Jacalin lectin At5g28520 is regulated by ABA and miR846

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Plant microRNAs (miRNAs) are important regulators of development and stress responses and are oftentimes under transcriptional regulation by stresses and plant hormones. We recently showed that polycistronic MIR842 and MIR846 are expressed from the same primary transcript which is subject to alternative splicing. ABA treatment affects the alternative splicing of the primary cistronic transcript which results in differential expression of the two miRNAs that are predicted to target the same family of jacalin lectin genes. One variant of miR846 in roots can direct the cleavage of AT5G28520, which is also highly upregulated by ABA in roots. In this addendum, we present additional results further supporting the regulation of AT5G28520 by MIR846 using a T-DNA insertion line mapping upstream of MIR842 and MIR846. We also show that AT5G28520 is transcriptionally induced by ABA and this induction is subject to ABA signaling effectors in seedlings. Based on previous results and data presented in this paper, we propose an interaction loop between MIR846, AT5G28520 and ABA in roots.

Abbreviations: ABA, abscisic acid; AGO, Argonaute; bZIP, basic leucine zipper transcription factor; DCL, Dicer-Like; GUS, Escherichia coli β-glucuronidase gene uidA; PP2C, protein phosphatase type 2C; pri-miRNA, primary MIRNA transcript; Pro-promoter; RACE, Rapid Amplification of cDNA Ends; RNAi, RNA interference; SGS3, Suppressor of Gene Silencing3; TSS, transcription start site

Plant microRNAs (miRNAs) are important regulators of development and stress responses and are oftentimes under transcriptional regulation by stresses and plant hormones. We recently showed that polycistronic MIR842 and MIR846 are expressed from the same primary transcript which is subject to alternative splicing. ABA treatment affects the alternative splicing of the primary cistronic transcript which results in differential expression of the two miRNAs that are predicted to target the same family of jacalin lectin genes. One variant of miR846 in roots can direct the cleavage of AT5G28520, which is also highly upregulated by ABA in roots. In this addendum, we present additional results further supporting the regulation of AT5G28520 by MIR846 using a T-DNA insertion line mapping upstream of MIR842 and MIR846. We also show that AT5G28520 is transcriptionally induced by ABA and this induction is subject to ABA signaling effectors in seedlings. Based on previous results and data presented in this paper, we propose an interaction loop between MIR846, AT5G28520 and ABA in roots.

We found that polycistronic MIR842 and MIR846 are transcribed from the same primary transcript that produces three different splicing isoforms. ABA affects the alternative splicing of the pri-miRNA and thereby represses the expression of the miRNAs. We also showed that AT5G28520, a gene encoding a predicted target jacalin lectin, is cleaved, possibly by a variant “isoMIR” of miR846 in roots. RNA gel blots showed several-fold upregulation of AT5G28520 by ABA, but real-time PCR experiments demonstrate AT5G28520 is highly (~50-fold) upregulated within a few hours (Fig. 1A). Real-time PCR also showed that ABA treatment repressed pre-miR846 with the strongest (60% reduction) effect observed after three hr exposure to 10 μM ABA (Fig. 1B). Furthermore, in the absence of exogenous ABA, pre-miR846 levels decreased after 24 h (Fig. 1B) when the expression of AT5G28520 was elevated (Fig. 1A), which is consistent with a causal relationship. However, a 50-fold upregulation of AT5G28520 by ABA within hours raises questions about whether this induction is by miRNA action alone or if some other type of regulation, for example a positive feedback on transcription initiation, might be involved.

To further study the function of miR842 and miR846, we searched on TAIR (www.arabidopsis.org) and identified a T-DNA insertion line CS815868 which carries an insertion upstream of MIR842 and MIR846, suggesting it may...
AT5G28520 mRNA was reduced (Fig. 2D), supporting a causal effect of elevated miR842 and/or miR846 expression on downregulation of AT5G28520. This supports our claim\textsuperscript{13} that miR846 can direct the cleavage of AT5G28520, which is subsequently degraded.

To study the transcriptional regulation of AT5G28520, transgenic plants were engineered\textsuperscript{21} that express the reporter gene GUS under control of the AT5G28520 promoter. GUS staining of 10-d-old Pro-AT5G28520:GUS seedlings had no detectable GUS activity in the absence of exogenous ABA (Fig. 3A). When treated with ABA (30 μM, 4 hr), strong GUS activity was observed in roots, especially in lateral root primordia, but not in leaves (Fig. 3B and C). RNA gel blot analysis further showed that this induction was strongly suppressed by ABA-insensitive signal transduction mutants such as PP2Cs abi1-1 (75% reduction), abi2-1\textsuperscript{22} (84% reduction), APETALA2 domain abi4-1\textsuperscript{23} (35% reduction) and bZIP abi5-1\textsuperscript{24} (32% reduction), whereas B3 domain abi3-1\textsuperscript{25} mutant had a relatively weak effect (12% reduction) (Fig. 2D), consistent with previous results showing

function as an activation-tagged allele. Analysis of the flanking sequence and a genomic Southern blot showed that this T-DNA is inserted near the transcription start site (TSS) of MIR842 and MIR846 (Fig. 2A and B). 5’ RACE showed that the TSS is altered (a 40 nt rDNA-like insertion fragment; data not shown) but pre-miR842 is otherwise intact in CS815868 (Fig. 2A). Real-time PCR showed enhanced expression of pre-miR842 and pre-miR846 in roots of CS815868 (Fig. 2C) while

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**Figure 1.** Real-time PCR results showing the ABA-regulated expression of (A) AT5G28520 and (B) pre-miR846 in 10-d-old seedlings over 24 h. Numbers above the bars indicate fold changes (comparing to no ABA at the same time point). Error bars are ± s.d., n = 3.

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**Figure 2.** AT5G28520 is downregulated in CS815868 T-DNA insertion plants, which overexpress pre-miR842 and pre-miR846. (A) Genomic structure around MIR842 and MIR846 in T-DNA insertion line CS815868. TSS revealed by 5’ RACE is shown with a vertical black arrow (numbers are sequenced clones mapping to site out of the total clones sequenced). The zigzag below the arrow represents a 40 bp sequence found that does not map to the T-DNA and has homology to rDNA. Open boxes represent pre-miR842 and pre-miR846. The short gray rectangles above the open boxes represent miRNA* and short black rectangles represent mature miRNA sequences. Isoform 1, which is the most abundant splice variant expressed from this locus in wild type is shown below. Black boxes represent exons. Horizontal arrows represent primers used for real-time PCR of pre-miRNAs in (C). (B) Genomic Southern blot showing single insert in CS815868. Lanes 1 and 2 are HindIII-digested genomic DNAs from two individual CS815868 plants. Blot was probed with BASTA selectable marker gene fragment from the vector. (C and D) Real-time PCR results showing the different expression levels of AT5G28520 (C) and pre-miR842 and -miR846 (D) in roots of control Col-0 and CS815868 line. Error bar, ± s.d., n = 2.
that ABI3 is expressed in seeds and ABI5 expression decreases progressively after germination.26

Together with the results presented in our recent paper,13 we have shown that: (1) miR846 and AT5G28520 are co-expressed in roots; (2) a possible isomer variant (5' shifted) of miR846 directs the cleavage of AT5G28520 in the absence of ABA; (3) AT5G28520 is downregulated in an MIR842 and MIR846 T-DNA activation-tagged overexpression allele; (4) ABA upregulates AT5G28520 transcriptionally and this regulation requires the functions of ABI1, -2, -4 and -5; and (5) ABA downregulates miR846 post-transcriptionally and this regulation requires the functions of ABI3 expression decreases progressively after germination.26

Together with the results presented in our recent paper,13 we have shown that: (1) miR846 and AT5G28520 are co-expressed in roots; (2) a possible isomer variant (5' shifted) of miR846 directs the cleavage of AT5G28520 in the absence of ABA; (3) AT5G28520 is downregulated in an MIR842 and MIR846 T-DNA activation-tagged overexpression allele; (4) ABA upregulates AT5G28520 transcriptionally and this regulation requires the functions of ABI1, -2, -4 and -5; and (5) ABA downregulates miR846 post-transcriptionally by alternative splicing. Based on these results, we propose a model in which ABA, MIR846 and AT5G28520 form a regulatory network in roots (Fig. 4). In the absence of exogenous ABA (and presumably low concentrations of endogenous ABA), expression of AT5G28520 is low and the expression of miR846 would direct the Jacalin lectin mRNA to be endonucleolytically cleaved or possibly subject to translational repression. In the presence of exogenous ABA (or high endogenous ABA concentrations, e.g., in response to stress), transcription of AT5G28520 is elevated, and at the same time miR846 biogenesis is repressed by alternate splicing, which results in AT5G28520 mRNA accumulation. This synergistic effect could account for the rapid -50-fold induction of AT5G28520 by exogenous ABA at as low as 1 μM, which is within the physiological range found in plants.27 We speculate that biogenesis of pre-miR842/846 by complexes containing effectors of ABA sensitivity and splicing such as ABA hypersensitivity1/cap-binding protein80,28 hypotonic leaves,29 serrate,30 argonaute31 and others may be important for miR846 function. In addition, ABA may also repress the expression of the MIR842-MIR846 cistron transcriptionally. The function of jacalin lectins is poorly understood and recent studies link their activities to virus resistance and biotic stress.32,33 Further work is needed to understand the function of AT5G28520 and the importance of RNAi regulatory networks to ABA signaling in root development and environmental stress responses.

Materials and Methods

Plasmid construction. For the promoter analysis of AT5G28520, a 1.5 kb sequence upstream of the start codon was PCR-amplified using PrimeSTAR HS DNA polymerase (Takara) and inserted between the Hind III and BamHI sites in pBl121, replacing the 35S promoter which drives uidA GUS reporter gene expression. Oligonucleotide primers were designed using “Perlprimer” (perlprimer.sourceforge.net) and synthesized commercially (Sigma). A list of primers used in the study is provided in Table 1.

Plant material and growth conditions. The accessions used in this study are listed as follows: Col-0 [CS60,000], Ler-0 [CS20], Ws-2 [CS2360], abi1-1 [CS22], abi2-1 [CS23], abi3-1 [CS24], abi4-1 [CS8104], abi5-1 [CS8105], CS815868 and were obtained from the Arabidopsis Biological Resource Center (abrc.osu.edu). Arabidopsis thaliana seeds were soaked in water and kept in the dark at 4°C for 3 d before transferring to soil. The growth condition was 21°C with a 16 h light and 8 h dark cycle. For promoter analysis, plants were transformed with Pro-AT5G28520:GUS constructs first electroporated into Agrobacterium tumefaciens strain GV3101 and then transferred to the plants using the floral dip method.21

For ABA treatments, seeds were sown on plates containing 0.5× Murashige and Skoog salts (Research Products International) 1% sucrose and 0.5% phytagel (Sigma). Plants were kept in the dark at 4°C for 3 d before transferring into a growth chamber. The growth condition was 21°C with continuous light. Plants were kept in the chamber for 10 d before spraying with ABA solutions. ABA [(±)-Absciscic acid, 98%; ACROS Organics] solutions were diluted with water from a 100 μM stock in 70% ethanol. The same amount of 70% ethanol was added to water to spray the control group.

β-Glucuronidase GUS assays. Plant samples were developed at 37°C for 12 h with a 1 mM solution of the indigogenic GUS substrate 5-bromo-4-chloro-3-indolyl b-D-glucuronide (X-Gluc; Rose Scientific) in 50 mM KH2PO4 (pH 7.0), 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.05% sodium azide, 0.1% Triton X-100. After staining overnight,
samples were immersed in 70% ethanol overnight. Samples were then mounted in 30% glycerol on microscope slides for imaging with a dissecting microscope or with an Olympus BX41 light microscope for high magnification and documented using QCapture software (v2.68.6, Silicon Graphics).

**Real-time PCR.** Total RNA from roots of 10-d-old seedlings was extracted with Iso-RNA Lysis Reagent (5 PRIME). Real-time PCR was performed using FastStart Universal SYBR Green (Promega) and reverse transcribed by MMLV reverse transcriptase (Promega). Real-time PCR reactions were run on an ABI7000 (Applied Biosystems) with default settings and results were analyzed using the CFX Manager software (v1.6, Bio-Rad). PCR was performed using FastStart Universal SYBR Green (Promega) with random primers (Promega). 

**Transcript Preparation**

Reverse transcriptase-PCR except that random primers (Promega) was used in reverse transcription.

**5’ RACE.** RACE experiments were performed with GenRacer Kit (Invitrogen) according to the manufacturer’s specification.

**Southern hybridization.** Genomic DNA was isolated from Col-0 plants and two individual CS815868 plants using CTAB (Hexadecyl trimethyl-ammonium bromide, ACROS Organics). 15 μg genomic DNA was used for each HindIII digestion. The samples were resolved on a 0.7% agarose gel and blotted to a Hybond-N+ membrane (GE Healthcare) according to the supplier’s protocol. BASTA template was amplified using primers “BASTA_F” and “BASTA_R” from CS815868 genomic DNA and was eluted after excision of the band from an agarose gel. Probes were synthesized using Random Primer DNA Labeling Kit (Takara) with [α-32P]-dCTP (PerkinElmer). Hybridization was performed with the PerfectHyb Plus hybridization buffer (Sigma) according to the manufacturer’s instructions. A storage phosphor screen (GE Healthcare) was used for autoradiography and it was scanned using a Storm 860 PhosphorImager (GE Healthcare).

**Northern hybridization.** Samples of 10 μg total RNA were resolved on a 1.2% denaturing agarose gel and blotted to a Hybond-N+ membrane (GE Healthcare) according to the supplier’s protocol. At5g28520 template was amplified using primers “AT5g28520CDS_F” and “At5g28520CDS_R” from a cDNA library and was eluted after excision of the band from an agarose gel. Probes were synthesized and blots processed as described for genomic DNA gel blots.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

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**References**

3. Antonian I, Chua NH. IAA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. Plant J 2007; 49:592-606; PMID:17217461; http://dx.doi.org/10.1111/j.1365-313X.2006.02980.x
Table 1. Primers used in this study

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5’ RACE

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Northern

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18S primer pair same as 18S_F and 18S_R pair used in real-time PCR


