PCP
PLANT & CELL PHYSIOLOGY

MicroRNA402 Affects Seed Germination of *Arabidopsis thaliana* Under Stress Conditions via Targeting DEMETER-LIKE Protein3 mRNA

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(Received April 22, 2010; Accepted May 5, 2010)

The functional roles of miR402 in *Arabidopsis thaliana* were investigated under abiotic stress conditions. Overexpression of *miR402* accelerated the seed germination and seedling growth of Arabidopsis under salt stress conditions, while its overexpression promoted only seed germination but not seedling growth of Arabidopsis under dehydration or cold stress conditions. The expression of DEMETER-LIKE protein3 mRNA was down-regulated in miR402-overexpressing transgenic plants. These results imply that miR402 plays a role as a positive regulator of seed germination and seedling growth of Arabidopsis under stress conditions, and that microRNA-guided regulation of DNA demethylation is an adaptive process of plants to stress conditions.

Keywords: Abiotic stress • Arabidopsis • DEMETER-LIKE protein • Demethylation • MicroRNA.

Abbreviations: CaMV, cauliflower mosaic virus; DML3, DEMETER-LIKE protein 3; miRNA, microRNA; MS, Murashige and Skoog; RT–PCR, reverse transcription–PCR.

MicroRNAs (miRNAs) are one of the groups of non-coding RNAs that lack protein-coding capacity and exert their actions mainly or exclusively at the RNA level. Plant miRNAs are endogenous RNAs of 20–24 nucleotides (nt) in length that can play important regulatory roles by targeting mRNAs for cleavage or translational repression. The origin, biogenesis and activity of miRNAs have been extensively reviewed (Bartel 2009, Voinnet 2009). Many recent reports determined that miRNAs play diverse roles in growth, development and morphogenesis of plants (Palatnik et al. 2003, Zhou et al. 2007, Schommer et al. 2008, Voinnet 2009). In addition to the roles in development and morphogenesis of plants, it is increasingly evident that miRNAs are also involved in the response of plants to changing environmental conditions (Jones-Rhoades and Bartel 2004, Jung and Kang 2007, Liu et al. 2008, Lu et al. 2008, Arenas-Huertero et al. 2009, Zhao et al. 2009). It was reported that miR399 regulates phosphate homeostasis and is involved in the phosphate starvation response in *Arabidopsis thaliana* (Fujii et al. 2005, Chiou et al. 2006, Lin et al. 2008, Pant et al. 2008). It was also reported that Cu/Zn superoxide dismutase genes are regulated by miR398, which is important for oxidative stress tolerance in Arabidopsis (Sunkar et al. 2006, Jagadeeswaran et al. 2009), and that miR395 is involved in the response of plants to sulfate starvation conditions (Jones-Rhoades and Bartel 2004, Kawashima et al. 2009).

As it is increasingly evident that plant miRNAs are involved in the response of plants to diverse environmental cues, it is worth determining experimentally the functional role of a specific miRNA in the plant stress response. miR402 was initially identified as one of the stress-regulated miRNAs in Arabidopsis (Sunkar and Zhu 2004), and REPRESSOR OF SILENCING1 (ROS1)-like protein (At4g34060), a putative DNA glycosylase, was predicted as its target gene. The ROS1-like protein is now described as DEMETER-LIKE protein3 (DML3), which is involved in DNA demethylation (Ortega-Galisteo et al. 2008). Considering that DNA methylation and demethylation are the most important cellular processes in the epigenetic regulation of gene expression (Penterman et al. 2007a, Penterman et al. 2007b), it is of interest to determine whether DML3 influences the growth of Arabidopsis under stress conditions. This work reports the functional role of miR402 in seed germination and seedling growth of Arabidopsis under various abiotic stress conditions.

We first analyzed the stress-responsive expression patterns of *miR402* in germinating Arabidopsis seeds (**Fig. 1A**). The up-regulation of *miR402* expression by salt, dehydration or cold stress has previously been documented in 2-week-old seedlings (Sunkar and Zhu 2004). In this study, we aimed to determine the expression patterns of *miR402* in germinating seeds at day 3 under stress conditions. The transcript levels of *miR402* in germinating seeds were noticeably increased by cold, salt or dehydration stress (**Fig. 1A**). Under these stresses, the expression of the stress response marker *RD29A* or *RD29B* was significantly increased (data not shown), compared with the expression of *Actin* in which no noticeable changes were observed (**Fig. 1A**).

Plant Cell Physiol. 51(6): 1079–1083 (2010) doi:10.1093/pcp/pcq072, available online at www.pcp.oxfordjournals.org

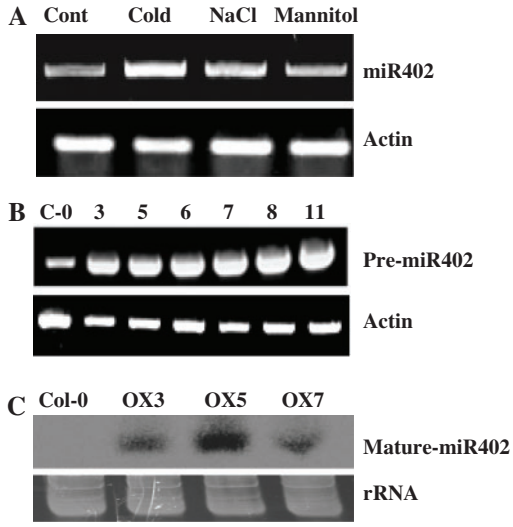

Fig. 1 Stress-responsive expression patterns of *miR402* and confirmation of miR402-overexpressing transgenic Arabidopsis plants. (A) Seeds were allowed to germinate on MS medium at normal (Cont) or cold temperature (12°C), or on MS medium supplemented with 150 mM NaCl or 300 mM mannitol, and transcript levels of *miR402* were determined on the third day. (B) Overexpression of 300 nt long pre-*miR402* was verified in miR402-overexpressing lines (3–11) by RT–PCR analysis. Actin was used as a reference to show that equal amounts of RNA were present in the samples. (C) Overexpression of 22 nt long mature *miR402* was confirmed by Northern blot analysis.

To determine the functional role of miR402 in the response of Arabidopsis to abiotic stress conditions, transgenic Arabidopsis plants that constitutively overexpress *miR402* under control of the cauliflower mosaic virus (CaMV) 35S promoter (35S::miR402) were generated, and their phenotypes under stress conditions were analyzed. Overexpression of both pre-*miR402* (300 nt) and mature *miR402* (22 nt) was verified via reverse transcription–PCR (RT–PCR) and Northern blot analysis (**Fig. 1B, C**). In addition to these transgenic plants, two loss-of-function mutant lines (SALK_056440 and CS871446) of *DML3*, the target mRNA for miR402, were obtained from the Arabidopsis Biological Resource Center, and their phenotypes under stress conditions were analyzed. SALK_056440 and CS871446 lines have T-DNA insertions in the fifth and 14th exon of *DML3*, respectively, and RT–PCR analysis confirmed knockout of *DML3* expression (data not shown). Since both mutant lines showed similar phenotypes under stress conditions, only the data of SALK_056440 are presented in the figures. The wild-type, *dml3* mutant and transgenic plants showed no differences in seed germination and subsequent growth under normal growth conditions (**Fig. 2A** and data not shown). When the seeds of wild-type, *dml3* mutant and 35S::miR402 plants were germinated on Murashige and Skoog (MS) medium supplemented with 150 mM NaCl or 300 mM mannitol, 35S::miR402 seeds and *dml3* mutant seeds germinated earlier that wild-type seeds under these stress conditions (**Fig. 2B, C**). No noticeable differences in seed germination were observed between wild-type, *dml3* mutant and 35S::miR402 plants in the presence of lower or higher concentrations of NaCl or mannitol (data not shown). When the seeds of wild-type, *dml3* mutant and 35S::miR402 plants were germinated at 12°C, the temperature which is generally used to test the effect of cold stress on seed germination and seedling growth of Arabidopsis plants (Kim et al. 2005, Kim et al. 2008), earlier germination of 35S::miR402 seeds and *dml3* mutant seeds compared with wild-type seeds was observed at this low temperature (**Fig. 2D**). Since no *DML3* expression is detected in *dml3* mutants, in contrast to 35S::miR402 plants showing partial down-regulation of *DML3* expression (**Fig. 3**), seed germination of *dml3* mutants was much faster than that of 35S::miR402 plants under stress conditions. It was evident that growth of the leaves but not the roots was markedly accelerated in 35S::miR402 and *dml3* plants compared with wild-type plants under salt stress conditions (**Fig. 2E**). The differences in seedling growth between the genotypes were most clearly observed in the presence of 150 mM NaCl, and no significant differences in seedling growth were observed between the genotypes in the presence of lower or higher concentrations of NaCl. The salt tolerance of 35S::miR402 and *dml3* plants was further reflected by the fact that the transgenic and *dml3* mutant plants survived approximately 10 d longer than wild-type plants in the presence of 150 mM NaCl. In comparison, no noticeable differences in seedling growth were observed between wild-type, *dml3* mutants and transgenic plants under dehydration (**Fig. 2F**) or cold stress conditions (data not shown). These phenotypes were consistently observed when the experiments were repeated with different batches of seeds. The findings imply that miR402 plays a role as a positive regulator of seed germination and seedling growth of Arabidopsis under salt stress conditions, but impacts positively only on seed germination of Arabidopsis under dehydration or cold stress conditions.

The putative target gene of miR402, DML3, was predicted in a previous report (Sunkar and Zhu 2004). When the transcript level of predicted target mRNA was analyzed in miR402-overexpressing transgenic plants, it was evident that the level of *DML3* transcript decreased in both germinating seeds and 2-week-old seedlings of transgenic plants compared with wild-type plants (**Fig. 3**). These results clearly show that *DML3* is the authentic target of miR402, and suggest that the observed phenotypes result from the down-regulation of *DML3* by miR402. DNA demethylation as well as DNA methylation are considered to be key regulatory processes of gene expression in eukaryotes (Penterman et al. 2007a, Penterman et al. 2007 b). DEMETER and DEMETER-LIKE proteins are required for appropriate distribution of DNA methylation marks, endosperm gene imprinting and seed viability in Arabidopsis (Choi et al. 2002, Ortega-Galisteo et al. 2008). Although the relevance of DNA methylation to the altered seed germination and seedling growth of the transgenic plants under stress conditions is not presently understood, some clues can be found from previous

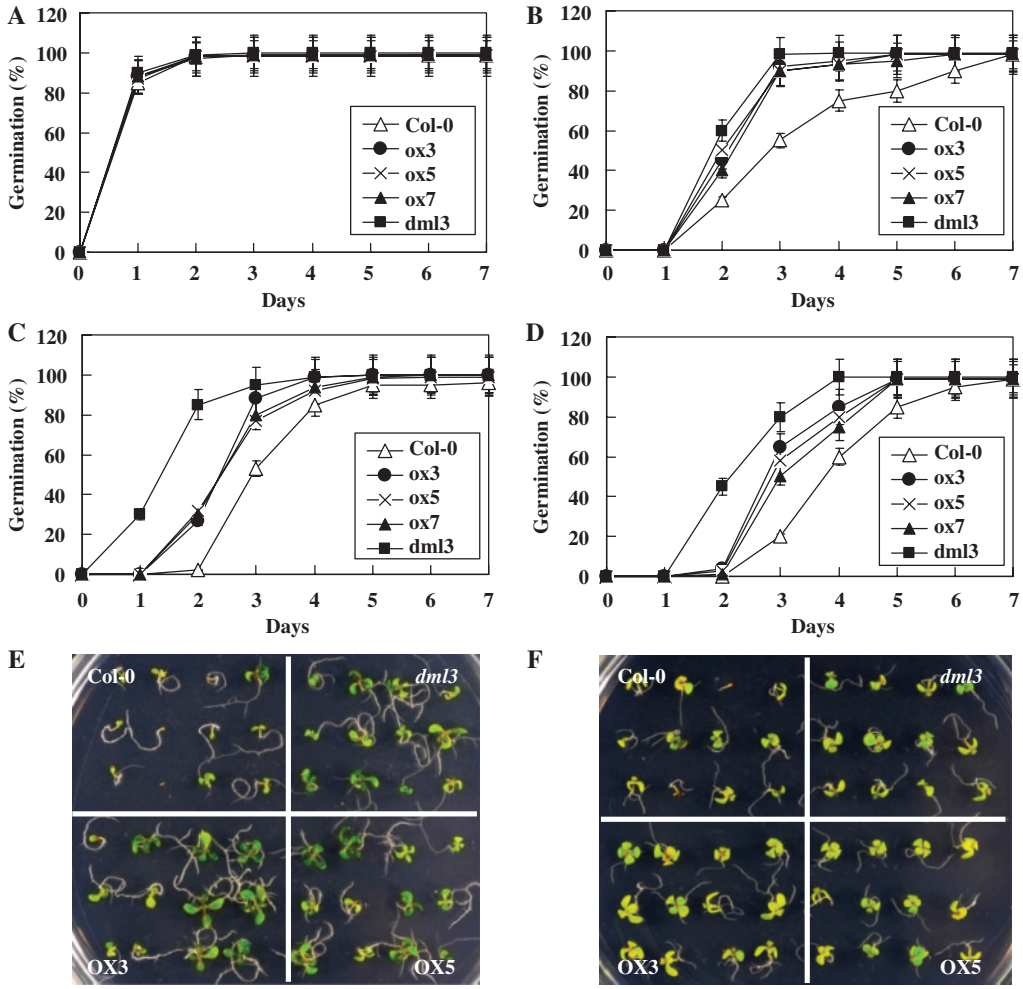

Fig. 2 Effect of different stresses on seed germination and seedling growth of wild-type, *dml3* mutant and miR402-overexpressing plants. Germination of wild-type, *dml3* mutant and 35S::miR402 seeds was measured in MS medium (A) and MS medium supplemented with 150 mM NaCl (B) or 300 mM mannitol (C), or at 12°C (D), and the germination rates scored on the indicated days. For seedling growth assay, fully germinated seedlings on normal MS medium were transferred to the medium supplemented with 150 mM NaCl (E) or 300 mM mannitol (F), and the pictures were taken 14 d after germination. Mean values and standard errors were obtained from five independent experiments (n = 40–50).

reports demonstrating that the level of DNA methylation decreases during seed germination (Follmann et al. 2000, Zluvova et al. 2001). It is likely that induction of *miR402* expression by stresses guides cleavage of *DML3*, which in turn maintains a higher DNA methylation level of the genes that play a negative role in seed germination. This results in accelerated seed germination of miR402-overexpressing plants under stress conditions. This hypothesis needs to be tested by analyzing the level of DNA methylation in the wild-type and miR402-overexpressing transgenic plants and by confirming the target genes whose DNA methylation levels are influenced by the down-regulation of *DML3* by miR402.

In conclusion, the present results demonstrate that miR402 has a positive impact on seed germination and seedling growth of Arabidopsis under stress conditions via cleavage of *DML3* mRNA, which indicates that miRNA-guided regulation of

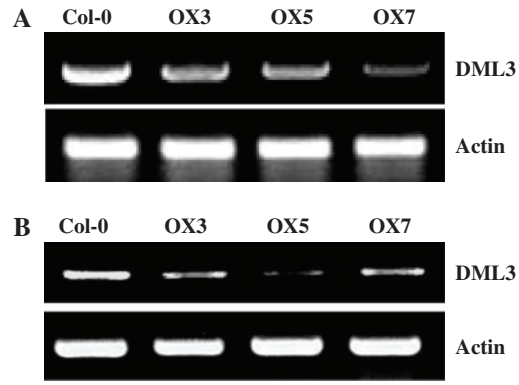

Fig. 3 Cleavage of putative mRNA target for miR402 in miR402-overexpressing transgenic plants. Total RNAs were extracted from (A) germinating seeds and (B) 2-week-old seedlings of wild-type (Col-0) and transgenic plants (ox3, ox5 and ox7), and the expression levels of *DML3* were analyzed by RT–PCR using the gene-specific primers.

DNA demethylation is an adaptive process of plants to abiotic stress conditions.

Materials and Methods

To analyze the stress-responsive expression patterns of *miR402* during seed germination of *A. thaliana* (Col-0 ecotype), seeds were germinated under cold stress at 12°C, under salt stress with 150 mM NaCl or under dehydration stress with 300 mM mannitol. The expression of *miR402* was determined by RT–PCR using the gene-specific primers: forward primer 5′-TCGGAAGGAGTTAGCATCACGTTG-3′ and reverse primer 5′-CATTCACTAAAGCTTCCCCTTCAC-3′. For the control reaction employing *Actin*, forward primer 5′-CTCCGTGTTG CTCCTGAGGAACATC-3′ and reverse primer 5′-ACCTCAGG ACAACGGAATCGCTC-3′ were used. All experiments were repeated at least three times.

To generate miR402-overexpressing transgenic Arabidopsis plants, the coding region of 300 nt long pre-miR402 was cloned under the CaMV 35S promoter in the pBI121 vector. Overexpression of both pre-*miR402* and mature *miR402* in transgenic plants was confirmed by RT–PCR with the gene-specific primers listed above and Northern blot analysis in which total RNAs (20 μg) were size-fractionated by electrophoresis on a 15% polyacrylamide gel, blotted, and cross-linked to a Hybond-N nylon membrane. A ^{32}P-labeled 22 nt *miR402* was used as a probe.

Germination assays were carried out on three replicates of 40–50 seeds essentially as described (Kim et al. 2008). Seeds were sown on half-strength MS medium supplemented with 1% sucrose, and the plates were placed at 4°C for 3 d in the dark and then transferred to normal growth conditions. Plants were grown at 23°C under long day conditions (16 h light/8 h dark cycle). To determine the effect of salt or dehydration stress on seed germination, the MS medium was supplemented with 100–200 mM NaCl or with 150–300 mM mannitol, respectively. To determine the effect of cold stress on germination, the MS plates were placed in an incubator maintained at 12°C. A seed was regarded as germinated when the radicle protruded through the seed coat.

To determine miR402-guided cleavage of putative target mRNA, gene-specific primers were designed across the miR402 target site on *DML3*. The expression of *DML3* was determined by RT–PCR using gene-specific primers; forward primer 5′-GTGTGATGAATCAGCACATCTTC-3′ and reverse primer 5′-GTCCGAGCCACGGATCTCTTAATC-3′.

Funding

This work was supported by the National Research Foundation (NRF) of Korea [a grant to the Agricultural Plant Stress Research Center (R11-2001-092-04002-0) of Chonnam National University]; the NRF of Korea [World Class University program funded by the Ministry of Education, Science and Technology (R32-20047-0)].

Acknowledgments

We thank the Arabidopsis Biological Resource Center for T-DNA mutant lines.

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