Biochemical and Biophysical Research Communications 427 (2012) 330–335

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 1 September 2012
Available online 18 September 2012

Keywords:
Drought tolerance
F-box protein
gma-MIR394a
Soybean

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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0006-291X/$ - see front matter © 2012 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2012.09.055

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