OVEREXPRESSION OF *ARABIDOPSIS* *AtmiR408* GENE IN TOBACCO

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MicroRNA miR408 is predicted to target the transcripts for the copper proteins plantacyanin and laccase in *Arabidopsis*. In this study it was found that miR408 is present in tobacco also. Its accumulation was positively induced by copper deficiency, providing additional evidence for the important role of miR408 in plant responses to nutrient conditions. To examine the functions of miR408 in tobacco, transgenic tobacco lines containing the *Arabidopsis* *AtmiR408* gene were created. Two transgenic lines with miR408 oversupply were chosen for functional characterization. The results showed that miR408 oversupply down-regulated the activities of antioxidant enzymes such as SOD, POD and CAT. As a result, transgenic lines exhibited altered chlorophyll content and seedling root growth, especially under high copper conditions. This suggests that miR408 accumulates in response to copper deficiency and influences plant growth by regulating antioxidant enzymes. It may be that miR408 exists and functions in tobacco in a conserved way similarly to miR408 in *Arabidopsis*.

**Key words:** MicroRNA, *AtmiR408* gene, overexpression, tobacco, copper deficiency.

INTRODUCTION

MicroRNAs (miRNAs), which remained unnoticed until recent years, are now known to play a number of important regulatory roles in plants (Jones-Rhoades et al., 2006). The miRNAs are a kind of short RNA about 21 nucleotides in length and are processed from stem-loop precursors by the Dicer enzymes (Bartel, 2004). They control mRNA stability, translation, or target epigenetic modifications to specific regions of the genome by complementarily binding to target nucleic acids (Jones-Rhoades et al., 2006). Many miRNAs are conserved evolutionarily across species boundaries (Bartel, 2004). For example, miR408 was first discovered in *Arabidopsis*, and is conserved in other flowering plants such as rice and poplar (Jones-Rhoades et al., 2006).

In *Arabidopsis*, miR408 targets transcripts for copper-containing proteins such as plantacyanin as well as laccases LAC3, LAC12 and LAC13, as confirmed by cleavage site analysis (Abdel-Ghany and Pilon, 2008). Plantacyanin (PC) proteins contain a single copper ion and belong to the phytocyanin family (Dong et al., 2005). It is essential for electron transfer between the cytochrome b6f complex and photosystem I, as shown in *Arabidopsis* mutants with insertions in both PC genes which are seedling-lethal (Weigel et al., 2003). Similarly, laccases have four copper atoms and catalyze the oxidation of their substrate molecules with the production of water and oligomers. The laccases belong to a multigene family in plants (LaFayette et al., 1999). In *Arabidopsis* there are 17 annotated laccase genes divided into four subgroups (McCaig et al., 2005). The proposed functions of laccases in plants are many, including lignin synthesis (Ranocha et al., 2002), wound healing (Dean and Eriksson, 1994), iron acquisition (Hoopes and Dean, 2004), response to stress (Liang et al., 2006), and maintenance of cell wall structure and integrity (Ranocha et al., 2002). Like miR408 itself, its target genes are also conserved in *Arabidopsis*, rice and poplar (Jones-Rhoades et al., 2006).

Plants have evolved sophisticated sensory receptors for copper nutrition, which in coordination monitor growth and development to regulate the different levels of copper responses throughout their life cycle. In *Arabidopsis*, miR408 mediates post-transcriptional regulation of plantacyanin and laccase transcripts. Its abundance is positively induced by copper deficiency, indicating that miR408 acts as a crucial regulator of copper homeostasis (Abdel-Ghany and Pilon, 2008). Considering the conservation of miR408 itself and its target genes, it raises the question of whether miR408-mediated regula-
tion of responses to copper is common in other plants besides Arabidopsis. In this study we used Northern blotting to determine whether miR408 is conserved in tobacco. Then the AtmiR408 gene was overexpressed in tobacco to verify its functions in response to copper. We discuss conservation of miR408 and its functions in tobacco.

MATERIALS AND METHODS

PLANT MATERIALS

Arabidopsis ecotype 'Columbia' grown at 20±1°C under a 16 h photoperiod was used for AtmiR408 gene cloning. Tobacco (Nicotiana tabacum L. 'NC89') was grown at 25±1°C under a 12 h photoperiod and used for genetic transformation. The transgenic tobacco was cultured under the same conditions for chlorophyll content and enzyme activity analysis.

RNA EXTRACTION AND NORTHERN BLOT ANALYSES OF MIR408 IN TOBACCO AND TRANSGENIC PLANTS

Total RNA was extracted with TRizol reagent (Invitrogen, U.S.A.) as described in the user's manual. Thirty micrograms of total RNA was loaded per lane, resolved on a denaturing 15% polyacrylamide gel, and blotted with 0.5×TBE buffer onto a Hybond-N+ membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were UV crosslinked and baked for 1 h at 80°C. A DNA oligonucleotide complementary to the miR408 sequence was end-labeled with 32P-dATP with Klenow kinase. Blots were prehybridized for at least 1 h and hybridized overnight using PerfectHYB Plus buffer (Sigma, St. Louis, MO) at 38°C. Blots were washed three times (twice with 2 × SSC [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS for 10 min, and once with 1 × SSC and 0.1% SDS for 10 min) at 50°C. The membranes were briefly air-dried and then autoradiographed.

AtmiR408 GENE CLONING AND VECTOR CONSTRUCTION

Genomic DNA was isolated from Arabidopsis with extraction buffer containing 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB (cetyltrimethyl ammonium bromide), 2% PVP and 0.4% β-mercaptoethanol as described by Sambrook et al. (1989). The AtmiR408 gene containing miR408 precursor was cloned using genomic DNA as template with forward primer 5'-CTCCAGCTAATTTAGGGGT-TAGG-3' and reverse primer 5'-GATGCAGGGAGAGAATCTGGAC-3'. The PCR reaction mixture contained 200 ng DNA, 1 μl of each primer (10 mM), 2.5 μl 10× Taq DNA polymerase buffer plus 20 mM Mg2+, 2 μl 2.5 mM dNTP and 2.5 U Taq DNA polymerase in a total volume of 25 μl. The reactions were initially denatured at 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension of 10 min at 72°C. PCR products were electrophoretically separated on 1% agarose gel and then subcloned into vector pMD18-T (TaKaRa, Dalian, China). Subsequently, AtmiR408 was cut with enzymes BamHI/SalI and ligated into pBI121 vector (Chen et al., 2003) downstream a cauliflower mosaic virus 35S promoter (Fig. 1). The resultant construct pBI121-AtmiR408 was introduced into Agrobacterium tumefaciens strain LBA4404 for tobacco transformation.

TOBACCO TRANSFORMATION

Tobacco (Nicotiana tabacum L. 'NC89') leaves were sterilized with 70% ethanol for 10 s and then 4% NaClO for 10 min. After washing with sterile water, the leaves were cut into pieces of explants which were then incubated on MS medium at 25°C under a 16 h photoperiod for 2 days. Then those explants were soaked in Agrobacterium LBA4404 cell cultures for 10 min followed by co-cultivation on MS medium for 2 d. After washing with sterile water supplemented with 250 mg l-1 carbenicillin Na2, the explants were transferred onto selection medium containing 0.2 mg l-1 1-naphthylacetic acid (NAA), 3 mg l-1 6-BA, 200 mg l-1 kanamycin and 250 mg l-1 carbenicillin Na2. After 3 weeks the regenerated shoots were transferred onto rooting medium containing MS salts, 1.5 mg l-1 NAA, 150 mg l-1 kanamycin and 250 mg l-1 carbenicillin Na2. Rooted plantlets were planted in soil and grown in a greenhouse at 23±1°C under a 12 h photoperiod. After harvesting, the seeds were selected on MS medium containing 250 mg l-1 kanamycin. T2 homozygous transgenic lines were selected for further investigation.

PCR AND RT-PCR CONFIRMATION OF TRANSGENIC PLANTS

For PCR amplification, genomic DNA was prepared from wild-type (WT) and transgenic lines as described by Sambrook et al. (1989). The reaction mixture and procedures were as described above.
For RT-PCR, total RNA was isolated from leaves with TRIzol reagent (Invitrogen, U.S.A.). Synthesis of cDNA was conducted using the PrimeScript First Strand cDNA Synthesis Kit according to the manufacturer’s instructions (Takara, Dalian, China). Then RT-PCR was performed. Each PCR reaction mixture contained 200 ng cDNA, 2.5 μl 10 Taq DNA polymerase buffer, 200 μmol of each dNTP, 10 pmol of each primer and 2.5 U Taq DNA polymerase in a total volume of 25 μl. 25 cycles of amplification were performed.

ANALYSIS OF GERMINATION AND ROOT LENGTH

Plants were grown on agar-solidified MS medium containing 3% sucrose under a 16 h photoperiod at 25°C. One- and two-week-old seedlings were used to evaluate germination and root growth respectively.

CHLOROPHYLL CONTENT AND ENZYME ACTIVITY

Chlorophyll was extracted from 0.2 g of leaves in 20 ml 80% acetone (Prochazkova et al., 2001). Absorbance was recorded at 665, 645 and 470 nm with a spectrophotometer (Shimadzu UV-2450, Japan).

Enzyme mixture containing superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) was extracted from 0.5 g of leaf samples in 4 ml 0.05 mol l⁻¹ phosphate buffer, pH 7.8. The mixture was centrifuged at 15,000 × g at 4°C for 20 min. Finally, the supernatant was used for enzyme activity assay as described by Prochazkova et al. (2001) with a spectrophotometer. Protein was quantified using the dye-binding assay.

STATISTICAL ANALYSIS

The results were analyzed with SAS software. Statistical analysis was performed with Duncan’s multiple range test.

RESULTS

ACCUMULATION OF miR408 IN TOBACCO

MicroRNA miR408 was highly conserved among different plant species (Jones-Rhoades et al., 2006). To examine whether miR408 is conserved in tobacco, total RNAs were extracted from mature leaves of tobacco ‘NC89’ with or without Cu²⁺ deficiency. Subsequently, RNA blotting was conducted using *Arabidopsis* miR408 sequence as probe. The results showed that the hybridization signal appeared only in tobacco samples with Cu²⁺ deficiency and not in the control, suggesting that tobacco miR408 is very similar to its *Arabidopsis* counterpart and that miR408 accumulation was positively induced with Cu²⁺ deficiency in tobacco (Fig. 2a, b).

creation and identification of transgenic tobacco overexpressing *AtmiR408* gene

To examine the function of *AtmiR408*, a construct containing the *Arabidopsis AtmiR408* gene driven
Overexpression of AtmiR408 gene in tobacco

by CaMV 35S promoter was introduced into tobacco with agrobacterium-mediated transformation. As a result, 17 independent tobacco lines were obtained on selection medium plus kanamycin. Genomic DNA PCR confirmed the integration of the AtmiR408 gene into the host genomes in 12 lines (Fig. 2c). Then 5 PCR-identified lines (L1, L3, L10, L11, L15) were randomly chosen to examine the expression levels of the exogenous AtmiR408 gene with semi-quantitative RT-PCR. The result indicated that the AtmiR408 gene was expressed at different levels depending on the line (Fig. 2d, e). Among them, lines 10 and 15 were chosen to check their miR408 accumulation levels with RNA blotting. The results showed that miR408 accumulation was much higher in L15 than L10 (Fig. 2a, b), in agreement with the expression level of AtmiR408 gene in two lines, suggesting that miR408 oversupply results from the overexpression of the AtmiR408 gene in transgenic tobacco. Thereafter, L10 and 15 were named LW and LS, referring to their weak and strong expression of the AtmiR408 gene, respectively. LW and LS were used for further investigation.

OVERSUPPLY OF MIR408 DOWN-REGULATES THE ACTIVITIES OF ANTIOXIDANT ENZYMES

As known in Arabidopsis, the target genes of miR408 encode laccases such as LAC3, LAC12 and LAC13, which have SOD or POD enzyme activity. To examine whether miRNA408 regulates those enzymes, the activities of SOD and POD and another antioxidant enzyme CAT were assayed in lines LW, LS and the nontransgenic WT control. The results showed that miR408 accumulation was much higher in L15 than L10 (Fig. 2a, b), in agreement with the expression level of AtmiR408 gene in two lines, suggesting that miR408 oversupply results from the overexpression of the AtmiR408 gene in transgenic tobacco. Thereafter, L10 and 15 were named LW and LS, referring to their weak and strong expression of the AtmiR408 gene, respectively. LW and LS were used for further investigation.

OVERSUPPLY OF MIR408 INHIBITS SEED GERMINATION AND ROOT GROWTH IN TRANSGENIC TOBACCO LINES

To determine whether miR408 functions in tobacco, seed germination was observed in LW, LS and WT one week after sowing on MS medium. Observations showed that miR408 oversupply inhibited seed germination to different extents, depending positively on the level of miR408 accumulation (Fig. 3a). To examine the effect of miR408 oversupply on root growth, tobacco seeds were sown on solid MS plates which were then placed vertically for root gravitropic growth. Two weeks later, root length was observed and quantified (Fig. 3b). Both transgenic lines (LW, LS) exhibited reduced root growth to different extents, correlated positively with their miR408 accumulation in a manner similar to that observed in seed germination.

To see whether miR408 oversupply affects plant growth at high Cu²⁺ concentration, root length of 2-week-old seedlings on MS medium containing 100 μM Cu²⁺ was measured; miR408 oversupply made the seedlings over-sensitive to high Cu²⁺ stress, as indicated by reduced root growth in LW and LS seedlings as compared with WT (Fig. 3b).

DISCUSSION

The high sequence conservation of some miRNAs within plants provides a means to identify conserved miRNAs from other plant species (Bartel, 2004; Fahlgren et al., 2007). Through methods of cloning, genetics and bioinformatics, 118 potential miRNA genes have been identified in Arabidopsis thaliana, which can be classified into 42 families based on their sequence similarity (Jones-Rhoades et al., 2006). Of those, 21 families are clearly con-
served in species such as rice and poplar, besides Arabidopsis (Jones-Rhoades et al., 2006). For example, miR408 has been identified in Arabidopsis and conserved in rice, Medicago and Populus (Sunkar and Zhu, 2004). In this study, RNA blotting showed that the miR408 probe from Arabidopsis detected the hybridization signal under conditions of copper deficiency, suggesting that miR408 is present in tobacco with high sequence similarity to its Arabidopsis counterpart, and that its accumulation is positively induced by copper deficiency. This provides new evidence of high conservation of miR408 among different higher plants.

In Arabidopsis, miR408 belongs to a one-gene family in chromosome II. Bioinformatics analysis of the Arabidopsis genome and target validation analysis have suggested that mature miRNA408 is predicted to target the coding sequences of plantacyanin, LAC3, LAC12 and the 5’-UTR of LAC13 (Abdel-Ghany and Pilon, 2008). Of those target genes, plantacyanin appears to play several roles in the biology of Arabidopsis, such as functioning as the last electron carrier in a chain between photosystems II and I (Weigel et al., 2003). Meanwhile, LACs encode enzyme laccases, which are copper-containing oxidase enzymes found in plants, fungi and microorganisms (McCaig et al., 2005). The miR408 expression pattern was negatively correlated with the accumulation of transcripts for plantacyanin and laccases in tobacco, also suggesting that the putative tobacco target genes contain the conserved miR408 recognition site.

Corresponding to the high similarity of sequences for the same miRNA, those miRNAs displayed conserved functions among different plant species. Proposed functions of laccases in plants include roles in lignin synthesis, wound healing, iron acquisition, response to stress, and maintenance of cell wall structure and integrity, suggesting its multiple functions in tobacco (McCaig et al., 2005). Taking copper acquisition as an example, tobacco miR408 was positively induced by copper deficiency, probably attributable to the enhanced cleavage efficiency of miR408 to the LAC gene, which allows copper to be utilized more efficiently in the production of more important proteins under conditions of copper deficiency. On the other hand, miR408 oversupply led to excessive cleavage to LAC genes under high copper conditions, which made the transgenic tobacco sensitive to heavy metal damage from high Cu²⁺. Transgenic tobacco overexpressing the miR408 gene exhibited more limited root growth than the WT control. This means that miR408 is involved in miRNA-mediated copper homeostasis, like miRNA395 and miRNA399 in sulfur and phosphate homeostasis, respectively (Jones-Rhoades et al., 2006).

CONCLUSION

Overexpression of the AtmiR408 gene made the transgenic tobacco lines hypersensitive to copper.
Our findings provide new evidence for conservation of miR408 functions in tobacco. Together with the progress in the tobacco genome project, our findings should help in identifying tobacco miR408 genes and their target genes.

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REFERENCES


