Deciphering miRNAs involved in crosstalk between auxin and cold stress in *Arabidopsis* roots

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Abstract

Background: The phytohormone auxin and microRNA-mediated regulation of gene expressions are key regulators for plant growth and development at both optimal and under low-temperature stress conditions. However, the mechanistic link between microRNA and auxin in regulating plant cold stress response remains elusive.

Results: To better understand the role of microRNA in the crosstalk between auxin and cold stress responses, we took advantage of the mutants of *Arabidopsis thaliana* with altered response to auxin transport and signal. Screening of the mutants for root growth recovery after cold stress at 4°C revealed that the auxin signaling mutant, solitary root 1 (slr1; mutation in Aux/IAA14), shows a hypersensitive response to cold stress. Genome-wide expression analysis of miRNA in wild-type and slr1 mutant roots using next-generation sequencing revealed 180 known and 71 novel cold-responsive microRNAs. Cold stress also increased the abundance of 26 nt-31 nt small RNA population in slr1 compared with wild-type. Comparative analysis of microRNA expression shows significant differential expression of 13 known and 7 novel miRNAs in slr1 at 4°C compared with wild-type. Target gene expression analysis of the members from one potential candidate miRNAs, miR169 revealed the possible involvement of miR169- NF-YA module in the auxin-mediated cold stress response.

Conclusions: Taken together, these results indicate that SLR/IAA14, a transcriptional repressor of auxin signaling, plays a crucial role in integrating miRNA in auxin and cold responses.

Background

Cold stress is a serious threat to the sustainability of crop yield. In 2009, the chilling temperature alone resulted in crop damage equivalent to approximately 158 billion yen in Japan. Similarly, early and late frost results in damaging the vegetable and fruit production equivalent to 5-6 billion yen every year in Japan [1]. Cold stress also limits the geographical distribution of many important crop species [2]. In response to cold stress, plants show various phenotypic symptoms, including poor germination, stunted growth, yellowing of leaves (chlorosis), reduced leaf expansion, wilting of leaves, necrosis and premature death [3]. Cold stress also severely affects the reproductive development of plants [4]. Exposure to stresses leads to changes in complex and interactive cellular and molecular processes that are required for physical adaptations and increased survival of the plants. Hence, a better understanding of the pathways that regulate plant growth during cold temperature stress is essential.

Hormonal regulation is one of the prime regulatory mechanisms involved in the survival of plants, which is a complex process comprises of interactions of various hormones at transcriptional, translational and cellular levels [5]. Among the hormones, auxin (indole-3-acetic acid, IAA) controls all aspects of plant growth and development, i.e., from embryogenesis to senescence [6]. Consistently, auxin has been shown to play an essential role in regulating plant growth and development under both high and low-temperature stresses [1, 7-11]. In *Arabidopsis*, cold stress seems to affect both auxin transport and signaling

processes. Long term cold stress results in coordinated down and upregulations of Aux/IAA and ARF protein family respectively [12], while short term cold stress affects the polar transport of auxin through modulating GNOM-mediated intracellular cycling of PIN2, a detrimental process for the functionality of PIN proteins [9, 13].

It was also demonstrated that cold stress alters the auxin homeostasis and considerably increases the auxin level in the root meristem resulting in root growth inhibition. Restoring the auxin level through increased transport could restore the root growth even under cold stress [9, 13]. Recently, the involvement of auxin maximum in the quiescent center (QC) has been shown to be an essential factor in preserving the root stem cells at a quiescent status under chilling stress [14]. The authors also demonstrated that this chilling stress-specific sacrifice-for survival mechanism not only protects the stem cell niche from chilling stress but also improves the root's ability to withstand the accompanying environmental pressures and to recover when ambient temperatures rise to an optimal level [14]. In rice, 4°C cold stress resulted in 1.2-1.6 fold increase in IAA level [15]. Differential expression of three auxin efflux carrier genes, 9 ARF genes and 10 *Aux/IAA* genes were observed under cold stress [16]. The genome-wide analysis of the early auxin-responsive gene families in rice under cold stress revealed both up and downregulation of several genes of *GH3*, *Aux/IAA*, *SAUR* and *ARF* families [17]. *OsGH3-2* overexpression-induced increase in cold tolerance was attributed to the combined effects of reduced free IAA content, alleviated oxidative damage, and decreased membrane permeability [18].

Additionally, it has also been shown that several known components of the cold signaling pathway are linked to auxin. For instance, *SIZ1*, a central regulatory component of cold response pathway, that stabilizes *ICE1*, is directly linked to auxin-mediated root architecture patterning [19, 20]. Another downstream component of cold signaling pathway, *AtNUP160*, which plays a critical role in the nucleocytoplasmic transport of mRNAs under cold stress [21], has also been shown to play an essential role in auxin signaling [22]. Collectively, these results demonstrate the importance of auxin in regulating plants cold response. However, what remains obscure are the molecular components that integrate auxin and cold stress response.

microRNAs (miRNAs), which are small non coding RNAs, usually consisting of 20–22 nucleotides for animals and 20–24 nucleotides for plants, have emerged as ubiquitous post transcriptional gene regulatory molecules [23]. miRNAs bind to complementary mRNA molecules and act as negative regulators of gene expression through endonucleolytic cleavage or translational repression of the cognate mRNA targets [24]. Lately, miRNA regulation of stress response pathway has been established in several plant species [25-28]. Consistently, a large number of cold stress responsive microRNAs have been identified in various plant species with variable results in different reports [27, 29, 30]. Upregulation of microRNAs viz. miR168, miR169, miR172, miR393, miR397 and miR395 during cold stress have been commonly observed in several plant species [31]. On the other hand, Lv et al. (2010) identified 18 cold-responsive miRNAs in rice, of which most were downregulated, indicating that both the up and/or downregulations of target genes controlled by miRNAs play an important role in plant's adaptation to cold stress [32]. For instance, overexpression of miR408, which targets cuproproteins belonging to the

phytoecyanin family and laccase, results in cold tolerance [33, 34]. Consistently, miR408 knockout lines show a hypersensitive response to cold stress [34]. Overexpression of miR397, which targets laccases and a casein kinase beta subunit 3, also results in increased freezing tolerance after cold acclimation [35]. Overexpression of miR394a and *LCR* have demonstrated the positive role of this miRNA-target pair in response to low-temperature stress [36]. In rice, miR319 overexpression lines show an increased survival rate under cold stress [37, 38]. In trifoliate orange, overexpression of the precursor of ptr-miR396b results in enhanced cold tolerance [39]. Collectively, these results suggest that miRNAs are potential regulators of cold stress response pathway across the plant species.

Reports on miRNAs have also demonstrated their strong potentials in modulating auxin signal transduction and several genes in auxin signaling have been reported as targets of miRNAs. For example, miR393 targets four closely related F-box genes, including the auxin receptor *TIR1* [40, 41]. miR393 also targets, a basic helix-loop-helix transcription factor from *Arabidopsis* that is homologous to GBOF-1 from tulip and annotated as an auxin-inducible gene [42]. Interestingly, Liu et al. (2017) showed that the heterologous expression of rice miR393a results in enhanced cold tolerance in switch grass (*Panicum virgatum* L.) [43]. Some auxin response factors (*ARFs*) have also been reported as a target of miRNAs [44, 45]. *ARF10*, *ARF16* and *ARF17* are regulated by miR160 [46, 47], while miR167 negatively regulates the expression of *ARF2*, *ARF3*, *ARF4*, *ARF6* and *ARF8* [48]. Few reports show that miR160 and the target *ARFs* are conserved between dicots and monocots [49, 50]. Liu et al. (2012) suggested that miR167 is essential for the appropriate expression of at least four *OsARFs* that contribute to the normal growth and development of rice [51]. Additionally, Lv et al. (2010) reported the involvement of miR167 during cold stress in rice, which has also been shown to be involved in regulating auxin signaling through modulating auxin response factors in several plant species [32, 51-54]. Moreover, miR164 fine-tunes the auxin signals by targeting the NAC domain transcription factors [55]. Taken together, these findings suggest that miRNA could be the potential link in integrating the auxin and cold stress responses.

In the present work, we tried to decipher the miRNAs that may regulate both auxin and cold stress responses by identifying auxin mutant that shows altered response to root growth after cold stress, followed by comparative analyses of genome-wide miRNAs in wild type and cold stress-sensitive auxin mutant by deep sequencing. Our results revealed that *Aux/IAA14* mutant *slr1* shows a hypersensitive response to cold-induced root growth inhibition. Comparative microRNA expression analysis displayed significant differential expression of 13 known and 7 novel miRNAs in *slr1* during cold stress compared with wild-type. Interestingly, majority of the differentially expressed miRNAs were downregulated in *slr1* in comparison to Col-0. The alteration of a significant number of miRNAs at 4°C in *slr1* background suggests that SLR/IAA14 plays a crucial role in integrating cold and miRNA responses. Further, expression analysis of the target genes of one of the potential candidates, miR169 revealed that miR169-*NF-YA* module may play an important role in integrating auxin signaling, miRNA and cold stress.

Results

Auxin signaling mutant *slr1* is susceptible to low temperature

To decipher microRNA mediated auxin signaling during plant cold stress response, we first focused on the identification of the cold-responsive auxin mutants. For the selection of cold-responsive auxin mutants, we used the root growth recovery assay developed in our lab earlier [9]. 12 h cold treatment at 4°C typically results in approximately 40-50% root elongation inhibition during the recovery phase at 23°C [9]. Hence, we selected 12 h incubation at 4°C as an optimal treatment for analyzing the effect of cold stress. Both auxin signaling mutants *slr1*, *tir1*, *axr1-3*, and *afb2-1*, and auxin transport mutants *aux1-7*, *eir1-1*, *pin3-3* and *pin4-3* were subjected to cold stress screening [56-63]. Percent root elongation recovery, compared to control (grown at 23°C) of each genotype was used as a measure of screening. 5-day-old seedlings grown at 23°C were transferred to new plates and kept at 4°C for 12 h and brought back at 23°C for recovery. The root elongation recovery was analyzed after 6 h and 24 h of recovery (Fig. 1) [9]. Consistent with previous results, we also found that cold stress inhibits root growth recovery by approximately 50% in the wild-type after 6 h (Fig. 1A). Through root growth recovery screening, we could identify *slr1*, an *Aux/IAA14* mutant as a potential candidate as *slr1* showed slower root elongation recovery at both 6 h and 24 h time point (Fig. 1, Supplementary Fig. S1). The other mutants did not show any significant difference compared with wild-type for root elongation recovery except auxin transport mutant *pin4-3*, which shows a slight but statistically significant slower root recovery response at 24 h (Fig. 1). To further confirm whether *slr1* response to cold stress persists for a longer time, we measured the root growth recovery till 24 h, and found that *slr1* shows slower root growth recovery at all-time points we tested (Fig. 2A and 2B). Because the *slr1* response to cold stress was consistent at all-time points, we selected *slr1* for miRNA study.

High-throughput sequencing of small RNA libraries

To identify the microRNAs that are responsive to auxin-mediated cold stress response, root tissues from 5 days old seedling grown on modified Hoagland media followed by treatment at 4°C for 12 h, and control grown at 23°C were used to construct small RNA libraries. We made eight small RNA libraries; four from wild-type (two each from 23°C and 4°C) and four from auxin mutant *slr1* (two each from 23°C and 4°C). By performing high-throughput sequencing on Illumina platform, a total of 270,303,139 reads (47,318,921 and 39,919,856 reads from Col-0 23°C; 30,230,648 and 23,124,483 reads from Col-0 4°C; 22,247,497 and 43,063,180 reads from *slr1*-23°C; 25,807,408 and 45,591,146 reads from *slr1*-4°C respectively) were generated. After trimming the adaptor and low-quality reads, the sequence reads were generated (Table 1). A total of 2,556,265 different tags were found from the trimmed reads, which comprises 933,996 different tags from Col-0 23°C; 441,338 different tags from Col-0 4°C; 626,812 different tags from *slr1*-23°C and 554,119 different tags from *slr1*-4°C respectively. The nucleotide length distribution in all the libraries showed that the majority of sequences ranged from 19 to 29 nt in size. We found 21 nt long small RNAs were the most abundant in all four libraries, followed by 24 nt. The total abundance of 2 types of small RNA population, i.e. 21 nt and 24 nt were 46.26% in Col-0 23°C, 56.44% in Col-0 4°C, 42.66% *slr1*-23°C and 30.43% in *slr1*-4°C respectively (Table 2). The significant change in small RNA abundance in the cold stressed *slr1* compared with wild-type suggests the importance of auxin signaling in miRNA modulated cold stress response. The populations of 26 nt-31 nt small RNAs were drastically affected in *slr1* backgrounds by cold stress as an increase in 26 nt-31 nt were observed (Table

2). We also found a significant decrease in the 21 nt small RNA population in *slr1* under cold stress, while in Col-0 this was increased. These results further reinforce the idea that auxin directly regulates miRNA expression (Fig. 3, Table 2). We did not observe any significant changes in 24 nt reads for treatments and genotypes (Table 2, Fig. 3). The significant change in the major population of small RNAs in the *slr1* mutant under cold stress confirms the involvement of auxin response in regulating miRNA function linked to cold stress response pathway, and also provides a possible explanation for the hypersensitive response of *slr1* to cold stress.

Identification of known and novel miRNAs

To identify the number of known miRNAs in small RNA libraries, we used CLC genomics workbench v12.0 and aligned unique reads to known *Arabidopsis* miRNA sequences in miRBase v22.1, allowing up to 0 mismatches. A total of 180 known miRNAs, representing 70 families, were identified in eight small RNA libraries made from root tissue (Fig. 4, Fig. 5, Supplementary Table S1). In all the eight libraries, miR166 family was the most abundant, followed by miR165 and miR168 families. During low-temperature stress, we found differential expression patterns of miRNAs in wild type and *slr1* (Fig. 4, Supplementary Table S1). The comparative analysis between cold treated Col-0 and *slr1* revealed that the expression of 13 miRNAs significantly changed in *slr1* during cold stress (Table 3). We also observed altered expression of 10 miRNAs in *slr1* background between control and cold treatment (Table 3). Interestingly, except for one, all the miRNAs expression was downregulated in *slr1* mutant in comparison to Col-0.

For the identification of novel microRNAs, mirDeep-P pipeline was used. A total of 71 sequences were predicted to be potentially novel microRNAs from unannotated small RNAs (Supplementary Table S2 and S3, Supplementary Fig. S2). The abundance of novel miRNAs was lower compared to conserved miRNAs and their length varied from 19 nt to 30 nt. The comparative analysis of novel miRNAs between Col-0 and *slr1* revealed that 7 of them significantly changed in *slr1* under cold stress (Table 4, Supplementary Fig. S2).

Validation of miRNA expression patterns

The consistency in microRNA expression identified by deep sequencing was validated using quantitative realtime-PCR. We selected 13 differentially expressed miRNAs that were regulated in response to cold in the root. The validation was performed using 10 known miRNAs (miR156, miR164b-3p, miR169a-5p, miR171-5p, miR390-5p, miR5642a, miR408-5p, miR398a-5p, miR472-3p and miR774a-5p) and 3 novel miRNAs (miR_Pred7, miR_Pred27 and miR_Pred37). In general, qRT-PCR validation results of miRNAs expression patterns were in agreement with the deep sequencing data, confirming the accuracy of the sequencing data (Fig. 6). Collectively, the qRT-PCR data confirm that the observed differences in miRNA expression in NGS are consistent and reproducible.

miRNA Target Prediction

The degree of sequence complementarity between miRNA and its binding site within the target determines the mode of action of miRNA. High sequence complementarity results in cleavage of targets [47, 64, 65], while low sequence complementarity results in translational inhibition [66, 67]. Several online resources such as psRNATarget use the same strategy to identify the plant miRNA targets. We carried out target prediction to understand the function of identified miRNAs by using psRNATarget server with preset values. The predicted targets for these miRNAs were from different classes of proteins associated with development, transport, auxin regulation, signaling and stress response (Fig. 7, Supplementary Table S1 and S2). For instance, cold stress response and signaling related proteins were targeted by miRNAs such as miR396b-3p, which targets MYB like transcription factors and miR156, which targets *SQUAMOSA-PROMOTER BINDING-LIKE (SPL)* transcription factor (Table 5). miRNAs like miR390a/b-5p targets the *TASI-ARF*, which is involved in auxin signaling. The possible roles of the target proteins in integrating auxin and cold stress responses have been discussed in detail in the discussion section. In general, we predict that the regulation of essential proteins contributing to cold stress tolerance in *slr1* is possibly linked to the cold susceptible phenotype of the mutant.

miR169-NF-YA module is altered in *slr1* under low-temperature stress

To understand the biological significance of the RNAseq results, we selected one of the potential miRNA candidates, miR169, which is evolutionarily conserved, reported to be present in various plant species including monocots, dicots, ferns and gymnosperms [68-70], and has been shown to be a central regulator of various abiotic stresses, including drought, salt, cold, heat, oxidative and hypoxia [71]. The miR169 family of *Arabidopsis* has 14 members, that matures into four types of different isoforms, differing only 1 or 2 nucleotides [71]. Phylogenetic analysis of miR169 revealed that apart from miR169a, b, c, and h, there are three obvious clades: clade I (miR169d, e, f, g), clade II (miR169i, k, m) and clade III (miR169j, l, n) [71]. The miR169 family members show distinct temporal and differential expression patterns and thus regulate diverse target genes [71, 72]. One of the major targets of miR169 for abiotic stress response is NUCLEAR FACTOR Y (NF-YA), a heterotrimeric transcription factor composed of NF-YA, NF-YB and NF-YC proteins [73]. The link of the NF-Y family members in regulating plant developmental and stress response pathways has been demonstrated in several studies [73-81]. Earlier, a direct effect of temperature on miR169h and NF-YA was demonstrated [82]. miR169h abundance is directly influenced by temperature; while at high temperature the abundance was high, at low temperature the abundance was considerably low. As expected, miR169 target gene NF-YA expression was reciprocal to the abundance of the miR169 expression [82]. They further demonstrated that NF-Y complex regulates the temperature-dependent flowering and petiole length through directly binding to the promoters of flowering regulator *F7* and the auxin biosynthesis gene *YUC2*. These results make an elegant model linking miR169, NF-Y and auxin. We tested whether a similar module works for cold stress response in *slr1*. For better clarification of the role of the miR169 family, we selected at least one member from each clade, and miR169a, b and h. Beside miR169m, in cold stressed *slr*, all the tested miR169 members showed either a significant reduction in expression (miR169a, miR169d, miR169e, miR169h) or no changes in expression (miR169b, miR169g) (Fig. 8). The expression data suggest that among miR169 family members, miR169a, miR169d and miR169h function as major regulators to link auxin response and cold.

Next, we investigated whether cold stress-induced change in miR169 affects the *NF-YA* abundance reciprocally. In Col-0, under cold stress, we observed a decrease in *NF-YAs* abundance (8, 15 and 22 fold decrease respectively for *NF-YA3*, *NF-YA5* and *NF-YA8* compared with 23°C, while in *slr1*, there was a higher accumulation of *NF-YA* transcripts compared with Col-0 (3.94, 4.3 and 4.4 fold increase for *NF-YA3*, *NF-YA5* and *NF-YA8* respectively, Fig. 8). Based on the above findings, we speculate that the altered miR169-*NF-YA* module in *slr1* is possibly contributing to its susceptible phenotype to cold stress. Further, miR169-*NF-YA* module might be playing a pivotal role during cold stress recovery, and miR169 regulates the expression of this module in an SLR dependent manner.

Discussion

Several studies indicate that auxin and microRNAs play essential roles in plant cold stress response [27, 30, 83]. Auxin plays a pivotal role in regulating temperature stress response, and high-temperature stress response directly affects the auxin biosynthesis through altering phytochrome interacting factors (PIFs) expression [84]. More recently, phytochromes have been shown to function as thermosensors in *Arabidopsis* [85, 86]. Additionally, auxin transport and auxin signaling have also been shown to be altered in response to high temperature [10, 87, 88]. Under cold stress, polar and lateral auxin transports are altered, resulting in slower growth [1, 9, 89]. As for microRNAs, a large number of miRNA has been implicated in regulating the low-temperature response in different plant species [30, 90-93]. Although these studies represent a link 1) between auxin and cold stress, and 2) between miRNA and cold stress, the mechanistic link between microRNA and auxin in regulating plant cold stress response remains elusive. Here we report that 1) downstream auxin signaling response is crucial for miRNA mediated cold stress response in *Arabidopsis* root, 2) loss of auxin response resulted in altered expression of specific miRNAs under cold stress, 3) the hypersensitive response of *slr1* to cold stress is possibly linked to the differential expression of cold regulated miRNAs, and 4) miR169-*NF-YA* module possibly plays a major role in integrating auxin signaling, miRNA and cold stress.

Intracellular auxin response and miRNA-mediated gene expression tightly regulate the root growth developmental process in plants. For instance, root elongation is inhibited by the accumulation of auxin in the cell elongation zone [94]. Similarly, the timely regulation of expression of several genes by miRNA is indispensable for proper root growth [95, 96]. Earlier it has been reported that the cold-induced inhibition of root growth is linked to the accumulation of auxin in the root meristematic zone, which results from the GNOM-regulated dysfunction of auxin efflux carrier, PIN2 [9]. In rice, genes linked to auxin signaling such as *Aux/IAA* and *ARF* were found to be altered by cold stress [16, 17]. Consistently, in our current screening, we found *slr1* showing hypersensitive response to cold treatment during root elongation recovery process. *SLR* encodes IAA14 protein, which functions as a repressor for auxin-induced gene expression, and a downstream auxin signaling component [56]. Mutation in *slr1* results in stable IAA14 protein, which does not degrade in response to IAA, resulting in auxin insensitivity [97]. The finding that *slr1* shows hypersensitive response to cold stress indicates the importance of downstream auxin signaling pathway in the process.

miRNA modulates the plant development, plant response to various environmental challenges and auxin response by regulating the gene expression post-transcriptionally. From the beginning of plant miRNA study, its role in low temperature and other stresses has been reported [25, 98-100]. Consistently, in the present work, we also identified a genome-wide change in small RNA expression. Cold stress resulted in a definite shift in the expression of small RNAs with longer nucleotide length as well as altered expression in several miRNAs (Fig. 3, Table 2). Several proteins help to regulate the multiple steps of miRNA biogenesis in optimal and stress conditions. The biogenesis of miRNA is precisely regulated during stress to regulate gene expression using the gene silencing machinery [101]. During the biogenesis, the pri-miRNAs sequentially get processed in miRNA-miRNA* duplexes by protein complex of DICER-LIKE 1 (DCL1), HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE). These miRNA-miRNA* duplexes are then methylated, and ultimately the mature miRNAs incorporate with AGO1 and loaded to miRNA-induced silencing complexes (miRISCs)[102]. In *Arabidopsis*, one of the four Dicer-like (DCL1-4) proteins process the double stranded RNA into distinct size (18-21 nt long by DCL1, whereas DCL2, DCL3 and DCL4 produce 22 nt, 24 nt and 21 nt long, respectively) of small RNA duplexes[24]. The abundance of 21 nt small RNA in all the libraries suggest that during the *Arabidopsis* miRNA biogenesis, the pri- and pre-miRNAs are mainly processed by DCL1 (Table 2). Till date, majority of the reported miRNAs are of 21 nt long in size followed by 24 nt (<http://www.mirbase.org>). It is also reported that even a single nt change in miRNA length affects its efficiency of target slicing [103]. The significant change in 21 nt small RNA abundance and an increase in nonspecific 26nt-31nt small RNAs in the cold stressed *slr1* compared with wild-type suggests that SLR might be playing a pivotal role in the biogenesis of miRNAs during cold stress (Fig. 3, Table 2). The SLR mediated auxin response could be regulating the 21 nt miRNA biogenesis under cold stress.

The findings that at least 13 known miRNAs among 180 identified miRNAs were significantly regulated in *slr1* under cold stress indicate the possible involvement of miRNAs in low-temperature stress response and recovery (Fig. 4, Table 3). Among the altered miRNAs, miR398, miR171, miR169, and miR396 have already been shown to be regulated by cold stress [34, 39, 104]. Few miRNAs that were found to be regulated by cold are also involved in auxin response viz. miR169, miR852 and miR390 [105, 106].

Previous studies suggest that during stress conditions, an increase in miRNA expression deregulates the negative regulators of stress. In contrast, a decrease in miRNAs expression leads to the accumulation of positive regulators of stress [107, 108]. Significant reduction in the expression of several miRNAs in the *slr1* mutant compared to Col-0 suggests that there could be an increased activity of negative regulators in mutant leading to a susceptible phenotype (Table 3). The observed differences in post-transcriptional regulation of *NF-YA* transcripts by miR169 in Col-0 and *slr1* under cold stress supports this notion (Fig. 8). The miR169-*NF-YA* module, which is one of the stress-regulated miRNA target modules, showed altered expression pattern in *slr1* under cold stress. Our results demonstrate that miR169a, miR169d and miR169h are the primary targets of cold in *slr1* mutant (Fig. 8). This is consistent with the idea that the temporal and differential expressions of the members within the same microRNA family widely varies depending on the growth stages and acquired stresses [109]. miR169m showed a complete opposite expression pattern in *slr1* under cold stress compared to other members. This is interesting, but at present

the significance of this result is unknown. The reduced expression of miR169a, miR169d and miR169h directly influenced the expression of *NF-YA* transcripts, resulting in higher accumulation in *slr1* compared with Col-0 under cold stress (Fig. 8). miR169-*NF-YA* module has already been shown to be linked in the regulation of the temperature response in previous studies [82, 110, 111]. In addition, NF-YA has been shown to modulate auxin response by regulating its biosynthesis [82]. Taken together, these findings suggest that the miR169-*NF-YA* module, which functions in a SLR/IAA14 dependent manner could function as a major regulator to integrate auxin, miRNA and cold stress response.

Beside miR169, several other microRNAs may be involved in regulating auxin- mediated cold stress response. Low-temperature stress also significantly decreased the expression of miR390 in *slr1* mutant compared to Col-0. miR390 has been suggested to direct the production of tasiRNAs from *Trans-acting siRNA3 (TAS3)* transcripts, which regulate the *ARF* genes essential for auxin signaling [105, 112]. Recently it has been demonstrated that the miR390/*TAS3/ARFs* module plays a key role in regulating lateral root development in salt-stressed poplar through modulating the auxin pathway [113]. Moreover, miR390b-3p targets *AtVps11*, an essential component for endosome organization, intracellular protein transport, vacuole biogenesis and pollen tube growth [114]. It also targets clathrin heavy chains which mediate endocytosis, intracellular transport, and are required for proper polar distribution of PINs [115]. Differentially regulated miR390 in wild-type and *slr1* under low-temperature stress may also function in linking auxin and miRNAs during low-temperature stress response.

The significantly altered miRNAs were mostly downregulated in the mutant (Table 3, Supplementary Table S1). The differential expression of microRNA under cold stress in wild-type and auxin signaling mutant *slr1* indicates that the cold susceptible phenotype of the mutant is possibly linked to the miRNA targets. For instance, the members of miR156 family, which is highly induced by heat stress, were significantly downregulated in *slr1* mutant background under cold stress, indicating their possible involvement in temperature responses [116]. miR156 also targets *SPL* transcription factor genes that play an essential function in *Arabidopsis* growth and development, including vegetative phase change, lateral root development, different plant organ and response to stress [117-119]. Another downregulated miRNA in *slr1* mutant was miR396b-3p, whose expression was 2-3 fold more in the wild-type compared to the mutant. miR396b-3p targets the putative MYB transcription factors, which have been shown to regulate various developmental processes, including auxin homeostasis, biotic and abiotic stress responses [120-122]. Additionally, miR396a-5p targets growth-regulating factors that are involved in plant growth and development [123, 124].

Another important miRNA that could be contributing to the susceptible phenotype of *slr1* mutant is miR398, that positively regulates the heat tolerance in *Arabidopsis* [125]. Moreover, enhancement of freezing tolerance in *Chrysanthemum dichrum* by overexpression of ICE1 was also linked to the downregulation of miR398 [126]. Besides, miR398a-5p targets SETH2 protein involved in GPI anchor protein (GAP) biosynthetic pathway and pollen germination [127]. GAPs are found abundantly in the plasma membrane of plants and display similarities with plasma membrane receptors, peptides, and lipid transfer-like proteins [128, 129]. Also, miR399, known to participate in phosphate homeostasis, is

regulated by changes in ambient temperature [130, 131]. Additionally, miR399b,c-3p targets the wall-associated kinase involved in cell surface receptor signaling, could also be involved in low-temperature stress signaling. Undoubtedly, during low-temperature stress, the membrane is a primary site of injury, and consistently the freezing stressed plants show higher electrolyte leakage [132]. To combat low-temperature stress, cold-hardy plants modify their membrane as a necessary physiological process [133]. The possible downregulation of proteins required for membrane components by miRs in mutant may contribute as a significant factor for its susceptibility to cold.

The essential amino acid tryptophan (Trp) is well known for its requirement in auxin biosynthesis. The plant needs Trp for the synthesis of various proteins and many metabolites. Interference in the Trp biosynthesis leads to various developmental defects in plants [134]. The targeting of tryptophan synthetase by miRNA5642 during low temperature suggests a possible crosstalk of low temperature and auxin biosynthesis during the cold stress response. Moreover, the miR774a, which targets several F-box proteins, could also be attenuating the auxin signaling as several F-box proteins play indispensable roles in auxin signaling and response [135]. Taken together, these results confirm a complex triangular relation of miRNA, cold and auxin response.

Intriguingly, the differentially regulated miRNAs target a wide range of proteins involved in response to the stimulus, developmental processes, cellular component organization and biogenesis, biological regulation, cellular processes, metabolic processes and stress signaling (Supplementary Table S1, Fig 7). Although many of the proteins targeted by specific miRNAs are still functionally not characterized, few of the target proteins have already been reported for their role in regulating various processes, including abiotic stresses such as cold [30, 108]. Characterization of the other target proteins in this list will help to reveal the functional significance of these altered miRNAs in integrating auxin response and cold stress.

In agreement with the notion that often the miRNAs are expressed at a lower level, majority of the novel miRNAs displayed a lower expression compared to known miRNAs (Supplementary Table S2). As per miRBase 22.1, these miRNAs have not been described previously in *Arabidopsis*, which could be due to their low non-detectable expression level. In the present study, we possibly discovered most of the miRNAs in *Arabidopsis* during cold stress. The novel predicted miRNAs as well as known miRs viz. miR5656, miR774a and miR8181 that show altered expression patterns in wild-type and *slr1* target the transposable elements (TE) and transposons (Table 5 and Supplementary Table S2). TEs can have a myriad of effects when they insert into new locations [136-138]. These effects vary depending on the sequence of the TEs and where precisely the TEs are inserted. TEs are also responsive and susceptible to environmental changes. Stress-activated TEs might generate the raw diversity that species require over evolutionary time to survive stressful situations [139]. From bacteria to mammals, TE-induced mutations are associated with environmental adaptations (For review, see E Casacuberta and J Gonzalez [139]. In the plants, TE-induced mutations result in adaptation to high altitude in soybean, adaptation to changing light environment in *Arabidopsis* and adaptation to a wide range of environments in wheat [104, 140-143]. Interestingly, TEs have also been shown to contribute to duplication of *Aux/IAA* genes in soybean

[144]. The findings that the cold stress stimulates miRNAs that potentially target TEs in *slr1* suggest a possible involvement of TEs in integrating auxin and cold stress responses and need further studies.

Conclusions

The present study provides a basic platform to explore the genetic and cellular mechanisms of auxin and miRNAs regulating the cold stress response. Identification of handful number of target miRNAs, including miR169 from the comparative RNA seq analyses between wild-type and auxin signaling mutant *slr1* indicates that auxin regulated miRNAs play essential roles in maintaining the cellular signaling system to ensure an optimal cold stress response. Future overexpression or loss of function studies with the specific miRNAs that are altered in *slr1* mutant and their targets will further clarify how the auxin and miRNA mediated pathways contribute in regulating the cold stress response.

Methods

Plant materials and growth condition

All lines are in the Columbia background of *Arabidopsis thaliana* (L.) Heynh., *axr1-3*, *aux1-7*, *eir1-1*; *pin3-3*, *pin4-3* and *tir1-1* were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). *afb2-1* was a kind gift of Nihal Dharmasiri (Texas State University, San Marcos, USA). *slr1* was described in Fukaki et al. (2002) [56].

Surface sterilized seeds were placed in round, 9-cm petri plates on modified Hoagland medium [1, 9, 145] containing 1 % (w/v) sucrose and 1 % (w/v) agar. The plates were kept at 4°C in the dark for 2 days for seed stratification. After stratification, the plates were transferred to the growth chamber (LPH-220S, NK System) at 23°C under continuous white fluorescent light at an intensity of 100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ and seedlings were grown vertically for 5 days.

Cold stress treatment and analysis of root growth recovery

Cold stress treatment and growth recovery were performed as described earlier [9]. Briefly, 5-day-old seedlings were transferred to new plates containing the modified Hoagland medium and kept at 4°C for 12 h in the growth chamber (NK System; LH-1-120.S). After cold stress, plates were put back at 23°C for recovery, whereas the control plates were kept continuously at 23°C. Primary roots were analyzed after 6 h and 24 h of recovery. The experiments were repeated at least three times, with eight seedlings per treatment. To measure cold stress recovery, seedlings were photographed by a digital camera (Canon Power Shot A640) and root growth recovery was analyzed by ImageJ software (<http://rsbweb.nih.gov/ij/>).

Chemicals

Difco Bacto Agar™ was purchased from BD Biosciences, Japan. Other chemicals were from Wako Pure Chemical Industries, Japan.

Small RNA isolation and sequencing

The total RNA was isolated from the roots of cold stress treated and control samples by using the RNeasy Kit (Qiagen, USA) according to the manufacturer's instructions. Eppendorf BioPhotometer plus (Germany) was used to detect the quality and the concentration of RNA. Construction of the sRNA libraries and deep sequencing were carried out by BGI (Beijing, China). Briefly, RNA with lengths of 16–36 nt was separated and purified using denaturing polyacrylamide gel electrophoresis, followed by sequential 3' and 5' RNA adaptor ligation to the small RNAs using T4 RNA Ligase. The adaptor-ligated samples were then reverse transcribed and amplified by PCR to construct the final libraries. Then, the prepared libraries were sequenced using an Illumina HiSeq 4000 platform (Illumina, USA).

Data Deposition Information

The sequencing data that support the findings of this study have been deposited in NCBI SRA database with the SRA accession code: PRJNA579274. The SRA record is accessible with the following link: <https://www.ncbi.nlm.nih.gov/sra/PRJNA579274>

Bioinformatic analysis of the sRNAs sequencing data

After sequencing, the raw reads were filtered and adapter sequences were removed along with contamination and low-quality reads from raw reads. The remaining unique sequences (clean reads) were then processed to identify known and novel microRNAs.

Known miRNAs from *Arabidopsis* root after cold stress were identified using CLC Genomics Workbench v12.0 (CLC Bio, Denmark). Briefly, clean reads processed from raw sequencing reads after trimming adaptor sequences and removing low-quality reads were further analyzed by CLC workbench to extract and group sRNA. Sequences shorter than 16 nt and larger than 36 nt along with non-coding RNA such as rRNA, tRNA, and snRNA were excluded from further analysis. The remaining reads were and then annotated to identify the known *Arabidopsis* miRNAs. To identify known miRNAs, small RNA sequences were annotated against miRBase 22.1 (<http://www.mirbase.org/index.shtml>) using CLC Genomics Workbench 12.0 based on their sequence homology. Finally, the mapped miRNAs were obtained, which then was normalized using the reads per million reads (RPM) method. Normalized reads were then used to determine the fold change between the control and stressed samples.

Novel miRNAs were identified by using mirDeep-P, a plant-specific miRNA identification pipeline [146]. Briefly, for each sequenced small RNA library, reads were filtered by length and only those between 16 nt and 36 nt were retained. FASTA-formatted reads were then analyzed by mirDeep-P using the *Arabidopsis* genome as a reference.

Prediction of miRNA targets

The targets of identified miRNAs were predicted using psRNATarget (the plant small RNA target server, 2011 Release; <http://plantgrn.noble.org/psRNATarget/>) by aligning with *Arabidopsis* transcripts and default parameters which included a threshold cut-off of 3.0, a complementarity scoring length of 20 bp, and the energy required for target accessibility equal to 25 kcal/mole.

Quantitative RT-PCR validation of selected deferentially expressed miRNAs

Analysis of miRNAs expression was performed using the poly(T) adaptor RT-PCR method by Mir-X miRNA First-Strand Synthesis kit (Clontech, Takara Bio, USA) as per manufacturers instruction. Briefly, for polyadenylation and cDNA synthesis 1 µg of DNaseI treated total RNA was incubated at 37°C for 60 min in a 10 µl reaction volume containing mRQ enzyme then the reaction was terminated at 85°C for 5 min to inactivate the enzymes. Quantitative RT-PCR (qRT-PCR), was run on a TaKaRa Dice Real Time apparatus (Takara, Japan) with the SYBR Green I Master kit (Bio-Rad, USA). The reaction conditions for qRT-PCR included following steps: 10 sec at 95°C followed by 40 cycles of denaturation for 10 s at 95°C and annealing for 20 s at 60°C, and extension for 15 s at 72°C. miRNAs were quantified using specific primer pairs with the translation initiation factor elongation factor 1-α (*EF1α*) as the normalization controls. Relative transcript abundance was calculated using $2^{-\Delta\Delta C_T}$ method [147]. All experiments were performed using three biological replicates and three technical replicates. The primers used in the study are listed in the supplementary Table S4.

Statistical Analysis

Results are expressed as the means ± SE from the appropriate number of experiments. A two-tailed Student’s *t*-test was used to analyze statistical significance.

Abbreviations

miRs	:	MicroRNAs
qRT-PCR	:	Quantitative real time PCR
EF1α	:	Elongation factor 1-α
TEs	:	Transposable elements
nt	:	Nucleotide
IAA	:	lindole-3-acetic acid
ARF	:	Auxin response factor
QC	:	Quiescent center

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

All the data and materials that are required to reproduce these findings can be shared by contacting the corresponding author. The sequencing data can be found in NCBI SRA database with the SRA accession code: PRJNA579274.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

M.A., Y.Q. and A.R. designed the study; M.A. and K.S. carried out the experiments. M.A. and A.R. analyzed, discussed the data and wrote the paper. All authors have read and approved the manuscript.

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Tables

Table 1: Summary of small RNA libraries after deep sequencing.

Sl. No	Sample Name	Number of reads	Average length	Number of reads after trim	Percentage trim	Average length after trim
1	Col0 23°C_Rep1	47,318,921	21.6	46,240,335	97.72%	21.6
2	Col-0 23°C_Rep2	38,919,856	22.4	38,635,669	99.27	22.4
3	Col-0 4°C_Rep1	30,230,648	23.8	30,004,070	99.25%	23.8
4	Col-0 4°C_Rep2	23,124,483	21.9	22,863,639	98.87%	21.9
5	<i>slr1</i> 23°C_Rep1	22,247,497	21.4	21,471,561	95.51%	21.4
6	<i>slr1</i> 23°C_Rep2	43,063,180	22.1	42,081,401	97.72%	22.1
7	<i>slr1</i> 4°C_Rep1	25,807,408	22.9	24,711,139	95.75	22.4
8	<i>slr1</i> 4°C_Rep2	43,591,146	23.0	43,069,325	98.8%	23.0

Table 2: Distribution of frequency percent of nucleotide length

	Sample Name	Frequency percent of nt length							
		21nt	24nt	26nt	27nt	28nt	29nt	30nt	31nt
1	Col-0 23°C	32.76	13.50	3.46	2.25	1.37	0.71	0.56	0.41
2	Col-0 4°C	42.49	13.95	3.02	1.31	0.69	0.30	0.16	0.06
3	<i>slr1</i> 23°C	30.52	12.14	3.75	2.06	1.08	0.48	0.26	0.16
4	<i>slr1</i> 4°C	20.12	10.31	6.72	4.42	4.00	2.97	2.68	2.43

Table 3: List of known miRNAs whose expression changed significantly during cold stress. Blue color represents the downregulation and the red color represents upregulation in the expression in the mutant.

Data were obtained from two independent biological replicates. Statistical significance between the treatments and genotype was obtained using Students *t*-test. ^aDenotes miRNAs showing significantly altered expression between wild-type and *slr1* at 4°C.

miRNA ID	Col-0			<i>slr1</i>			p-value Col-0 vs. <i>slr1</i>	
	23°C	4°C	p-value	23°C	4°C	p-value	23°C	4°C
			23°C vs 4°C			23°C vs 4°C		
miR156a,b,c,d,e,f,	114.53	100.9	0.809	78.61	54.51	0.654	0.648	0.002 ^a
miR164b-3p	22.5	13.47	0.611	3.64	2.79	0.447	0.338	0.006 ^a
miR169a-5p	26.49	30.81	0.613	15.9	12.7	0.587	0.296	0.059 ^a
miR170,miR171a-5p	4.7	2.96	0.638	3.17	0.92	0.051	0.652	0.27
miR390a,b-5p	1385	812.14	0.423	383.72	263.68	0.341	0.223	0.029 ^a
miR390b-3p	65.18	49.73	0.583	25.47	16.48	0.254	0.237	0.029 ^a
miR396b-3p	6.34	2.97	0.389	2.72	1.38	0.009	0.36	0.018 ^a
miR398a-5p	0.56	0.48	0.854	2.87	1.88	0.603	0.297	0.013 ^a
miR399a	4	3.15	0.165	5.65	2.31	0.049	0.148	0.224
miR399b,c-3p	25.8	23.93	0.845	17.41	10.81	0.027	0.413	0.029 ^a
miR408-5p	14.55	33.15	0.249	42.01	37.83	0.684	0.034	0.764
miR447a-3p,miR447b	21.39	23	0.864	15.29	6.72	0.036	0.43	0.105
miR472-3p	379.33	154.02	0.539	63.04	45.1	0.241	0.41	0.04 ^a
miR5642a,b	77.64	62.74	0.059	82.11	34	0.239	0.891	0.028 ^a
miR5656	1.31	1.33	0.982	0.81	0.18	0.044	0.235	0.257
miR773a	419.47	248.54	0.605	167.81	77.04	0.032	0.465	0.019 ^a
miR774a	0.21	0.3	0.496	0.85	0.51	0.327	0.028	0.495
miR775	26.11	15.41	0.621	9.78	5.68	0.266	0.472	0.04 ^a
miR8180	0.85	0.5	0.257	0.83	0.13	0.048	0.914	0.257
miR8181	1.49	2.08	0.567	1.98	0.73	0.018	0.25	0.243
miR824-3p	177.08	365.65	0.267	99.59	171	0.037	0.484	0.149
miR850	8.16	6.72	0.685	3.71	1.52	0.103	0.017	0.238
miR852	3.7	1.72	0.468	1.15	0.9	0.384	0.372	0.058 ^a

Table 4: List of predicted miRNAs whose expression changed significantly during cold stress. Blue color represents the downregulation and the red color represents upregulation in the expression in the mutant. Data were obtained from two independent biological replicates. Statistical significance between the treatments and genotype was obtained using Students *t*-test. ^aDenotes miRNAs showing significantly altered expression between wild-type and *slr1* at 4°C.

miRNA ID	Col-0			<i>slr1</i>			p-value Col-0 vs. <i>slr1</i>	
	23°C	4°C	p-value	23°C	4°C	p-value	23°C	4°C
			23°C vs 4°C			23°C vs 4°C		
Pred_7	30.68	16.18	0.521	36.20	69.86	0.033	0.84	0.015 ^a
Pred_9	27.75	21.95	0.483	17.38	12.98	0.274	0.294	0.007 ^a
Pred_11	10.06	6.75	0.637	15.59	21.14	0.289	0.506	0.018 ^a
Pred_19	10.24	6.64	0.271	6.71	4.67	0.434	0.383	0.0031 ^a
Pred_25	3.38	1.59	0.185	2.15	0.98	0.002	0.305	0.011 ^a
Pred_26	6.32	5.14	0.647	2.90	1.74	0.503	0.304	0.056 ^a
Pred_27	4.01	4.49	0.774	3.03	2.17	0.302	0.040	0.280
Pred_35	2.80	1.26	0.099	1.49	0.48	0.071	0.029	0.294
Pred_36	2.19	2.92	0.755	3.08	1.81	0.039	0.107	0.638
Pred_37	2.30	1.41	0.007	3.57	2.38	0.247	0.010	0.313
Pred_44	1.47	0.78	0.015	1.01	0.43	0.466	0.551	0.076
Pred_47	0.99	0.66	0.387	0.30	0.33	0.827	0.012	0.411
Pred_53	0.64	1.03	0.743	1.20	1.27	0.941	0.058	0.868
Pred_60	0.31	0.33	0.835	0	0	0	0.000	0.040 ^a
Pred_62	0.22	0.14	0.060	0.001	0.147	0.116	0.001	0.983
Pred_65	0.26	0.28	0.966	1.21	0.647	0.475	0.015	0.658

Table 5: miRNA target prediction

miRNA ID	Target locus
miR156a,b,c,d,e,f,	Squamosa promoter binding protein-like 15 (AT3G57920), Squamosa promoter-like 11 (AT1G27360), SPL13B (AT5G50670), SPL9 (AT2G42200), SPL2 (AT5G43270), SPL10 (AT1G27370), SPL13A (AT5G50570), SPL10 (AT1G27370), SBP domain transcription factor (AT1G69170), SPL3 (AT2G33810), Protein kinase superfamily protein (AT3G28690), SPL4 (AT1G53160), SPL5 (AT3G15270), Transposable element gene (AT1G16660), Cysteine/Histidine-rich C1 domain family protein (AT2G21840)
miR164b-3p	PPR repeat protein (AT5G14770)
miR169a-5p	Nuclear factor Y, subunit A8 (AT1G17590,AT1G54160)
miR170,miR171a-5p	Transposable element gene (AT2G06790, AT3G30393, AT1G36470, AT1G50860, AT3G29732, AT2G12305), MATE efflux family protein (AT1G15180), F-box family protein (AT5G3946)
miR390a,b-5p	TASIR-ARF (AT5G57735), Transmembrane kinase-like 1 (AT3G24660)
miR390b-3p	Vacuolar protein sorting 11 (AT2G05170), RNA-binding (RRM/RBD/RNP motifs) family protein (AT3G07810), Transposable element gene (AT2G41570, AT1G35990), Clathrin, heavy chain (AT3G11130)
miR396b-3p	MYB76 (AT5G07700), ATBTAF1 (AT3G54280),RNA helicase family protein (AT1G58060)
miR398a-5p	SETH2 UDP-Glycosyltransferase superfamily protein (AT3G45100), Alpha/beta-Hydrolases superfamily protein (AT3G48080), ARM repeat superfamily protein (AT5G06120)
miR399a	PHO2/UBC24 (AT2G33770), CYP705A30 (AT3G20940), Terpenoid cyclases family protein (AT1G78500), Sodium Bile acid symporter family (AT2G26900), Transposable element gene (AT3G43867), Nucleic acid binding (AT1G27750)
miR399b,c-3p	PHO2 (AT2G33770), Wall-associated kinase 2 (AT1G21270)
miR408-5p	Oxidoreductase, 2OG-Fe(II) oxygenase (AT4G02940, AT4G25310), Nucleotide/sugar transporter family (AT4G03950), glutathione S-transferase TAU 25 (AT1G17180), Leucine-rich repeat protein kinase family protein (AT1G04210), Unknown protein (AT4G37030), pseudogene (AT2G31860), Calmodulin-binding protein-related (AT5G10660), Early-responsive to dehydration stress protein (AT4G35870)
miR447a-3p,miR447b	P-loop containing nucleoside triphosphate (AT5G60760),FAD-dependent oxidoreductase family protein (AT2G22650), lncRNA (AT5G05905)
miR472-3p	Disease resistance protein (CC-NBS-LRR class) family (AT5G43740, AT1G12290, AT1G15890), RPS5 Disease resistance protein (CC-NBS-LRR class) family (AT1G12220)
miR5642a,b	Tryptophan synthase beta-subunit 1 (AT5G54810),VHA-E3 vacuolar H ⁺ -ATPase subunit E isoform 3 (AT1G64200)
miR5656	Mitochondrial editing factor 9 (AT1G62260), Zinc finger (C3HC4-type RING finger) family protein (AT5G60710), Transposable element gene (AT3G29787)
miR773a	Remorin family protein (AT3G57540), root FNR 1 (AT4G05390), Acyl-CoA N-acyltransferase with RING/FYVE/PHD-type zinc finger protein (AT4G14920), transposable element gene(transposable element gene), RAD3-like DNA-binding helicase protein (AT1G79950), Oleosin3 (AT5G51210)
miR774a	F-box and associated interaction domains-containing protein (AT3G17490), F-box family protein (AT3G19890), Transposable element gene (AT1G34405, AT2G01024,AT4G16910, AT3G42996,AT2G07660)
miR775	Dicer-like 1 (AT1G01040), Galactosyltransferase family protein (AT1G53290),

	Galactosyltransferase family protein (AT1G53290)
miR8180	Alpha/beta-Hydrolases superfamily protein (AT3G55190), Plastid division2 (AT2G16070), ATP-dependent helicase family protein (AT2G28240), lncRNA (AT3G08825), Fatty acid reductase 1 (AT5G22500),
miR8181	Ovate family protein (AT2G36026),Cysteine/Histidine-rich C1 domain family protein (AT1G55430),LOB domain-containing protein 39 (AT4G37540), transposable element gene (AT3G30713)
miR824-3p	Concanavalin A-like lectin protein kinase family protein (AT3G08870),Pentatricopeptide (PPR) repeat-containing protein (AT5G27300)
miR850	Chloroplast RNA binding (AT1G09340), threonyl-tRNA synthetase (AT2G04842), lncRNA (AT2G08250), AtSWEET4 (AT3G28007)
miR852	IAA-leucine resistant (ILR)-like 1(AT5G56650) , IAA-leucine resistant (ILR)-like 2 (AT5G56660), TIR1 F-box/RNI-like superfamily protein (AT3G62980), K ⁺ transporter 1 (AT2G26650), H(+)-ATPase 11 (AT5G62670)

Figures

A) **B)** **Figure 1**

Screening of auxin mutants for root growth recovery response after cold stress A) Percent root growth recovery at 6 h after 12 h of cold stress B) Percent root growth recovery at 24 h after 12 h of cold stress. Five-day old *Arabidopsis* seedlings were transferred to new agar plates and kept at 4°C for 12 h. Plates were brought back to 23°C for recovery after cold stress. Percent root recovery compared to control was analyzed from primary root growth. Vertical bars represent mean $\pm$ S.E. Data are from at least three independent experiments (n=3 or more) with 8-10 seedlings per treatment. Asterisks represent the

statistical significance between control and treatment as judged by the Student's t-test (* $P < 0.05$ and *** $P < 0.001$).

Figure 2

Auxin mutant *slr1* shows a hypersensitive response to cold stress A) Five-day old *Arabidopsis* seedlings were transferred to new agar plates and kept at 4°C for 12 h. Plates were brought back to 23°C for recovery after cold stress. Root growth was analyzed from primary root growth elongation 6 h, 12 h and 24 h of recovery. Vertical bars represent mean $\pm$ SE. Data are from at least three independent experiments ($n=3$ or more) with 8-10 seedlings per treatment. *slr1* root growth recovery at 4°C was statistically significant at all-time points (*** $P < 0.001$) as judged by the Student's t-test. B) Root phenotype of Col-0 and *slr1* during 24 h recovery period after cold stress at 4°C for 12 h. Tick marks indicate the starting point of the recovery at 23°C. Scale bar = 10mm.

Figure 3

Nucleotide length distribution in Col-0 and *slr1* Nucleotide length distribution of small RNAs libraries made from five-day old control (23°C) and 12 h cold treated (4°C) Col-0 and *slr1* roots. The results are obtained from two independent biological replicates. Vertical bars represent mean $\pm$ SD. Asterisks represent the statistical significance between the treatments as judged by the Student's t-test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Figure 4

Known miRNAs expression profiles in the root of Col-0 and slr1. The heat map shows the hierarchical cluster analysis of miRNAs regulated in the cold stressed root of Col-0 and slr1. The color bars of the heatmap represent the gradient scale of normalized log2-TPM values for each miRNA. Red color indicates high levels of miRNAs abundance, and green color indicates low abundance. The analysis was performed using two independent biological replicates.

A)

B)

Figure 5

Venn diagram analysis showing the overlap of miRNAs among four libraries (A) Known microRNA identified in auxin-mediated cold stress response. (B) Novel miRNA identified in auxin-mediated cold stress response. Venn diagram was used for fishing overlapping and non-overlapping miRNAs in Col-0 and slr1 mutant.

Figure 6

Validation of miRNAs expression in response to cold stress A) RT-qPCR validation of selected miRNAs from cold stressed NGS library. RNA was isolated from the roots of 5-day-old 12 h cold (4°C) stressed seedlings. microRNA expression is expressed in fold change of expression against *ef1α* calculated by $2^{-\Delta\Delta CT}$. Vertical bars represent the mean $\pm$ SE of three biological replicates. Asterisks represent the statistical significance between control and treatment as judged by the Student's t-test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

Figure 7

Pie chart showing the class of proteins targeted by identified microRNAs (A) Protein class targeted by known microRNAs (B) Protein class targeted by novel miRNAs.

Figure 8

Relative expression of miR169 precursors and NF-YA3, NF-YA5, NF-YA8 in Col-0 and slr1 after 12 h of cold (4°C) stress RNA was isolated from the roots of 5-day-old 12 h cold (4°C) stressed and control (23°C) seedlings. The fold change of expression is calculated by $2^{-\Delta\Delta CT}$ against 23°C using ef1α as internal control. Vertical bars represent the mean $\pm$ SE of two biological replicates. Asterisks denote the statistical significance between the Col-0 and slr1 at 4°C as judged by the Student's t-test (***) $P < 0.001$).

Supplementary Files

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