云 南 植 物 研 究 2009, **31** (5): 421~426
Acta Botanica Yunnanica DOI: 10.3724/SP.J.1143.2009.09044

高表达拟南芥 miR396 提高烟草抗旱性*

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摘要: MiR396是一个由21个核苷酸组成的单链非编码RNA小分子。烟草内的miR396受干旱诱导说明其可能参与烟草的干旱应答。在35S强启动子作用下我们将miR396转入到烟草体内获得高表达转基因植株, 生理学测试表明高表达miR396的转基因烟草耐旱性增强, 同时叶片表现出比野生型较低的失水率和较高的相对含水量, 进一步分析表明转基因植株除了叶片变得更为窄小外, 其气孔密度和气孔系数都比野生型降低, 这些都表明miR396作为一个正调节因子参与烟草的干旱胁迫应答。

关键词: miR396; 烟草; 干旱; 气孔
中图分类号: Q 945 文献标识码: A 文章编号: 0253-2700(2009)05-421-06

Overexpression of *Arabidopsis* MiR396 Enhances Drought Tolerance in Transgenic Tobacco Plants

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Abstract: MiR396 was a single-stranded noncoding small RNA with 21 nucleotides, and the expression of MiR369 in leaves was strongly induced in water deficit condition in tobacco, which suggested a possible role of miR396 in drought stress response. Under the control of 35S promoter, *MIR*396 was introduced into tobacco mediated by *Agrobacterium tumfaciens*. Physiological tests indicated that the elevated levels of miR396 increased drought tolerance in tobacco accompanying with lower water loss rate and higher relative water content. Further more miR396-overexpressing plants exhibited visible reductions both in stomatal density and stomatal index as well as a narrow and small leaf phenotype in comparison with wild-type plants. The present study indicated that miR396 was a positive regulator in response to drought stress in tobacco.

Key words: miR396; Tobacco; Drought; Stomata

Water deficit is one of the most universal environmental stresses that affect almost all plant functions (Zhu, 2001). When water supply is limited, in order to survive under such unfavorable growth condition, plants have evolved diverse mechanisms for adapting to drought challenge (Flexas and Medrano, 2002). Increasing evidence has indicated that the molecular trait of genes has the potential to overcome lots of limitations in improving drought-tolerance in tobacco, such as mannitol-1-phosphate dehydrogenase (*mtlD*) (Abebe *et al.*, 2003; Karakas *et al.*, 1997), beanie aldehyde dehydrogenase (*BADH*) (Holmstrom *et al.*, 1996), pryroline-5-carboxylase (*P5C*) (Kishor and Miao, 1995) and late embryogenesis abundant protin (*LEA*))

* Foundation items: National High Technology Research and Development Program of China (863 Program) (2006AA02Z129), National Natural Science Foundation of China (90408022), Science Foundation of Yunnan Province (2004C0051M), and "Hundred talents" Program of the Chinese Academy of Sciences

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Received date: 2009-03-09, Accepted date: 2009-06-01

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(Zhang *et al.*, 2000). Although the physiological and molecular basis of bio-water saving or water uptaking were complicated and diverse (Chaves and Oliveira, 2004), the control of water loss by stomata has, in particular, received attention since plants loss more than 90% of the water through the stomatal pore, and stomata characteristics are highly linked to drought tolerance (Flexas and Medrano, 2002).

Recently, strong evidence suggested that miRNAs, which was a class of about 22nt, single-stranded RNAs, regulating genes expression by targeting mRNAs for cleavage or transcriptional repression (Juarez *et al.*, 2004; Chen, 2004; Griffiths-Jones *et al.*, 2006), are hypersensitive to abiotic or biotic stress as well as to diverse physiological process (Sunkar and Zhu 2004; Lu *et al.*, 2005; Jones-Rhoades *et al.*, 2006; Navaro *et al.*, 2006). In *Arabidopsis*, miR398 was found to target CSD1 and CSD2 to improve tolerance of plant under oxidative stress conditions (Sunkar *et al.*, 2006). MiR395 and miR399 were identified to be involved in sulfate and inorganic phosphate starvation responses, respectively (Jone-Rhoades and Bartel 2004; Fujii *et al.*, 2005; Kawashima *et al.*, 2009). Additionally, miR169 was investigated to function in drought resistance as a negative regulator by targeting NFYA5 in *Arabidopsis* (Li *et al.*, 2008).

In this study, we found that miR396 was markedly induced by drought stress in tobacco. To further our understanding of the role of miR396 as a conserved sequence, we analyzed two transgenic plant lines overexpressing *AtMIR396a* in tobacco (*Nicotiana tabacum* L.) showing that the increased levels of miR396 enhanced drought tolerance in tobacco. Meanwhile narrower leaf and lower stomatal density than those of wild-type were observed in transgenic tobacco, it suggested that the reduced leaf size and the decreased number of stomatal pore might partly make a contribution to the enhanced drought resistance in tobacco.

Materials and methods

Plant materials and treatments Tobacco seeds (*Nicotiana tabacum* CV. Xanthi NC) were surface sterilized, seeded in culture dish containing MS medium, solidified with 0.65% (w/v) agar. After two weeks, the seedlings then transferred into the soil in a greenhouse at 24℃-28℃. For PEG treatments, a-week-old tobacco seedlings were transferred into half-strength MS solution after germination; the nutrient solution in the plastic growth containers was continuously aerated with pumps and renewed every week. For drought treatment, 8-week-old seedlings grown in soil were held without water for 3 weeks before the plants were rewatered. For ABA, GA, and NAA treatment, samples of 8-week-old seedlings were exposed to 100 μmol L^{-1} ABA, 100 μmol L^{-1} NAA, and 1 μmol L^{-1} GA for 4 hours, respectively.

Plasmid construction and tobacco transformation A fragment of 544 bp (containing miR396a precursor of 151 bp) was amplified from *Arabidopsis* genomic DNA by PCR using specific primers (miR396a: 5′-TGC TGT AAA AGA ATG ACC CTT-3′ and 5′-AAA CTC ATA GAC AGA AGT TAG GGT T-3′), and then was inserted into a plant transformation vector (pOCA30), downstream of the constitutive 35S promoter. The construct were introduced into tobacco by the leaf-disk method as Curtis described (Curtis *et al.*, 1995) mediated by *Agrobacterium tumefaciens* strain GV3101. Transgenic plants were screened on MS medium containing 300 μm L^{-1} kanamycin. Two independent lines of T_2 plants were used for detailed analysis.

RNA gel Blotting Total RNA was extracted from samples using Trizol reagent (Invitrogen) Samples of 10 μg total RNA were resolved on a 15% denaturing polyacrylamide/1x TBE/7 M urea gel and subjected to blot-hybridization analysis using [^{32}P] ATP-labeled single-stranded anti-miR396a DNA probe (CAGTTCAAGAAAGCTGTGGAA), performed as described by Akbergenov (Akbergenov *et al.*, 2006).

Confocal Microscopy and Statistics To visualize stomata outlines, the leaves were immersed in 0.2 mg mL^{-1} propidium iodide for 30 min, with the blade adaxial side facing up. A laser scanning confocal microscope LSCM was used to take images. Statistics analysis followed the method described by Thomas (Thomas *et al.*, 2003).

Results

MiR396 Expression Induced by Water Deficit

MiR396 accumulated mainly in leaves and flowers and to a lower extent in roots and stems in tobacco (unpublished data). To study the expression pattern of miR396 in stress conditions, we detected the response of miR396 expression to drought, ABA, GA and NAA treatment by northern blot analysis. An obvious increase in miR396 levels was observed when the seedlings were exposed to water deficit condition for 4 days and the accumulation reached to the peak at the day 7, and then at the first day after re-watered miR396 re-

turned to a normal level (Fig. 1B), while no visible increase of miR396 was observed in wide-type plants treated with ABA, GA or NAA (Fig. 1C). These results indicated that miR396 were up-regulated by drought stress suggesting a potential role of miR396 in the drought resistance in tobacco.

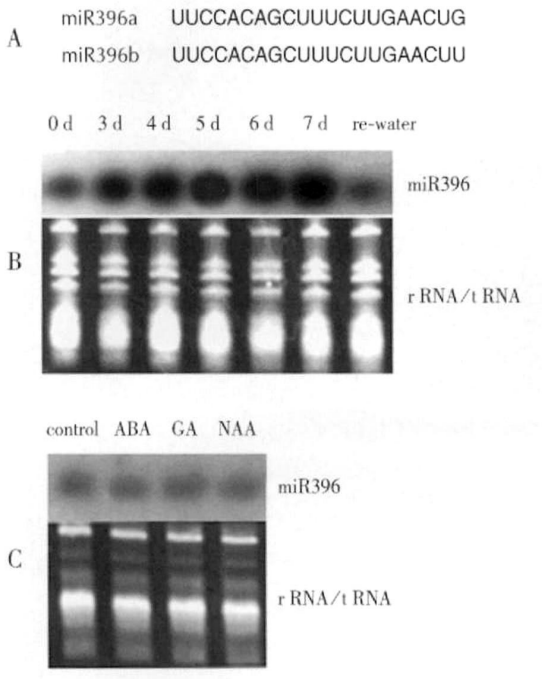

Fig. 1 Expression of miR396 in tobacco with drought, ABA, GA and NAA treatment (A) Mature miR396 sequence. (B) Expression of miR396 in 8-week-old tobacco withheld from water for a week. (C) Expression of miR396 in leaves of 8-week-old seedlings exposed to 100 μmol L^{-1} ABA, 1 μmol L^{-1} GA and 100 μmol L^{-1} NAA for 4 hours, respectively

MiR396 Overexpression Improved Drought Tolerance in Tobacco

The expression of miR396 induced by drought stress in tobacco prompted us to determine whether miR396 was involved in plant drought resistance. Given the miR396 was conserved in distantly related plant species, both in terms of primary and mature miRNAs (Zhang *et al.*, 2006), and miR396a differs from miR396b just by one nucleotide (Fig. 1A), we generated transgenic tobacco overexpressing *AtMIR396* under the cauliflower mosaic virus 35S promoter (*35S:: AtMIR396a*). More than 10 transgenic lines were obtained and confirmed by PCR analysis and northern blot annalysis, and the lines 2 and 5 were selected for further studies (Fig. 2).

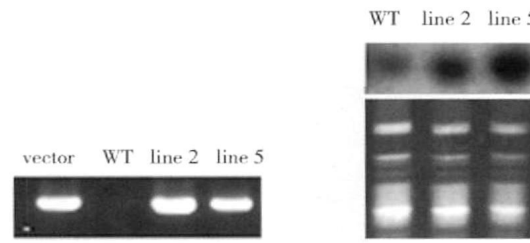

Fig. 2 Identification of *35S:: AtMIR396a* plants by PCR (A) and northern blot analysis using miR396a antisense probe (B)

We utilized two types of water deficit conditions to investigate the role of miR396 in drought stress response. 8-week-old seedlings of transgenic and wild-type plants were withheld from water for 3 weeks. The wild-type plants leaves were clearly wilted compared to those of transgenic plants (Fig. 3A). In the parallel experiments, PEG6000 (20%) was used to produce a more stringent water stress condition that did not permeate the roots but only imitated soil dehydration. The leaves of wild-type plants wilted quickly upon 3 h PEG6000 treatment, while those of the transgenic plants were normal (Fig. 3B). To determine whether the response to osmoticum stress in seedling root was influenced by miR396, we analyzed root development and germination rate under normal and drought conditions (MS medium with 200 mmol L^{-1} mannitol). Both of them were not significantly different between miR396-overexpressing and wild-type seedlings (data not shown), indicating that the modulation of miR396 in tobacco drought stress tolerance was not associated with the early stage of seedling development directly.

MiR396 Modulates the Drought Stress Response in Tobacco Through Regulating Leaf and Stomata Development

To further elucidate the water stress resistance of transgenic plants, we then detected daily water loss in 8-week-old transgenic and wild-type plants. As shown in Figure 4, water loss rates of leaves detached from both two lines of transgenic plants were significantly lower than that of wild-type plant (Fig. 4A). In addition, under both the well-watered condition and drought stress condition, relative water contents (RWC) of the transgenic lines were higher than those of wild-type samples and the distinction was more remarkable under

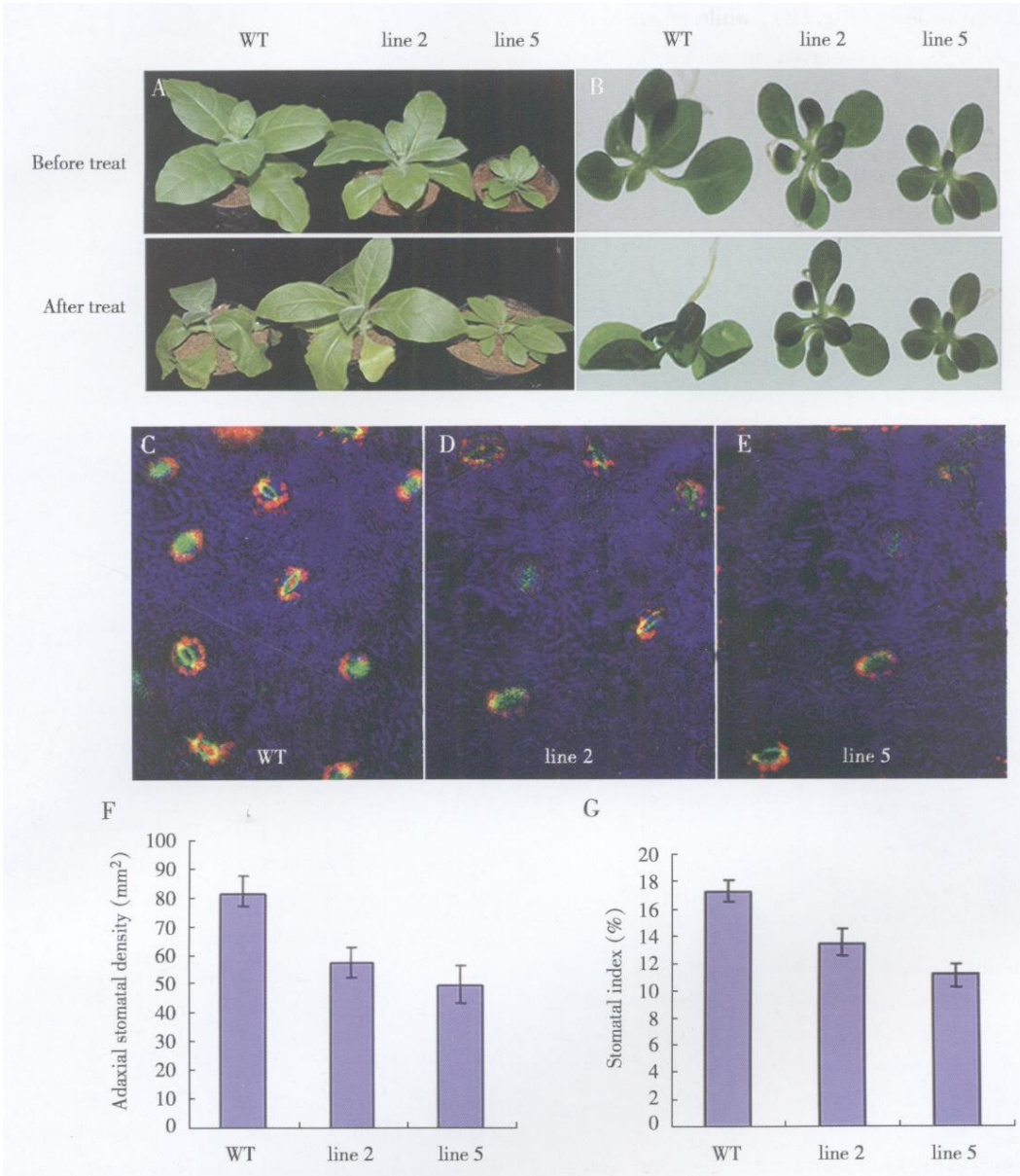

Fig. 3 A and B Growth statuses of different transgenic lines under stress conditions. (A) 8-week-old seedlings wild-type (WT) and two T_2 transgenic plants (line 2 and line 5) in greenhouse were withheld from water for 3 weeks; (B) 3-week-old seedlings were treated with 20% PEG6000 for 3 hours; G-G. Measurement of stomatal number in wild-type and transgenic plants. C. gital images of impressions from adaxial leaf surface of wide type; D and E. transgenic lines; analysis of stomatal density (F) and index (G) of abaxial surface of mature leaves from wild-type and transgenic plants. 8-week-old plants were used. ($P < 0.05$)

water deficit conditions (Fig. 4B). These results suggested that transgenic plants enhanced drought tolerance, at least partly, due to an enhanced ability of bio-water-saving which was reported to be mainly controlled by leaf area and stomata activity (Hill, 1994).

Our previous study showed that miR396 accumulation in tobacco led to a reduction in leaf size by targeting three *GRF*-like genes (unpublished data), which might contribute to plants water-saving by decreasing transpiration (Hill, 1994; Schoch *et al.*, 1984). In consistent with the small and narrow leaf, lower water-loss rate and higher relative water content, a lower stomatal density and stomatal index were observed in transgenic plant. As shown in Figure 3 (G-G), the stomatal density on the abaxial surface of the fifth leaves of *35S:: MIR396a* transgenic line 2 and line 5 were

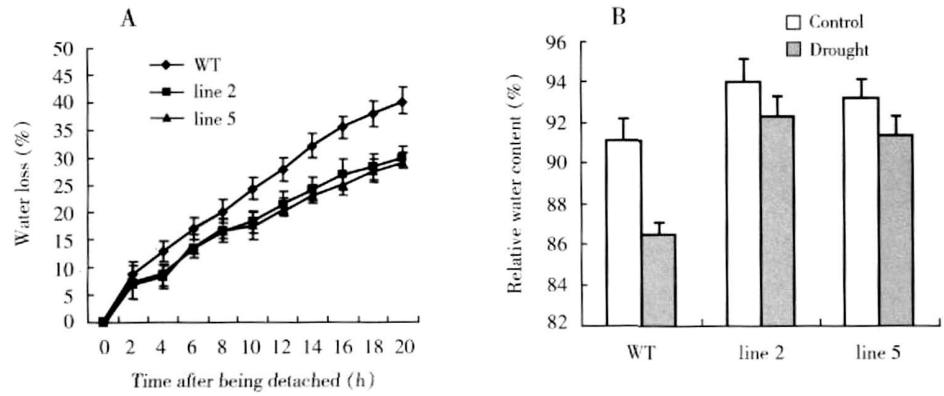

Fig. 4 Effect of miR396 over expression on water loss and relative water content in wild-type and transgenic plants.
(A) Water loss of the detached leaves from 8-week-old plants. (B) Measurement of leaf relative water content of 8-week-old wild-type and transgenic plants at normal condition and water deficit condition (a-week water withdrawal)

clearly decreased by 30% and 40%, respectively, when compared with that of control plants grown under the same condition (Fig. 3E). Meanwhile an obvious reduction in stomatal index: number of stomata related to total epidermal cell number (Ticha, 1982), in transgenic plants was observed (Fig. 3G). All of these results suggest that miR396 function as a positive regulator in tobacco response to drought resistance.

Discussion

Drought is the primary limitation to plant production worldwide, to cope with such unfavorable situation, most plants conserve water by reducing leaf size and/or decreasing stomatal index (Hill, 1994; Schoch *et al.*, 1984; 1980), since water conservation in plants are regulated to a large extent via the opening and closure of stomata (Schurr *et al.*, 1992). Stomata closure is the primary cause of the reduction in photosynthetic rate under mild drought (Cornic, 2000; Medrano *et al.*, 2002; Chen *et al.*, 1997). Recent advances in stomata development and the identification of a number of genes regulating stomatal density make it possible to generate transgenic plants with different stomatal densities (Xu and Zhou, 2008).

In this work, drought-induced miR396 accumulation has been detected, and the wild-type plants showed sensitive physiological responses (e.g., leaf rolling) to the sudden or continuous drought stress (10% PEG treatment for 4 h or water-withheld for 3 weeks), while no apparent external change was observed in the transgenic plants (Fig. 3). Meanwhile, we observed lower water loss rate and higher relative water content in consistent with a lower stomatal density and stomatal index compared to the wild-type plants as well as small and narrow leaves, while no distinction in root development or germination rate in transgenic tobacco compared to that in the wild-type under normal or drought stress. These data indicated that miR396 could improve drought tolerance in tobacco with enhanced ability of water conservation through reducing leaf area and stomatal index but had no markedly influence in early stage of seedling growth. It also offered a potential strategy to create drought-tolerant plants, for miR396 was evolutionarily conserved both in terms of primary and mature miRNAs and it would be feasible to obtain drought-tolerance transgenic plants by introducing *35S: miR396* into tobacco.

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