and morphogenesis in plants.

The differences in the patterns of *EXPANSIN* expression have different effects on plant morphology, e.g., the expansion of *Arabidopsis* leaves (Cho and Cosgrove, 2000), soybean roots (Lee et al., 2003), rice internodes (Choi et al., 2003), and wheat coleoptiles (Zhao et al., 2011). Similarly, AT-hook nuclear localized (AHL) proteins also play important roles in plant development (Lim et al., 2007; Xiao et al., 2009). They are widely distributed in a range of plant species namely, *Arabidopsis* (*A. thaliana*), rice (*Oryza sativa*), poplar (*Populus trichocarpa*), pea (*Pisum sativum*), Madagascar periwinkle (*Catharanthus roseus*), maize (*Zea mays*), and grape vine (*Vitis vinifera*). About 29 AHL proteins have been identified in *A. thaliana* (Fujimoto et al., 2004; Grasser et al., 2007; Vom Endt et al., 2007), and are expressed in most plant organs (Matsushita et al., 2007; Street et al., 2008).

In the present study, we successfully downregulated *AtExpA8* and *AHL25* expression using artificial miRNA (amiRNA) to target these genes in individual *A. thaliana* plants and analyzed the effect of down regulated transcript levels on the growth of etiolated hypocotyls in response to low pH and high salt concentrations compared with that of the wild-type control. The reduction in the

expression level was determined by quantitative real-time polymerase chain reaction (qRT-PCR). The resulted lines of amiRNA for *AtExpA8* were sensitive to stresses such a slow pH and high salt concentrations as compared with the wild-type control.

The objective of the present study was to downregulate gene expression, i.e., *AHL25* and *AtExpA8* and investigate their effect on the phenotype of *A. thaliana* transgenic lines.

MATERIAL AND METHODS

General strategy

Standard methods such as plasmid isolation, restriction analysis of nucleic acids, gel electrophoresis, cloning and transformations, were performed as described by Hanahan (1983) and Sambrook and Russell (2001).

The oligonucleotides (primers) were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Sequences of the primers used in this study for qRT-PCR were designed using online tool of QuantPrime (Arvidsson et al., 2008).

The primers used to generate the amiRNA constructs from *AtEXPA8* and *AHL25* are as follows: A8-miR-s, 5'-GATCTAAGCTAATTAAGCGGGTCTCTCTCTTTGTATTC-3'; A8-miR-a, 5'-GAGACCCGCTTAATTAGCTTAGATCAAAGAGAATCAATGA-3'; A8-miR*s, 5'-GAGAACCGCTT AATTTGCTTAGTTCACAGGTCGTGATATG-3'; A8-miR*a, 5'-GAACTAAGCAAATTAAGCGGTTCT CTACATATATATTCCT-3'; and miRAHL-s, 5'-GATCTAGTTATAATCGTCGGACGTCTCTCTTTGT ATTCC-3'; miAHLR-a, 5'-GACGTCCGACGATTATACTAGATCAAAGAGAATCAATGA-3'; miRAHL*s, 5'-GACGCCCCGACGATTAAACTAGTTCACAGGTCGTGATATG-3'; miRAHL*a, 5'-GAACTAGTTTAAATCGTCGGGCGTCTACATATATATTCCT-3', with asterisks indicating adapted as described by Schwab et al. (2006).

Plant material and growth conditions

A. thaliana (L.) Heynh. ecotype Col-0 was used in these experiments. Seeds were surface sterilized with a sterilization solution (1% sodium hypochlorite, 0.02% Triton X-100), first treated with 500 µL 70% ethanol for 5 min and then 500 µL sterilization solution was added in 2-mL Eppendorf tubes and subjected to continuous shaking for 10 min. Subsequently, they were washed three times with sterile distilled water and dried on Whatman filter paper. All steps were performed under sterile conditions in a flow-hood clean bench. Sterile seeds were plated on MS medium with 2% sucrose and 0.7% agar (Murashige and Skoog, 1962). The MS medium was supplemented with appropriate selection markers for the selection of positive transformants. Seeds were then exposed to vernalization (16 h light at 140-285 µE, 22°C; 8 h dark; 8°C, 70% humidity) conditions for 1 week and then placed in Percival growth chambers (16 h light at 140-285 µE, 22°C; 8 h dark, 22°C, 70% humidity). The plants were later transplanted to soil (type GS90; Gebr. Patzer, Sinntal-Jossa) in 6-cm pots and maintained in a phytotron growth chamber (16 h light at 80-120 µE, 20°C; 8 h dark, 16°C; 70% humidity).

Isolation of total RNA

Total RNA was extracted using either TRIzol Reagent (Gibco Life Technologies; Eggenstein, Germany) or Qiagen RNeasy Plant Mini Kit (Qiagen Hilden, Germany) following manufacturer

instructions. The fresh or frozen plant material (50-200 mg) was ground in liquid nitrogen and subsequently treated according to the protocol described by the manufacturer. The RNA pellet was dissolved in 30 μ L RNase-free water for TRIzol, or 25-30 μ L elution buffer for Qiagen RNeasy.

First-strand cDNA synthesis and RT-PCR

First-strand cDNA was synthesized from freshly isolated RNA by reverse transcriptase using Fermentas First strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer protocol.

qRT-PCR

After synthesis of cDNA, it was used to analyze the relevant abundance of a particular mRNA. Efficiency of cDNA synthesized was controlled by qRT-PCR amplification of house-keeping genes *UBIQUITIN10* (At4g05320) and *ACTIN2* (At3G18780). An ABI PRISM 7300 sequence detection system (Applied Biosystems Applied Biosystems, Darmstadt, Germany) was used for the amplification and detection of PCR fragments as described by Caldana et al. (2007). The reactions were performed in triplicate and the SDS 2.2.1 software (Applied Biosystems Applied Biosystems) was used to determine the abundance of each transcript in the respective samples. C_T (threshold cycle) values were obtained by analysis of amplification curves with a normalized reporter (R_n : the ratio of the fluorescence emission intensity of SYBR Green to the fluorescence signal of the passive reference dye) threshold of 0.2. The obtained data were normalized to the reference gene *UBIQUITIN10*; $\Delta C_T = C_T$ (gene under consideration) - C_T (*UBQ10*). The expressions were measured in three replicates in a single PCR run, average C_T was used for relative expression analyses.

Measurement of hypocotyl length

Seeds were germinated on agar containing MS medium plates. Plates were maintained in dark for 3-5 days at 4°C to synchronize seed germination and then placed for 1 day in the dark before being exposed to normal growth conditions. Hypocotyl length was measured 3-5 days after germination using the Leica software and a stereomicroscope.

Statistical analysis

Data were first recorded and then analyzed. Statistical analysis was performed using a *t*-test. Statistically significant differences were confirmed at 0.05 or 0.01.

RESULTS

Selected genes for artificial miRNA

Two genes were selected for the generation of amiRNA constructs: *AtExpA8* (At2g40610) and *AHL25* (At4g35390). The coding sequence of *AtExpA8* comprised of 762 bp encoding a protein of 253 amino acids (Figure 1A). The predicted molecular weight of *AtExpA8* is 27.27 kDa. The first 25 amino acids are annotated as signal peptides. There are two conserved characteristic domains present in *AtExpA8* that classify it as a member of the expansin family. The first domain

is 103 amino acids long (amino acids 48-160), while the second domain includes 81 amino acids (positions 170-250). The N-terminal domain 1 has structural and sequence similarity to family-45 endoglucanases (EG45-like domain). This domain has a distant homology to the catalytic domain of glycoside hydrolase family 45 (GH45) proteins. Fungal β -1,4-D-endoglucanases represent most members of this family; members from this family have been characterized and their mechanism of action has been determined (Davies et al., 1995). The C-terminal domain 2 is designated as a putative polysaccharide-binding domain (PP-BD).

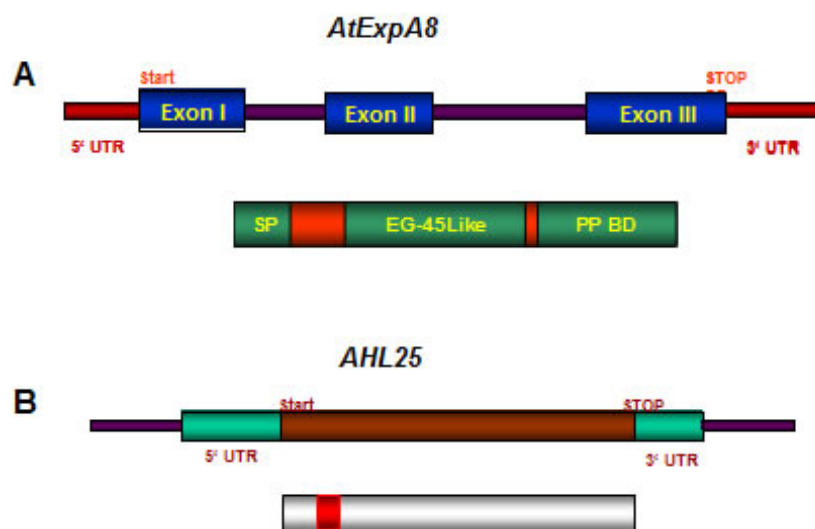


Figure 1. Schematic diagram of the *AtExpA8* and *AHL25* genes and proteins. **A.** Pictorial representation of the *AtExpA8* gene (**A** upper) and the protein 27.27 kD (**A** lower). The SP (amino acids 1-25, signal peptide), conserved EG45-like domain (amino acids 48-160 with structural and sequence similarity to family-45 endoglucanases), and the PP-BD (amino acids 170-250, putative polysaccharide binding domain) are shown. **B.** Pictorial representation of the *AHL25* gene. Start and stop codons, as well as untranslated regions (5'UTR and 3'UTR) are indicated (**B** upper). The conserved AT-hook motif in the protein is shown in red. The PPC (plants and prokaryotes conserved) domain is underlined (**B** lower).

The coding sequence of the AT-hook motif protein *AHL25* (At4g35390) comprised 897 bp encoding a protein of 299 amino acids (Figure 1B). The predicted molecular weight of *AHL25* is 30.5 kDa. *AHL25* is an intron-less gene. The 5' untranslated region (UTR) and 3'UTR of *AHL25* are 277 and 190 bp long, respectively. A number of homologs of *AHL25* were also found in other plant species such as *Z. mays*, *O. sativa*, *V. vinifera*, *Ricinus communis*, and *P. trichocarpa* (<http://blast.ncbi.nlm.nih.gov>).

Generation of artificial miRNA lines

One of the factors in the functional analysis of a gene is knockout, i.e., to completely diminish or reduce its expression. Therefore, to downregulate *AtExpA8* and *AHL25* expression, the artificial miRNA method was adopted. Primers were generated using an online tool (<http://wmd.weigelworld.org/cgi-bin/mirnatools.pl>). Sequences of the primers used for amplification and generation of the ami*AtExpA8* and ami*AHL25* are provided in the Material and Methods section.

The amiRNA for *AtExpA8* and *AHL25* were generated by overlapping PCR. In the first round of PCR, fragments (a) to (c) were separately amplified using plasmid pRS300 as a template (Schwab et al., 2006). In the fourth round, the amplified fragment was obtained by overlapping PCR (Table 1), where the products of the previous PCRs were used as templates.

The amplified fragments were digested with *Xho*I and *Spe*I and subsequently cloned individually in a binary vector pJFH1 under the control of the CaMV 35S promoter. A total of 9 and 7 transgenic lines were selected for *AtExpA8* and *AHL25*, respectively, through *Agrobacterium tumefaciens* transformation and by subsequent selection on kanamycin supplemented media (Figure 2).

Table 1. Scheme of PCRs.

	Primer pair	Template
(a)	A and IV	pRS300
(b)	III and II	pRS300
(c)	I and B	pRS300
(d)	A and B	(a)+(b)+(c)

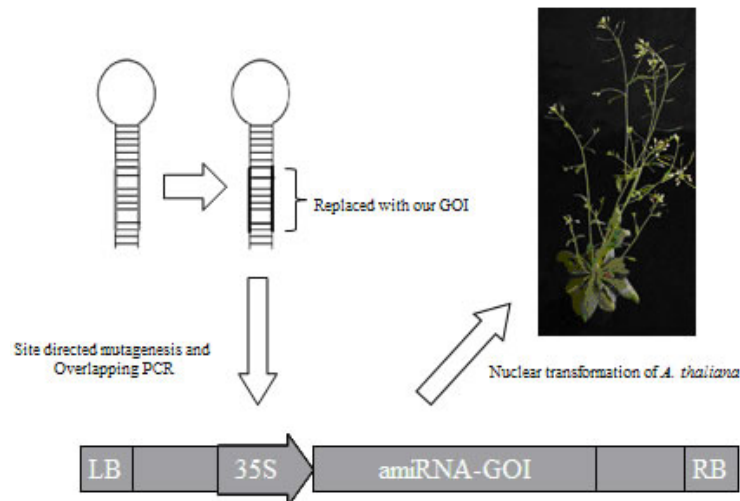


Figure 2. Schematic diagram of artificial miRNA generation. *Arabidopsis* micro-RNA *MIR319a* was used as a precursor for the construction of artificial micro-RNA for the gene of interest (GOI) through site-directed PCR followed by overlapping PCR (Schwab et al., 2006). The amplified fragment was cloned under CaMV 35S promoter in a binary vector. *Arabidopsis thaliana* (Col-0) plants were transformed with the cassette through *Agrobacterium tumefaciens*-mediated nuclear transformation. Third generation (T3) transformed lines were used for further experiments.

Analysis of transcript level through qRT-PCR

The reduction in the transcript level by artificial miRNA was investigated through qRT-PCR in *pamiRNA::ExpA8* and *pamiRNA::AHL25* lines respectively (Figures 3 and 4). Primers used for qRT-PCR were 5'-ACATCTCTCTGTTTTGGGTGCT-3' and 5'-CAACCATTGAAGCAATAATTGCG-3' for *AtExpA8* and 5'-GGCTCTTCCTCCGGACGTCG-3' and 5'-GAGACTGCCTCGGATATGTCC-3' for *AHL25*.

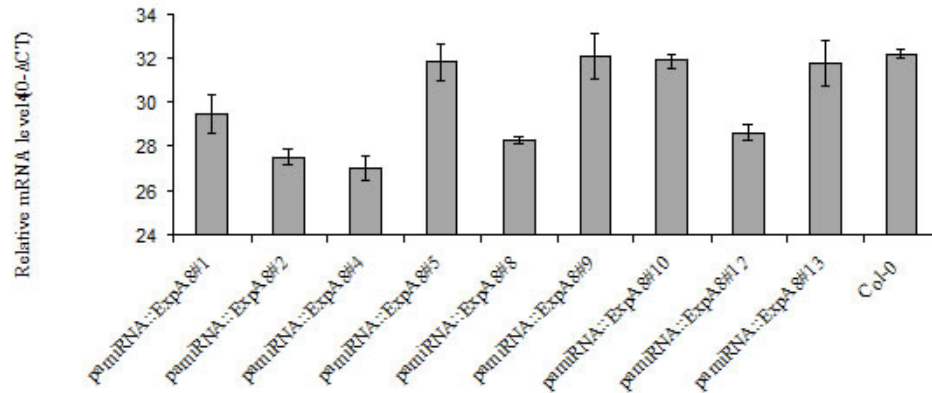


Figure 3. Expression analysis of *AtExpA8* in artificial micro-RNA lines in *Arabidopsis thaliana* Col-0 background. Expression level of *AtExpA8* was analyzed through qRT-PCR and normalized to the expression of *UBQ10* in different *pamiRNA::AtExpA8* transgenic lines (ami#) and compared with the wild-type (Co-0). Total RNA was extracted and cDNA was made from two week-old seedlings. The mean value of the expression level is given from three technical replicates while error bars indicate the standard deviation.

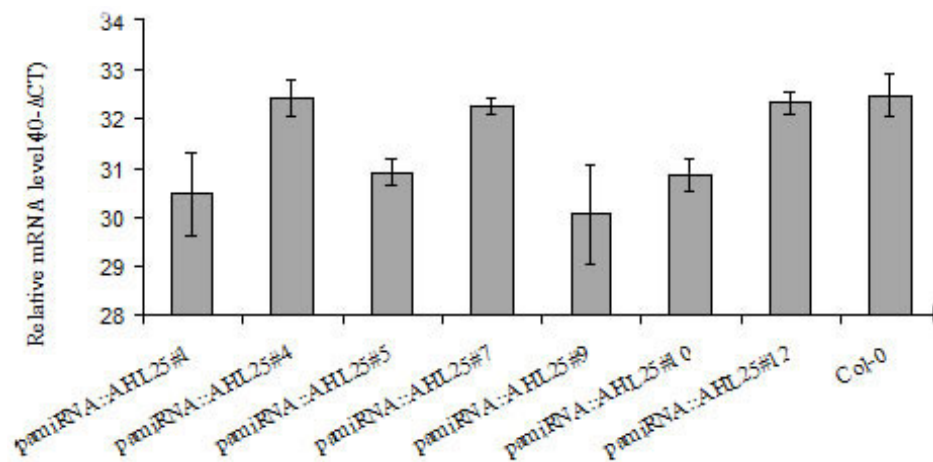


Figure 4. Expression analysis of *AHL25* in artificial micro-RNA lines in *Arabidopsis thaliana* Col-0 background. Expression level of *AHL25* was analyzed through qRT-PCR and normalized to the expression of *UBQ10* in different *pamiRNA::AHL25* transgenic lines and compared with the wild-type (Co-0). Total RNA was extracted and cDNA was made from two-week-old seedlings. The mean value of the expression level is given from three technical replicates while standard deviation is displayed by error bars.

Analysis of the qRT-PCR data showed that *AtExpA8* expression was reduced in five lines (*pamiRNA::ExpA8#1*, *pamiRNA::ExpA8#2*, *pamiRNA::ExpA8#4*, *pamiRNA::ExpA8#8*, and *pamiRNA::ExpA8#12*), while there was no significant reduction in *AtExpA8* transcript abundance in *pamiRNA::ExpA8#5*, *pamiRNA::ExpA8#9*, *pamiRNA::ExpA8#10*, and *pamiRNA::ExpA8#13*.

Similarly, *AHL25* expression was reduced in *pamiRNA::AHL25#1*, *pamiRNA::AHL25#5*, *pamiRNA::AHL25#9*, and *pamiRNA::AHL25#10*, while there was only slight or no reduction

in *AHL25* transcript abundance in lines *pamiRNA::AHL25#4*, *pamiRNA::AHL25#7*, and *pamiRNA::AHL25#12*. Transgenic lines with reduced transcript levels for *AtExpA8* and *AHL25* were used for further analysis.

Abiotic stress and phenotypic response of the artificial miRNA lines

The effect of low pH and high NaCl concentration on the growth of *AtExpA8* artificial miRNA seedlings compared to Col-0 as the wild-type control was investigated. We germinated the seeds in darkness for 3 days and measured the final hypocotyl length. The pH of the MS medium was adjusted to 5. The results revealed that there was a significant reduction in hypocotyl length in *AtExpA8* miRNA seedlings (Figure 5A). Similarly, NaCl (150 mM) reduced seedling growth, with reduced *AtExpA8* expression than the wild-type Col-0 (Figure 5B). The results demonstrated that reduction in transcript levels of *AtExpA8* in miRNA lines reduced their tolerance to low pH and high salt concentration. These results revealed the negative effects of low pH and high salt concentration in response to reduced *AtExpA8* transcript levels in artificial miRNA lines.

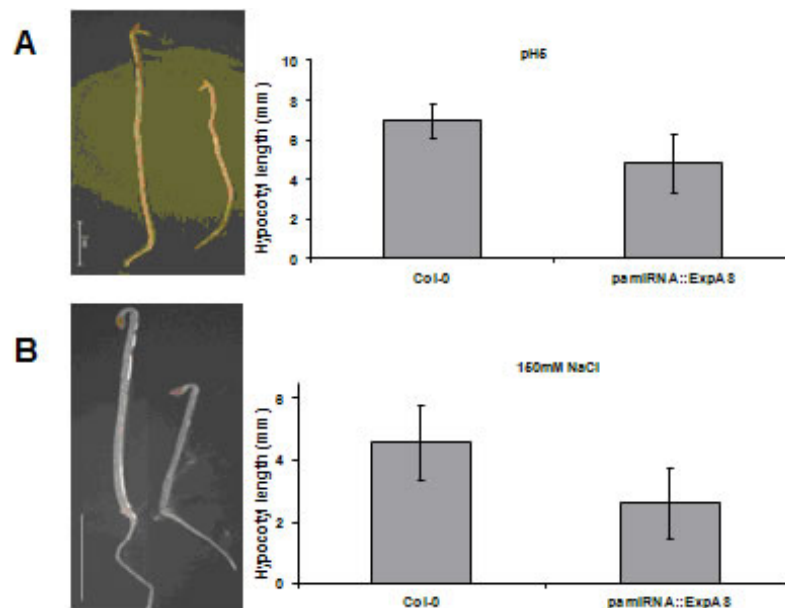


Figure 5. Downregulation of *AtExpA8* resulted in high sensitivity to low pH and high NaCl concentration. Seeds were germinated on plates containing MS medium with pH 5 (A) or supplemented with 150 mM NaCl (B). Plants were germinated for 3 days in complete darkness and then the length of hypocotyls was measured. Error bars represent the standard deviation of at least 25 seedlings. Length of hypocotyls is indicated in mm (y-axis). Scale bars represent 2 mm.

DISCUSSION

An amiRNA approach was considered particularly important because it can knock down the expression of a gene via RNA interference (RNAi) with high specificity (Schwab et al., 2006). Therefore, we used this technique to downregulate the expression of two genes, i.e., *AtExpA8* and *AHL25* in *A. thaliana*. Analysis of the qRT-PCR data showed greater reduced expression of these genes in the

transgenic plants harboring the amiRNA cassettes than in the control. There were about five individual lines for each of the genes that showed reduced transcript levels indicated in Figures 3 and 4.

The functional role of *AHL25* was investigated by its reduced expression in the transgenic lines generated through amiRNA. However, no significant phenotypic differences compared to Col-O were observed in the transgenic lines with reduced mRNA levels for *AHL25*, although the level of downregulation of *AHL25* in some *pamiRNA::AHL25* lines was more than 50% (Figure 5B). This may be due to the redundancy among the *AHL* genes, which has also been reported by Xiao et al. (2009), where no phenotypic differences were observed between the wild type and RNAi lines for *AHL18* and *AHL22*.

Cell wall extension is relative to the growth of the tissue and organ, one of the properties of the cell wall is the ability to extend at acidic pH (Rayle and Cleland, 1992). In the present study, the downregulation of *AtExpA8* resulted in more sensitive behavior of the plants towards low pH and high salt concentration (Figure 5). This indicates the importance of this gene for the normal growth of the plants. This shows that expansin-mediated cell wall enlargement is required for the complex and highly coordinated pattern of growth and morphogenesis in plants.

amiRNAs serve as an effective tool to study the effects of post-transcriptional gene silencing. Previously, naturally occurring antisense transcripts were identified as important regulators of the corresponding sense transcript (Borsani et al., 2005). amiRNAs have an edge over conventional hair-pin mediated RNAi as they are specific in targeting either the sense or antisense RNA. Furthermore, amiRNA can be designed to target a specific splice form of the same gene. Some genes are fundamental for normal growth and development of an organism; hence their loss of function as a knockout mutant is lethal, which makes the use of amiRNA technology an appropriate potential method for determining the downregulation of gene expression and its effect on the studied organism.

Another fundamental aspect of artificial miRNA is that it has been used to downregulate the expression of exogenous genes e.g., the invading virus pathogen in wheat, such as the wheat streak mosaic virus (Fahim et al., 2012).

In conclusion, miRNAs are naturally occurring small RNAs that regulate the expression of genes at the post-transcriptional level; in the present study we used this technology by making amiRNAs against two genes in *Arabidopsis*. The expression of these genes was successfully downregulated, which was evident from the analysis of the transcript of these genes through qRT-PCR.

Conflicts of Interest

The authors declare no conflict of interest.

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