www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 52/2: 26–31, 2010
DOI: 10.2478/v10182-010-0020-x

OVEREXPRESSION OF *ARABIDOPSIS ATMIR408* GENE IN TOBACCO

XIAO-MING FENG, YU QIAO, KE MAO, YU-JIN HAO, CHUN-XIANG YOU*

State Key Laboratory of Crop Biology, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai-An, Shandong 271018, China

Received July 1, 2010; revision accepted October 10, 2010

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 nutriment 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). Pantacyanin (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-

*e-mail: youchunxiang@sdau.edu.cn

PL ISSN 0001-5296

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 tobaccum* 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 cross-linked and baked for 1 h at 80°C. A DNA oligonucleotide complementary to the miR408 sequence was end-labeled with ^{32}P-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'-CTCCAGCTAATTTAGAGGGTTAGG-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 Mg^{2+}, 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) downstreaming a cauliflower mosaic virus 35S promoter (Fig. 1). The resultant construct *pBI121-AtmiR408* was introduced into *Agrobacterium tumefaciens* strain LBA4404 for tobacco transformation.

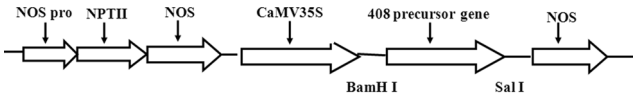

Fig. 1. Construction of overexpression vector *pBI121-AtmiR408*.

TOBACCO TRANSFORMATION

Tobacco (*Nicotiana tobaccum* 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 Na_2, 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 Na_2. 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 Na_2. 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.

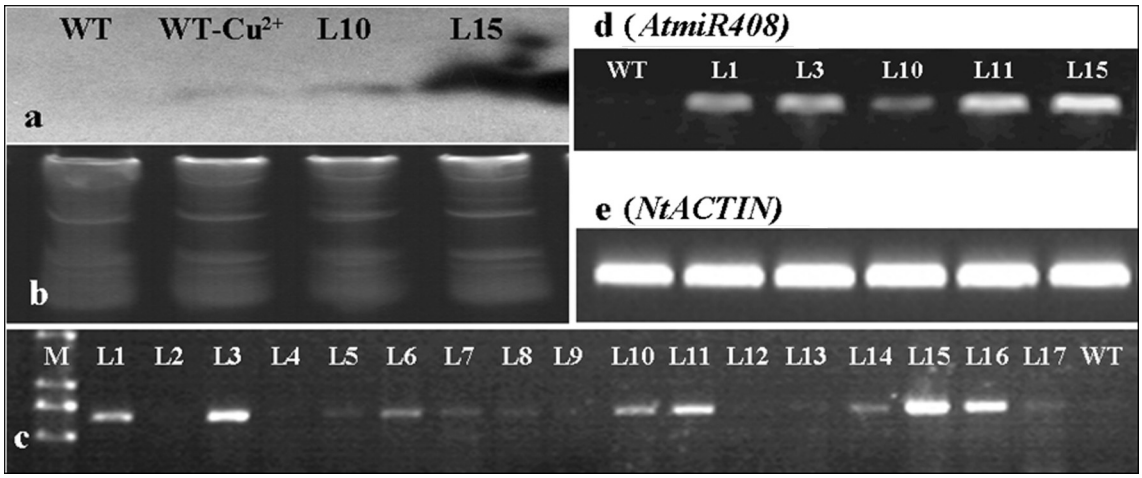

Fig. 2. Accumulation of miR408 and identification of transgenic tobacco. (**a**) Northern blotting for miR408 in tobacco, (**b**) Loading control for Northern blotting; In a and b, lane 1 is WT under normal conditions, lane 2 is WT under copper deficiency, L10 and L15 are transgenic tobacco lines LW and LS, respectively, (**c**) Colony PCR of putative transgenic tobacco plants; lanes L1-L17 are PCR products of different plants regenerated from selection medium; WT is the nontransgenic control; M is DNA marker, (**d**) Semi-quantitative RT-PCR analysis of 5 transgenic tobacco lines. L1, L3, L10, L11 and L15 are transgenic lines, while WT is the nontransgenic control, (**e**) *NtACTIN* gene was used as loading control for semi-quantitative RT-PCR.

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 Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} 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

TABLE 1. Chl content and SOD, CAT and POD activities in transgenic and control plants

	Chl*a*+Chl*b* (mg·g^{-1})	SOD (U·g^{-1}·FW)	CAT (Δ240·g^{-1}·FW·min)	POD (U·min^{-1}·g^{-1}·FW)
WT	15.73±0.96^{a}	104.63±5.21^{a}	8.37±0.51^{a}	9.08±0.70^{a}
LS	12.47±2.70^{b}	50.11±6.04^{c}	6.13±0.49^{b}	8.19±0.30^{a}
LW	12.87±0.90^{ab}	94.89±1.08^{b}	7.31±1.27^{ab}	9.02±0.39^{a}

WT – wild type nontransgenic control; LS and LW – transgenic line with strong and weak expression of *AtmiR408* gene, respectively. Data are means ±SD of three independent experiments with three replicates each. Values followed by different letters differ significantly at $p < 0.05$ by Duncan's multiple range test.

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 all three antioxidant enzymes exhibited decreased activities in both LW and LS relative to the WT control, and that the activities correlated negatively with the accumulation level of miR408 in the two transgenic lines (Tab. 1), suggesting that oversupply of miR408 underlies the down-regulation of enzyme activities.

Theoretically, down-regulation of antioxidant enzyme activity should impair the plant's capacity to scavenge reactive oxygen species, which are harmful to chlorophyll in plants. To examine whether chlorophyll was impaired in the transgenic lines, total content of chlorophyll *a* and *b* was detected. We found that the transgenic lines produced reduced chlorophyll in a manner depending positively on miR408 accumulation (Tab. 1).

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 Cu^{2+} concentration, root length of 2-week-old seedlings on MS medium containing 100 μM Cu^{2+} was measured; miR408 oversupply made the seedlings over-sensitive to high Cu^{2+} 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-

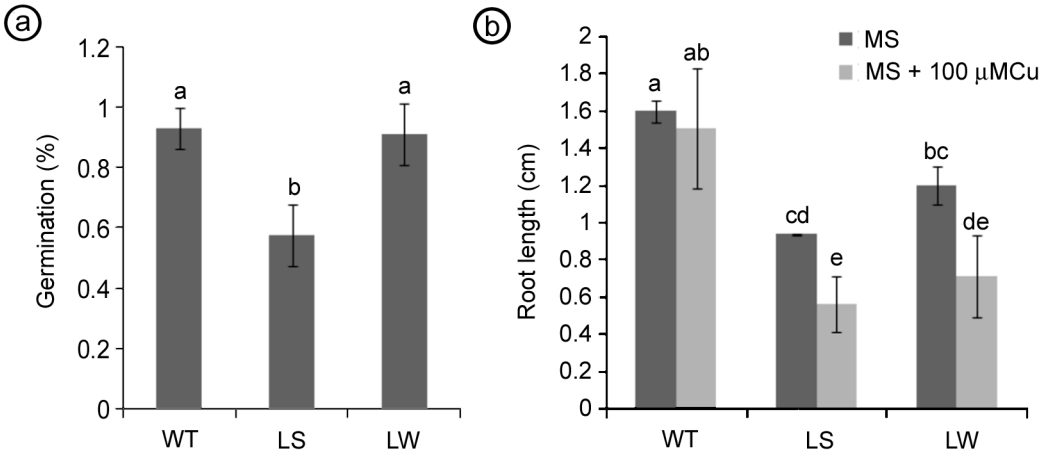

Fig. 3. Seed germination and root growth of transgenic tobacco lines. (**a**) Seed germination of transgenic lines and WT control on MS medium, (**b**) Root length of transgenic lines and WT control under normal and high copper conditions. LS and LW are transgenic tobacco lines with strong and weak expression of *AtmiR408* gene, respectively. Each column represents means ±SD of three independent experiments with three replicates each. Values followed by different letters differ significantly at $p < 0.05$ by Duncan's multiple range test.

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, miR408 oversupply led to remarkable decreases in chlorophyll content as well as SOD, POD and CAT activities, which might be due to specific cleavage of miR408 to the endogenous target gene encoding 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 Cu^{2+}. 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.

ACKNOWLEDGEMENTS

This work was supported by Programs for New Century Excellent Talents in University (NCET-06-607), Changjiang Scholars and Innovative Research Team in University (IRT0635).

REFERENCES

ABDEL-GHANY SE, and PILON M. 2008. MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*. *The Journal of Biological Chemistry* 283: 15932–15945.

BARTEL DP. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281–297.

DEAN JF, and ERIKSSON KE. 1994. Laccase and the deposition of lignin in vascular plants. *Holzforschung* 48: 21–33.

DONG J, KIM ST, and LORD EM. 2005. Plantacyanin plays a role in reproduction in *Arabidopsis*. *Plant Physiology* 138: 778–789.

FAHLGREN N, HOWELL MH, KASSCHAU KD, ELISABETH JC, SULLIVAN CM, CUMBIE JS, GIVAN SA, LAW TF, GRANT SR, DANGL JL, and CARRINGTON JC. 2007. High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE* 2: e219.

HOOPES JT, and DEAN JFD. 2004. Ferroxidase activity in a plant multicopper oxidase. *Plant Physiology and Biochemistry* 42: 27–33.

JONES-RHOADES MW, and BARTEL DP. BARTEL B. 2006. MicroRNAs and their regulatory roles in plants. *Annual Review of Plant Biology* 57: 19–53.

LAFAYETTE PR, ERIKSSON KE, and DEAN JF. 1999. Characterization and heterologous expression of laccase cDNAs from xylem tissues of yellow-poplar (*Liriodendron tulipifera*). *Plant Molecular Biology* 40: 23–35.

LIANG M, DAVIS E, GARDNER D, CAI X, and WU Y. 2006. Involvement of AtLAC15 in lignin synthesis in seeds and in root elongation of *Arabidopsis*. *Planta* 224: 1185–1196.

MCCAIG BC, MEAGHER RB, and DEAN JFD. 2005. Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. *Planta* 221: 619–636.

PROCHAZKOVA D, SAIRAM RK, SRIVASTAVA GC, and SINGH DV. 2001. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Science* 161: 765–771.

RANOCHA P, CHABANNES M, CHAMAYOU S, DANOUN S, JAUNEAU A, BOUDET AM, and GOFFNER D. 2002. Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Plant Physiology* 129: 145–155.

SUNKAR R, and ZHU JK. 2004. Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* 16: 2001–2019.

WEIGEL M, VAROTTO C, PESARESI P, FINAZZI G, RAPPAPORT F, SALAMINI F, and LEISTER D. 2003. Plastocyanin is indispensable for photosynthetic electron flow in *Arabidopsis thaliana*. *The Journal of Biological Chemistry* 278: 31286–31289.