Supporting Information

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SI Text

Results

Confirmation of the *CmMYB1*-null Mutant Strain. To verify the expected recombination in SI282, we conducted PCR, Northern blot, and immunoblot analyses. These results are shown in Fig. 1*C*, Fig. S2, and Fig. S4. When a set of primers, J282_TF_Check_F1 (F1 in Fig. S2*A* and Table S2) and J282_TF_Check_R1 (R1), were used, we detected 1.6-kb and 2.3-kb bands with M4 and SI282 genomic DNAs as template DNA, respectively, as was predicted (Fig. S2*B*, left). When a set of primers, J282_TF_Check_F2 (F2) and J282_TF_Check_R2 (R2), were used, we detected 4.6-kb and 5.8-kb bands with M4 and SI282 genomic DNAs, respectively, as was predicted (Fig. S2*B*, middle). When a set of primers, URA_UP_R2 (F3) and R2, were used, we only detected a band of 1.8-kb with SI282 genomic DNA, as was predicted (Fig. S2*B*, right). Furthermore, the expression of *CmMYB1* at transcription and protein levels were not detected in SI282 cells (Fig. 1*C* and Fig. S4). These results clearly showed that this recombination involved a double-crossover reaction and successfully disrupted *CmMYB1* gene in SI282.

Complementation Test in the *CmMYB1*-Null Mutant Strain. To confirm that the deficiency of the nitrogen transcriptional regulation is actually resulted from the *CmMYB1* mutation, a plasmid harboring the hemagglutinin (HA)-tagged *CmMYB1* gene, pBSHA-J282 (see *SI Methods*), was constructed and transiently introduced into SI282 cells for the complementation analysis. To verify the expression of HA-tagged CmMYB1 in SI282 cells, we conducted immunoblot analysis using anti-HA and anti-CmMYB1 antibodies (Fig. S3*A* and *B*). When anti-HA antibody was used (Fig. S3*A*), bands of the expected size were detected from pBSHA-J282-introduced SI282 cells irrespective of the nitrogen status. The amount of HA-fused CmMYB1 was significantly increased in response to the nitrogen-depletion, although the protein was detected even under the nitrogen-replete condition, which is different from the results shown in Fig. 1*E*. The reason for these differences is unknown, however, it may be due to the plasmid-based expression of CmMYB1. As the control experiment, no band was observed when the vector plasmid was introduced in the cells. When we used anti-CmMYB1 antibody (Fig. S3*B*), bands were detected similarly as when anti-HA antibody was used (Fig. S3*A*). The protein level under the nitrogen-depleted condition in the complemented cells appeared relatively low as compared with that in the parental strain. Subsequently, we quantified the transcripts of nitrogen assimilation genes, *CmNRT*, *CmNIR*, and *CmGS*, as well as *CmMYB1* gene, in the complemented cells. The results (Fig. S3*C*) indicated that amounts of those transcripts were well correlating to those of CmMYB1 in the complemented cells, indicating the complementation by the plasmid-born CmMYB1. Thus, the result of the complementation analysis indicated that CmMYB1 was actually responsible for the expression of nitrogen assimilation genes.

Specificity of CmMYB1 Antibodies. Antibodies raised against the recombinant CmMYB1 specifically recognized proteins of apparent mass of 82 kDa in *C. merolae* wild type and M4 cells (Fig. 1*C*, −N condition, and Fig. S4, lane 2). This apparent molecular weight was slightly smaller than the signal corresponding to the recombinant CmMYB1, which harbors approximately 10 kDa of extra peptide derived from the His-tag and expression vector linker sequence (Fig. S4, lane 4). The nitrogen depletion-induced CmMYB1 was not detected with the rabbit IgG purified from the preimmune serum (Fig. S4, lane 1). Moreover, the band disappeared when a total protein prepared from SI282 cells was used (Fig. S4, lane 3), indicating that the prepared antibody correctly recognizes the endogenous CmMYB1 protein.

Determination of Transcription Start Sites of *CmNRT*, *CmNIR*, and *CmGS*. The transcription start sites (TSSs) of *CmNRT*, *CmNIR*, and *CmGS* genes were determined by 5′-massively parallel signature sequence (MPSS) analysis: 92 bp for *CmMYB1*, 131 bp for *CmNRT*, 75 bp for *CmNIR*, and 149 , 141 , and 134 bp for *CmGS* upstream from the initiation codon (134-bp upstream point was inferred as the transcription start site for *CmGS* in this study). The MPSS results were consistent with results obtained by primer extension analysis and the full-length EST analysis (1).

Preparation of Recombinant CmMYB1 Protein. For the preparation of a recombinant CmMYB1 protein, we first over-produced His-tagged CmMYB1, however, the recombinant protein forms an insoluble inclusion body. Thus we attempted to over-produce trigger factor-fused CmMYB1, as it is known that trigger factor prevents aggregation of recombinant proteins in *E. coli* (2). Consequently, we over-produced and purified the trigger factor-fused CmMYB1 from the soluble fraction, and used in the EMSA analysis.

Methods

Microarray Analysis. Synthesis of dye-labeled probes was performed using amino-allyl RNA (aRNA) synthesis kit, ver. 2 high yield type (SIGMA). Ten μg synthesized aRNA was coupled with Cy3 or Cy5 dyes and purified with an Amicon YM-30 column (Millipore). Hybridization of the probes on the DNA microarray was done at 57 °C for 16 h, followed by appropriate washing with 2× SSC/0.05% SDS for 10 min at 48 °C, followed by 0.05× SSC at room temperature for 5 min. Arrays were then dried by air-spray before scanning. Detection of the signals on the DNA microarray and calculation of the signal intensities were performed as described (3). Each signal value was normalized by the global normalization method (4). The control experiment with normal growth cells demonstrated that the range of experimental errors in the induction factor was <2.0 and >0.5.

Preparation of DNA for Construction of *CmMYB1*-Null Mutant. The DNA fragment was produced by a 2-step PCR method. First PCR, fragment 1 (1.5 kb of upstream region of *CmMYB1* gene, −1,500 to −1, +1 as the initiation codon) was amplified by PCR with the following primer set: J282_1500up_F (5′- CTGAGCTTGTTGAAGGGTGACAGTG-3′) and J282_R_5′URACm-Gs (5′- *CCTCAGTTCGGTACC*TATGGTTCGTCGGCAAGAGTACGCA -3′, adaptor sequence in italics), and *C. merolae* genomic DNA as a template; fragment 2 (1.5 kb of downstream region of *CmMYB1* gene, +1570 to + 3069) was amplified by PCR with the following primer set: J282_F_5′URACm-Gs (5′- *GTTGAATGAAAGCTT*TAAGTTCGCGCCAATACCGCGCGAT -3′, adaptor sequence in italics) and J282_1500down_R (5′- ATGGCCAGTATCCTGACAAGGAAAATC -3′), and *C. merolae* genomic DNA as a template; fragment 3 (2.3 kb of a selectable marker UMP synthase gene cassette), was amplified by PCR with the following primer set:

5′URACm-Gs_F (5′- *TGCCGACGAACCATA*GGTAC-CGAACTGAGGGGCGAAC-3′, adaptor sequence in italics) and 5′URACm-Gs_R (5′-*ATTGGCGCGAACTTA*AAGCTTTCAT-TCAACGTATTCTTCAAGTCG-3′, adaptor sequence in italics), and pKFURACm-Gs as a template DNA. The second PCR was performed with a set of primers, J282_1500up_F and J282_1500up_R, using fragments 1, 2, and 3, as the template DNAs. The resultant 5.3-kb DNA fragment (the *CmMYB1* upstream region + the selection marker cassette + the *CmMYB1* downstream region from the 5′-end to the 3′-end in this order, Fig. S2*A*) was used for the transformation.

Construction of Chromosomal Deletion Mutant. General and detailed procedures for the transformation and selection of the mutants will be described elsewhere.

Complementation Test. For construction of plasmids for HA epitope-tagged CmMYB1 expression in *C. merolae* cells, ORF of *CmMYB1* was amplified together with 5′-franking region (1.5 kb) from *C. merolae* total DNA with set of primers, 5′-GTACTAGTCTGAGCTTGTTGAAGGGTGACAGT-3′ and 5′-GTACTAGTGACGCCACTCAGGAGCCAGC-3′. Resultant fragment was digested with *Spe*I, and cloned into the *Spe*I site of pBSHAb-T3′ (5) to construct pBSHA-J282. *CmMYB1* gene was inserted in the same orientation as the HA epitope tag, so as to express the CmMYB1 protein as the carboxy-end HA-tagged protein. pBlueScriptSK was used a control plasmid as described previously (5). Plasmids were transiently transformed to *C. merolae* as described (5) with some modifications. After the PEG treatment, cells were incubated overnight in MA2 medium, collected by centrifugation (2,000 × *g* for 5 min), and gently resuspended in either MA2 or the nitrogen free medium. After cultivation for 6 h, gene expressions were examined by quantitative real-time PCR (QRT-PCR) analysis (6) and immunoblot analysis (5) as described previously. Primers used for the cDNA synthesis and QRT-PCR analysis are indicated in Table S2 and Table S3, respectively.

Preparation and Purification of Polyclonal Antibodies for CmMYB1. The *CmMYB1* structural gene was amplified by PCR using *C. merolae* genomic DNA as a template and a set of primers: CMJ282C_En_F, CACCATGGACGACGTGGAGCCTT, and CMJ282C_En_R, GACGCCACTCAGGAGCCA. The PCR-amplified fragment was inserted into pENTR/D-TOPO (Invitrogen) according to the manufacturer's instructions to create pJ282. For preparation of the antigen for production of polyclonal antibodies against CmMYB1, the CmMYB1 structural gene in pJ282 was recombined into the destination vector pDEST-HIS (7) to create pDEST-HIS-J282. The over-production and purification of His-tagged CmMYB1 from insoluble inclusion bodies, and the preparation of polyclonal antibodies against CmMYB1 were performed as described previously (8). For affinity purification of CmMYB1 antibodies, the CmMYB1 structural gene in pJ282 was recombined into the destination vector pDEST-Cold-TF (7) to over-produce the fusion protein as a soluble protein. Over-production and purification of trigger factor-tagged CmMYB1 was achieved as for the cyanobacterial transcription factor NtcA described previously (6). The purified protein (1.5 mg) was conjugated to 2 mL NHS-activated Sepharose 4 Fast Flow (GE Healthcare) and the CmMYB1 antibody was purified with the affinity column according to the manufacturer's instructions. Purification of IgG from the preimmune serum of rabbit was performed with Protein A Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's instructions.

Preparation of DNA Fragments for EMSA Analysis. Each probe for EMSA analyses was amplified by PCR with a set of primers (Table S4) and *C. merolae* genomic DNA as a template, and 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara). Protein-DNA complexes were separated by electrophoresis on 5% polyacrylamide gel in 0.6xTBE at room temperature. The gels were dried, and the signals were visualized by a BAS1000 Bio-Image Analyzer (Fujifilm).

1. Matsuzaki M, et al. (2004) Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. *Nature* 428:653–657.
2. Nishihara K, Kanemori M, Yanagi H, Yura T (2000) Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Appl Environ Microbiol* 66:884–889.
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5. Ohnuma M, Yokoyama T, Inouye T, Sekine Y, Tanaka K (2008) Polyethylene glycol (PEG)-mediated transient gene expression in a red alga, *Cyanidioschyzon merolae* 10D. *Plant Cell Physiol* 49:117–120.
6. Imamura S, Tanaka K, Shirai M, Asayama M (2006) Growth phase-dependent activation of nitrogen-related genes by a control network of group 1 and group 2 factors in a cyanobacterium. *J Biol Chem* 281:2668–2675.
7. Imamura S, Hanaoka M, Tanaka K (2008) The plant-specific TFIIB-related protein, pBrp, is a general transcription factor for RNA polymerase I. *EMBO J* 27:2317–2327.
8. Imamura S, et al. (2003) Purification, characterization, and gene expression of all sigma factors of RNA polymerase in a cyanobacterium. *J Mol Biol* 325:857–872.

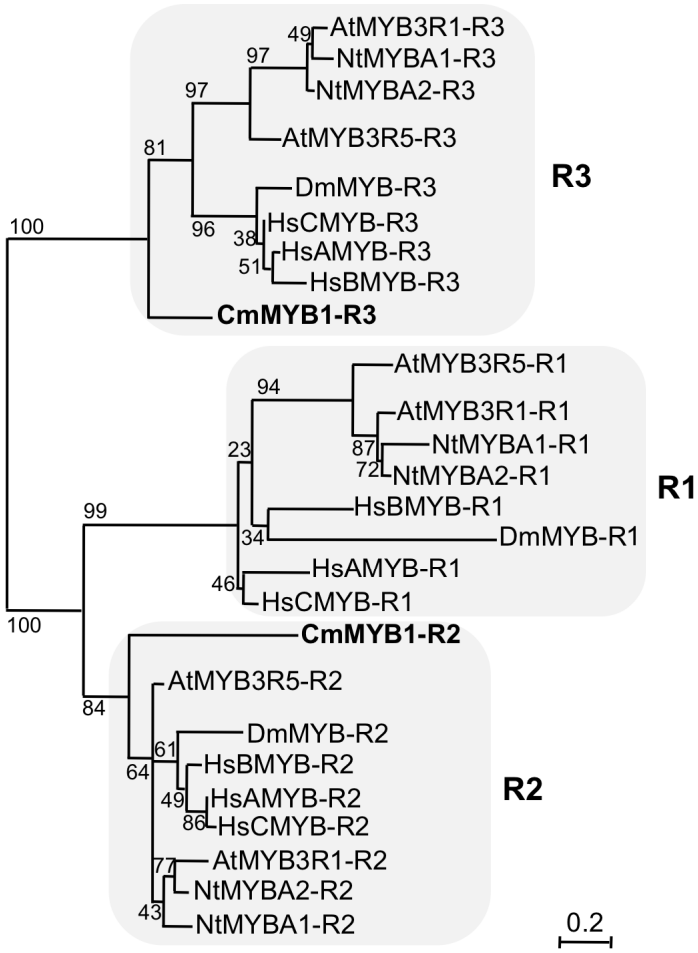

Fig. S1. Evolutionary relationship of MYB domain proteins. A maximum-likelihood tree based on 47 unambiguously aligned amino acid positions of 26 MYB domain proteins was constructed. Numbers at each node represent the percentage of trees supporting the specific branching pattern in the bootstrap analysis. Branch lengths are proportional to the number of amino acid substitutions, indicated by the scale bar below the tree. Designations and GenBank accession numbers for sequences of the MYB domain proteins are as follows: CmMYB1 for *Cyanidioschyzon merolae* CMJ282C (http://merolae.biol.s.u-tokyo.ac.jp/); AtMYB3R1 and AtMYB3R5 for *Arabidopsis thaliana* MYB3R-1 (NM_202989) and MYB3R-5 (NM_120310); NtMYBA1 and NtMYBA2 for *Nicotiana tabacum* NtmybA1 (AB056122) and NtmybA2 (AB056123); DmMYB for *Drosophila melanogaster* Myb (NM_206734); and HsAMYB, HsBMYB, and HsCMYB for *Homo sapiens* MYBL1 (NM_001080416), MYBL2 (NM_002466), and MYB (NM_005375). R1, R2, and R3 with hyphen (−) after each designation indicate R1-, R2-, and R3-type MYB domain, respectively.

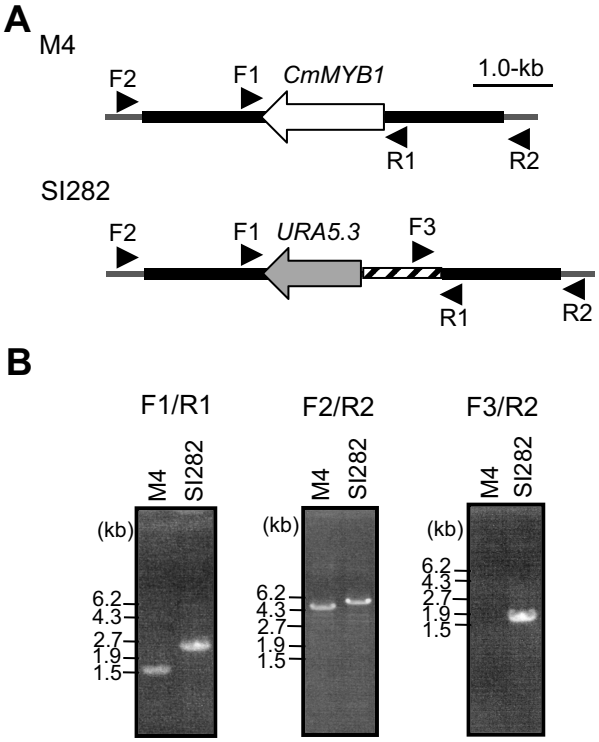

Fig. S2. Confirmation of a *CmMYB1* null mutant strain. (*A*) Schemas for the loci of *CmMYB1* in M4 (the parental strain) and SI282 strains. White and gray arrows indicate *CmMYB1* and the marker UMP synthase gene (*URA5.3*), respectively. Thick black and hatched lines indicate upstream or downstream region of *CmMYB1* gene and upstream region of the marker cassette, respectively. The positions of primers for PCR are shown with arrowheads. The bar, 1.0 kb. (*B*) Confirmation of the transformation. The genomic DNA was analyzed by PCR with a set of primers (Table S2), F1/R1 (*Left*), F2/R2 (*Center*), or F3/R2 (*Right*). The PCR products were resolved by 1.0% agarose gel electrophoresis. The positions of a molecular size marker are indicated as kb at the left.

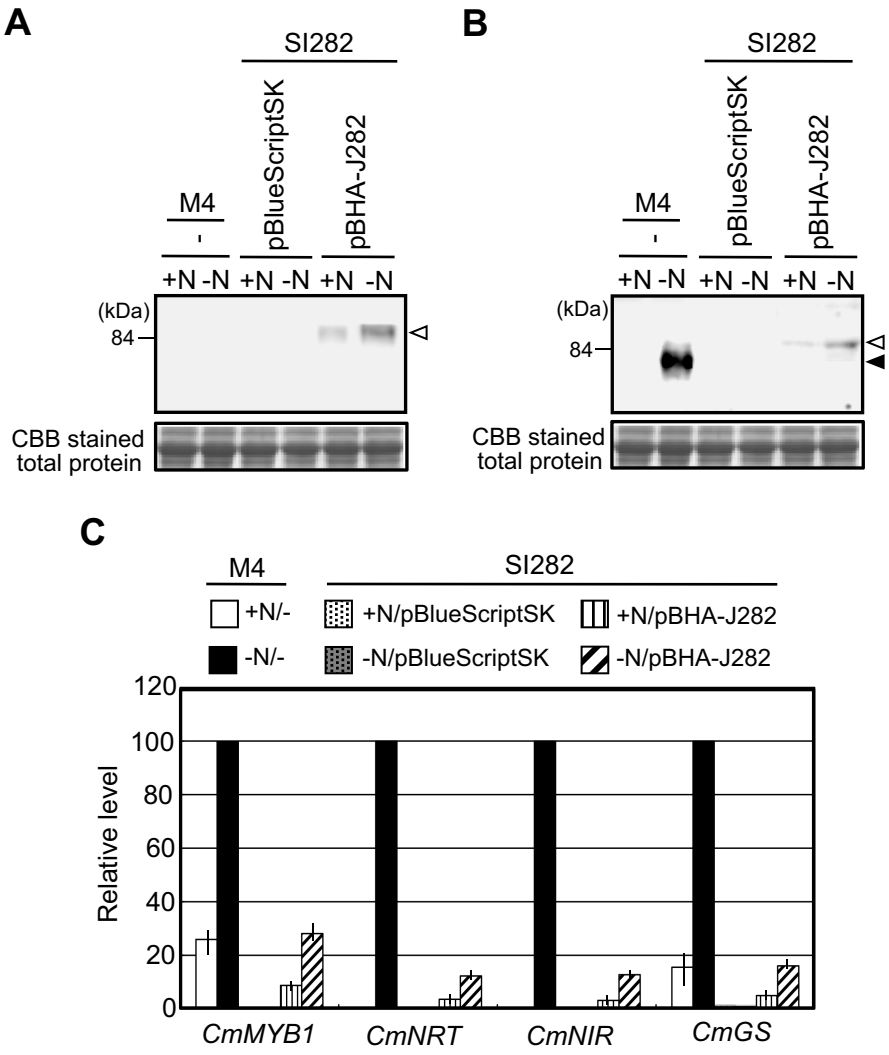

Fig. S3. Complementation test in SI282 mutant. (*A* and *B*) Expression of the HA-tagged CmMYB1 protein in SI282. The combination of used cells and plasmids for the transformations and nitrogen conditions were indicated at the Top. Aliquots containing 20 μg total protein prepared from the cells were subjected to immunoblot analysis with antibodies to HA-epitope (*A*) or CmMYB1 (*B*). White and black arrowheads indicate positions of HA-fused CmMYB1 and CmMYB1, respectively. Total protein ($\approx$ 55 kDa) stained with Coomassie Brilliant Blue (CBB) is shown as a loading control (*Bottom*). (*C*) Transcripts of *CmMYB1*, *CmNRT*, *CmNIR*, and *CmGS* in SI282 mutant in which HA-fused CmMYB1 was transiently expressed. The combination of used cells and plasmids for the transformations and nitrogen conditions were indicated at the Top. Total RNA (5 μg) was prepared from the cells and subjected to cDNA synthesis followed by QRT-PCR analysis. The levels of transcripts of *CmMYB1*, *CmNRT*, *CmNIR*, and *CmGS* are presented (n = 3, means $\pm$ SD.) as relative values (value for M4/–N/– as 100%).

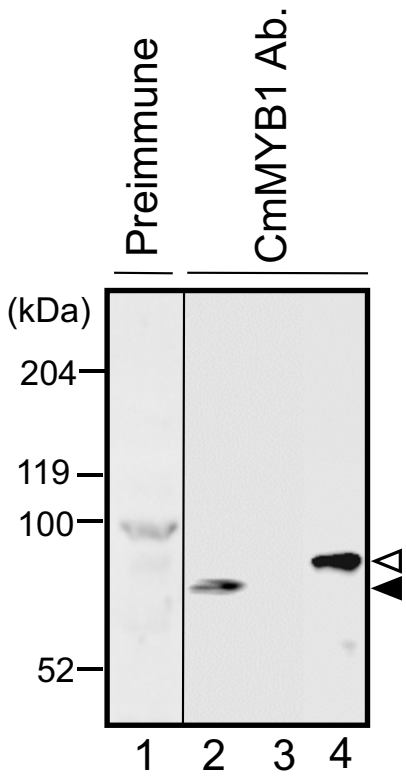

Fig. S4. Preparation and specificity of CmMYB1 antibody. M4 or SI282 cells were harvested at 4 h in −N conditions and the total protein was isolated from the cells. Aliquots containing 20 μg of total protein prepared from M4 (lanes 1 and 2) or SI282 cells (lane 3), or His-tagged CmMYB1 (15 ng) were subjected to immunoblot analysis with indicated antibodies. Black and white arrowheads indicate the position of endogenous CmMYB1 and His-tagged CmMYB1, respectively. His-tagged CmMYB1 harbors approximately 10 kDa extra peptide derived from the His-tag and expression vector linker sequence, expressed in *E. coli* (lane 4).

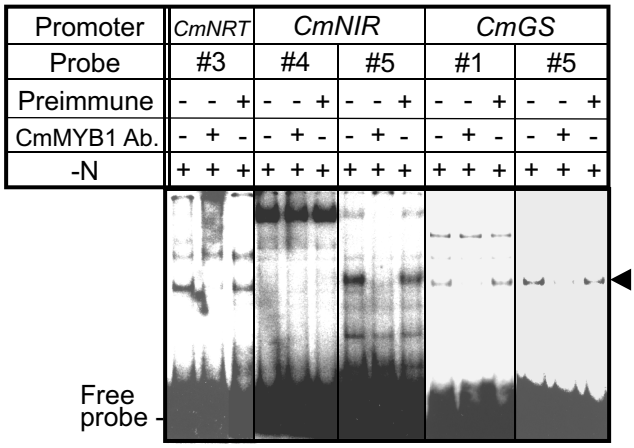

Fig. S5. Effect of CmMYB1 antibody against the protein-DNA complexes. EMSA analysis was performed as Fig. 4 with the indicated antibodies. The protein extract was incubated for 30 min with 0.2 μg purified anti-CmMYB1 antibody (CmMYB1 Ab.), or the purified IgG from the preimmune serum (Preimmune), at 4 °C either before or after the addition of the probe. Arrowheads indicate positions of specific protein-DNA complexes that were specifically detected when proteins prepared from the −N condition were used.

Other Supporting Information Files

Table S1 (PDF)
Table S2 (PDF)
Table S3 (PDF)
Table S4 (PDF)