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Overexpression of *gma-MIR394a* confers tolerance to drought in transgenic *Arabidopsis thaliana*

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ABSTRACT

MicroRNAs, key posttranscriptional regulators of eukaryotic gene expression, play important roles in plant development and response to stress. In this study, a soybean *gma-MIR394a* gene was functionally characterized, especially with regard to its role in drought stress resistance. Expression analysis revealed that *gma-MIR394a* was expressed differentially in various soybean tissues and was induced by drought, high salinity, low temperature stress, and abscisic acid treatment in leaves. One target gene of gma-miR394a, *Glyma08g11030*, was predicted and verified using a modified 5′ RLM-RACE (RNA ligase-mediated rapid amplification of 5′ cDNA ends) assay. Overexpression of *gma-MIR394a* resulted in plants with lowered leaf water loss and enhanced drought tolerance. Furthermore, overexpression of *gma-MIR394a* in *Arabidopsis* reduced the transcript of an F-box gene (*At1g27340*) containing a miR394 complementary target site. These results suggest that the *gma-MIR394a* gene functions in positive modulation of drought stress tolerance and has potential applications in molecular breeding to enhance drought tolerance in crops.

1. Introduction

MicroRNAs (miRNAs) are 20- to 24-nucleotide (nt) small RNAs that are key posttranscriptional regulators of eukaryotic gene expression [1]. miRNAs downregulate the expression of target genes through cleavage or repression of translation. Numerous miRNAs have been discovered in both plants and animals [2]. miRNAs are now known to have greatly expanded roles in a variety of plant developmental processes [3], signal transduction [4], and responses to environmental stressors and pathogen invasions [5,6].

Plants have evolved sophisticated anatomical, physiological, and molecular responses to environmental stresses [7]. Abiotic stresses, such as drought, salinity, and extreme temperatures, regulate the expression of thousands of genes in plants at both the transcriptional and posttranscriptional levels. Recent evidence indicates that plant miRNAs have an important function in adaptive responses to abiotic stresses [8]. The first indication of such roles came from miRNA research on stressed *Arabidopsis thaliana*, which revealed miRNAs that had not been cloned previously from *Arabidopsis* grown under normal conditions [9,10]. At present, many miRNAs have been predicted and some have been confirmed experimentally to be involved in a variety of abiotic stress responses. For example, miRNA417 has a negative impact on seed germination and survival rate of *Arabidopsis* under high salt stress [11]. Overexpressing ath-miR169a enhances leaf water loss and makes plants more sensitive to drought stress compared to wild-type (WT) plants [12]. In contrast, transgenic plants overexpressing Sly-miR169c display reduced stomatal opening, a decreased transpiration rate, lowered leaf water loss, and enhanced drought tolerance [13]. Overexpressing *osa-MIR393* in rice and *Arabidopsis* results in plants with enhanced sensitivity to salinity and alkaline stress and are hyposensitive to auxin, in addition to an increase in tillers and early flowering [14,15]. Likewise, transgenic rice and *Arabidopsis* plants constitutively overexpressing *osa-MIR396c* show reduced salt and alkali stress tolerance compared to WT plants [16].

MiR394 is a conserved miRNA that has been found in several plant species [9,17–19]. A recent study found that expression of miR394 is upregulated by high salinity in *Arabidopsis* [20]. Expression of miR394b in roots and miR394a and b in shoots is initially upregulated and then downregulated during a period of iron deficiency [21]. In *Brassica napus*, miR394a, b, and c in roots and stems are upregulated by sulfate deficiency [18]. Similarly, treatment with cadmium induces expression of miR394a, b, and c in all tissues [18]. Recent studies indicate that miR394 and its target, the F-box gene *At1g27340*, are involved in the regulation of leaf

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curling-related morphology in *Arabidopsis* [22]. These results suggest that miR394 is involved in both development and abiotic stress regulation.

Soybean (*Glycine max*) is one of the most important crops worldwide. Drought is a major abiotic stress factor that negatively affects soybean productivity, and improving the drought tolerance of soybean is a major goal of many breeding programs. Here, we characterized *gma-MIR394a* expression patterns under diverse environmental stresses and in various soybean tissues. The target gene of gma-mi394a was predicted, and it was verified using a modified 5′ RLM-RACE (RNA ligase-mediated rapid amplification of 5′ cDNA ends) assay. To further characterize *gma-MIR394a*, we generated transgenic *A. thaliana* plants constitutively overexpressing *gma-MIR394a* and found that transgenic plants had enhanced drought stress tolerance. This study sought to elucidate the role of *gma-MIR394a* in drought stress responses.

2. Materials and methods

2.1. Plant materials, growth conditions, and treatments

Soybean cultivar Williams 82 was used to isolate the miRNA precursor (pre-miRNA) of *gma-MIR394a* and to examine its expression patterns under various treatments. Seeds were germinated in pots containing vermiculite, and 20-day-old seedlings were used in the following treatments. For salt, dehydration, and abscisic acid (ABA) treatments, the roots of the seedlings were immersed in solutions containing 250 mM NaCl, 20% polyethylene glycol (PEG), and 100 μM ABA, respectively, for various lengths of time. For the cold treatment, seedlings were kept at 4 °C for the indicated time period. Leaves from plants in each treatment were harvested and stored at −80 °C for RNA isolation. The roots, stems, leaves, and cotyledons from 20-day-old seedlings and flowers from mature plants were also separately harvested for RNA isolation and used for tissue-specific expression analysis.

2.2. Quantitative real-time PCR (qPCR)

For miRNA analysis, validation of mature miRNA expression by qPCR was carried out as previously described [23]. Briefly, total RNA was isolated from different tissues as described above using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and treated with RNase-free DNase I (Fermentas, Vilnius, Lithuania). First-strand cDNA synthesis of miRNA was then performed using a miRcute miRNA first-strand cDNA synthesis kit (Tiangen, Beijing, China) according to the manufacturer's instructions. qPCR was carried out using the Eco Real-Time PCR system (Illumina, San Diego, CA, USA) and the SYBR Green PCR master mix (miRcute miRNA qPCR Detection Kit; Tiangen), which contained antisense adaptor primers, and applying the corresponding miRNA sequences as sense primers. Soybean miR1520d was used as an internal standard. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated at least three times. The PCR primers for qPCR of miR394a and miR1520d were as follows: 394a primer (5′-TTGGCATTCTGTCCACCTCCA-3′) and miR1520d primer (5′-ATCAGAACATGACACGTGACAA-3′).

For mRNA analysis, cDNA was synthesized from 100 ng of total RNA using RevertAid Reverse Transcriptase (Fermentas). SYBR green qPCR Master Mix (Fermentas) was used for real-time PCR. A *ubiquitin* (UBQ3) transcript of *Arabidopsis* was used to quantify the expression levels of *gma-MIR394a* and the target gene in transgenic *Arabidopsis* plants. Primer sequences were as follows: AtUBQ3-F, 5′-CGGAAAGACCATTACTCTGGA-3′; AtUBQ3-R, 5′-CAA GTGTGCGACCATCCTCAA-3′; At1g27340-F, 5′-GTTGATGCTGGTGG GTCTAC-3′; At1g27340-R, 5′-AGACAATTCATCCTAATGTGCTTT-3′.

2.3. Generation of transgenic Arabidopsis plants

To generate an overexpression construct that constitutively overexpressed *gma-MIR394a* under the control of a cauliflower mosaic virus 35S promoter, a 157-bp fragment flanking the miRNA sequence including the fold-back structure was amplified from soybean genomic DNA with the following primers: forward 5′-AT CATGAGGGTTTAGCAAAGTGTT-3′ and reverse 5′-ATCATGAAGGC TTTACAAAGTGTAGC-3′. The amplified fragment was introduced into the pJET1.2 vector (Fermentas) for sequencing confirmation and was subcloned into the pCAMBIA3301 vector (Cambia, Canberra, Australia).

The construct was transferred into *Agrobacterium tumefaciens* GV3101 and was transformed into Columbia (Col-0) ecotype *Arabidopsis* plants using the vacuum infiltration method. T_3 generation plants were used for further analysis.

Transgenic *Arabidopsis* plants were detected by a GUS activity assay. GUS activity was assayed histochemically using a modification of the method of Jefferson et al. [24].

2.4. Root growth assay

For the root growth assay, transgenic and WT seeds were placed on Murashige and Skoog (MS) agar plates for germination. Three days later, 30 germinated seedlings from each line were carefully transferred to new MS agar plates supplemented with 0, 100, 200, 300, or 400 mM mannitol. After 7 days of upright growth in the treatment medium, seedling root length was measured.

2.5. Water loss measurement

For the water loss measurement, leaves were detached from plants at the rosette stage and weighed immediately on weighing paper. The weight was measured at designated time intervals at 25 °C and a relative humidity of 70% under normal light conditions. Three replicates were done for each transgenic line. Water loss was represented as the percentage of initial fresh weight at each time point.

2.6. Drought stress treatment in transgenic Arabidopsis

Drought tolerance assays were performed on seedlings. Transgenic and WT seeds were germinated on MS medium. One-week-old seedlings were planted in identical pots containing mixed soil (vermiculite:humus, 1:1) and well watered. The seedlings were cultured in a greenhouse (22 °C, 70% humidity, 150 $\mu M\ m^{-2}\ s^{-1}$, 12 h light/12 h dark cycle) without watering until phenotypic differences were evident between transgenic and WT plants; then they were rewatered. Drought tolerance experiments were conducted in triplicate. The numerical data were subjected to statistical analyses using Excel 2003 (Microsoft, Redmond, WA, USA).

2.7. Target prediction and validation

The gma-miR394a putative targets were predicted using psRNATarget (http://bioinfo3.noble.org/psRNATarget/). Targets were validated using a modified 5′ RLM-RACE assay. Briefly, total RNA was isolated from roots of 3-week-old soybean plants using the Plant RNA reagent (Invitrogen) according to the manufacturer's recommended protocol. The GeneRacer Kit (Invitrogen) was used to process the total RNA and map the 5′ terminus of the primary transcript. Total RNA was directly ligated to the RNA oligonucleotide and reverse transcribed with SuperScript III reverse transcriptase using oligo(dT) primer (as provided in the kit). The cDNA samples were amplified with nested PCR according to the manufacturer's protocol. Initial PCR was carried out using the GeneRacer

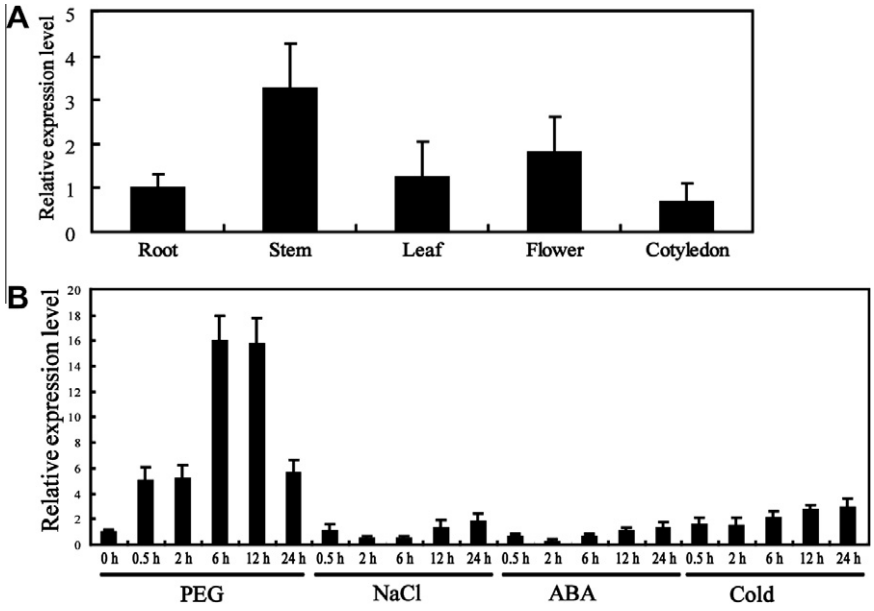

Fig. 1. Expression patterns of gma-miR394a in various tissues and in response to various stresses. (A) Expression patterns of gma-miR394a in different soybean tissues. (B) Expression patterns of gma-miR394a under various stress conditions. gma-miRNA1520d was used as an internal control. Values represent the means of three biological replicates and error bars represent standard deviations.

5′-primer and gene-specific outer primers (08g11030: 5′-CAA GTGACACCAGACTGCAAGGCCAGG-3′). Nested PCR was carried out using 1 μL of the initial PCR reaction, GeneRacer 5′-nested primer, and gene-specific inner primers (08g11030: 5′-GAGCCAACCCC AGTTTGGACCTTCCAGC-3′). After amplification, 5′ RACE products were gel purified and cloned to a pJET1.2 vector (Fermentas), and at least 10 independent clones were sequenced for the PCR product.

3. Results

3.1. Expression profiles and stress-responsive expression patterns of gma-miR394a in soybean

qPCR was used to analyze the expression patterns of gma-miR394a. As shown in Fig. 1A, gma-miR394a was constitutively expressed in stems, roots, leaves, cotyledons, and flower tissues. Expression was higher in stems than in the other tissues examined.

The expression pattern of gma-miR394a under various stresses in leaves was also investigated (Fig. 1B). Following drought treatment, the expression of gma-miR394a increased gradually, reached a maximum after 6 h, and then decreased. Following NaCl stress, expression increased slightly in the first 0.5 h; it was lower after 2 h and 6 h of stress and reached a maximum at 24 h. The expression of gma-miR394a was initially downregulated and then upregulated to high levels upon ABA treatment. The transcript level of gma-miR394a moderately increased and reached a maximum after 24 h under cold stress.

3.2. Overexpression of gma-MIR394a confers drought tolerance in Arabidopsis

To elucidate *gma-MIR394a* functions in plants, transgenic *Arabidopsis* plants overexpressing *gma-MIR394a* were generated. Three homozygous transgenic lines were selected for functional analysis. Transgenic *Arabidopsis* plants were detected by reverse transcription (RT)-PCR and the GUS activity assay. *gma-MIR394a* was overexpressed in the three transgenic *Arabidopsis* lines (Fig. 2A and B). The expression of miR394a in all three *35S:gma-MIR394a* transgenic lines was upregulated at least by threefold compared to WT plants (Fig. 2C). Three transgenic lines were used for analysis of root growth inhibition under mannitol treatment. Three-day-old seedlings of transgenic and WT *Arabidopsis* were transferred to MS medium plates supplemented with mannitol or without. When grown on MS medium, the root length of *35S:gma-MIR394a* transgenic lines was similar to that of WT plants. However, seedlings of *35S:gma-MIR394a* transgenic lines had longer roots than WT seedlings when exposed to 100 mM mannitol. Root growth remained superior in *35S:gma-MIR394a* transgenic lines as the mannitol concentration increased to 200, 300, and 400 mM (Fig. 2D).

To examine the role of *gma-MIR394a* in drought stress tolerance, 19-day-old *Arabidopsis* plants were grown in pots. When the soil was allowed to dry by withholding water for 22 days, WT plants displayed severe wilting (Fig. 3A). After rewatering for 3 days, most control plants were unable to recover and eventually died (25% survival). In contrast, a majority of the *35S:gma-MIR394a* transgenic lines appeared to be healthy before and after rewatering, and they survived and continued to grow, unlike WT plants, under severe water stress (Fig. 3B). Consistent with these results, detached leaves of *35S:gma-MIR394a* transgenic lines lost water more slowly than those of the WT (Fig. 3C). Taken together, these results show that overexpression of *gma-MIR394a* confers drought tolerance in *Arabidopsis*, which may be partially due to the improvement of tolerance against osmotic stress.

3.3. Identification of the gma-miR394a targets

The putative target genes for gma-miR394a were identified using psRNA Target, and a putative target gene (*Glyma08g11030*) was selected. To confirm regulation of *Glyma08g11030* transcripts by gma-miR394a *in vivo*, we used 5′ RACE to identify their cleavage products in RNA samples obtained from roots of soybean. Fig. 4A shows the detection of cleavage products for the *Glyma08g11030* transcript; it cleaved between the ninth and tenth nucleotides complementary to gma-miR394a.

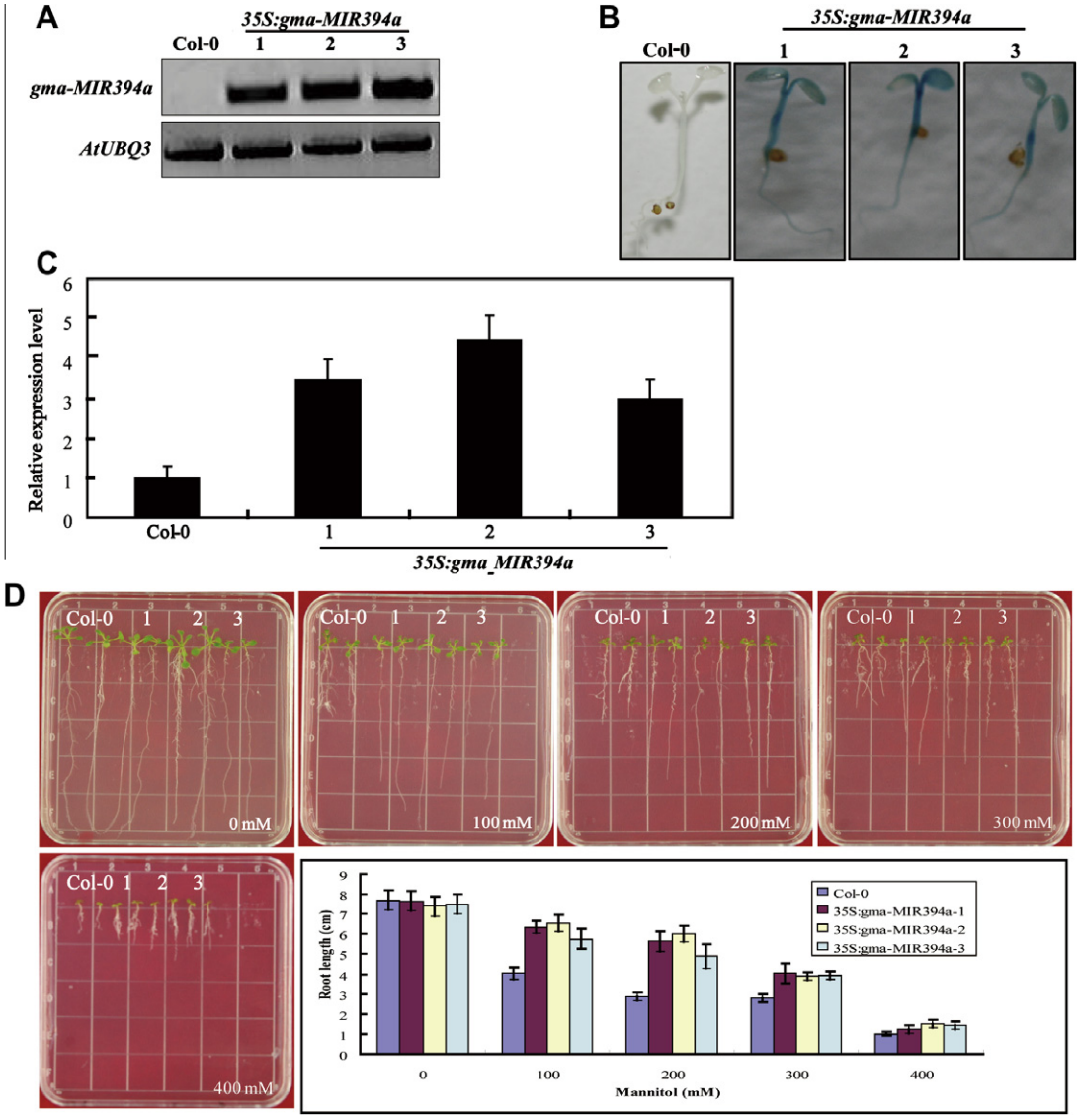

Fig. 2. The effect of *gma-MIR394a* expression on osmotic tolerance in transgenic *Arabidopsis* plants. (A) Expression pattern of *gma-MIR394a* precursor in wild-type and transgenic lines by reverse transcription (RT)-PCR. cDNAs were normalized using the *AtUBQ3* gene. (B) GUS activity analysis of transgenic *Arabidopsis*. (C) Verification of gma-miR394a overexpression in *gma-MIR394a*-overexpressing transgenic *Arabidopsis* lines by real-time RT-PCR. (D) Three-day-old seedlings were transferred to medium containing various concentrations of mannitol for 7 days before the images were taken. Data are given as root lengths. All values are the mean (±SE) for 90 seedlings from three independent experiments (30 seedlings per experiment).

3.4. Repression of putative target genes by overexpression of gma-miRNA394a

Previous studies have identified two loci generating miR394a and miR394b, and a putative target gene (*At1g27340*) in *Arabidopsis* [9]. The gma-miR394a sequence was nearly perfectly complementary to the target site of the *At1g27340* mRNAs. Theoretically, gma-miR394a oversupply should silence or downregulate *At1g27340* mRNAs in *Arabidopsis*. To further determine whether the increased level of gma-miR394a transcript could cause degradation of *At1g27340* mRNAs, we compared the expression of *At1g27340* in *35S:gma-MIR394a* transgenic lines and WT plants using qPCR. The three transgenic lines produced lower levels of *At1g27340* transcripts than the WT control (Fig. 4B). The extent of downregulation was positively correlated with the accumulation gma-miR394a in the three transgenic lines (Fig. 2C), suggesting that gma-miR394a oversupply underlies the decline in *At1g27340* transcripts.

4. Discussion

Understanding the spatial and temporal dynamics of miRNA activity during development is central to understanding miRNA functions. Recently, microarray technology has been adapted to rapidly survey expression profiles of plant miRNAs [20,25]. Some miRNAs are broadly expressed, whereas others are expressed most strongly in particular organs or developmental stages [25,26]. In this study, we found that *gma-MIR394a* is broadly expressed in stems, roots, leaves, cotyledons, and flowers (Fig. 1A). Similarly, in *Arabidopsis*, miR394 shows a broad expression profile with peak expression within stems and long-day seedlings and depressed expression within siliques [25]. In *B. napus*, miR394 is expressed in roots, stems, and leaves [18].

Numerous studies have shown that several miRNAs that participate in stress responses have adapted to environmental challenges [8]. Previous studies have reported that miR394 is involved in abiotic stress regulation [18,20,21]. In the present study, expression of

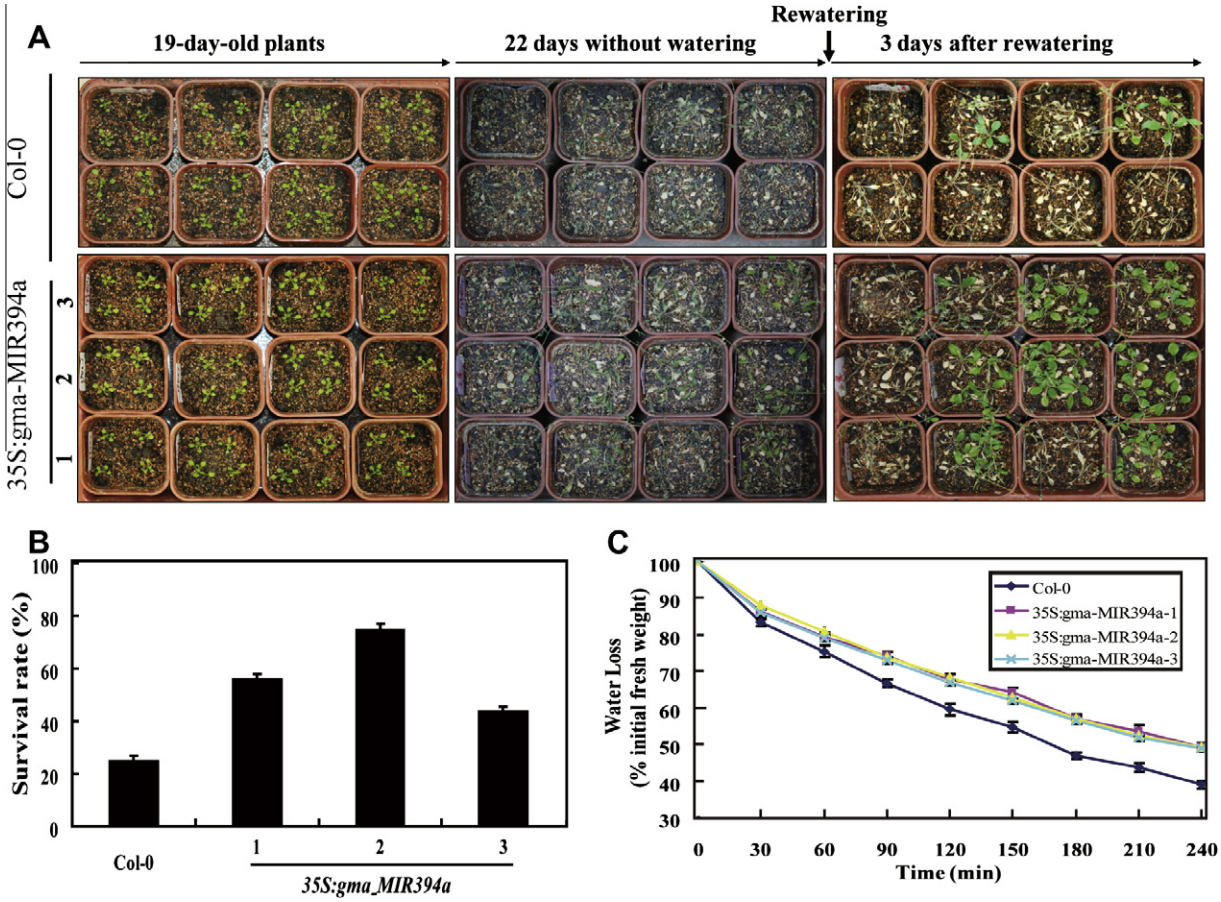

Fig. 3. Improved drought resistance in *35S:gma-MIR394a* plants. (A) Drought resistance of *35S:gma-MIR394a* plants (lines 1, 2, and 3). Healthy 19-day-old wild-type (WT) and *35S:gma-MIR394a* plants were grown for 22 days without water, followed by rewatering for 3 days. Dehydration tolerance was assayed as the capability of plants to resume growth when returned to normal conditions following water stress. (B) The survival rate of WT and three independent transgenic lines are shown. Error bars represent the standard error (SE) for three independent experiments. (C) Water loss from detached leaves of WT and *35S:gma-MIR394a* plants. Water loss was expressed as the percentage of initial fresh weight. Values are means from 10 leaves for each of three independent experiments.

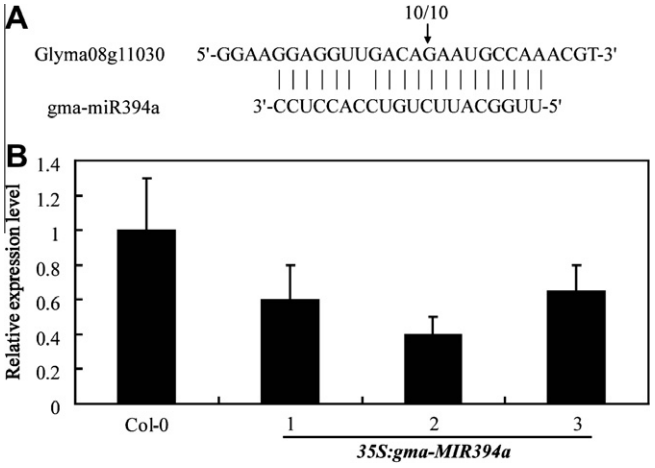

Fig. 4. Targets were validated using a modified 5′ RLM-RACE assay and real-time RT-PCR. (A) miRNA target gene cleavage site validated by 5′ RACE. The gma-miR394a cleavage site on its target gene *Glyma08g11030* is highlighted by the arrow. The number is the frequency of accurate clones when validating the cleavage sites of target mRNAs. (B) Expression identification of the predicted target *At1g27340* mRNAs in *35S:gma-MIR394a* transgenic plant lines by real-time RT-PCR. Quantifications were normalized to the expression of *AtUBQ3*. Error bars represent the standard error (SE) for three independent experiments.

gma-MIR394a was induced by drought, salt, cold, and exogenous ABA (Fig. 1B), suggesting that *gma-MIR394a* is involved in an intricate network for multi-environmental stress responses. Our findings are consistent with those of Li et al., who found miR394a is upregulated in response to drought stress but downregulated in response to salinity stress in soybean by deep sequencing and qPCR methods [27]. Comparing these expression patterns, we found that the *gma-MIR394a* transcript was markedly elevated in response to 20% PEG in leaves compared with other stresses, suggesting that *gma-MIR394a* might be involved in plant tolerance against drought stress. These results prompted us to conduct further experiments to characterize the function of *gma-MIR394a* in the plant drought response. As transgenic work is extremely difficult in soybean, in this study, we overexpressed the *gma-MIR394a* in *Arabidopsis* under the control of the 35S CaMV promoter to examine its role in drought stress. As shown in Fig. 2D, seedlings overexpressing *gma-MIR394a* grown in MS medium containing mannitol for 7 days exhibited longer roots compared with control plants. Furthermore, *35S:gma-MIR394a Arabidopsis* plants exhibited higher survival rates compared with WT plants under drought stress (Fig. 3). These results strongly suggest that *gma-MIR394a* is a positive regulator of drought tolerance.

The rate of water loss from excised leaves, presumably a measure of cuticular transpiration, has been suggested as an important indicator of water status [28]. In this study, the water loss rate of detached leaves from *35S:gma-MIR394a* transgenic lines was lower

than that of the WT plants (Fig. 3C), strongly indicating that the transgenic lines had a higher water retention ability.

Investigation of the target mRNAs of the miRNAs identified can assist us in understanding their biological roles. A growing number of plant miRNA targets predicted through bioinformatics have been experimentally confirmed [9]. In this study, we searched for putative target genes for gma-miR394a using a Web-based computer program, and found it targets *Glyma08g11030* mRNAs that encode a putative F-box protein. The cleavage of target mRNAs appears to be the predominant mode of gene regulation by plant miRNAs [1]. Perhaps the most useful method of miRNA target validation uses 5′ RACE to detect *in vivo* products of miRNA-mediated cleavage [2]. In the present study, 5′ RACE experiments demonstrated that gma-miR394a directs the cleavage of *Glyma08g11030* mRNA molecules (Fig. 4A).

Although pre-miRNAs of miR394 vary among plant species, the length (20 bp) and the sequence of mature miRNA are conserved among different species. Not only are miRNA genes conserved across all plant lineages, their targets also are conserved in different plant families [25]. For example, the ath-miR396 sequence is complementary to the four *NtGRF*-like mRNAs that encode a portion of the conserved N-terminal WRC domain of proteins. Overexpression of ath-miR396 in tobacco reduces the levels of three *NtGRF*-like genes containing a miR396 match site [29]. In *Arabidopsis*, miR394 targets a putative F-box gene (*At1g27340*) [9]. As one of the largest gene families, F-box domain proteins have important roles in regulating various developmental processes and stress responses [30]. For example, overexpression of a rice F-box gene (*Os02g44990*) reduces the sensitivity of rice to ABA and abiotic stress tolerance and promotes root growth [31]. Likewise, a mutation in *At2g31470* encodes a putative F-box protein resulting in a hypersensitive ABA response of stomatal closing and a substantial increase in drought tolerance; in contrast, transgenic plants overexpressing *At2g31470* are more susceptible to drought stress [32]. In this study, the gma-miR394a sequence was complementary to the *At1g27340* mRNAs that encode an F-box protein. Overexpression of *gma-MIR394a* confers tolerance to drought in transgenic *A. thaliana*. Furthermore, the expression of *At1g27340* mRNAs significantly decreased in *35S:gma-MIR394a Arabidopsis* plants (Fig. 4B). These results imply that *At1g27340* may be similar to *At2g31470* and *Os02g44990* in that it plays a negative role in the response to drought stress in *Arabidopsis*.

In conclusion, our data indicate that drought-induced *gma-MIR394a* expression might downregulate its target F-box gene, which may encode negative regulators of drought responses. When we overexpressed *gma-MIR394a* in *Arabidopsis*, the transgenic plants showed enhanced tolerance to drought stress. These results suggest that *gma-MIR394a* likely functions as a positive regulator of drought tolerance.

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