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Endogenous Small-Noncoding RNAs and Potential Functions in Desiccation Tolerance in *Physcomitrella Patens*

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Early land plants like moss *Physcomitrella patens* have developed remarkable drought tolerance. Phytohormone abscisic acid (ABA) protects seeds during water stress by activating genes through transcription factors such as ABSCISIC ACID INSENSITIVE (ABI3). Small noncoding RNA (sncRNA), including microRNAs (miRNAs) and endogenous small-interfering RNAs (endo-siRNAs), are key gene regulators in eukaryotes, playing critical roles in stress tolerance in plants. Combining next-generation sequencing and computational analysis, we profiled and characterized sncRNA species from two ABI3 deletion mutants and the wild type *P. patens* that were subject to ABA treatment in dehydration and rehydration stages. Small RNA profiling using deep sequencing helped identify 22 novel miRNAs and 6 genomic loci producing trans-acting siRNAs (ta-siRNAs) including TAS3a to TAS3e and TAS6. Data from degradome profiling showed that ABI3 genes (*ABI3a/b/c*) are potentially regulated by the plant-specific miR536 and that other ABA-relevant genes are regulated by miRNAs and ta-siRNAs. We also observed broad variations of miRNAs and ta-siRNAs expression across different stages, suggesting that they could potentially influence desiccation tolerance. This study provided evidence on the potential roles of sncRNA in mediating desiccation-responsive pathways in early land plants.

Early land plants such as moss *Physcomitrella patens* have developed remarkable drought tolerance for survival, whereas modern vascular plants have only retained drought tolerance in a few specialized tissues like seeds and spores¹. During seed development, an essential plant phytohormone abscisic acid (ABA) protects seeds by inducing a variety of genes including those encoding proteins for seed storage as well as genes that encode late embryogenesis abundant (LEA) proteins^{2,3}. ABA is prevalent in the developmental stages of land plants and plays a critical role in mediating plant growth and development as well as promoting desiccation tolerance⁴. Abundant ABA typically leads to arrest of growth under abiotic stress conditions⁵.

ABA activates genes that have stress-tolerance functions through transcription factors such as ABSCISIC ACID INSENSITIVE 3 (*ABI3*)⁶. In *P. patens*, deletion mutants of *ABI3* are not able to recover from desiccation despite being treated with ABA, indicating that *ABI3* is required for *P. patens* vegetative tissues to survive desiccation¹. The role of *ABI3* has been postulated to maintain the expression of ABA-inducible genes in recovery stages during rehydration, as the loss of *PpABI3* has little effect on most ABA up-regulated genes before rehydration. For instance, ABA induces *lea group3* which remains expressed after a 24-hour ABA treatment and a 24-hour dehydration in both wild-type (WT) and *ABI3* deletion mutants. In the WT, *lea group3* maintains its expression after 5 and 15-minute rehydrations but exhibits dramatically reduced expression levels in the *ABI3* mutants during rehydration¹, indicating its dependence on the activity of *ABI3*. Transcription factors *ABI3* and *ABI5* are known regulators of ABA responses in *Arabidopsis thaliana* germination^{7,8}.

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P. patens is capable of producing small RNAs, including microRNAs (miRNAs) and endogenous small-interfering RNAs (endo-siRNAs)⁹. Plant miRNAs are normally derived from hairpin-shaped structured pre-miRNAs which are processed by Dicer-like (DCL) proteins in the nucleus to release ~22-nt double-stranded RNAs with ~2-nt 3' overhangs, namely miRNA/miRNA* duplexes. Mature miRNAs are then loaded into the RNA-induced silencing complexes (RISC) that contain Argonautes (AGO) in the cytoplasm in order to exert their regulatory effect by guiding the RISC to target transcripts through complete or partial complementary base pairing¹⁰. Endogenous small interfering RNAs (endo-siRNAs) can arise from long double stranded RNAs (dsRNAs) of overlapping antisense transcripts or from products of RNA-dependent RNA polymerases (RdRP)^{11–14}. In plants, different classes of endo-siRNAs have been described based on their distinct characteristics, biogenesis pathways, and functions^{15,16}. For example, noncoding TAS genes generate trans-acting siRNAs (ta-siRNAs)^{17–20} by consecutive Dicer processing in a phased fashion¹⁰. In addition, long hairpins are prevalent and have the capacity to host both miRNAs and siRNAs²¹. While DCL1 processes multiple phased miRNAs on long hairpin substrates, the other Dicer-like enzymes (DCL2, DCL3, and DCL4) are able to process long hairpins which give rise to endo-siRNAs of various sizes²².

It has been shown that miRNAs play important roles in gene regulation in response to ABA treatment in plants. In germinating *A. thaliana* seeds, ABA induces accumulation of miR159 in an *ABI3*-dependent fashion and miR159 mediates the cleavage of two transcription factors MYB101 and MYB33 *in vivo*⁵. Additional evidence has indicated that miR159 functions through a homeostatic mechanism to maintain sensitivity to hormone signaling during seedling stress responses⁵. However, miR159 is not encoded in moss. Instead, miR319 is known to regulate MYB genes in moss⁹. Interestingly, miR477h has been predicted to target *ABI3* gene⁹. In *P. patens*, miRNAs have been shown to induce hypermethylation of their target genes and consequential transcriptional silencing upon ABA treatment²³. For example, ABA-induced accumulation of miR1026 subsequently leads to hypermethylation and down-regulation of the *PpbHLH* gene, indicating that miRNAs also function in epigenetic control of stress-responsive genes²³. Like miRNAs, endo-siRNAs in plants also play important roles in gene regulation. Trans-acting siRNAs derived from well-conserved TAS3 genes regulate auxin response factors (ARF) in plants¹⁷.

Despite the known gene regulatory activities of miRNAs triggered by ABA, most of their functions have yet to be analyzed. We performed a comprehensive study of small-noncoding RNAs in response to ABA in *P. patens* through Next-Generation Sequencing (NGS) based profiling of small RNA species. We compared the WT and the *ABI3*-deleted mutants in ABA treated and untreated tissues as well as in dehydration and rehydration stages. The results showed that moss *ABI3* genes (*ABI3a/b/c*) were potentially regulated by miR536, a basal plant-specific miRNA that is conserved but not beyond *Selaginella moellendorffii*⁹ in the green plant lineage. We also identified novel miRNAs and endo-siRNAs derived from long hairpin RNAs, miRNA-cleaved transcripts, and putative target genes of small RNAs by investigating data from degradome profiling of *P. patens*. Differential expression analyses revealed a broad alteration of small RNA expressions across different conditions, indicating the involvement of small RNAs in the plant's adaptation to dynamic environmental changes.

Results

Profiling of small-noncoding RNAs in ABA-treated and dehydrate mosses. Eight small-RNA libraries from normal and *ABI3* deletion mutant *P. patens* at 7-day ABA-treated, dehydrated, and then rehydrated stages were prepared and sequenced through NGS using the Illumina Genome Analyzer IIX (GAIIx) (Table S1). These libraries produced more than 190 million raw sequencing reads total, among which 108 million (~57% of the total) are high-quality, adapter-trimmed reads (qualified reads). A total of 65% of the qualified reads can map to the *P. patens* reference genome (<http://www.phytozome.net>) allowing no mismatches (Table S1).

miRNAs regulate ABA responsive genes. NGS profiling detected a total of 226 miRNAs in the conditions we examined (see Methods). Among the expressed miRNAs, we found 22 novel miRNAs (Table S2) and 204 of the 229 *P. patens* miRNAs that are currently annotated in miRBase (version 21, Tables S2 and S3). Two novel miRNAs are shown in Fig. 1 with their characteristic hairpin structures and RNA-RNA duplexes with ~2-nt 3' overhangs. We failed to find homologous hairpin sequences of the 22 novel miRNAs in other plant species using conservation analysis, which indicates that these novel miRNAs may be *P. patens* specific.

The overall abundance of the detected miRNAs amounted to around 7 million reads, accounting for ~5.6% of the total genome-mapped, qualified reads. This ratio of miRNA abundance is similar to that in rice (5.2%), but less than in *Arabidopsis* (14%)²⁴. Overall, the 5 most abundant mature miRNAs were miR156, miR319, miR535, miR904, and miR1028 (Table S3). The most abundant novel miRNA, novel-miR12, has more than 3 K reads in all four libraries combined (Table S2), making it moderately abundant among all 226 detected miRNAs.

The expression of detected miRNAs indicated their roles in desiccation tolerance. A substantial number of miRNAs exhibited dramatic variations in expression levels across different conditions. Overall, 46 expressed miRNAs were differentially expressed (DE) by at least 2-fold in two different conditions (Fig. 2A and Table S4). On the other hand, only a few miRNAs were differentially expressed during dehydration versus ABA-treatment in both the WT and mutant, indicating that miRNAs are less perturbed under dehydration stress after ABA treatment. These observations are consistent with changes in protein-coding gene expressions, as ABA-induced genes have been shown to maintain their expression in a relatively stable fashion during the dehydration stage¹. In contrast, a large number of miRNAs were DE during rehydration in both the WT and mutants (Fig. 2A), suggesting that miRNAs are involved in the rehydration process.

miRNAs directly regulate several genes that are critical for ABA responses, including ABA receptors²⁵ and those that are induced by ABA¹. We identified a total of 230 miRNA binding sites on 35 ABA-relevant genes using computational methods (see Methods, Table S5). The most significant of these putative target genes were those related to ABA responses, particularly the genes in the *ABI3*, *MYB* and *LEA* families that are known to

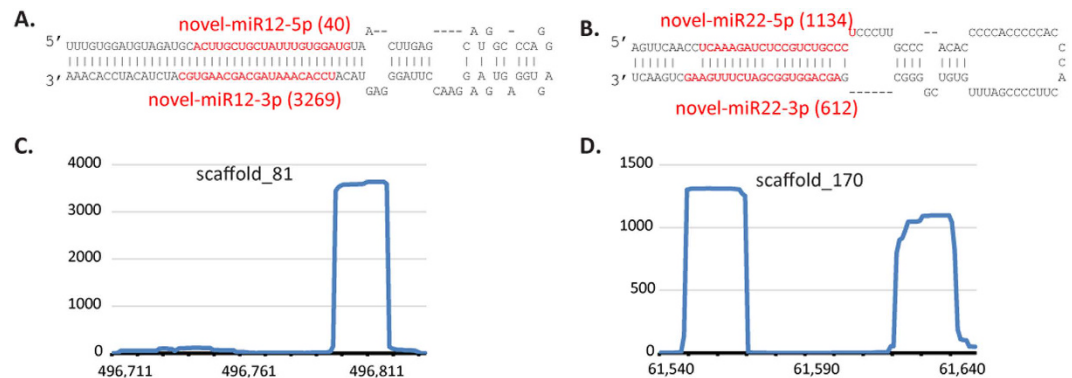


Figure 1. Examples of two novel miRNAs in *P. patens*. The hairpin structures of novel-miR12 (A) and novel-miR22 (B) are shown with mature miRNA-5p and miRNA-3p highlighted in red. The mature miRNAs form RNA duplexes with ~2-nt 3'-overhangs. The number in parenthesis represents the number of reads from sequencing profiling. The genomic loci for novel-miR12 (C) and novel-miR22 (D) and the read distributions across the two loci.

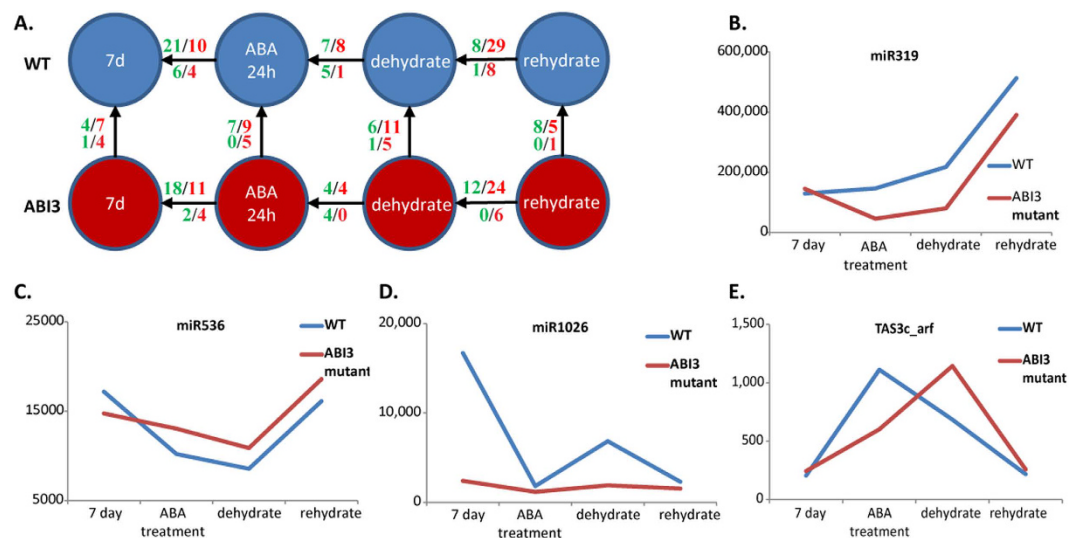


Figure 2. (A) The number of differentially expressed miRNAs in various comparisons. The arrow indicates the comparison direction (source as case and destination as control). The numbers on an edges are the number of upregulated sncRNAs (green) and the number of downregulated sncRNAs (red). The first row is the number of the DE miRNAs and the second row is the number of the DE siRNAs. A large amount of DE miRNAs were observed after ABA treatment for 24h versus the control, and rehydration versus dehydration stages. (B to D) Normalized digital expression levels of miRNAs at four different conditions for the WT and mutant.

protect the seed during desiccation. A total of 22, 5, and 20 miRNAs targeted ABI3, MYB, and LEA genes, respectively (Table S5).

miR536 may potentially cleave the genes in the ABI3 family. ABI3 has three paralogs in *P. patens* (i.e. ABI3a/b/c) and the highly expressed miR536 family bound to all three paralogs at specific loci which exist downstream of the B3 domain of the ABI genes (Fig. 3A). As shown, a sufficient number of reads from RNA degradation product profiling (i.e. degradome data GSE16367) were uniquely mapped to the binding regions of ABI3a and ABI3c. Note that the 5'-ends of the degradome reads were enriched at the canonical miRNA cleavage sites, indicating that miR536 endogenously cleaves the ABI3 genes upon binding (Fig. 3B). The three binding sites showed strong sequence conservation at the cleavage sites but displayed weak conservation outside of the miRNA binding regions (Fig. 3C). miR536 is a basal plant-specific miRNA previously identified in moss (*P. patens*) and lycopod (*S. moellendorffii*). It was highly abundant in our current profiling data, thus suggesting that the members of the miR536 family behave as genuine regulators of ABA responses.

miR319 is known to target three paralogous MYB genes in moss, including the two paralogs, Pp1s143_30V6.1 and Pp1s391_54V6.1⁹, and one target we newly identified in our study, Pp1s66_200V6.1. Some of the reads from the degradome profiling were aligned to the miR319 cleavage site on the new miR319 target (MYB, Pp1s66_200V6.1, Fig. 3D). Importantly, miR319 was highly expressed in the current sequencing data and exhibited down-regulation (3.2-fold) in the ABI3 knockout mutant. However, it also displayed a 1.3-fold up-regulation

Furthermore, degradome profiling can also help detect siRNA cleavage events, exemplified by several known ta-siRNAs in *Arabidopsis*^{29,30}. Here, we utilized the degradome data to identify targets of highly expressed siRNAs derived from long hairpins and TAS transcripts (see Methods). We detected 22 targets for the set of siRNAs, listed in File S2, along with functional annotations and alignment of reads from degradome profiling. One example is the siRNA species from hpRNA-2, which putatively targets and cleaves a gene encoding ribosomal protein S17, i.e., Pp1s76_75V6.1 (Figure S4). This result shows that these siRNAs have the potential of regulating cellular genes.

Discussion

ABA is a potent regulator of abiotic stress signaling in plants including moss, and ABA pretreatment is required for moss (*P. patens*) to confer desiccation tolerance³¹. In our study, we showed that this desiccation tolerance in moss was in large part attributed to small RNA, particularly miRNA mediated gene expression regulation.

Our results showed that the miR536 family functioned as regulators of the ABI3 genes on the ABA signaling pathway. We hypothesize that the acquisition of miR536 regulation on ABA signaling contributes significantly to the vegetative desiccation tolerance in moss, a trait that vascular plants do not possess. Several lines of evidence support this hypothesis. First, *P. patens* carries three ABI3 paralogs, while other vascular plants contain a single copy of this critical gene³². Second, there are 6 paralogous copies of miR536 in *P. patens*, while there is only one copy of miR536 in *Selaginella moellendorffii* and miR536 is completely missing beyond bryophytes. In other words, compared to other plant species *P. patens* contains a larger number of ABI3 and miR536 paralogous genes. This discrepancy may be attributed to the robustness and complexity of ABA signaling in moss and contribute to vegetative desiccation tolerance in the plant. Third, a conservation analysis of miRNA regulation of ABI3 genes in lycopod (*Selaginella moellendorffii*), rice (*Oryza sativa*), and *Arabidopsis thaliana* showed that the regulation of ABI3 genes by miRNAs is missing in these species (data not shown). As a previous study has shown, miR536 in *P. patens* directly cleaves the F-box genes⁹, which are auxin receptors³³. Due to the abundant number of ABI3 genes and miR536 members in *P. patens* as well as desiccation tolerance of all moss vegetative and haploid tissues, we conclude that each ABI3 gene and each member of the miR536 family are involved in different ABA signaling pathways in each tissue. These results also indicate that these genes might have contributed to plant early adaptation to harsh land environments, which was later recognized to link to the seed strategy for survival on land for Angiosperms. Since ABI3 genes are a major regulator of gene expression in the seeds of most vascular plants which lack miR536, our results also suggest that higher plants must have introduced alternative miRNA strategies on ABI3 regulation, for example through miR159 regulation on ABI3 in *Arabidopsis*⁵.

Finally, recent studies also showed that ABA treatment induced overexpression of miR1026 and consequently led to the down-regulation of PpbHLH gene, a target of miR1026²³. In addition, PpbHLH has been experimentally validated as a target of miR902-5p⁹, indicating that PpbHLH is tightly regulated by multiple miRNAs. miR1026 was highly expressed in our sequencing data and was dramatically down-regulated by more than 8-fold in the WT after 24-hour ABA-treatment (Table S4) but not in the mutant, which indicates a large alteration to the gene regulatory circuit involving ABA, PpbHLH and miR1026.

Targets of miRNAs in moss have been studied using both bioinformatic and experimental methods^{34–37}. Most bioinformatic approaches follow restrictive rules focusing mainly on misalignments between a miRNA and a target. As a result, these methods are not as effective as expected. Indeed, recent studies have revealed non-canonical target sites of plant miRNAs which are oversights in the original studies^{38,39}. These non-canonical binding sites form unconventional complementary sequences to miRNA sequences but with evident miRNA cleavage activities. For example, miR398 cleaves blue copper-binding protein (BCBP) at a non-canonical complementary site in the 5'-untranslated region (UTR) of BCBP with a bulge of six nucleotides opposite to the 5' region of the miRNA³⁹. In our current study, the target sites of miR536 on ABI3 genes showed atypical annealing (Fig. 3), as there are two mismatches and one wobble match occurring from the 2-nd to 8-th nucleotide (the so called seed region of a miRNA in animal). We also observed a low degree of complementarity within the seed region in the binding of miR319 and MYB gene (Fig. 3D), whereas experiments demonstrate that miR319 is able to cleave MYB gene in moss⁹. Taken together, our results suggest a broad existence of non-canonical target sites of plant miRNAs that may not have a high degree of complementarity within seed regions.

Materials and Methods

Plant materials. *Physcomitrella patens* subspecies *patens* (Gransden) was used as the wild type (WT) strain. The ABI3 deletion mutants were two independent lines (Δ abi3-1 and Δ abi3-2) in which all three *P. patens* ABI3 genes (A, B and C) were deleted (see ref. 1 for details). The WT and mutants were cultured at 25 °C under a 16 h light and 8 h dark regime. Under these conditions, the protonemal filaments are not under water stress. For ABA treatments, protonemal tissue grown on cellophane was transferred to BCDA medium containing 50 μ M ABA and incubated for the indicated times.

Desiccation assay. Protonemal tissues grown for 7 days under standard conditions (on cellophane in direct contact with the medium) are reflective of growth under non-stressed conditions. Protonemal tissues grown on a cellophane were subjected to water stress by transferring onto a filter paper (Whatman, USA) in an empty Petri dish and desiccating inside a laminar flow hood for 24 h. The desiccated tissues were transferred to BCDA plate and 2 mL of sterile water or different concentrations of ABA were added, similar to the protocol used for the moss *Funaria*. These rehydrated tissues were then incubated under standard growth conditions described above.

RNA profiling using NextGen deep sequencing. RNA from protonemal tissue of the WT and mutant lines were grown for 7 days then subjected to 24 hours of ABA treatment, dehydration, and rehydration (see Fig. 2A). These samples were extracted separately, following the protocol in¹. The protonemal tissues from

the two mutant lines at corresponding stages were mixed before RNA extraction. Total RNA was isolated using the Plant RNA Isolation Mini kit (Agilent Technologies, Inc., DE, USA) following the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed by ThermoScript™ RT-PCR System (Invitrogen Co., CA, USA) with oligo-dT(20) primer following the manufacturer's instruction. PCR was carried out using 1 µL of cDNA with GoTaq® Green Master mix (Promega Co., WI, USA). Small-RNA libraries were prepared for Illumina sequencing following the manufacturer's instructions⁴⁰. miRNeasy Mini Kit (Qiagen) was used for RNA extraction, with on-column DNase I digestion, followed by sequencing on the Illumina GAIIx platform using Small RNA Sample Prep Kit (Illumina).

Preprocessing of sequencing data. Raw sequence reads that contained no 3' sequencing adaptor, were of low quality, or were shorter than 17-nt were discarded. The adaptor trimming was done by an in-house method that recursively searches for the longest substring of the adaptor appearing within a sequence read. If a raw sequence read did not have a substring of the adaptor longer than 6-nt, it was considered to carry no adaptor. The adaptor-trimmed sequences with no ambiguous reads, which were referred to as qualified reads, were then mapped to the *P. patens* reference genome using Bowtie⁴¹. The genome and gene sequences were downloaded from Phytozome <http://www.phytozome.net> (release v1.6 of *Physcomitrella patens*). InterPro database⁴² was used to extract the B3 DNA binding domain of ABI3 genes (access number IPR003340).

Identification of novel miRNAs. The method for novel miRNA identification has been described in detail in previous studies of plant miRNAs^{43,44}. Here, we briefly discuss the key steps of the method. First, qualified reads from all libraries that mapped to the known moss miRNAs (miRBase release 21) were excluded from the analysis. The remaining reads were then mapped to the *P. patens* reference genome using Bowtie⁴¹ and neighboring loci were merged if they had reads overlapping one another. The (merged) loci were extended 300-nt on both ends and segments of 250-nt were extracted using a sliding window starting from the 5'-end. Possible secondary structures of each of the segments were found using RNA-fold⁴⁵. Candidate miRNAs were chosen based on four key criteria, including presence of more than 10 reads, existence of hairpin structures, appearance of miRNA* sequences and RNA-RNA duplex structures with ~2-nt 3' overhangs.

Identification of siRNAs. The remaining qualified reads from miRNA discovery were used to search for siRNAs by investigating both genomic and cDNA sequences for clusters with 21-nt reads enriched as siRNA-yielding candidates. Specifically, qualified reads from all small-RNA libraries that were aligned to miRNA loci were removed first. The remaining reads were then aligned to genomic and cDNA sequences with Bowtie (version 0.12.7)⁴¹ allowing no mismatches. Genome-aligned reads were clustered within a window size of 50-nt to form a putative candidate region. Two stringent criteria were applied to those candidate transcripts and regions with mapped reads. First, candidates with less than 10 mapped reads were removed to ensure a sufficient level of expression. Second, the majority (over 70%) of the mapped reads within a candidate transcript must be 21-nt long. These two criteria aimed to filter out false positive candidates due to random RNA degradation or other types of endogenous small RNAs that do not possess the characteristic of 21-nt enrichment. For ta-siRNAs, we further looked for the presence of at least one miRNA binding site on a siRNA-yielding candidate transcript. These criteria were previously adopted in studies of miRNAs in rice and *Arabidopsis*^{20,46}.

Identification of targets of miRNAs and siRNAs. We first applied the target prediction tool, TargetFinder⁴⁷, to identify putative targets of miRNAs and siRNAs. As for siRNAs, we modified the scoring for mismatches (i.e. no double penalties in the 2-nd to 7-th nucleotide of a siRNA sequence) in the TargetFinder method because siRNAs may not obey the seed-region rule as miRNAs. We then integrated degradome data (NCBI GEO GSE16367) with the results from (modified) TargetFinder for detecting candidate target sites with evidence from some sequencing reads. Degradome profiling is a high-throughput method for capturing and profiling 5'-ends of uncapped mRNAs that include miRNA and siRNA cleavage products³⁷. To increase detection specificity, we increased the binding score of TargetFinder to 8, which was normally set at 4 (see⁴⁷ for detail of the scoring metric).

References

- Khandelwal, A. *et al.* Role of ABA and ABI3 in Desiccation Tolerance. *Science* **327**, 546 (2010).
- Zhang, W., Ruan, J., Ho, T. D., You, Y., Yu, T. & Quatrano, R. S. Cis-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. *Bioinformatics* **21**, 3074–3081 (2005).
- Hoth, S. *et al.* Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the *abi-1* mutant. *J Cell Sci* **115**, 4891–4900 (2002).
- Finkelstein, R. R., Gampala, S. S. L. & Rock, C. D. Abscisic Acid Signaling in Seeds and Seedlings. *Plant Cell Online* **14** (suppl 1), S15–S45 (2002).
- Reyes, J. L. & Chua, N.-H. ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. *Plant J* **49**, 592–606 (2007).
- Finkelstein, R., Reeves, W., Ariizumi, T. & Steber, C. Molecular Aspects of Seed Dormancy*. *Annu Rev Plant Biol* **59**, 387–415 (2008).
- Lopez-Molina, L., Mongrand, S., McLachlin, D. T., Chait, B. T. & Chua, N.-H. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *Plant J* **32**, 317–328 (2002).
- Zhang, X., Garretton, V. & Chua, N.-H. The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes Dev* **19**, 1532–1543 (2005).
- Axtell, M. J., Snyder, J. A. & Bartel, D. P. Common Functions for Diverse Small RNAs of Land Plants. *Plant Cell Online* **19**, 1750–1769 (2007).
- Allen, E. & Howell, M. D. miRNAs in the biogenesis of trans-acting siRNAs in higher plants. *Semin Cell Dev Biol* **21**, 798–804 (2010).
- Mosher, R. A. *et al.* Uniparental expression of PolIV-dependent siRNAs in developing endosperm of *Arabidopsis*. *Nature* **460**, 283–286 (2009).

12. Wierzbicki, A. T., Haag, J. R. & Pikaard, C. S. Noncoding Transcription by RNA Polymerase Pol IVb/Pol V Mediates Transcriptional Silencing of Overlapping and Adjacent Genes. *Cell* 635–648 (2008).
13. Pikaard, C. S., Haag, J. R., Ream, T. & Wierzbicki, A. T. Roles of RNA polymerase IV in gene silencing. *Trends Plant Sci*, 390–397 (2008).
14. Rajagopalan, R., Vaucheret, H., Trejo, J. & Bartel, D. P. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev* 20, 3407–25 (2006).
15. Vazquez, F. *Arabidopsis* endogenous small RNAs: highways and byways. *Trends Plant Sci* 460–468 (2006).
16. Chapman, E. J. & Carrington, J. C. Specialization and evolution of endogenous small RNA pathways. *Nat Rev Genet* 8, 884–896 (2007).
17. Axtell, M. J., Jan, C., Rajagopalan, R. & Bartel, D. P. A two-hit trigger for siRNA biogenesis in plants. *Cell* 127, 565–77 (2006).
18. Ronemus, M., Vaughn, M. W. & Martienssen, R. A. MicroRNA-Targeted and Small Interfering RNA-Mediated mRNA Degradation Is Regulated by Argonaute, Dicer, and RNA-Dependent RNA Polymerase in *Arabidopsis*. *Plant Cell Online* 18(7), 1559–1574.
19. Cuperus, J. T., Fahlgren, N. & Carrington, J. C. Evolution and functional diversification of MIRNA genes. *Plant Cell* 23, 431–42 (2011).
20. Howell, M. D. *et al.* Genome-Wide Analysis of the RNA-DEPENDENT RNA POLYMERASE6/DICER-LIKE4 Pathway in *Arabidopsis* Reveals Dependency on miRNA- and tasiRNA-Directed Targeting 19 (March), 926–942 (2007).
21. Axtell, M. J., Westholm, J. O. & Lai, E. C. Vive la différence: biogenesis and evolution of microRNAs in plants and animals. *Genome Biol* 12, 221 (2011).
22. Rajagopalan, R., Vaucheret, H., Trejo, J. & Bartel, D. P. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev* 20, 3407–3425 (2006).
23. Khraiweh, B. *et al.* Transcriptional Control of Gene Expression by MicroRNAs. *Cell* 140, 111–122 (2010).
24. Zhang, X. *et al.* Genome-wide analysis of plant nat-siRNAs reveals insights into their distribution, biogenesis and function. *Genome Biol* 13, R20 (2012).
25. Fujii, H. *et al.* *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature* 462, 660–664 (2009).
26. Bologna, N. G., Mateos, J. L., Bresso, E. G. & Palatnik, J. F. A loop-to-base processing mechanism underlies the biogenesis of plant microRNAs miR319 and miR159. *EMBO* 28, 3646–3656 (2009).
27. Sunkar, R. & Zhu, J. K. Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* 16, 2001–2019 (2004).
28. Zhou, L. *et al.* Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *J Exp Bot* 61, 4157–4168 (2010).
29. German, M. A. *et al.* Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. *Nat Biotech* 26, 941–946 (2008).
30. Addo-Quaye, C., Eshoo, T. W., Bartel, D. P. & Axtell, M. J. Endogenous siRNA and miRNA targets identified by sequencing of the *Arabidopsis* degradome. *Curr Biol* 18, 758–762 (2008).
31. Frank, W., Ratnadewi, D. & Reski, R. *Physcomitrella patens* is highly tolerant against drought, salt and osmotic stress. *Planta* 220, 384–394 (2005).
32. Cuming, A. C. Mosses as model organisms for developmental, cellular, and molecular biology. In *Bryophyt Biol*. Second. Edited by Shaw A. J. Cambridge University Press, 199–236 (2008).
33. Jones-Rhoades, M. W., Bartel, D. P. & Bartel, B. MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* 57, 19–53 (2006).
34. Talmor-Neiman, M. *et al.* Identification of trans-acting siRNAs in moss and an RNA-dependent RNA polymerase required for their biogenesis. *Plant J* 48, 511–521 (2006).
35. Talmor-Neiman, M., Stav, R., Frank, W., Voss B. & Arazi, T. Novel micro-RNAs and intermediates of micro-RNA biogenesis from moss. *Plant J* 47, 25–37 (2006).
36. Fattash, I., VoSZ, B., Reski, R., Hess, W. & Frank, W. Evidence for the rapid expansion of microRNA-mediated regulation in early land plant evolution. *BMC Plant Biol* 7, 13 (2007).
37. Addo-Quaye, C. *et al.* Sliced microRNA targets and precise loop-first processing of MIR319 hairpins revealed by analysis of the *Physcomitrella patens* degradome. *RNA* 15, 2112–2121 (2009).
38. Zheng, Y., Li, Y.-F., Sunkar, R. & Zhang, W. SeqTar: an effective method for identifying microRNA guided cleavage sites from degradome of polyadenylated transcripts in plants. *Nucleic Acids Res* 40, e28 (2012).
39. Brousse, C. *et al.* A non-canonical plant microRNA target site. *Nucleic Acids Res* 42, 5270–5279 (2014).
40. Joyce, C. E. *et al.* Deep sequencing of small RNAs from human skin reveals major alterations in the psoriasis miRNAome. *Hum Mol Genet* 20, 4025–4040 (2011).
41. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 10, R25 (2009).
42. Hunter, S. *et al.* InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Res* 40, D306–12 (2011).
43. Sunkar, R., Zhou, X. F., Zheng, Y., Zhang, W. X. & Zhu, J. K. Identification of novel and candidate miRNAs in rice by high throughput sequencing. *BMC Plant Biol* 8, 25 (2008).
44. Zhang, W. *et al.* Multiple distinct small RNAs originate from the same microRNA precursors. *Genome Biol* 11, R81 (2010).
45. Hofacker, I. L. Vienna RNA secondary structure server. *Nucleic Acids Res* 31, 3429–31 (2003).
46. Johnson, C. *et al.* Clusters and superclusters of phased small RNAs in the developing inflorescence of rice. *Genome Res* 19(8), 1429–40 (2009).
47. Fahlgren, N. *et al.* High-Throughput Sequencing of *Arabidopsis* microRNAs: Evidence for Frequent Birth and Death of MIRNA Genes. *PLoS One* 2, e219 (2007).

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Author Contributions

W.Z. and R.Q. initiated the project and W.Z., R.Q. and P.-F.P. designed the overall study. X.W. and P.-F.P. prepared moss plants and small-RNA libraries for sequencing profiling. Y.H. provided reagents. J.X. processed and analyzed all sequencing data. J.X. and W.Z. analyzed the miRNA-based gene regulation and wrote the manuscript.

Additional Information

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