Identification of two tobacco genes encoding MYB3R proteins with repressor function and showing cell cycle-regulated transcript accumulation

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Abstract MYB3R family transcription factors play a central role in the regulation of G2/M-specific gene transcription in Arabidopsis thaliana. Among the members of this family, MYB3R3 and MYB3R5 are structurally closely related and are involved in the transcriptional repression of target genes in both proliferating and quiescent cells. This type of MYB3R repressor is widespread in plants; however, apart from the studies on MYB3Rs in Arabidopsis thaliana, little information about them is available. Here we isolated tobacco cDNA clones encoding two closely related MYB3R proteins designated as NtmybC1 and NtmybC2 and determined the nucleotide sequences of the entire coding regions. Phylogenetic analysis suggested that NtmybC1 and NtmybC2 can be grouped into a conserved subfamily of plant MYB3Rs that also contains MYB3R3 and MYB3R5. When transiently expressed in protoplasts prepared from tobacco BY-2 cells, NtmybC1 and NtmybC2 repressed the activity of target promoters and blocked promoter activation mediated by NtmybA2, a MYB3R activator from tobacco. Unlike MYB3R3 and MYB3R5, NtmybC1 and NtmybC2 showed cell cycle-regulated transcript accumulation. In synchronized cultures of BY-2 cells, mRNAs for both NtmybC1 and NtmybC2 were preferentially expressed during the G2 and M phases, coinciding with the expression of NtmybA2 and G2/M-specific target genes. These results not only broadly confirm our fundamental view that this type of MYB3R protein acts as transcriptional repressor of G2/M-specific genes but also suggest a possible divergence of MYB3R repressors in terms of the mechanisms of their action and regulation.

Key words: BY-2 cells, Cell cycle, MYB3R, synchronized culture, transcriptional regulation.

Plant organ growth relies on the proper progression of the cell cycle, which is entirely responsible for increasing the number of somatic cells during development (Komaki and Sugimoto 2012). For the cell cycle to progress, maintaining a specific order of events is believed to be important. Therefore, regulating sets of genes so that they are expressed during defined periods of the cell cycle is critical. There are two main sets of genes that are regulated in a cell cycle-specific manner, G1/S-specific and G2/M-specific genes, which are known to be regulated by the E2F and MYB3R families of transcription factors, respectively (Berckmans and De Veylder 2009). MYB3R transcription factors are characterized by the presence of a Myb domain that is repeated three times and known to constitute a relatively small subfamily within the large family of Myb transcription factors in plants (Feng et al. 2017; Katiyar et al. 2012). MYB3R proteins bind to cis-acting elements, called the mitosis-specific activator (MSA) elements, which are present in the majority of G2/M-specific genes (Haga et al. 2011; Kato et al. 2009). Some reports have recently demonstrated that plant cells express multiprotein complexes called DREAM complexes, which contain both E2F and MYB3R; however, the precise roles of these complexes and their mechanisms of action are not fully understood (Kobayashi et al. 2015; Ning et al. 2020).

The MYB3R family also contains members that activate or repress the transcription of target genes (Kobayashi et al. 2015). The Arabidopsis thaliana (Arabidopsis) genome contains five MYB3R genes, among which MYB3R4 encodes transcriptional activators and products of MYB3R3 and MYB3R5 act as repressors. The MYB3R4 expression is restricted to a short time

Abbreviations: Arabidopsis, Arabidopsis thaliana; CaMV, cauliflower mosaic virus; LUC, luciferase; MSA, mitosis-specific activator; PCNA, proliferating cell nuclear antigen; RACE, rapid amplification of cDNA ends.
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window during the G2 and M phases and is required for sufficient levels of target transcription during G2/M (Haga et al. 2007). MYB3R repressors exhibit a relatively constant level of expression throughout the cell cycle (Menges et al. 2005) and are important in suppressing the transcription of targets outside G2/M in the cell cycle and after exit of cell proliferation (Kobayashi et al. 2015). The functions of MYB3Rs in cell cycle regulation have, to date, been exclusively studied in Arabidopsis, although some reports have suggested potential roles for MYB3Rs in plant responses to environmental stress in rice (Dai et al. 2007; Ma et al. 2009). Therefore, the study of plant species other than Arabidopsis is required to fully understand the roles played by these transcription factors and how they are regulated during the cell cycle and plant development.

We previously identified cDNA clones for MYB3Rs from *Nicotiana tabacum* (tobacco): *NtmybA1*, *NtmybA2*, and *NtmybB* (Ito et al. 2001). *NtmybA1* and *NtmybA2* are closely related to each other and to MYB3R1 and MYB3R4 in their amino acid sequences, and they were shown to have an ability to activate the transcription from target promoters such as *CYCB1* and *NACK1* in BY-2 protoplasts (Haga et al. 2007; Ito et al. 2001). No clear ortholog of *NtmybB* has been found in Arabidopsis or rice genomes (Feng et al. 2017), but it can competitively repress target promoters against *NtmybA1* and *NtmybA2* in BY2 protoplasts (Ito 2005; Ito et al. 2001). These findings in tobacco cells provided a basic understanding of MYB3Rs, in that their common functions are closely related to the cell cycle and that different members have separate physiological roles. However, there has been no report on the tobacco homologs of MYB3R3 and MYB3R5 that supports the evidence for their function and regulation found in Arabidopsis.

In order to identify tobacco homologs of MYB3R similar to but different from *NtmybA1*, *NtmybA2*, and *NtmybB*, we conducted a BLAST search using protein sequences of these tobacco MYB3Rs as queries, and found a short (160 bp) cDNA fragment from BY-2 cells in the GenBank database (accession number: AJ717905). To determine the full sequence of the corresponding mRNAs, we performed 3' rapid amplification of cDNA ends (RACE) using cDNAs prepared from BY-2 cells and identified two different sequences containing regions identical or highly similar to those of the original cDNA. Subsequent experiments using 5' RACE enabled us to identify two different cDNA clones containing long open reading frames encoding 528 and 552 amino acids, which were designated as *NtmybC1* and *NtmybC2*, respectively (DDBJ accession numbers: LC600140 for *NtmybC1* and LC600141 for *NtmybC2*). 3' and 5' RACE was performed using a FirstChoice RLM-RACE Kit (Thermo Fisher Scientific) according to manufacturer's instructions. The encoded amino acid sequences contained N-terminally located Myb domains with three imperfect repeat sequences (Figure 1) that distinguish MYB3Rs from other types of Myb proteins in plants (Ito 2005). When the amino acid sequences of *NtmybC1* and *NtmybC2* were compared, high similarity was found within the Myb domain (92.3% identity), but less similarity was observed outside the Myb domain (53.1% identity), similarly to case for *NtmybA1* and *NtmybA2* (Figures 1, 2A, B). The Myb domains in *NtmybC1* and *NtmybC2* showed high sequence similarity to those in *NtmybA1*, *NtmybA2*, and *NtmybB* (Figure 2A). However, there was negligible similarity when the protein sequences C-terminal to the Myb domains were compared. This suggested that *NtmybC1* and *NtmybC2* may have equivalent physiological roles, which may differ from other MYB3Rs in tobacco. We then compared the protein sequences of *NtmybC1* and *NtmybC2* with MYB3Rs in Arabidopsis and found that they showed

![Figure 1. Schematic structures of NtmybC1, NtmybC2, and other MYB3R proteins from tobacco. The numbers between the MYB3R proteins indicate the amino acid identity within and outside the Myb domain. The length of each MYB3R protein is shown on the right. The phylogenetic relationships among the MYB3R proteins from tobacco are also shown on the left.](image-url)
the highest similarity to MYB3R3 and MYB3R5. Amino acid sequence similarity was observed along the entire proteins, both within and outside the Myb domains (Figure 2A, B). Phylogenetic analysis showed that NtmybC1 and NtmybC2 are grouped into the same clade that also contains MYB3R3 and MYB3R5 from Arabidopsis and Os01g62410 and Os05g38460 from rice (Figure 2C). These results confirmed NtmybC1 and NtmybC2 to be new members of the tobacco MYB3R family that constitute a separate subgroup together with MYB3R3 and MYB3R5.

To analyze the roles of NtmybC1 and NtmybC2, we created expression constructs, 35S:NtmybC1 and 35S:NtmybC2, in which their full-length cDNAs were placed under the strong cauliflower mosaic virus (CaMV) 35S promoter, and performed luciferase (LUC) reporter assays using protoplasts prepared from tobacco BY-2 cells. As a reporter construct, we used the NACK1 promoter placed upstream of the LUC reporter gene (pNACK1:LUC). When 35S:NtmybC1 was co-transfected, we observed significantly decreased LUC activities compared with the control without co-transfection of the expression construct (Figure 3A). Confirming our previous results (Ito et al. 2001), transfection of the expression plasmid for NtmybA2 (35S:NtmybA2) resulted in a clear increase in LUC activity. We additionally made an effector construct, 35S:C1MD-VP16, which contained the Myb domain of NtmybC1 fused to the VP16 activation domain placed downstream of a CaMV 35S promoter. Co-transfection of this construct resulted in a clear increase in LUC activity. The extent of this increase was even higher than that caused by 35S:NtmybA2 co-transfection (Figure 3A). These results suggested that NtmybC1 has an ability to bind to the target promoter through the Myb domain to modulate transcriptional activity. It was also suggested that the NtmybC1 may also have a repressing effect on the target promoter, which requires the regions outside the Myb domain, as was previously observed for NtmybB (Ito et al. 2001).

In our previous transfection assays, NtmybB showed an antagonistic repressing effect on transcriptional activation by NtmybA2 (Ito et al. 2001). To test the interactions between different types of MYB3Rs for their action, we transfected two different effector constructs in combination, and the resulting LUC activities were compared with those seen following the transfection of each individual construct. When 35S:NtmybB and 35S:NtmybA2 were co-transfected in combination, we observed intermediate LUC activity compared with that resulting from the separate transfection of either NtmybA2 or NtmybB, which is consistent with our previous view that NtmybA2 and NtmybB may act antagonistically (Figure 3B). The transfection of 35S:NtmybC2 alone significantly decreased LUC activity.

Figure 2. NtmybC1 and NtmybC2 are grouped into a separate MYB3R subfamily, together with the MYB3R repressors from Arabidopsis. (A) Comparison of the amino acid sequences within the Myb domain of NtmybC1 and NtmybC2, in which their full-length cDNAs were placed under the strong cauliflower mosaic virus (CaMV) 35S promoter, and performed luciferase (LUC) reporter assays using protoplasts prepared from tobacco BY-2 cells. As a reporter construct, we used the NACK1 promoter placed upstream of the LUC reporter gene (pNACK1:LUC). When 35S:NtmybC1 was co-transfected, we observed significantly decreased LUC activities compared with the control without co-transfection of the expression construct (Figure 3A). Confirming our previous results (Ito et al. 2001), transfection of the expression plasmid for NtmybA2 (35S:NtmybA2) resulted in a clear increase in LUC activity. We additionally made an effector construct, 35S:C1MD-VP16, which contained the Myb domain of NtmybC1 fused to the VP16 activation domain placed downstream of a CaMV 35S promoter. Co-transfection of this construct resulted in a clear increase in LUC activity. The extent of this increase was even higher than that caused by 35S:NtmybA2 co-transfection (Figure 3A). These results suggested that NtmybC1 has an ability to bind to the target promoter through the Myb domain to modulate transcriptional activity. It was also suggested that the NtmybC1 may also have a repressing effect on the target promoter, which requires the regions outside the Myb domain, as was previously observed for NtmybB (Ito et al. 2001).

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Tobacco MYB3R proteins with repressor function

albeit to a smaller extent, compared with NtmybB, suggesting that NtmybC2, similarly to NtmybC1, has a repressing function (Figure 3B). When 35S:NtmybC2 was transfected together with 35S:NtmybA2, LUC activity dramatically decreased compared with that resulting from the transfection of 35S:NtmybA2 alone but was higher than that produced by the transfection of 35S:NtmybC2 alone (Figure 3B). This finding suggested that, similar to NtmybB, NtmybC2 may have an ability to block the activation of the target promoter that is mediated by NtmybA2, further raising the possibility of an antagonistic interaction between NtmybC2 and NtmybA2. Co-transfection of NtmybC2 and NtmybB in combination resulted in a decrease in LUC activity in comparison with the control, but the extent of this decrease was equivalent to that caused by NtmybB transfection alone (Figure 3B). The absence of an additive effect of NtmybC2 and NtmybB on the target promoter was consistent with an idea that NtmybB and NtmybC2
act in the same pathway, probably by binding to the same promoter element.

To characterize the expression patterns of \textit{NtmybC1} and \textit{NtmybC2}, we analyzed BY-2 cells with different activity for cell proliferation. We used BY-2 cells at different time points after being subcultured. In our culture conditions, cells started to rapidly proliferate by 1 d and showed an exponential increase in cell numbers until 4 d after subculturing. Subsequently, the rate of increase in cell numbers gradually declined, and the cells entered the stationary phase at 7 d, after which no clear change in the cell number was observed (Figure 4A). We collected cells every 24 h after subculturing up to 9 d and used them in quantitative RT-PCR analyses. As a control, we analyzed the transcript levels of representative cell cycle genes including \textit{proliferating cell nuclear antigen (PCNA)} as a G1/S-specific gene and \textit{CYCB1} as a G2/M-specific gene. The levels of their transcripts rapidly increased by 1 d and stayed high for 1–4 d after

![Figure 5. Accumulation patterns of mRNAs for \textit{NtmybC1} and \textit{NtmybC2} in BY-2 cells synchronized by aphidicolin. (A) BY-2 cells were synchronized by treatment with aphidicolin and its removal. Seven-day-old BY-2 cells were treated with aphidicolin (5 mg ml\(^{-1}\)) for 24 h and washed with fresh medium as previously described (Ito et al. 1997). After release from the aphidicolin block, cells were sampled every hour up to 17 h and used to extract the total RNA and measure the mitotic index. (A) Change in the mitotic index during the culture period. (B) RNA gel blot analysis of \textit{NtmybC1}, \textit{NtmybC2}, \textit{NtmybA2}, and \textit{CYCB1}. RNA gel blot analysis and synthesis of randomly primed probes were performed as previously described (Ito et al. 1997). Gene-specific probes for \textit{NtmybA2}, \textit{NtmybC1}, and \textit{NtmybC2} were generated by PCR amplification of the 3’ untranslated region of each cDNA. \textit{rpL25} mRNA was similarly analyzed as a control, showing the equivalent quantity and quality of mRNA loaded in each lane.](#)

![Figure 6. Accumulation patterns of mRNAs for \textit{NtmybC1} and \textit{NtmybC2} in BY-2 cells synchronized by the two-step method. (A) BY-2 cells were synchronized by sequential treatment with aphidicolin and propyzamide. After the cells had been treated with aphidicolin (5 mg ml\(^{-1}\)) for 24 h, they were cultured in fresh medium for 5 h. The cells were then treated with propyzamide (3 \(\mu\)M) for 4 h and washed with fresh medium (Kumagai-Sano et al. 2006). After release from the propyzamide block, the cells were sampled every hour up to 17 h. (A) Change in the mitotic index during the culture period. (B) RNA gel blot analysis of \textit{NtmybC1}, \textit{NtmybC2}, \textit{NtmybA2}, and \textit{CYCB1}. An RNA gel blot analysis was performed as in Figure 5.](#)
synchronous cell cycle progression (Figure 5A). An RNA gel blot analysis indicated that NtmybC1 and NtmybC2 mRNAs showed similar expression changes during the cell cycle (Figure 5B). The amounts of mRNAs from both genes were relatively low just after aphidicolin removal, but they subsequently increased to maximal levels at 6–8h, coinciding with the peak of the mitotic index (Figure 5A). The observed time-course changes in the mRNA levels were similar to those of NtmybA2 and the G2/M-specific CYCB1 gene (Figure 5B). To confirm the cell cycle-dependent expression changes, we applied a two-step induction method to synchronize BY-2 cells, in which cells were first synchronized by aphidicolin treatment and its removal and subsequently treated with propyzamide, a drug that inhibits tubulin polymerization (Kumagai-Sano et al. 2006). During propyzamide treatment, cells were arrested in the mitotic metaphase, and subsequent removal of propyzamide induced the rapid decline of the mitotic index, indicating a synchronous exit from mitosis (Figure 6A). At 13h after release from the propyzamide block, we observed an increase in the mitotic index, which may have corresponded to the onset of mitosis in the next round of the cell cycle. RNA gel blot analysis again showed that the mRNA levels of NtmybC1 and NtmybC2 changed in parallel with the change in the mitotic index throughout the culture period (Figure 6B). Their expression patterns were also similar to those of NtmybA2 and CYCB1. Our expression analysis using the two-step synchronization method again supported their preferential expression at G2/M, excluding the possibility that the observed expression changes were due to the artificial effects of the drugs applied for synchronization and instead were due to the progression of the cell cycle itself.

In this study, we identified two tobacco MYB3R homologs, NtmybC1 and NtmybC2 that constitute a distinct subgroup of MYB3Rs, which also contains MYB3R3 and MYB3R5, with repressing functions on G2/M-specific genes. High sequence similarity outside the conserved Myb domain suggested that similar biological functions are shared among the members in this subgroup. As expected from our knowledge of the role played by Arabidopsis homologs (Kobayashi et al. 2015), NtmybC1 and NtmybC2 showed repressing effects on target promoter activity in transient expression assays of BY-2 protoplasts. In addition, we showed that fusion between the DNA-binding Myb domain from NtmybC1 and the VP16 activation domain activated the NACK1 promoter in a transient expression assay. We interpreted this result as that NtmybC1 with no transactivation ability bound the target promoter through the Myb domain, but when transactivation ability was provided by fusion with VP16, it activated the target promoter. Therefore, in a broad sense, these results confirmed our fundamental view that this group of MYB3Rs may be involved in the negative regulation of target transcription, and this function may be conserved among different plant species.

The expression patterns of NtmybC1 and NtmybC2 in BY-2 cells were also consistent with this view. When mRNA accumulation was examined in asynchronously cultured BY-2 cells, the levels of both NtmybC1 and NtmybC2 mRNAs were roughly equivalent between cells in the logarithmically proliferating phase and those in the stationary phase. In contrast, NtmybA2 transcripts showed a dramatic difference in expression levels before and after the cessation of cell proliferation. These results suggested that, unlike NtmybA2 with a specific function at G2/M, NtmybC1 and NtmybC2 may have functions in both proliferating and quiescent cells as previously reported for MYB3R3 and MYB3R5 in Arabidopsis (Kobayashi et al. 2015).

Our data from the protoplast transfection assays were consistent with the idea that repression mediated by NtmybC1 and NtmybC2 seems to be due to the antagonistic action against NtmybA2 on the target promoter. One explanation for this interaction may be the potential competitive binding of NtmybC2 to the target promoter, which may inhibit the binding of the NtmybA2 activator to the same target. However, contrary to this observation, we previously concluded that there may be no competitive interaction between MYB3R activators and repressors in Arabidopsis (Kobayashi et al. 2015). We observed that cytokinesis defects caused by the loss of MYB3R activators through mutations were not recovered by the additional loss of MYB3R repressors. In our explanation for this observation, we suggested that the activation and repression of target transcription by MYB3Rs do not occur in the same cells at the same time; thus, they do not compete each other. Instead, we hypothesized that the repressing activity of MYB3R may be activated when MYB3R activators are not active outside the G2/M phase of the cell cycle or after exit from cell proliferation (Kobayashi et al. 2015). Therefore, there is a disagreement between the results from tobacco cells presented here and those obtained from our previous genetic studies in Arabidopsis. A number of studies will be required to fully resolve this issue and understand the conserved and divergent aspects of MYB3Rs in plants. Indeed, there may be many possible explanations for this discrepancy. For example, it is possible that the above disagreement is based on the divergence of MYB3R action among different plant species, i.e., tobacco and Arabidopsis. Alternatively, it may be due to the different molecular events that occur in intact plants and cultured cells. It is worth noting that both NtmybC1 and NtmybC2 showed cell cycle-regulated changes in mRNA levels in synchronized cultures of BY-2 cells. This result is in clear contrast to the expression of Arabidopsis homologs, MYB3R3 and MYB3R5 that showed relatively constant
mRNA levels throughout the cell cycle in synchronized MM2d cells (Menges et al. 2005) (Supplementary Figure S1). This suggests that NtmybC1 and NtmybC2 may have some physiological roles during G2/M in the cell cycle and possibly act on G2/M-specific target genes. Because the transcriptional activator NtmybA2 is also expressed during the same phase of the cell cycle, there may be antagonistic interactions between the activator and repressor to quantitatively regulate target transcription. It may be further speculated that such competitive interaction occurs between MYB3R activators and repressors in Arabidopsis when plants are placed under unusual conditions such as environmental stress. We recently showed that MYB3R repressors play a role in active growth repression in Arabidopsis under moderate salt stress by downregulating G2/M-specific genes (Okumura et al. 2021). To comprehensively understand MYB3R action, further study on MYB3R-mediated growth repression under salt and other stress conditions is needed. It would also be of interest to analyze the consequences of the loss-of-function of MYB3R repressors in tobacco and other plant species using the CRISPR-Cas9 system, which is becoming increasingly accessible even for non-model plant species. Such studies would provide a clearer view on the conserved and divergent aspects of the roles and action of MYB3Rs in plants.

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