Short Communication

Virus-induced silencing of *NtmybA1* and *NtmybA2* causes incomplete cytokinesis and reduced shoot elongation in *Nicotiana benthamiana*

Satoshi Araki[1,a], Yasunori Machida[1], Masaki Ito[2,*]

[1]Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Aichi 464-8602, Japan; [2]Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan
* E-mail: masakito@agr.nagoya-u.ac.jp   Tel & Fax: +81-52-789-4168

Abstract   In the plant cell cycle, a group of genes with mitotic functions is transcribed specifically during G2 and M phases. Such G2/M-specific genes are regulated by a common mechanism involving a *cis*-acting element called mitosis-specific activator (MSA) and R1R2R3-class Myb transcription factors that bind to the element. We have previously shown that a group of structurally related R1R2R3-Myb proteins acts as transcriptional activators, which include NtmybA1 and NtmybA2 from tobacco and MYB3R1 and MYB3R4 from *Arabidopsis thaliana*. Loss-of-function mutations in *MYB3R1* and *MYB3R4* showed reduced shoot elongation and significant impairment in cytokinesis during somatic cell division in *A. thaliana*. However, it remained unclear if this type of Myb transcription factors also have equivalent developmental roles in evolutionarily distant species. Here, we showed virus-induced silencing of both *NtmybA1* and *NtmybA2* causes similar defects in cytokinesis and severely dwarfed phenotype. Our results showed that a group of evolutionarily conserved R1R2R3-Myb transcriptional activators has common physiological functions in the promotion of cytokinesis and shoot elongation in different plant species.

Key words: Cell cycle, cytokinesis, Myb transcription factor, virus-induced gene silencing, tobacco.

For plant growth and development, it is important to maintain proper balance between cell division, expansion and differentiation. Division of each cell is controlled by cell cycle regulators, such as cyclins and cyclin-dependent kinases (CDKs), which are further controlled during the cell cycle and also in the developmental context (Inzé and De Veylder, 2006; Inagaki and Umeda, 2011). In *Arabidopsis thaliana* (Arabidopsis), 61 core cell cycle regulators have been defined, among which 16 genes with possible important functions for mitosis are expressed specifically at G2 and M phases (Vandepoele et al. 2002; Menges et al. 2005). In addition, there are many other genes that are transcribed in similar timing of the cell cycle (Menges et al. 2003), and we have previously defined 185 G2/M-specific genes in the Arabidopsis genome (Haga et al. 2011). Most of these G2/M-class genes commonly contained *cis*-acting element, called the mitosis-specific activator (MSA) element, which activates cell cycle-regulated transcription in tobacco cells (Ito et al. 1998; Ito, 2000). It has been shown that a group of Myb transcription factors, the so-called R1R2R3-Myb,

binds to the MSA elements and regulates transcription of various target genes (Ito et al. 2001). There are five genes encoding this type of Myb transcription factors in Arabidopsis, two of which, MYB3R1 and MYB3R4, act as transcriptional activators. A double *myb3r1 myb3r4* mutation resulted in downregulation of many G2/M-class genes, which causes incomplete cytokinesis during somatic cell division and impaired shoot elongation (Haga et al. 2007).

We have previously isolated cDNA clones for three different R1R2R3-Myb proteins, NtmybA1, NtmybA2 and NtmybB, from tobacco (Ito et al. 2001). NtmybA1 and NtmybA2 (hereafter NtmybA1/A2) are structurally closely related to each other and to MYB3R1 and MYB3R4 (hereafter MYB3R1/4) in Arabidopsis, thus constituting an evolutionarily conserved subfamily within a family of R1R2R3-type Myb (Haga et al. 2007). Like MYB3R1/4, NtmybA1/A2 are also considered as transcriptional activators, because they can activate MSA-containing promoters in transient expression assays of tobacco BY2 cells (Ito et al. 2001). NtmybA2

contains a negative regulation domain that inhibits its own transactivation activities, and deletion of this domain dramatically enhanced activity of NtmybA2 for transactivation (Araki et al. 2004). Our microarray analysis revealed that stable overexpression of this hyperactive form of NtmybA2 selectively and significantly upregulates various G2/M-class genes in BY2 cells (Kato et al. 2009). Therefore, it is suggested that both NtmybA1/A2 and MYB3R1/4 may activate transcription of many target genes and may positively regulate initiation and progression of mitosis. Although MYB3R1/4 were shown to be required for completion of cytokinesis (Haga et al. 2007), it has remained unclear if similar cytokinetic function of this class of R1R2R3-Myb is conserved in other plant species as well. To analyze phenotypic changes caused by loss-of-function of *NtmybA1/A2* in tobacco, we exploited a virus-induced gene-silencing (VIGS) system using potato virus X (PVX; Angell and Baulcombe, 1999), and showed that NtmybA1/A2 have indeed a critical function for cytokinesis in tobacco.

Because possible functional redundancy between NtmybA1 and NtmybA2 might attenuate the severity of defects, we attempted to repress expression of both genes simultaneously. Fragments of NtmybA1 and NtmybA2 cDNAs were amplified by PCR using primer pairs, 5′-ATA GTT CTG TTA AAA AGA AAC TG-3′ and 5′-TAA CAT TGA ACA AGA AAC ATC TTG -3′ for NtmybA1, and 5′-ACA AAG TCT TCT CTA ACT ACG -3′ and 5′-AGC TTC GAG TCG TCT AGC G-3′ for NtmybA2. These fragments of approximately 350 bp are cloned in tandem between *Sal*I and *Sma*I sites of a PVX vector (pP2C2S) to create PVX:mybA1/A2 (Figure 1, upper). A control vector, PVX:GFP, containing a green fluorescent protein (GFP) fragment was generated previously (Nishihama et al. 2002) (Figure 1, lower). Recombinant PVX vectors were used to infect to *Nicotiana benthamiana* plants as described previously (Nishihama et al. 2002). Infectious PVX RNAs were transcribed *in vitro* with PVX:mybA1/A2 or PVX:GFP DNA as template, which were used for inoculation onto leaves of *N. benthamiana* plants. Inoculated plants were then grown at 23°C in a growth chamber, and used for quantitative RT-PCR experiments and phenotypic analysis.

By 4–7 days after inoculation, typical vein clearing and mild mosaic symptoms appeared on the upper leaves in half of the inoculated plants of both the PVX:mybA1/A2 and PVX:GFP series. After the appearance of symptoms, retardation of growth became apparent in plants infected with PVX:mybA1/A2, which finally stopped growing (Figures 2A, B). In such arrested plants, leaf growth was inhibited even at the early stage of leaf development, generating small, humped leaves at a position above inoculated leaves (Figure 2C). Formation of flower buds was also attenuated in PVX:mybA1/A2-infected
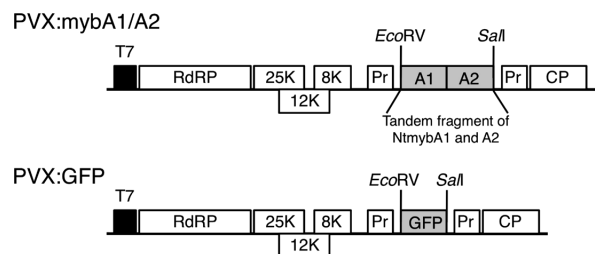

Figure 1. VIGS constructs used for simultaneous suppression of *NtmybA1* and *NtmybA2*. The tandemly arrayed fragments from NtmybA1 and NtmybA2 cDNAs were inserted into pP2C2S vector derived from PVX (PVX:mybA1/A2). The GFP construct (PVX:GFP) was similarly generated and used as a control. The PVX open reading frames are shown as RdRp (RNA-dependent RNA polymerase), 25K (25K protein), 12K (12K protein), 8K (8K protein), and CP (coat protein). Pr indicates coat protein duplicated promoter regions. T7 promoter was used for *in vitro* synthesis of infectious transcripts.

plants, which sometimes formed abnormal flower buds possessing morphologically normal sepals but lacking other floral organs possibly due to premature termination of their growth (Figure 2E, compare with the control flower bud in Figure 2D). By contrast, when PVX:GFP was infected, plants grew normally and generated normal flower buds by 33 days after inoculation (Figures 2A, B). Normal growth was also observed for PVX:mybA1/A2-inoculated plants without symptoms of virus infection (Figure 2B), suggesting that infection, but not inoculation itself, is responsible for growth retardation. Therefore, the growth defect seemed to be induced by VIGS effects caused by infection of PVX:mybA1/A2 and not by disease due to PVX.

We investigated cellular defects in PVX:mybA1/A2-infected plants by microscopic observation of arrested leaves. Leaves were fixed in an 8:2 mixture of ethanol and acetic acid and then stained with 1% orcein in a 1:1 mixture of lactic acid and propionic acid as described previously (Nishihama et al. 2001). We observed the occurrence of severely defected stomata with multiple nuclei that did not have ventral walls (Figure 3B, compare with the stoma of control plants in Figure 3A). This suggests that these abnormal stomata resulted from a failure of the guard mother cells to undergo cytokinesis. Similar defects in stomata have been found in Arabidopsis plants lacking functional *MYB3R1/4* genes and other cytokinesis-defective mutants (Yang et al. 1999; Söllner et al. 2002; Falbel et al. 2003; Haga et al. 2007). Less frequently, cytokinesis-defective guard cells contain two nuclei with different sizes, suggesting that chromosome segregation may be also impaired during mitosis (Figure 3C). In addition to guard cells, binucleation was occasionally observed in some pavement cells (Figure 3D).

To investigate the nuclear DNA content of arrested leaves in PVX:mybA1/A2 plants, flow cytometric analysis was performed as described previously (Haga

et al. 2011). As expected, arrested leaves showed a higher frequency of 4C nuclei in comparison with control leaves infected with PVX:GFP (Figure 3E). In addition, 8C nuclei were also detected, which were never observed in control leaves. This observation suggests that VIGS-induced inhibition of G2/M transition
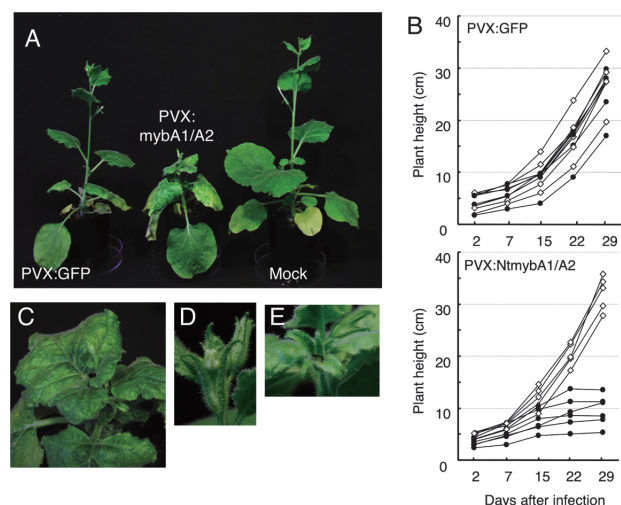

Figure 2. Growth inhibition by VIGS-induced downregulation of *NtmybA1* and *NtmybA2*. (A) Gross morphology of typical *N. benthamiana* plants at 33 days after inoculation of PVX:GFP (left) and PVX:mybA1/A2 (middle). A mock-inoculated plant is also shown as a control (right). (B) Time–course analysis of plant height after inoculation of PVX:GFP (upper) and PVX:mybA1/A2 (lower). For each construct, independently inoculated plants with and without symptoms were separately analyzed. Plants without symptoms serve as control without VIGS effects after inoculation of PVX:mybA1/A2. Closed circles, plants with symptoms; open diamonds, plants without symptoms. (C) Close-up view of shoot apex in a PVX:mybA1/A2-infected plant. (D) Normal flower bud formed in PVX:GFP-infected plants. (E) Malformed flower bud in PVX:mybA1/A2-infected plants.

in PVX:mybA1/A2 plants might result in frequent occurrence of endoreduplicatoin, which is characterized by atypical cell cycle lacking M phase (Breuer et al., 2010). Alternatively, polyploid somatic cells might be generated as a secondary effect of defective cytokinesis, which is presumably due to nuclear fusion or division of binucleate cells. Generation of such polyploid somatic cells has been also observed in cytokinesis-defective Arabidopsis mutants including *knolle* and *atnack1/hinkel* (Lukowitz et al. 1996; Strompen et al. 2002).

To confirm reduced expression of *NtmybA1/A2* in PVX:mybA1/A2-infected plants, semiquantitative RT-PCR was performed using RNA isolated from shoot apices (containing small leaves less than 10 mm in length) and young leaves (about 10 mm in length). RNA extraction, cDNA synthesis and PCR reaction were performed as described previously (Iwakawa et al. 2002). Sequences of primers used for RT-PCR are listed in Supplemental Table 1. In young leaves, levels of *NtmybA1* and *NtmybA2* mRNAs of endogenous genes were clearly decreased in PVX:mybA1/A2-infected plants compared with those of control plants, although detectable amounts of transcripts were still present (Figure 4A). On the other hand, such a reduction of transcript levels was not observed in shoot apices, possibly due to the inability of PVX to invade shoot meristems (Schwach et al. 2005). Therefore, for gene expression analysis, we used young leaves as materials to see the effects of *NtmybA1/A2* downregulation. As shown in Figure 4B, NtmybB, the other member of the R1R2R3-Myb gene family from tobacco (Ito et al. 2001), was not affected by infection of PVX:mybA1/A2. To see the effect on cell cycle-related genes, PCR primers were designed for *CDKA*, which represents a canonical class of plant CDK (Setiady et al.
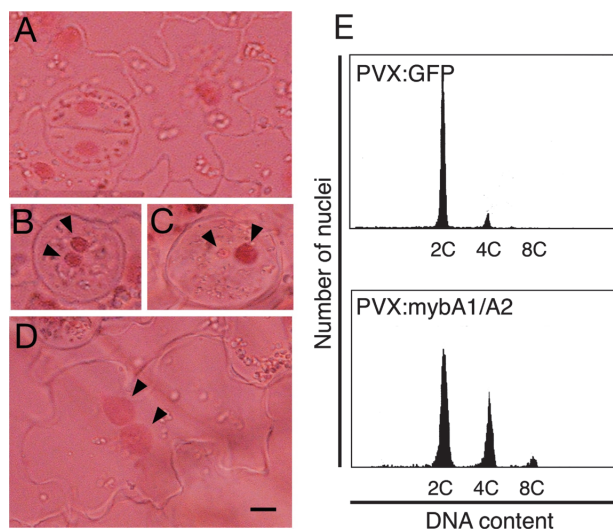

Figure 3. Cellular abnormalities found in PVX:mybA1/A2-infected plants. (A) Orcein-stained leaf epidermis from PVX:GFP-infected plants. (B–D) Orcein-stained leaf epidermis from PVX:mybA1/A2-infected plants. Binucleate guard cells without ventral walls (B, C) and a binucleate pavement cell (D) are shown. Positions of nuclei are indicated by arrowheads. (E) Flow cytometric analysis of leaves from PVX:GFP- and PVX:mybA1/A2-infected plants. Young leaves around shoot apices were detached from plants at 20 days after inoculation and subjected to flow cytometric analysis.
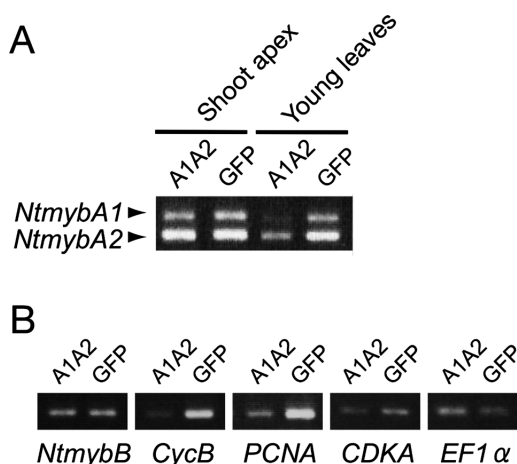
Figure 4. Semiquantitative RT-PCR analysis. (A) mRNA levels of *NtmybA1* and *NtmybA2* in shoot apices and young leaves from PVX : GFP- and PVX : mybA1/A2-infected plants. (B) mRNA levels of cell cycle-related genes in young leaves from PVX : GFP- and PVX : mybA1/A2-infected plants. Levels of EF1α mRNA were analyzed as an internal control.

1996), *PCNA* as a marker gene of S phase (Sekine et al. 1999), and *CYCB1* as a marker gene for G2/M (Ito et al. 1997). We found that all these genes were downregulated in arrested leaves from PVX : mybA1/A2-infected plants (Figure 4B). This suggests that VIGS-induced reduction of *NtmybA1/A2* expression inhibited overall cell division activity in young leaves and possibly in other organs.

Taken together, our data showed that NtmybA1/A2 are required for proper shoot elongation, normal development of floral organs, and maintenance of diploidy in somatic cells, some of which may be due to reduced division activity and cellular defects in cytokinesis. These abnormalities closely resembled phenotypic defects found in Arabidopsis plants with double *myb3r1 myb3r4* mutations, which include incomplete cytokinesis of somatic cells and reduced shoot elongation. Therefore, our study demonstrated that a conserved group of R1R2R3-Myb transcription factors in different plant species may have a common important roles both at cellular and whole plant levels.

Acknowledgments

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