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Plant Gene 5 (2016) 56–64

Contents lists available at ScienceDirect

Plant Gene

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miR394 and its target gene LCR are involved in cold stress response in *Arabidopsis*

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 5 December 2015
Accepted 8 December 2015
Available online 18 December 2015

Keywords:
Arabidopsis
miR394
LCR
Cold stress
CBF

ABSTRACT

MicroRNA394 (miR394) and its target gene *LEAF CURLING RESPONSIVENESS* (LCR) are known to mediate plant growth and development. However, whether miR394 and LCR regulate plant adaptation to low temperature has not been investigated. Here, we showed that miR394a overexpressing (*35S*::*MIR394a*) plants and a *LCR* loss of function *lcr* mutant exhibited more tolerance to cold stress (4 to −11 °C), whereas *LCR* overexpressing (*35S*::*m5LCR*) plants displayed a cold hypersensitive phenotype as compared to wild-type. Both *35S*::*MIR394a* and *lcr* plants accumulated more proline and total soluble sugars under cold stress (4 °C) than wild-type. Furthermore, a lower rate of electrolyte leakage was observed in *35S*::*MIR394a* and *lcr* plants. These results indicated that the physiological response was changed due to the expression of miR394. However, a contrasting phenotype was found in *LCR* overexpressing plants. Analysis of the cold-responsive genes revealed that transcripts of *CBF1*, *CBF2* and *CBF3* (C-repeat/dehydration binding factor) were increased in the *35S*::*MIR394a* plants relative to the wild-type, suggesting that miR394-regulated cold tolerance was involved in the CBF-dependent cold responsive pathway. Taken together, our results showed that both miR394 and its target gene *LCR* are involved in plant response to low temperature.
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1. Introduction

As plants (or crops) grow in changing environments, their growth and development are constantly challenged by various environmental stresses such as drought, salinity and unfavorable temperature. Low temperature is one of the most serious climate issues that drastically restrict the worldwide plant growth and crop production (Guy, 1990; Thakur et al., 2010). Under cold stress, plant biochemical and physiological processes are altered, cellular aqueous and ionic equilibriums are disturbed, and gene expression for the biological processes is reprogrammed (Xiong et al., 2002; Jeon and Kim, 2013). Plants have evolved delicate mechanisms to cope with the cold stress via perceiving the stress signal, transmitting it through a variety of signal transduction pathways, and consequently making adjustment of their metabolic processes (Zhou et al., 2007). To date, a variety of cold-responsive genes have been identified and most of them are found to be involved in synthesis of dehydrins and osmolytes, enzymes for antioxidation, and components for abscisic acid (Chinnusamy et al., 2010).

Plants under low temperature display varying degrees of freezing tolerance through cold acclimation (Sakai and Larcher, 1987). The cold acclimation is a complex process involving numerous physiological, biochemical and molecular responses (Xiong et al., 2002; Zhao et al., 2015). Physiological adaptation concerns stability of subcellular structures, antioxidation, and osmotic changes (Medina et al., 2011). Additionally, most of the alterations are associated with a huge amount of gene expression, as transcriptome analyses have shown that around 1000 genes in *Arabidopsis* are regulated by low temperature (Zeller et al., 2009; Park et al., 2015). Like other abiotic stresses such as salt and drought, cold stress triggers many responsive genes encoding transcription factors, indicating that the cold stress response is involved in multiple regulatory pathways (Lee et al., 2005). Among the cold-induced transcription factors, several transcription factors known as C-repeat-binding factors (CBFs)/dehydration-responsive element-binding factors 1 (DREB1s) are of particular attention because this group of genes can bind to the C-repeat/dehydration responsive element (CRT/DRE) and activate numerous downstream genes (Liu et al., 1998; Fowler and Thomashow, 2002; Medina et al., 2011).

Recently, a large number of genes coding for small RNAs or microRNAs (miRNAs) have been identified in response to cold stress (Jones-Rhoades and Bartel, 2004; Zhou et al., 2008; Zhang et al., 2009; Lv et al., 2010; Barakat et al., 2012; Chen et al., 2012b; Wang et al., 2014; Ma et al., 2015). miRNAs are a class of short (~21 nt) endogenous non-coding small RNAs that base pair sequences complementary with specific target genes to repress their translation or induce their degradation. Currently, hundreds of miRNAs have been discovered in

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http://dx.doi.org/10.1016/j.plgene.2015.12.001
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Arabidopsis, rice and other plant species, and some of them have been found to mediate plant response to abiotic stresses (Khraiwesh et al., 2012). These stress-responsive miRNAs represent a newly found mechanism for plant adaptation to abiotic stresses (Phillips et al., 2007; Khraiwesh et al., 2012; Zhou et al., 2012a, 2012b; Yang and Chen, 2013). Constitutive overexpressing Sly-miR169c enhanced drought tolerance by reducing stomatal opening and leaf water loss (Zhang et al., 2011). In *Arabidopsis*, miR858 targets a gene coding for a transcription factor MYB20, and overexpression of a miR858-resistant version (*35S*::*m5AtMYB20*) enhanced sensitivity to drought and showed ABA resistance phenotypes (Gao et al., 2014). miR319 was reported to confer rice tolerance to cold stress in its over-expression transgenic plants (Yang et al., 2013). However, conflicting results have been reported. For instance, expression of miR319 was up-regulated in roots and shoots of sugarcane plantlets under 4 °C condition (Thiebaut et al., 2012), whereas both miR319a and miR319b were found to be down-regulated in rice plants under the same condition (4 °C) (Yang et al., 2013). These observations indicate that the regulatory mechanism of miRNAs in plants is more complicated than expected. The biological roles and regulatory networks in plant response to cold stress remain to be elusive.

miR394 is one of the highly conserved miRNAs existing in many monocot and dicot plant species (Jones-Rhoades and Bartel, 2004, Huang et al., 2010; Ni et al., 2012; Zhou et al., 2012b). Recently, miR394 and its target gene *LCR* were reported to involve the regulation of leaf morphological development (Song et al., 2012) and stem cell identity (Knauer et al., 2013) in *Arabidopsis*. Interestingly, miR394 was also found to be involved in regulation to plant response to drought and salinity stresses; overexpression of miR394a or miR394b resulted in hypersensitivity to salt stress, whereas *LCR* overexpression plants display salt-tolerant phenotypes (Song et al., 2013). To date, several miRNAs in plants have been shown to respond to low temperature (Jones-Rhoades and Bartel, 2004; Liu et al., 2008). However, only miR319 has been functionally identified as a regulator of plant cold stress response (Yang et al., 2013). In this report, we described our recent characterization of miR394 that regulates plant response to cold stress. Our results indicate that miR394 is positively involved in plant adaptive response to low temperature. To our knowledge, this is the first report indicting a role of miR394 in improving plant tolerance to low temperature.

2. Materials and methods

2.1. Plant materials and treatments

Arabidopsis ecotype Col-0 was used throughout the study. The *lcr* (*At1g27340*) T-DNA insertion mutants *lcr-1* (SALK_016763c) and *lcr-2* (SALK_136833c) were obtained from the *Arabidopsis* Biological Resource Center. Identification of the mutants and expression of *LCR* in the *lcr-1* and *lcr-2* was described by Song et al. (2013). The mutant *lcr-1* has a T-DNA insertion at 5′ UTR, while *lcr-2* has an insertion in the intron towards the first exon. The two mutants were verified by diagnostic PCR using LCR gene-specific and T-DNA border primers. The *lcr-1* and *lcr-2* alleles were confirmed by RT-PCR and Western blot, by which the expression of both LCR mRNA and proteins were severely suppressed.

Seeds were soaked in a 30% bleach solution for 10 min and rinsed thoroughly with sterilized water. Seeds were germinated on Murashige and Skoog (MS) solid medium with 1–3% sucrose and 0.8% phytoagar (pH 5.7) or in soil (sunshine mix 5; SunGro) in a growth chamber at 22 °C with 100 μE m^{-2} s^{-1} photosynthetically active radiation and a 16 h light/8 h dark cycle.

The survival experiment was performed based on the method of Miura et al. (2007). Plants were grown in soil at 22 °C for 3 weeks and subjected to 4 °C treatment for 30 min, followed by 0 °C for 1 h. After that, the temperature was successively decreased by 1 °C h^{-1} until −8 °C, and held at −8 °C for 4 h. Following the freezing acclimation, the plants were kept at 4 °C for 12 h and transferred to 22 °C. The survival ratio was determined one week later.

2.2. Plant transformation

The plasmid construction and plant transformation were performed based on the methods described previously (Song et al., 2012). Briefly, pCAMBIA1304 was used as the plant expression vector with or without CaMV35S as a promoter and NOS terminator as transcriptional termination sequences. The target genes (including primary MIR394a/b, and LRC) were PCR-amplified using primers with restriction enzyme sites at the 5′-end of forward and reverse primers, respectively (Supplementary data 1). The PCR amplified products were first cloned to a T/A vector (pMD19, Takara), sequenced and digested with enzymes. The digested segments were cloned into pCAMBIA1304, and the cloning was confirmed by sequencing and restriction analysis. The confirmed clones were transformed into *Agrobacterium tumefaciens* strain LBA4404 following the standard freeze thaw method. All vectors containing the corresponding genes were transferred into *Arabidopsis* via *Agrobacterium*-mediated transformation. All homozygous transgenic plants (T_4) were used in this study.

2.3. GUS assay

Histochemical detection of GUS activity was performed using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc) as a substrate. Plant tissues were placed in X-Gluc solution containing 750 mg mL^{-1} X-Gluc, 0.1% Nonidet P-40, 3 mM K_3F_3 $(CN)_6$, 10 mM EDTA and 100 mM $NaPO_4$ (pH 7) under a vacuum at room temperature for 10 min and then incubated at 37 °C overnight (Song et al., 2012).

2.4. RT-PCR analysis

Real time-PCR and semi-quantitative PCR were performed to analyze gene transcripts based on the methods described previously (Guo et al., 2008; Shen et al., 2011). Briefly, total RNA was isolated and 1.0 μg RNA was used as template for cDNA synthesis. Quantitative RT-PCR (qRT-PCR) was conducted on CFX96 Real-Time PCR Detection System (Bio-Rad). Amplification reaction was performed in a 25 μL mixture containing 5 ng template, 12.5 μL SYBR-Green PCR Mastermix (Toyoba, Japan) and 10 pmol primers. The temperature profile was 98 °C for 30 s, followed by 40 cycles at 98 °C for 2 s, 60 °C for 5 s and melt curve at 65 °C for 5 s. Data were analyzed using CFX Data Analysis Manager Software. The relative expression level was normalized to *ACTIN2*, which was used as the internal control, with the $2^{-\triangle CT}$ method representing the relative quantification of gene expression. The primers used for analysis are presented in Supplementary data 1.

2.5. Western blot analysis

LCR protein extracts were prepared by grinding tissues on ice in extraction buffer (5% glycerol, 4% SDS, 1% polyvinylpolypyrrolidone, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris, pH 8.0), followed by centrifugation at 4 °C and 14,000 g for 15 min. Proteins (15 μg) were separated by electrophoresis on a 12% SDS polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. The immunobloting analysis was performed with an affinity-purified LCR monoclonal antibody (1:100 dilution) which is specifically for LCR in *Arabidopsis*, and the horseradish peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody (1:10,000 dilution) (Song et al., 2013). LCR antibodies were prepared by Abmart Company. The color was developed with 50 mM TBS (pH 7.6) solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride as the horseradish peroxidase substrate. Coomassie brilliant staining was used to show the equal amounts of proteins loaded.

2.6. Electrolyte leakage from leaves

Electrolyte leakage was assessed using fully developed rosette leaves of 3-week-old plants as described previously (Zhu et al., 2004). Leaves (5th or 6th rosette leaves) were placed into a tube containing 200 mL deionized water. An ice chip was added to initiate nucleation. The tube was incubated in a refrigerated circular bath. The temperature of the bath was programmed for a temperature decrease from 0 to −11 °C by a continuous reduction of −1 °C over 30 min until the desired temperature was reached. Each tube was removed from the refrigerated bath and placed immediately into ice to facilitate gradual thawing. Deionized water (14 mL) was added to the sample that was then shaken overnight, after which the conductivity of the solution was determined with the Accumet model 20 pH/conductivity meter. The tube was then incubated at 90 °C for 1.5 h and cooled to room temperature, and conductivity of the solution was determined (i.e., 100% conductivity).

2.7. Proline determination

Lyophilized leaf material (30 mg) was extracted with 3 mL of deionized water at 80 °C for 15 min. The samples were shaken for approximately 1 h at room temperature and then allowed to stand overnight at 4 °C. The extracts were filtered through glass wool and analyzed for proline content using the acid ninhydrin method (Troll and Lindsley, 1955). Briefly, 500 μL of the aqueous extract was mixed with 500 μL of glacial acetic acid and 500 μL of acid ninhydrin reagent (12.5 mg of ninhydrin, 0.3 mL of glacial acetic acid and 0.2 mL of 18 N orthophosphoric acid) and heated at 100 °C for 45 min. After cooling, 500 μL of the reaction mix was partitioned against toluene (2 mL) and the absorbance of the organic phase was determined at 515 nm. The resulting values were compared with a standard curve constructed using known amounts of proline.

2.8. Sugar determination

A sample of 0.5 g of harvested leaves was crushed in 5 mL of 95% (*v*/*v*) ethanol. The insoluble fraction was washed twice with 5 mL of 70% (*v*/*v*) ethanol, and all soluble fractions were centrifuged at 3500 × *g* for 10 min. The supernatants were collected and stored at 4 °C. Total soluble sugars were analyzed by reacting 0.1 mL alcoholic extract with 3 mL freshly prepared anthrone and 100 mL of 72% (*v*/*v*) H_2SO_4, followed by immersion in boiling water for 10 min. After cooling, the A_{625} was determined in a spectrophotometer (Gilmour et al., 2000).

2.9. Statistical analysis

All experiments in the study were independently performed three times. Each result shown in the figures was the mean of at least three replicated treatments and each treatment contained at least 12–30 seedlings. Unless indicated, the equal amount of mixed transgenic line seeds was used and samples for analysis were randomly selected from all transgenic lines. The significant differences between treatments were statistically evaluated by standard deviation ($n = 3$) and one-way analysis of variance (ANOVA). The data between differently treated groups were compared statistically by ANOVA, followed by the least significant difference (LSD) test if the ANOVA result was significant at $P < 0.05$.

3. Results

3.1. Expression of miR394 under cold stresses

To identify whether miR394 expression responded to cold stress, two week-old *Arabidopsis* seedlings grew under the normal (22 °C) or cold (4 °C) conditions for 0, 6 and 12 h. Using semi-quantitative RT-

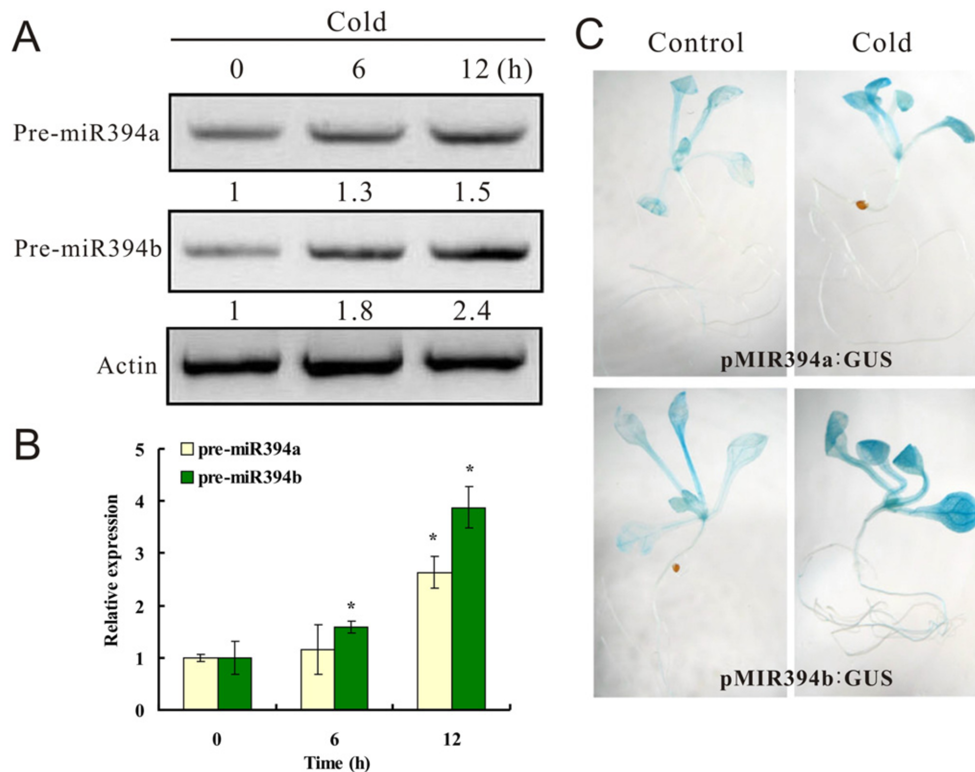

Fig. 1. Transcriptional expression of precursor miR394a/b (pre-miR394a/b) (A and B) and *pMIR394*::*GUS* (C) in wild-type seedlings under low temperature. Two week-old wild-type *Arabidopsis* seedlings were treated with cold (4 °C) for 0, 6 and 12 h. The expression data were obtained using semi-quantitative RT-PCR (A) and qRT-PCR (B) analysis. The number below the band indicates the relative abundance of the corresponding genes with respect to the loading control Actin. C: GUS staining in two week-old *pMIR394a*/*b*::*GUS* transgenic seedlings treated with cold (4 °C) for 12 h.

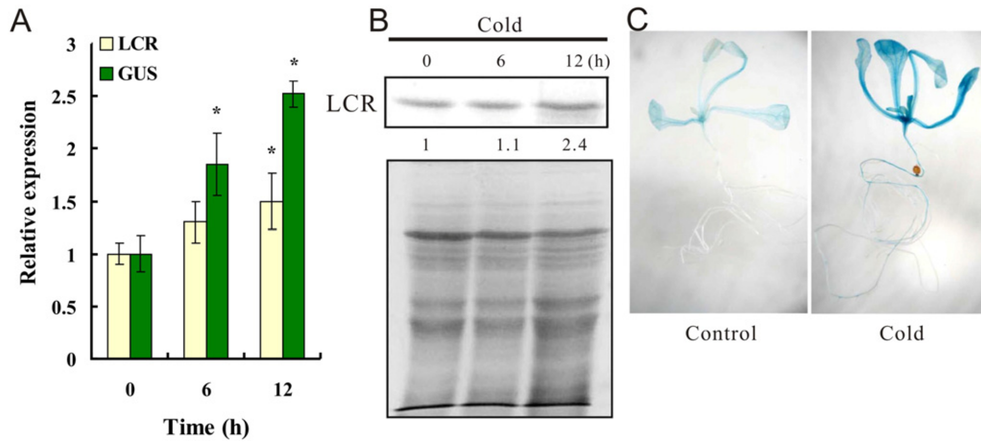

Fig. 2. Expression patterns of miR394 target gene *LCR* (*at1g27340*) by cold stress in wild-type *Arabidopsis*. Two week-old wild-type seedlings were treated with cold (4 °C) for 0, 6 and 12 h. A: Quantitative real time RT-PCR analysis of *LCR* transcripts and *GUS* expression in *pLCR*::*GUS* plants. Vertical bars represent standard deviation of the mean. Asterisks indicate the significant difference between the treatments (6 and 12 h) and control (0 h) ($P < 0.05$). B: Western-bolt analyses of protein level of LCR in plants with cold (4 °C) for 12 h. The loaded protein was normalized. C: GUS staining in *pLCR*::*GUS* transgenic seedlings treated with cold (4 °C) for 12 h.

PCR and real-time RT-PCR (qRT-PCR), we analyzed expression of primary miR394a/b and showed that both pri-miR394a and pri-miR394b were up-regulated under the cold stress (Fig. 1A, B). Moreover, the promoter sequences of miR394a and miR394b were retrieved, fused to the *β-glucuronidase* (*GUS*) reporter gene, and introduced into transgenic plants individually. Histochemical analysis showed that heavy GUS staining was detected in *pMIR394a*/*b*::*GUS* seedlings under cold stress, whereas the relatively light GUS staining was observed in the control plantlets (Fig. 1C).

To investigate whether LCR was regulated by the abiotic stress, the same seedlings were exposed to cold (4 °C) for 0, 6 and 12 h. Transcription of *LCR* was also induced under the cold stress (Fig. 2A). To understand whether LCR proteins at a translational level were regulated by low temperature, a western blot study was carried out. Using a monoclonal antibody specifically against the LCR protein (52 kDa), we showed that the amount of LCR protein could be induced by cold treatments; compared to the control, the expression of LCR increased by 2.4 fold after treatment for 12 h (Fig. 2B). We generated a construct encoding a GUS reporter gene fused to *LCR* under the control of *LCR* promoter sequence (1.45 kb). This vector was transformed into *Arabidopsis*. Histochemical staining indicated that the GUS activity in *pLCR*::*GUS* plants was higher than that of the control under the cold stress (Fig. 2C). When comparatively analyzing the transcripts of GUS in *pLCR*::*GUS* plants and *LCR* in wild-type using qRT-PCR (Fig. 2A), we noticed that although *LCR* was induced under cold stress, the GUS level was higher than the level of *LCR* expression. This result suggests that *LCR* mRNA was partially silenced by miR394 in plants under the cold stress condition.

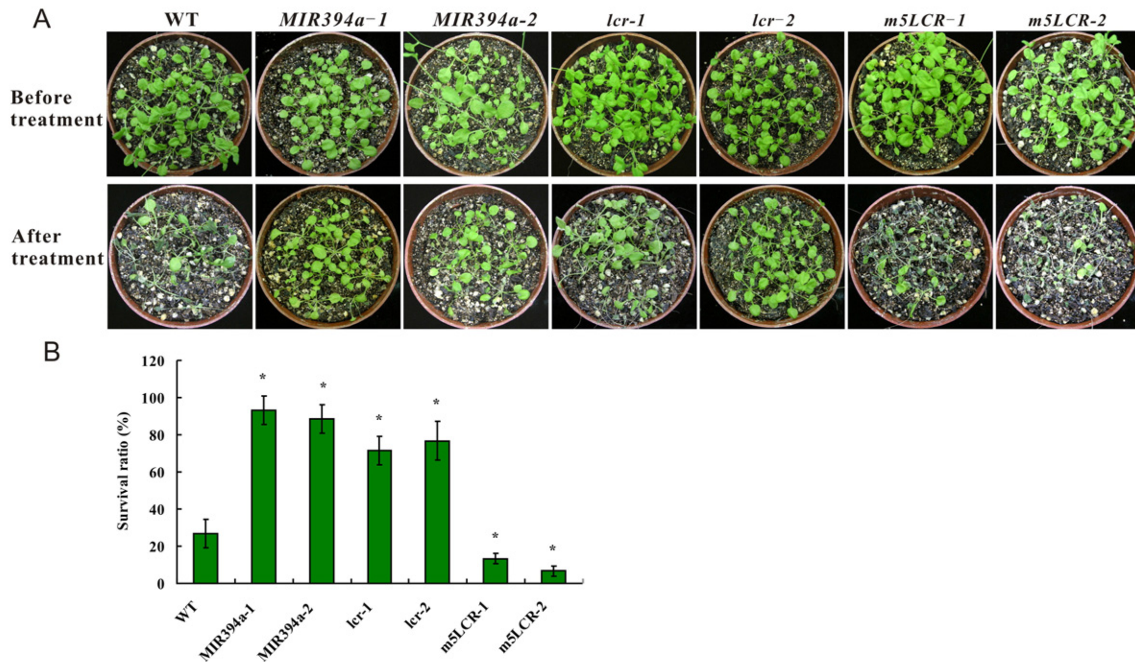

Fig. 3. Cold tolerance analysis of WT (wild-type), *35S*::*MIR394a-1*, *35S*::*MIR394a-2*, *lcr-1*, *lcr-2*, *35S*::*m5LCR-1* and *35S*::*m5LCR-2*. Plants were grown in soil at 22 °C for 3 weeks and subjected to 4 °C treatment for 30 min, followed by 0 °C for 1 h. After that, the temperature was successively decreased by 1 °C h^{-1} until −8 °C, and held at −8 °C for 4 h. Following the cold acclimation, the plants were kept at 4 °C for 12 h and transferred to 22 °C. After 7 d, photograph was taken and survival rate was analyzed. The control plants constantly grew at 22 °C. A: phenotypes; B: The survival ratio was determined after treatments. Vertical bars represent standard deviation of the mean. Asterisks indicate the significant difference between the transgenic plants or mutants and WT ($P < 0.05$).

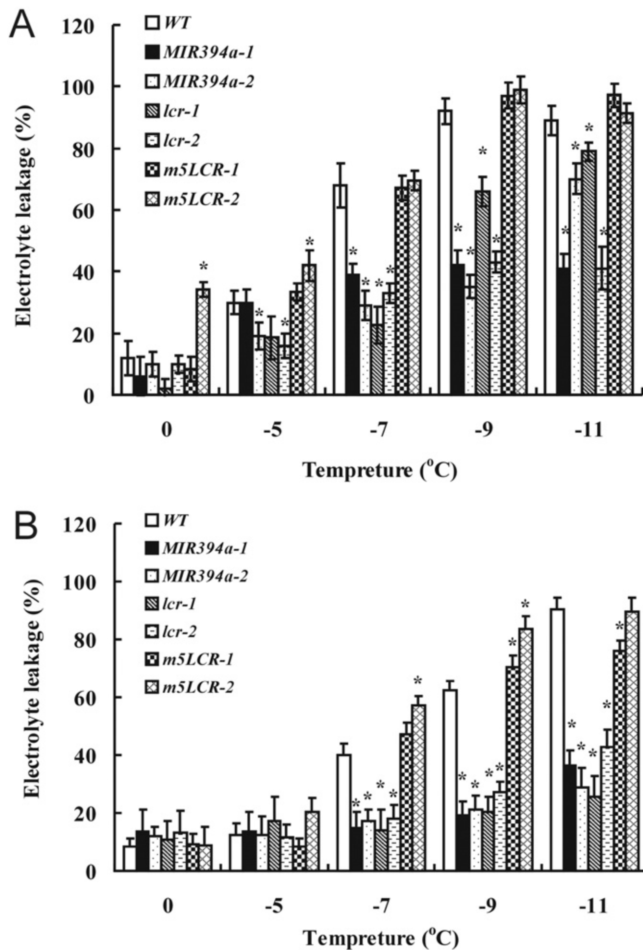

Fig. 4. Effect of cold stress on electrolyte leakage of WT (wild-type), *35S::MIR394a-1*, *35S::MIR394a-2*, *lcr-1*, *lcr-2*, *35S::m5LCR-1* and *35S::m5LCR-2*. Plants were grown in soil at 22 °C for 3 weeks. For non-acclimated experiment (A), plants continued to grew at 22 °C for 7 d. For acclimated experiment (B), plants grew at 4 °C for 7 d. After that, all plants stood at 0 °C for 1 h. Following the step, the temperature was successively decreased by 1 °C per 0.5 h until −5, −7, −9 and −11 °C and held at the indicated temperature for 0.5 h, respectively. The 5th and 6th rosette leaves of treated plants were collected and the electrolyte leakage for the plants was determined (see Materials and methods section). Vertical bars represent standard deviation of the mean. Asterisks indicate the significant difference between the transgenic plants or mutants and WT ($P < 0.05$).

3.2. Overexpression of miR394a enhances plant tolerance to cold stress

To identify the role of miR394 in regulating plant response to cold stress, transgenic *Arabidopsis* plants overexpressing miR394 were generated based on the method described previously (Song et al., 2012). DNA fragments corresponding to the precursors of both *MIR394a* (403 bp) and *MIR394b* (296 bp) were fused to the cauliflower mosaic virus 35S promoter, and transformed into ecotype Columbia (Col-0). *MIR394* overexpression in the transgenic plants was well confirmed by RNA gel blot and qRT-PCR analyses (Song et al., 2012). To assess the importance of the level of *LCR* mRNA, a cleavage-resistant version (*35S::m5LCR*) was generated by introducing five silent mutations in the miR394 binding site but without changing the protein sequences; qRT-PCR analysis indicated that the level of *LCR* mRNA in *35S::m5LCR* plants was sufficiently higher (34.4–40.5 folds) than that of wild-type (Song et al., 2012).

We performed a whole plant survival test using transgenic plants overexpressing miR394a to evaluate the effect of miR394 expression on plant cold tolerance. After a successive decrease of temperature from 22 °C to −8 °C and then back to 22 °C, most of wild-type plants died (Fig. 3). The survival rate of the wild-type plants was only 26.6%, whereas *35S:MIR394a* plants had an 88.3–99.3% higher survival rate. The higher tolerance to cold stress was also found in *lcr* mutant plants, with 71.7–76.6% survival. However, the miR394 cleavage resistant version *35S::m5LCR* plants displayed a more severe phenotype of cold stress compared to the wild-type.

We further carried out an electrolyte leakage test to validate the cold tolerance of *35S:MIR394a* plants. Our analysis showed that the freezing tolerance of non-acclimated miR394a overexpressing plants was greater than that of non-acclimated wild-type plants (Fig. 4A). The non-acclimated wild-type had an EL_{50} (temperature that causes a 50% leakage of electrolyte) of approximately −6 °C, whereas the *35S:MIR394a* plants had EL_{50} values of approximately −9–11 °C. The freezing tolerance of cold-acclimated miR394a overexpressing plants was significantly greater than that of both non-acclimated *35S:MIR394a* plants and cold-acclimated wild-type. *35S:MIR394a* plants with cold-acclimation for 7 d had EL_{50} values of −11 °C or lower (Fig. 4B). The cold-acclimated control plants had EL_{50} values of approximately −8 °C. In all experiments, we did not find any cold-acclimated wild-type plants with EL_{50} values of as low as those obtained with the cold-acclimated *35S:MIR394a* plants. In the two experiments, the phenotype of *lcr* mutant plants was generally in agreement with *35S:MIR394a* plants, whereas the *35S::m5LCR* plants always showed a phenotype different from *35S:MIR394a* lines. These results indicated that overexpression of miR394 was able to confer plant tolerance to cold stress.

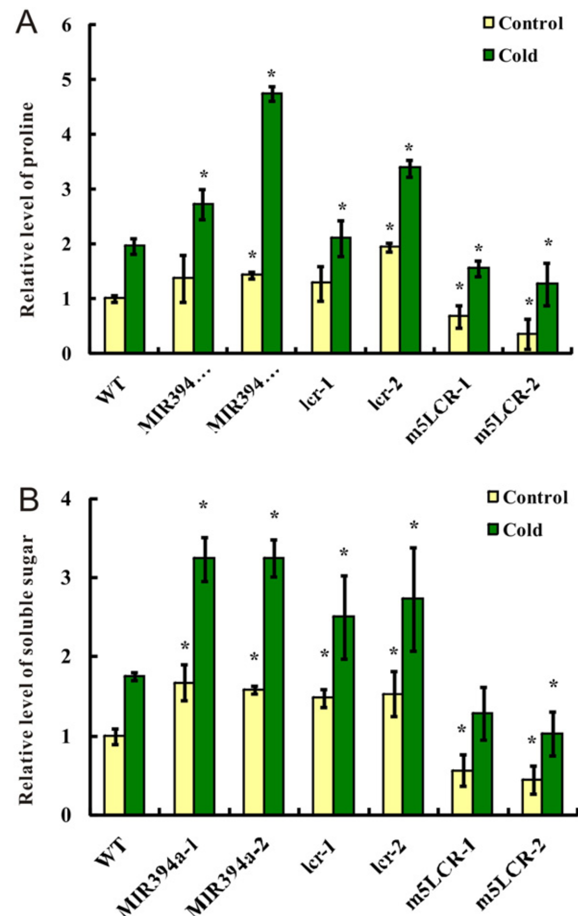

Fig. 5. Proline (A) and soluble sugar (B) accumulation in WT (wild-type), *35S::MIR394a-1*, *35S::MIR394a-2*, *lcr-1*, *lcr-2*, *35S::m5LCR-1* and *35S::m5LCR-2* under cold stress. Plants grew at 22 °C for 3 weeks and then continued to grow under the same condition (control) or subjected to low temperature (4 °C, cold) for 7 d. Vertical bars represent standard deviation of the mean. Asterisks indicate the significant difference between the transgenic plants or mutants and WT ($P<0.05$).

3.3. miR394a overexpressing plants accumulate proline and soluble sugar under cold stress

One of the most remarkable symptoms of cold stress in plants is the accumulation of proline (Gilmour et al., 2000). Thus, the content of the metabolite in the transgenic plants was determined. The *35S*:*MIR394a* plants had a significantly higher level of free proline than the wild-type under the cold stress (4 °C, for 7 d)(Fig. 5A). The degree of increased proline levels in *35S*:*MIR394a* plants was 2.0–3.3 folds over that in wild-type plants.

Accumulation of simple sugars is another commonly observed metabolic change occurring in cold-stressed plants (Gilmour et al., 2000). Under the normal condition, the content of total soluble sugars in *35S*:*MIR394a* plants was slightly higher than that of wild-type (Fig. 5B). With the lower temperature (4 °C), the difference of the sugar content between *35S*:*MIR394a* plants and wild-type was more pronounced. There were 1.9–2.1 fold higher total soluble sugars in the *35S*:*MIR394a* plants over the wild-type. A similar result was observed for the *lcr* mutant plants, which accumulated 1.7–1.8 fold higher total sugars than the wild-type. For *35S*::*m5LCR* plants, the levels of both proline and total sugars were lower than those of the wild-type. Taken together, miR394 overexpressing plants produced more proline and sugars than wild-type under the cold stress condition.

3.4. miR394a overexpressing plants promote expression of major cold-responsive genes

To get an insight into the plant tolerance to cold stress mediated by miR394, three major genes *CBF1*, *CBF2* and *CBF3* (C-repeat/dehydration binding factor) regulating plant cold stress response were analyzed. The *CBFs* encode transcriptional activators that control the expression of genes with the CRT/DRE responsive element in their promoters and are rapidly induced in response to low temperature (Stockinger et al., 1997). Expression of *CBF1* or *CBF3* in transgenic *Arabidopsis* plants has been shown to enhance freezing tolerance in acclimated or non-acclimated plants (Gilmour et al., 2000). The present study revealed that expression of all these genes increased in *35S*:*MIR394* plants after a six-hour cold treatment (Fig. 6). Compared to wild type, expression of *CBF1*, *CBF2* and *CBF3* in *35S*:*MIR394a* plants after 3 h cold treatment increased by 1.34, 1.32 and 1.10 fold, respectively. The higher expression of *CBF1*, *CBF2* and *CBF3* was also found in *lcr* mutant plants. In contrast, expression of these genes was lower in *35S*::*m5LCR* plants.

We further analyzed several stress-responsive genes downstream of CBF including *RD29A*, *COR15a*, *COR15b* and *KIN1* belonging to the DRE/CRT (drought responsive/C-repeat) elements containing class of genes (Xiong et al., 2002). In a similar way, these genes were induced in *35S*::*MIR394a* or *lcr* plants; conversely, expression of *RD29A*, *COR15a*, *COR15b* and *KIN1* were down-regulated in *35S*::*m5LCR* plants, although the degree of the gene depression varied differently (Fig. 6). These results indicated that miR394 overexpression could enhance the transcription of stress-responsive genes.

4. Discussion

With recent advance in high-throughput sequencing technologies and availability of genomic sequences of various plant species, a large number of miRNAs corresponding to cold and other abiotic stresses have been identified (Zhou et al., 2008; Barakat et al., 2012; Chen et al., 2012a). Of these, most of the miRNAs besides miR319 and miR393, are involved in environmental signaling (Chen et al., 2012a; Thiebaut et al., 2012; Yang et al., 2013). miR394 plays an important role in plant leaf development and response to abiotic stresses (Song

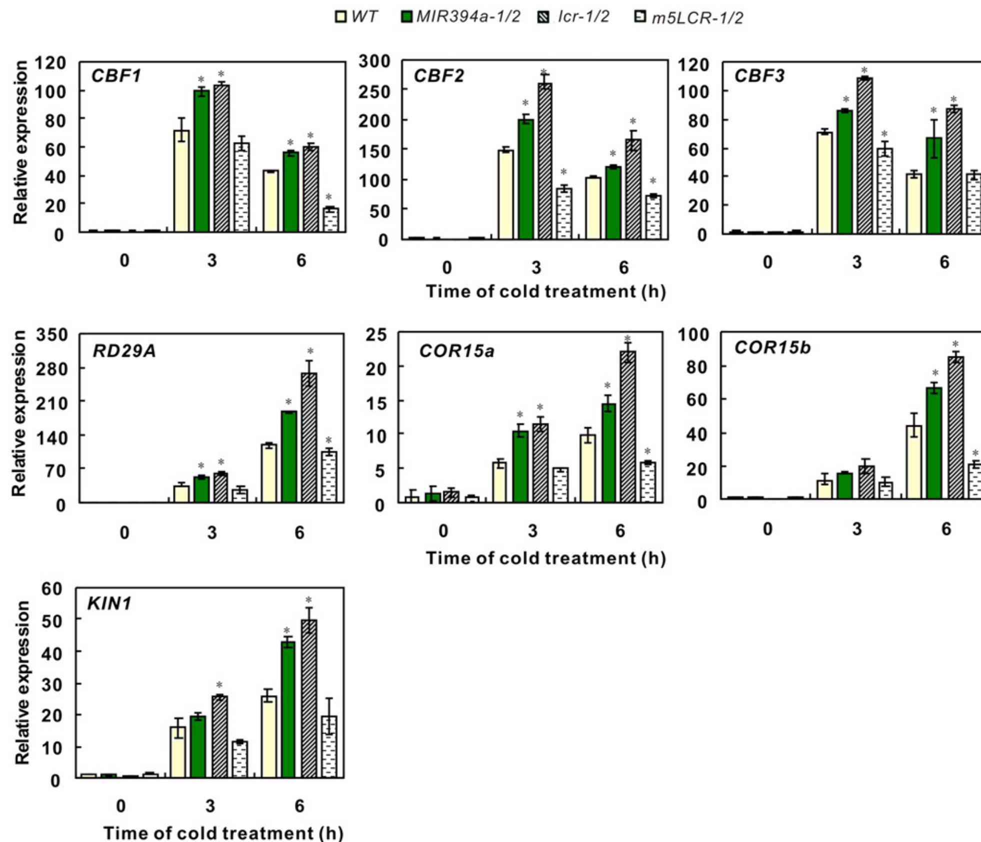

Fig. 6. Expression of cold-responsive genes in WT (wild-type), *35S*::*MIR394a*, *lcr* and *35S*::*m5LCR* (*m5LCR*) seedlings. Plants grew at 22 °C for 2 weeks and subjected to low temperature (4 °C, Cold) for 0, 3 and 6 h. The equal amount of mixed transgenic line and mutant seeds was used and samples for analysis were randomly selected from all transgenic lines or mutants. Vertical bars represent standard deviation of the mean. Asterisks indicate the significant difference between the transgenic plants or mutants and WT ($P < 0.05$).

et al., 2012, 2013). Considering its multi-functions, we hypothesized that miR394 and *LCR* may also involve plant response to cold stress. Our analysis showed that both miR394 and *LCR* could be independently induced by cold stress in wild type plants, and no clear counterpart correlation between miR394 and *LCR* expression was observed. This suggests that co-expression of miR394 and *LCR* may be necessary for proper response to cold stress. In the point of fact, the transcriptional expression of *LCR* in wild-type plants was lower than the *GUS* expression in *pLCR*::*GUS* plants under cold stress, indicating that the *LCR* transcripts were indeed cleaved by miR394. Such an expression pattern of miR394 and *LCR* may be a fine balance between *LCR* and miR394, that is essential for controlling *LCR* transcripts at a certain level when plants are subjected to cold stress. The mechanism leading to the phenomenon needs more investigation.

The present study demonstrated that miR394a overexpressing plants and *LCR* loss of function *lcr* mutants exhibited more tolerance to low temperature (4 to −11 °C). Both *35S*::*MIR394a* and *lcr* plants accumulated more proline and total soluble sugars than the wild-type under cold stress. Furthermore, a reduced electrolyte leakage was observed in *35S*::*MIR394a* and *lcr* plants. These results indicate that the physiological and molecular changes should be attributed to the overexpression of miR394. To highlight the contribution of miR394 target genes, we constructed a miR394 cleavage resistance version of *35S*::*m5LCR* and showed that *LCR* overexpressing plants have a cold hypersensitive phenotype, with an elevated electrolyte leakage and lower amount of proline and total soluble sugars in plants. The altered

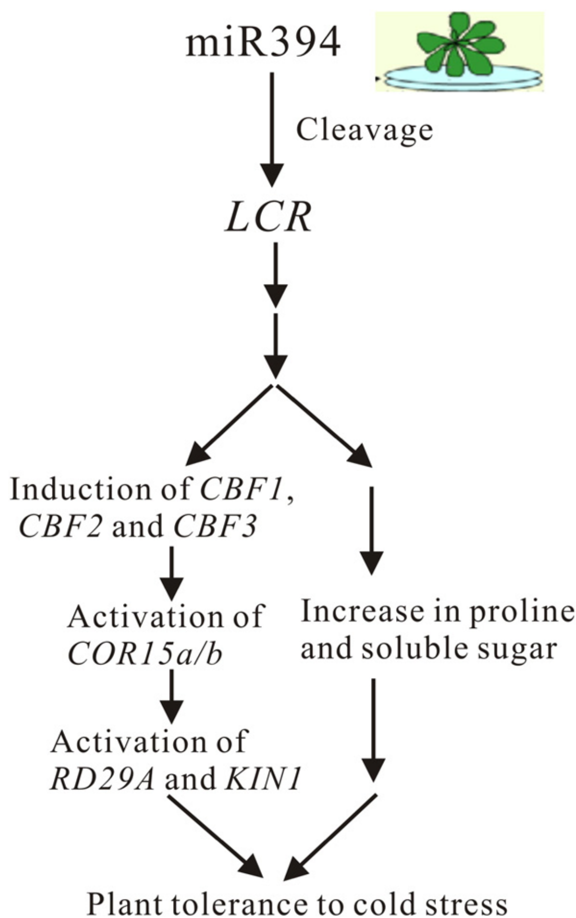

Fig. 7. The possible model for miR394 regulating *Arabidopsis* response to cold stress by its target gene *LCR*. The schematic model reflects that overexpression of miR394 down-regulates LCR expression, which activates cold-responsive genes *CBF1*, *CBF2*, *CBF3*, *COR15a*, *COR15b*, *RD29A* and *KIN1*; miR394 overexpression also enhances accumulation of proline and soluble surge, all of which are favorable to plant tolerance to cold stress.

phenotypes in *35S*::*m5LCR* plants indicate that LCR plays a negative role in response to low temperature.

Cold stress is one the major environmental factors that adversely affects the plant growth and development. As one of the indispensable organs of plants that sense various environmental changes, leaves play critical roles in photosynthesis, substance metabolism and water recycling. When encountered by low temperature, leaves may alter their morphogenesis to make adjustment to the changing environment. For example, transgenic turfgrass overexpressing *Osa-miR319a* exhibits wider and thicker leaves, increased weight–area ratio, and improved total leaf cuticle wax coverage under abiotic stresses (Zhou et al., 2013). Also, *Osa-MIR319* overexpression rice plants showed increased leaf width and enhanced cold tolerance at seedling stage (Yang et al., 2013). In similar, transgenic *Arabidopsis* overexpressing miR394 usually has enlarged leaf blade and upward curling leaf phenotype (Song et al., 2012). Furthermore, miR394 overexpressing plants are more tolerant to drought as compared to the wild-type control (Song et al., 2013). Based on the phenotypes of *35S*::*MIR394* plants, we may assume that the leaf morphological change is closely associated with the low temperature tolerance, supporting the notion that thicker and wider leaves contribute the enhanced plant resistance to abiotic stress (Deák et al., 2011; Zhou et al., 2013). miR319 seems to have more target genes. In rice, two other OsmiR319-targeted genes *OsPCF6* and *OsTCP21* are showed to involve cold stress response in addition to the targets *OsPCF5* and *OsPCF8* that are targeted by OsmiR319 (Wang et al., 2014). Our result is functionally consistent with the previous observations on miR319, indicating that *35S*::*MIR394* plants have a better adaptation to very low temperature. Despite the observation that the adjustment of leaf morphology (e.g. leaf width) and cold stress tolerance in *35S*::*MIR394* plants is coordinated, the underlying molecular mechanisms are largely unknown.

In *Arabidopsis*, the C-repeat binding factor-dependent cold response pathway plays a critical role in cold acclimation (Chinnusamy et al., 2010). The *CBFs* encode transcriptional activators that control the expression of genes with the CRT/DRE-responsive element in their promoters and are rapidly induced by low temperature (Stockinger et al., 1997). Overexpression of the DREB1/CBF family genes driven by CaMV 35S promoter increases stress tolerance to freezing, drought, and high salinity in transgenic *Arabidopsis* plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998). The rice *CBF* homolog *DREB1* genes were shown to be involved in chilling acclimation and cold tolerance (Ito et al., 2006; Mao and Chen, 2012). However, there is a report indicating that the CBF genes in rice appear not to be involved in miR319-mediated cold stress response, because no difference was observed in expression of *DREB1* genes between miR319-overexpressing plants and wild-type plants under normal or low temperature condition (Yang et al., 2013). In this study, expression of *CBF1*, *CBF2* and *CBF3* was found to be increased in miR394a-overexpressing plants and *lcr* mutants as compared to the wild-type controls, suggesting that miR394-mediated cold tolerance in *Arabidopsis* is possibly dependent on *CBF* expression. CBF1/2/3 proteins belong to the family of APETALA2/ethylene-responsive transcription factors that bind to CRT/DRE (C-repeat/dehydration responsive element) and activate the transcription of many cold stress-inducible genes to confer plant tolerance to abiotic stresses (Stockinger et al., 1997; Akhtar et al., 2012). Contribution of CBF genes to cold acclimation is supported by the finding that constitutive overexpression of *CBF1*, *CBF2* or *CBF3* in transgenic plants that induces the expression of many COR genes, and some of COR proteins are thought to be important for freezing tolerance even without prior cold exposure (Chinnusamy et al., 2010; Medina et al., 2011). We analyzed the representative CBF-downstream genes *COR15a* and *COR15b* and showed the genes were activated in *35S*::*miR394a* and *lcr* plants under cold stress, suggesting that the two genes were also involved in *35S*::*miR394*-induced cold stress response. As both CBF and COR genes regulated by cold acclimation involve other cold-responsive regulons in a way of crosstalk or co-regulation during cold stress (Zhao et al., 2015), two important other

stress-responsive genes *RD29A* and *KIN1* were tested. As expected, expression of the genes was transcriptionally activated in *35S::miR394a* and *lcr* plants under cold stress, confirming the hypothesis that miR394a overexpression improved plant cold tolerance possibly through the abiotic stress-responsive signaling pathway (Xiong et al., 2002; Zhao et al., 2015). Based on our observation, a simple model could be proposed of how miR394 regulates plant response to cold stress (Fig. 7). The cold-regulated gene cascades are highly interconnected and much more complex than previously thought (Zhao et al., 2015). How miR394 and to what extent miR394 are involved in the cold stress responsive pathway remains to be investigated. Further investigation on the coordinated functions of miR394 with other genes in the cold-responsive networks will help to understand the molecular mechanisms underlying miR394-mediated plant cold tolerance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plgene.2015.12.001.

Authors' contributions

Z. M. Yang designed and carried out the study, and drafted the manuscript. Jian Bo Song and Shuai Gao carried out gene extraction and analysis. Ye Wang, Yong Li Zhang and Bo Wen Li participated in seedling culture and analysis of gene expression. All authors read and approved the final manuscript.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (31071343), Colleges and Universities in Jiangsu Province plans to graduate research and innovation (KYZZ_0181) and the Priority Academic Program Development of Jiangsu Higher Education Institution (200910).

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