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MYB-Related Transcription Factor NtMYB2 Induced by Wounding and Elicitors is a Regulator of the Tobacco Retrotransposon *Tto1* and Defense-Related Genes

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Transposition of the tobacco retrotransposon *Tto1* is regulated mainly by transcription from the long terminal repeat (LTR). Functional analysis of the LTR showed that the 13-bp motif is a *cis*-regulatory element involved in activation by tissue culture, wounding, and treatment with elicitors. The 13-bp motif contains a conserved motif (L box) that has been implicated in the expression of phenylpropanoid synthetic genes in response to defense-related stresses. To gain further insight into the regulatory mechanism of the retrotransposon and defense-related genes, cDNAs encoding four different proteins binding to the 13-bp motif have been isolated and characterized. One protein is identical to the previously reported NtMYB1, the RNA for which is induced by virus infection; the others are also MYB-related factors. One of these factors, NtMYB2, was analyzed in detail. *NtMYB2* mRNA was induced by wounding and by treatment with elicitors. NtMYB2 activated expression from the promoter with the 13-bp motif and from the promoter of the phenylalanine ammonia lyase gene (*Pv-PAL2*) in tobacco protoplasts. Overexpression of *NtMYB2* cDNA in transgenic tobacco plants induced expression of *Tto1* and a *PAL* gene. Together, these results indicate that NtMYB2 is involved in the stress response of the retrotransposon and defense-related genes.

INTRODUCTION

Retrotransposons (or class I elements) are widely distributed in eukaryotic genomes. Differing from DNA-type transposable elements (or class II elements) such as *Activator/Dissociation* (*Ac*/*Ds*), *Suppressor-Mutator* (*Spm-Mu*), and *Mu* elements of maize and *Tam* elements of snapdragon (Coen et al., 1989; Fedoroff, 1989), retrotransposons transpose through an RNA intermediate. By reverse transcription, DNA copies are synthesized and reinserted into the genome. Retrotransposons are classified into two groups: long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. Studies have revealed that LTR retrotransposons are major components of the plant genome (Pearce et al., 1996; SanMiguel et al., 1996; Bennetzen et al., 1998). Retrotransposons have contributed significantly to the remarkable variations in genome size and may also have contributed to genomic evolution by changing the structures and expression patterns of genes (White et al., 1994).

For understanding the process of the genome and gene evolution, elucidation of the mechanisms of regulation of retrotransposons is crucial. In plants, diverse sequences of retrotransposons have been found in >100 species (Flavell et al., 1992; Voytas et al., 1992; Hirochika and Hirochika, 1993). Despite this wide and abundant distribution in plants, most of retrotransposons are presumed to be inactive because of their defective structures. Only *Tnt1* and *Tto1* of tobacco and *Tos17* of rice have been demonstrated to be active (Grandbastien et al., 1989; Pouteau et al., 1991; Hirochika, 1993; Hirochika et al., 1996a). Both transposition and transcription of these retrotransposons are inactive under normal conditions. Under stress conditions, however, such as protoplasting (*Tnt1*) or tissue culture (*Tto1* and *Tos17*), transcription and transposition are induced. These data indicate that transposition of these retrotransposons is primarily regulated at the transcriptional level. The *Tnt1* LTR promoter is activated by several defense-related stresses in addition to protoplasting, such as pathogen infection, wounding, and treatment with microbial elicitors, salicylic acid (SA), and $CuCl_2$ (Pouteau et al., 1994; Grandbastien et al., 1997; Grandbastien, 1998, and references therein). Detailed analysis of the *Tnt1* LTR showed that the 31-bp repeated sequence named BII box is one of the *cis* elements involved in the response to protoplasting and treatment with elicitors (Casacuberta and Grandbastien, 1993; Vernhettes et al., 1997).

The *Tto1* LTR is also activated by defense-related stresses in addition to tissue culture, such as wounding, protoplasting, virus infection, and treatment with SA, methyl jasmonate, and fungal elicitors such as extracts of *Trichoderma viride* (cellulase Onozuka R10), chitin oligomer, and xylanase (Hirochika and Otsuki, 1995; Hirochika et al., 1996b; Takeda et

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al., 1998, 1999). *cis* elements in the *Tto1* LTR have been defined by deletion and gain-of-function analysis (Hirochika et al., 1996b; Takeda et al., 1999). One of the *cis* elements, a 13-bp motif repeated twice in the LTR, has been shown to be sufficient for the response to stresses (Takeda et al., 1999).

Interestingly, the *Tnt1* BII box and the *Tto1* 13-bp motif contain the H box ($CCTACC[N]_7CT$) and the complementary sequence of the L box (also called the AC-I motif, TCTCACCTACC), respectively, and share the core sequence of the H box (CCTACC). The L box and H box–related sequences are highly conserved in promoter regions among defense-related genes for enzymes involved in phenylpropanoid metabolism, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), and have been suggested to be involved in the regulation of stress-responsive or tissue-specific expression of these genes (Lois et al., 1989; Loake et al., 1992; Hatton et al., 1995; Faktor et al., 1996; Seki et al., 1997). The L box was originally identified as an inducible in vivo DNA footprint in the parsley *PAL-1* gene promoter after treatment of cells with UV light and treatment with an elicitor derived from the pathogenic fungus (Lois et al., 1989). Two protein factors, KAP-1 and KAP-2, which bind to three H boxes in the bean promoter, were purified by DNA affinity chromatography (Yu et al., 1993). A basic domain/leucine zipper–type DNA binding factor of soybean, G/HBF-1, was recently cloned as a factor that bound to both the G box and the H box of the promoter (Dröge-Laser et al., 1997). G/HBF-1 was rapidly phosphorylated by elicitation, and phosphorylation enhanced its binding to the *chs15* promoter. The L box and H box–related sequences of the bean *PAL2* gene, which are essential for expression in the petal, were found to be recognized and activated by a flower-specific R2R3-type MYB-related protein, Am MYB305 (Sablowski et al., 1994, 1995). R2R3-type MYB-related proteins have been suggested to play a role in the response to UV and pathogens.

In this study, we cloned cDNAs encoding three different proteins binding to the 13-bp motif of *Tto1*. One of these factors, NtMYB2, is transcriptionally regulated and induced by wounding and treatment with fungal elicitors. We also show that NtMYB2 activates the *Tto1* LTR promoter and the *PAL* gene promoter by binding to the 13-bp motif and the L box and H box–like motif, respectively, in tobacco protoplasts and also in transgenic tobacco plants. These results indicate a role for NtMYB2 in activating retrotransposons and phenylpropanoid biosynthetic genes in response to environmental stresses.

RESULTS

The cDNAs Isolated Encode Proteins That Interact with the 13-bp Motif

To understand the regulatory mechanisms of the 13-bp motif, cDNAs encoding proteins that associate with the 13-bp motif were isolated from tobacco by using a yeast one-hybrid system (Wang and Reed, 1993). We constructed a yeast reporter strain carrying an integrated copy of an *His3* allele with six copies of the 13-bp motif cloned upstream of the *His3* minimal promoter as a selectable marker gene. The cDNAs of a tobacco cell line BY2 protoplast in which the transcription of *Tto1* was most active (Hirochika, 1993) were expressed in the reporter strain as a translational fusion with a GAL4 activation domain (AD/cDNA library). Twenty-nine colonies that grew on the selection media were obtained from the AD/cDNA library, which contains 2.6×10^5 independent clones. To further select cDNA clones encoding proteins that bind to the 13-bp motif, proteins extracted from these colonies were analyzed by the electrophoretic mobility shift assay (EMSA). The results of the EMSA showed that extracts from seven of 24 colonies contained proteins that could bind to the wild-type probe but not to the mutant probe of the 13-bp motif. The seven cDNAs from these clones were classified into three groups by sequence analysis. Three of the proteins predicted by cDNA sequence have a putative DNA binding domain conserved among a class of transcription factors, the MYB family. Because one of these MYBs is identical to NtMYB1, the RNA for which was induced by SA treatment or tobacco mosaic virus (TMV) infection (Yang and Klessig, 1996), we named the others NtMYB2 and NtMYB3 (formerly called LBM1 and LBM2, respectively) (Figure 1A). Five, one, and one of the seven cDNAs obtained encode NtMYB2, NtMYB3, and NtMYB1, respectively. The five cDNAs encoding NtMYB2 are grouped into two types based on their polyadenylation sites; the longer cDNA is registered as AB028649 and the polyadenylation site in the shorter cDNA is located at nucleotide (nt) 1060, 377 bp upstream of the original site in the longer cDNA. Because the upstream polyadenylation site is located in the noncoding region, both cDNAs encode proteins of the same size. Sequence analysis revealed that none of the seven clones fused translationally with a GAL4 activation domain, indicating that NtMYB1, NtMYB2, and NtMYB3 work as positive regulators in yeast by themselves. Another cDNA clone, encoding a new MYB (NtMYB4; formerly called LBM4), was obtained by screening cDNA libraries on the basis of homology with *NtMYB2*. The EMSA showed that NtMYB4 can bind to the 13-bp motif (data not shown).

Computer analysis of amino acid sequence maximum matching showed that NtMYB2 is 70, 71, and 94% identical to NtMYB3, NtMYB1, and NtMYB4, respectively, and that NtMYB3 is 89% identical to NtMYB1. These NtMYB proteins contain two imperfect repeats of $\sim$50 residues (R2 and R3), as do most R2R3-type MYB-related proteins in plants (Martin and Paz-Ares, 1997). In contrast, MYB proteins from animals and the recently reported plant MYB-related proteins PpMYB3R-1 and AtMYB3R-1 (Kranz et al., 2000) contain three repeats (R1, R2, and R3). The multiple alignment of R2 and R3 sequences of NtMYB proteins and representative members of the MYB family is shown in Figure 1B. The amino acid sequences of the NtMYB family are highly

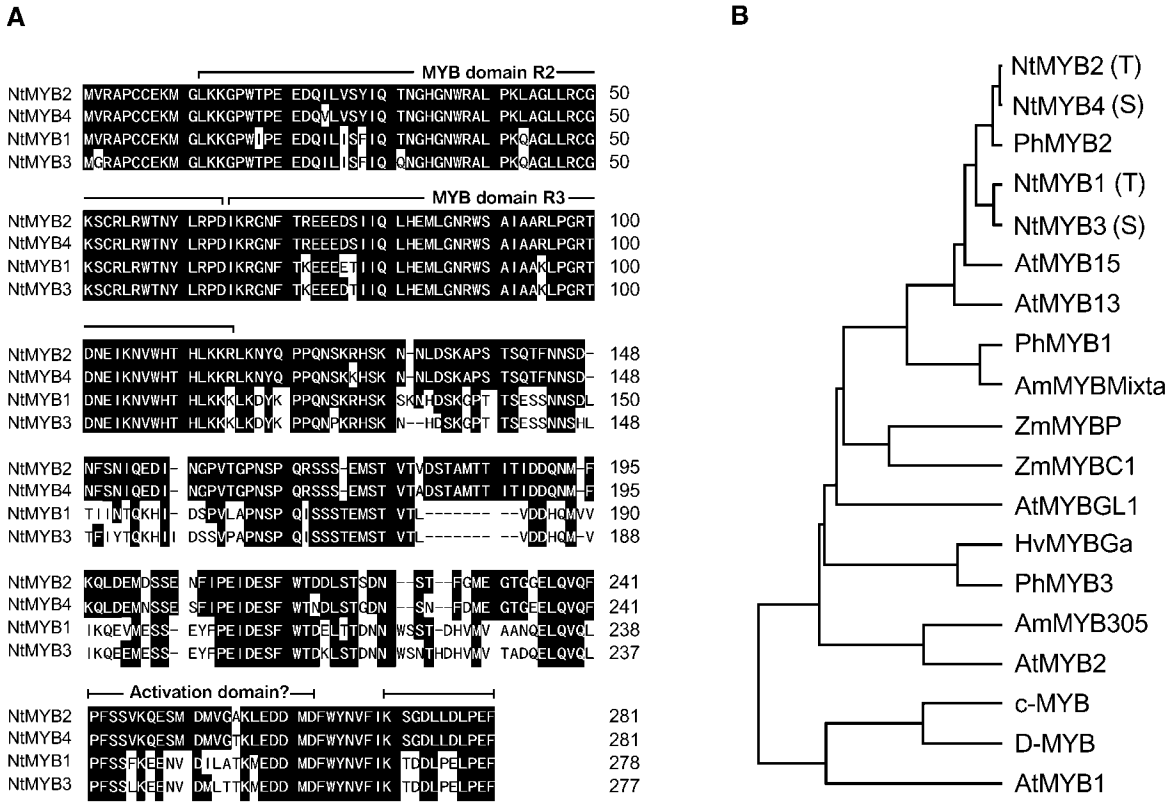

Figure 1. Amino Acid Sequences of NtMYB Proteins and Sequence Comparisons among MYB Proteins.

(A) Amino acid sequences of NtMYB2, NtMYB3, NtMYB1, and NtMYB4. The R2 and R3 of MYB domain and putative acidic activation domains are indicated by bars.

(B) Dendrogram relationships among R2R3 domain of MYB proteins constructed from the matrix of sequence similarities. MYB proteins included in this comparison are D-MYB from Drosophila (Katzen et al., 1985); c-MYB from human (Majello et al., 1986); C1 (ZmMYBC1; Paz-Ares et al., 1987) and P (ZmMYBP; Grotewold et al., 1991) from maize; Am305 (AmMYB305; Jackson et al., 1991) and MIXTA (AmMYBMixta; Noda et al., 1994) from *Antirrhinum majus*; GL1 (AtMYBGL1; Oppenheimer et al., 1991), AtMYB1 (Shinozaki et al., 1992), AtMYB2 (Urao et al., 1993), AtMYB13 (Kirik et al., 1998a), and AtMYB15 (Quaedvlieg et al., 1996) from Arabidopsis; PhMYB1, PhMYB2, and PhMYB3 from petunia (Avila et al., 1993); GA-MYB from barley (HvMYBGa; Gubler et al., 1995); NtMYB1 from tobacco (Yang and Klessig, 1996); NtMYB2, NtMYB3, and NtMYB4 from tobacco (this study). NtMYBs indicated with (T) and (S) are derived from *N. tomentosiformis* and *N. sylvestris*, respectively.

homologous with that of a plant MYB-related protein, PhMYB2, which is constitutively expressed in whole tissues of petunia (Avila et al., 1993). For example, NtMYB2 is 81% identical to PhMYB2.

In DNA gel blot analysis of genomic DNA from the amphidiploid *Nicotiana tabacum* and its diploid parental species, *N. sylvestris* and *N. tomentosiformis*, *NtMYB2*, *NtMYB3*, *NtMYB4*, and *NtMYB1* gene fragments (3′ region of each cDNA) were used as probes. The results showed that *NtMYB2* and *NtMYB1* are derived from *N. tomentosiformis*, whereas *NtMYB4* and *NtMYB3* are derived from *N. sylvestris* (data not shown). These results suggest that *NtMYB2* and *NtMYB1* are orthologous to *NtMYB4* and *NtMYB3*, respectively.

NtMYB2 Expression Is Induced by Wounding and Elicitors

Given that the 13-bp motif confers wound responsiveness, the expression of *NtMYB2*, *NtMYB3*, and *NtMYB1* in response to wounding was examined. The RNA gel blots prepared from leaf segments that had been incubated on 0.05% Mes buffer, pH 5.7, for 0.5, 1, 2, 4, or 12 hr were probed with *NtMYB2*, *NtMYB3*, and *NtMYB1* cDNA fragments. Among these samples, *NtMYB2* RNA was induced by wounding (Figure 2A), whereas the expression of *NtMYB3* and *NtMYB1*, if any, was below the detection limit in both wounded and control leaves (data not shown). The *NtMYB2* mRNA reached a maximum ~0.5 hr after wounding

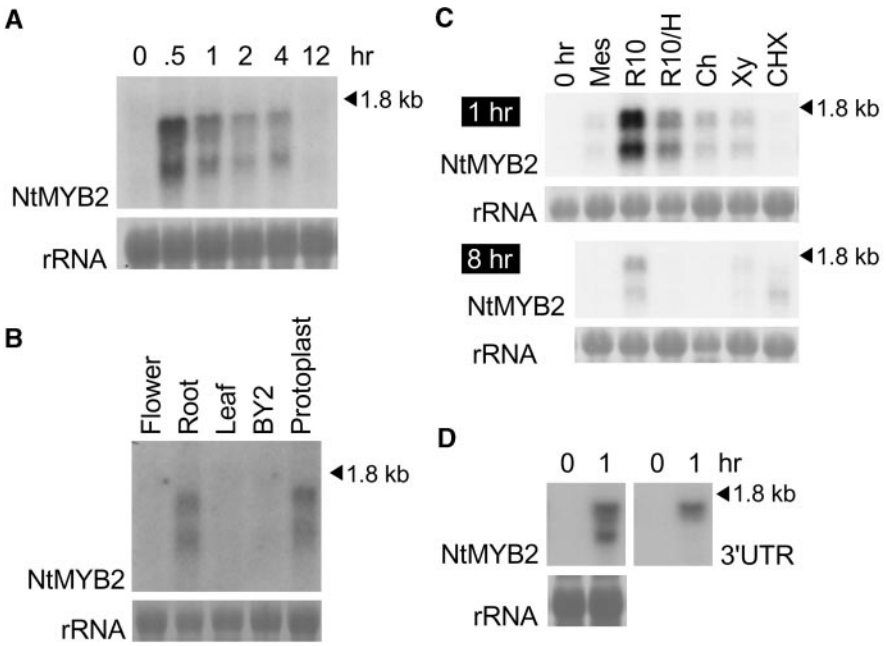

Figure 2. RNA Gel Blot Analysis of *NtMYB2* Transcripts.

(A) Each lane was loaded with 20 μg of total RNA from leaf segments harvested at 0, 0.5, 1, 2, 4, and 12 hr after cutting and was then probed with an *NtMYB2*-specific DNA fragment. The 28S rRNA stained with methylene blue is shown under each RNA gel blot to assure equal loading of RNA.

(B) Each lane was loaded with 5 μg of total RNA from flowers, roots, leaves, BY2 suspension-cultured cells, and protoplasts of BY2 and probed with an *NtMYB2* gene–specific DNA fragment.

(C) Five micrograms of RNA from detached whole leaves that had been treated for 1 or 8 hr with Mes buffer in the absence (Mes) or presence of 1 mg/mL Onozuka cellulase solution (R10), autoclaved Onozuka cellulase solution (R10/H), 100 μM chitin oligomer (Ch), 2.5 μg of xylanase per gram of leaf (Xy), and cycloheximide (CHX) were blotted and probed with an *NtMYB2*-specific DNA fragment.

(D) Five micrograms of RNAs from leaf segments was harvested at 0 and 1 hr after cutting and was probed with an *NtMYB2*-specific DNA fragment (NtMYB2) or a 3′-UTR. The position of the 18S rRNA is indicated by an arrowhead (1.8 kb).

and returned to its basal value within 12 hr. For further examination of the expression of *NtMYB2* RNA, total RNAs were extracted from different tissues and analyzed by RNA gel blotting. The *NtMYB2* RNA was detected in roots and protoplasts but was almost undetectable in nontreated leaves, flowers, and cultures of BY2 cell line (Figure 2B).

Because the 13-bp motif responds to elicitors such as cellulase Onozuka R10, chitin oligomer, and xylanase (Takeda et al., 1999), we also examined the induction of *NtMYB2* expression in response to such elicitors. RNAs were extracted from leaves 1 or 8 hr after the leaf petioles had been dipped into elicitor solutions and were analyzed by gel blot. *NtMYB2* RNA was rapidly induced by treatment with Onozuka cellulase solution (Figure 2C; R10). Heat-inactivated Onozuka cellulase solution also activated *NtMYB2* expression (Figure 2C; R10/H). Fungal cell wall components, such as chitin oligomer, are known to induce defense responses in plants (Ryan and Farmer, 1991). Treatment with 100 μM (and also 10 μM; data not shown) chitin oligomer (5- to 6-mer), which is probably one of the components of the Onozuka cellulase solution, also induced *NtMYB2* expression in leaves (Figure 2C; Ch). Onozuka cellulase solution also contains xylanase activity (Nagata et al., 1981; Fuchs et al., 1989). The elicitor activity of xylanase induces a range of defense responses in tobacco, inducing biosynthesis of ethylene (Fuchs et al., 1989) and of pathogenesis-related (PR) proteins (Lotan and Fluhr, 1990; Raz and Fluhr, 1993). Xylanase also induces *NtMYB2* RNA (Figure 2C; Xy).

Wounding induced two different *NtMYB2* RNAs, of ~1.2 and 1.5 kb, which probably correspond to two types of cDNAs. This was confirmed by RNA gel blotting as follows. Both RNAs were detected by the *NtMYB2*-specific probe (Figure 2D; *NtMYB2*), but the 1.2-kb RNA was not detected by the *NtMYB2* 3′-untranslated region (3′-UTR) probe (Figure 2D). Wounding also induced *NtMYB4*, an ortholog of *NtMYB2*, but only a 1.5-kb RNA was detected (data not shown).

NtMYB2 Activates Transcription from the *Tto1* LTR Promoter and the Minimal Promoter with the 13-bp Motif by Binding to the 13-bp Motif

The correlation between the expression patterns of *NtMYB2* and *Tto1* suggests that *NtMYB2* is involved in stress responses of *Tto1*. To address this point, we examined whether the overexpression of *NtMYB2* activates the transcription from the promoter having the 13-bp motif, depending on the affinity of *NtMYB2* to the 13-bp motif in vitro. The in vitro affinities of *NtMYB2* to the wild type and mutants of the 13-bp motif were compared by EMSA (Figure 3A). A complex formed between in vitro–translated *NtMYB2* and the ^{32}P-labeled fragment for *Tto1* LTR −92 to −52 (LTR4 probe), which contains one of the 13-bp motif repeats. The complex formation was competed with by an

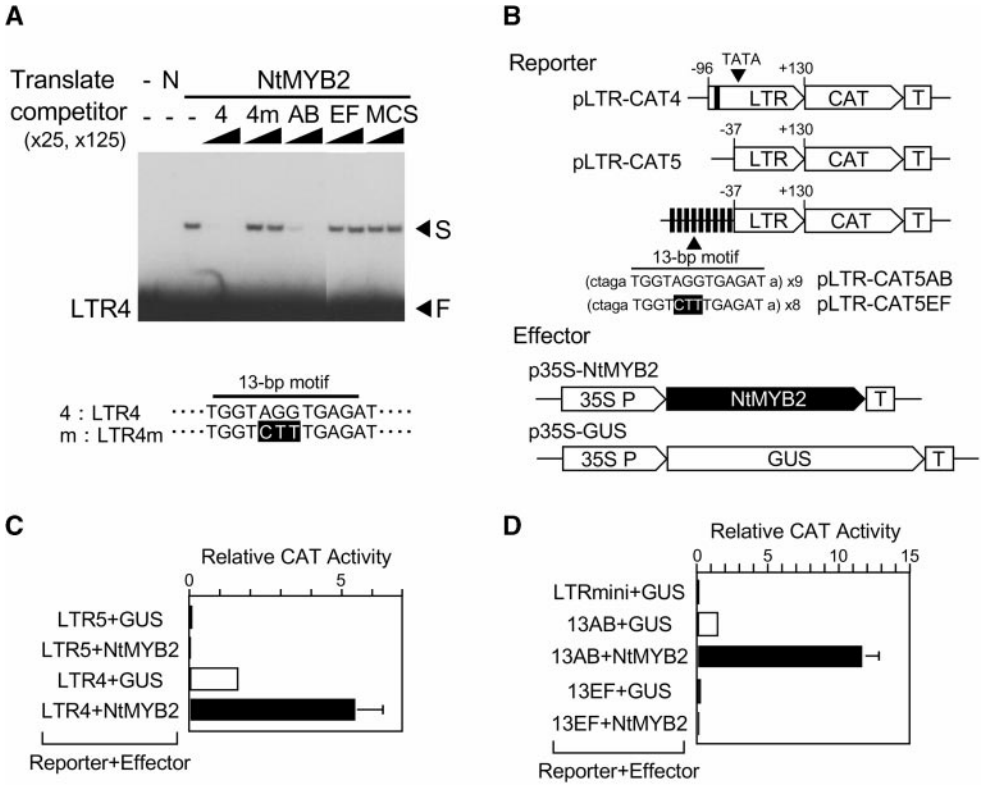

Figure 3. NtMYB2-Mediated *trans*-Activation Depends on the Binding to the 13-bp Motif.

(A) Competition assays for binding of in vitro–translated NtMYB2 to the labeled LTR (−92 to −52; LTR4) probe. EMSA was performed by preincubating 25-fold (×25; left lane under the triangle) or 125-fold (×125; right lane under the triangle) excess amounts (based on the number of binding site) of unlabeled competitor DNA fragments, LTR (−92 to −52) (4), mutant LTR (4m), multimerized wild type (AB), multimerized mutant 13-bp motif (EF), or a multicloning site sequence of pBlueScript SK+ (MCS). Dash, without translate or competitor; N, in vitro translate without NtMYB2 plasmid (pSK-NtMYB2); F, free probe; S, shifted probe. The difference in the sequence between the wild-type (4) and mutant (m) LTR fragments used as unlabeled competitors is shown, highlighting the substituted nucleotides.

(B) Structures of the reporter and effector plasmids. pLTR-CAT4, LTR (−96 to +130) promoter fused to the *CAT* gene; pLTR-CAT5, LTR (−37 to +130) minimal promoter fused to the *CAT* gene (Hirochika et al., 1996b). pLTR-CAT5AB and pLTR-CAT5EF, 13-bp motif (AB) or mutant (EF; substituted nucleotides are indicated on a black background), were multimerized (×9, nine copies; ×8, eight copies) and inserted upstream of the LTR. Nucleotides in uppercase correspond to the 13-bp motif. The effector plasmid consists of 35S promoter fused to the *NtMYB2* cDNA (p35S-NtMYB2). The plasmid consisting of the 35S promoter fused to the *GUS* gene (p35S-GUS) was used as a control.

(C) The effector plasmid (p35S-NtMYB2, NtMYB2) or the control plasmid (p35S-GUS, GUS) was cotransfected with reporter constructs (pLTR-CAT4, LTR4; pLTR-CAT5, LTR5) into tobacco protoplasts. Relative CAT activity (and standard deviation) obtained from three independent experiments is shown.

(D) The effector or the control plasmid was cotransfected with reporter constructs (pLTR-CAT5AB, 13AB; pLTR-CAT5EF, 13EF) into tobacco protoplasts.

Error bars indicate SE.

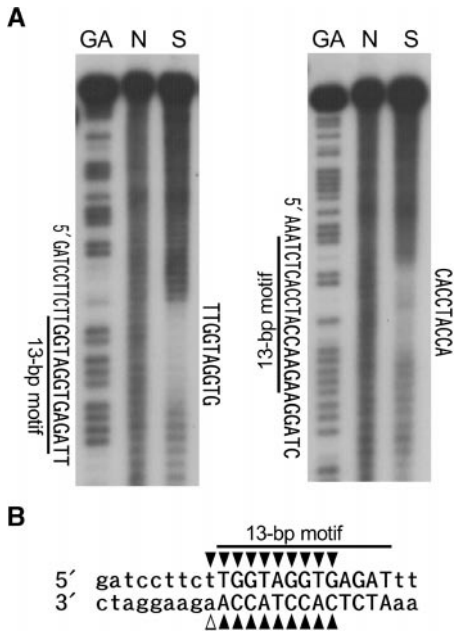

Figure 4. Hydroxyl Radical Interference Analysis of NtMYB2 Binding to the 13-bp Motif.

(A) Hydroxyl radical–treated LTR probe (−92 to −52), which was asymmetrically labeled with ^{32}P-dCTP, was incubated with in vitro–translated NtMYB2 and subjected to native PAGE. Shifted DNA (S) was compared with free probe (N) on the sequencing gel. G + A reactions were loaded as a marker (GA). Both DNA strands were analyzed.
(B) Summary of nucleotides for which a single-base deletion led to a decrease (open triangle) or failure (closed triangles) of the NtMYB2 binding. Nucleotides in uppercase letters correspond to the 13-bp motif.

excess amount of the unlabeled LTR4 probe and the wild-type 13-bp motif (AB). In contrast, the mutant motif (EF) and the mutant LTR4 (Figure 3A; LTR4m) lost their ability to compete, indicating that the mutated three bases are of critical importance for binding NtMYB2. This mutation (EF) also erased the reporter gene expression in response to wounding in transgenic tobacco (Takeda et al., 1999).

The reporter construct in which a chloramphenicol acetyl transferase (CAT) coding region is fused to the LTR promoter from nt −96 to +130 possesses one of the two NtMYB2 binding sites in the *Tto1* LTR (pLTR-CAT4; Figure 3B). The minimal LTR promoter from nt −37 to +130, which has the TATA box and has lost the NtMYB2 binding site, was used as a control reporter construct (pLTR-CAT5; Figure 3B). The CAT activity induced by pLTR-CAT4 in BY2 protoplasts was stimulated fourfold when the *NtMYB2* expression plasmid was cotransfected (p35S-NtMYB2; Figure 3B), whereas pLTR-CAT5, which has no NtMYB2 binding site, was not activated by *NtMYB2* overexpression (Figure 3C). To further confirm that the activation by *NtMYB2* overexpression depends on the 13-bp motif, we examined reporter constructs having nine or eight copies of wild-type or mutant 13-bp motifs inserted upstream of the LTR minimal promoter (−37 to +130) of the control construct (Figure 3B; pLTR-CAT5). These constructs (pLTR-CAT5AB and pLTR-CAT5EF) were cotransfected with either the *NtMYB2* expression construct or the control plasmid (p35S–β-glucuronidase [GUS]). The AB reporter was activated by *NtMYB2* expression, whereas the EF reporter was not (Figure 3D). These results show that NtMYB2 can activate the LTR promoter, depending on the binding to the 13-bp motif in tobacco protoplasts.

The NtMYB2 Binding Site Was Determined by Hydroxyl Radical Interference and Mutational Analysis

To further confirm NtMYB2 binding to the 13-bp motif, we performed a hydroxyl radical interference experiment (Hayes and Tullius, 1989). Hydroxyl radical–treated LTR4 DNA asymmetrically labeled with phosphorus-32 was incubated with in vitro–translated NtMYB2. The bound probe was separated by native PAGE and analyzed by sequencing gel electrophoresis. Because hydroxyl radical interference experiment indicates missing nucleotides are required for protein binding, the result shows that 10 bases corresponding to the left part of the 13-bp motif and 1 bp outside the 13-bp motif are essential for NtMYB2 binding (Figure 4).

NtMYB2 Also Activates Transcription from the *Pv-PAL2* Promoter by Binding to the Promoter

As we noted (Takeda et al., 1999), the 13-bp motif contains the complementary sequence of the L box, which is highly conserved in promoter regions among genes for enzymes of phenylpropanoid synthetic pathways and is involved in the regulation of these genes in response to defense-related stresses such as wounding and UV-irradiation (Lois et al., 1989; Hatton et al., 1995). The characteristics of NtMYB2 together with these data suggest that NtMYB2 may be involved in this regulation. This possibility was tested by using the *PAL2* gene of bean, *Pv-PAL2*, which has been well characterized in heterologous tobacco. Analysis of transgenic tobacco carrying the *Pv-PAL2* promoter fused to the reporter gene showed that *Pv-PAL2* expression was induced not only by mechanical wounding but also during normal development of the xylem and flower, as is the case in the natural host plant, bean (Bevan et al., 1989). Truncated *Pv-PAL2* promoter from −254 to +55 retains the localized elicitor response or wound response (Shufflebottom et al., 1993; Hatton et al., 1995). To examine whether or not NtMYB2 could interact with the *Pv-PAL2* promoter sequence, the promoter fragment was used as a probe for EMSA. Figure 5A shows that in vitro–translated NtMYB2 formed a complex with the *Pv-PAL2* promoter fragment.

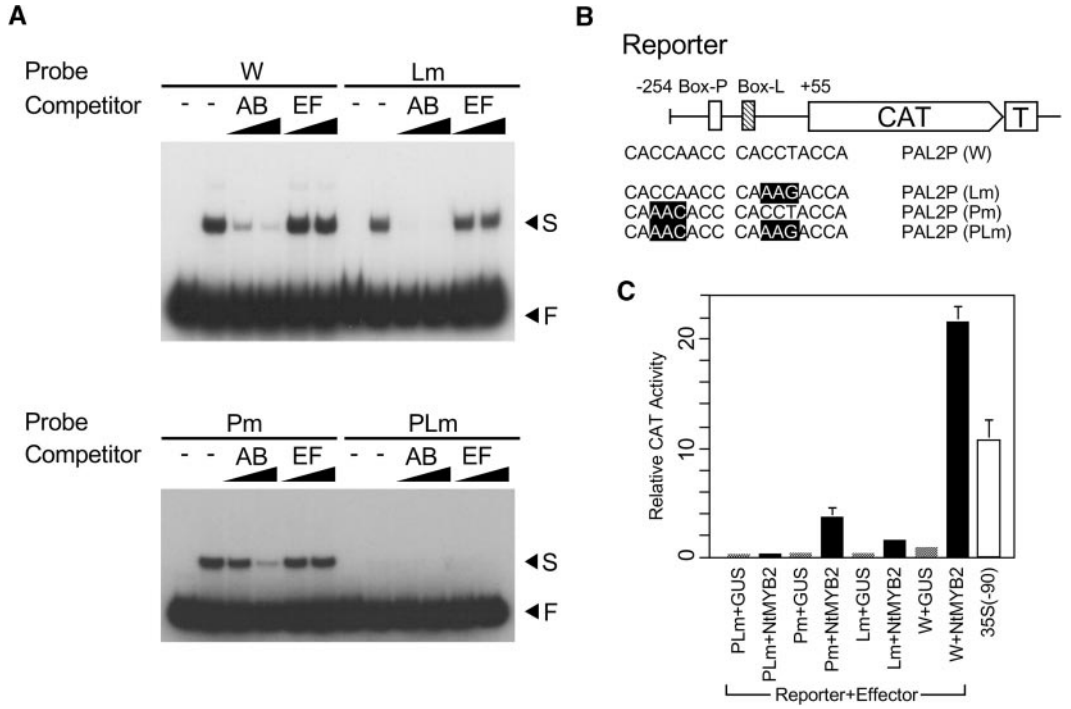

Figure 5. NtMYB2-Mediated *trans*-Activation of *Pv-PAL2* Promoter.

(A) EMSA was performed with in vitro–translated NtMYB2 and wild-type (W) *Pv-PAL2* promoter probe (−254 to +55) or mutant probes (Lm, Pm, and PLm). Competition assays were performed by preincubating none (–) or 25-fold (left lane under the triangle) or 125-fold (right lane under the triangle) excess amounts of unlabeled competitors, multimerized 13-bp motif (AB), or multimerized mutant motif (EF). Free probe (F) and mobility shift by NtMYB2 complex (S) are indicated.

(B) Reporter constructs. *Pv-PAL2* (-254 to +55, W) or mutant prompters (Lm, Pm, and PLm) were fused to the *CAT* coding region. Highlighted letters show the substituted nucleotides.

(C) The plasmid consisting of the cauliflower mosaic virus 35S promoter fused to *NtMYB2* cDNA (LBM; p35S-*NtMYB2* of Figure 3B) or to the GUS coding region (GUS; p35S-GUS of Figure 3B) was cotransfected with reporter constructs (W, Lm, Pm, and PLm) into tobacco protoplasts. Average CAT activity (and standard deviation) obtained from three independent transfection assays is shown.

When the 13-bp motif (AB) or the mutant (EF) was added in excess as the unlabeled competitor, the shifted band was competed with by AB but not by EF, indicating that NtMYB2 can bind to the *Pv-PAL2* promoter through 13-bp motif-like elements such as the L box (AC-I). To show that NtMYB2 binds to the L box, the ^{32}P-labeled mutant *Pv-PAL2* promoter fragment (Lm), which has a mutation in the L box, was also tested by EMSA. Figure 5A shows that NtMYB2 still forms the complex with the mutant *PV-PAL2* promoter. Because the P box (AC-II) is quite similar to the NtMYB2 binding sequence (Figure 5B), a mutation was introduced into the P box. Figure 5A shows that NtMYB2 cannot bind to a ^{32}P-labeled mutant fragment carrying mutations in both the P box and the L box (PLm).

To examine the effect of *NtMYB2* expression on *Pv-PAL2* promoter activation, reporter constructs of wild-type and mutant *Pv-PAL2* promoters fused to the *CAT* coding region (Figure 5B) were cotransfected with the *NtMYB2* expression plasmid into tobacco protoplasts (Figure 5C). The correlation between the affinity of NtMYB2 to the promoter fragments (Figure 5A) and the *trans*-activation of the promoter activity by NtMYB2 (Figure 5C) suggested that *NtMYB2* is also involved in the expression of *Pv-PAL2*.

Binding Activity from Wounded or Elicitor-Treated Leaf Nuclear Extracts Corresponds to That of NtMYB2 Protein

To verify that NtMYB2 protein exists in stressed tobacco nuclei, nuclear extracts were prepared from leaves harvested 1 hr after wounding and from leaves treated with Onozuka cellulase solution for 1 hr. Both wounded leaf–specific binding activity (Figure 6A) and elicitor treatment–specific binding activity (Figure 6B) were detected by EMSA with the LTR4 (−92 to −52) probe. Both binding activities

were competed with by an excess amount of the LTR4 fragment but not by the mutant LTR4 fragment in which the 13-bp motif is mutated (Figures 6A to 6C), which indicates that these proteins bind to the 13-bp motif. The mobility shift induced by nuclear extracts is the same as that induced by in vitro–translated NtMYB2 (Figures 6A and 6B)—a result suggesting that NtMYB2 or NtMYB4 (or both) in the nuclear extracts induced the mobility shift. This was further confirmed by the result that the mobility shifts induced by nuclear extracts were supershifted by the addition of immune serum raised against 57 amino acids of NtMYB2 (positions 174 to 231) (Figures 6A and 6B). This antibody, which was affinity-purified by antigen bound to Sepharose, can distinguish NtMYB2 from NtMYB3, NtMYB1, and even NtMYB4, which is 96% identical to NtMYB2 (in the antigen peptide, seven of the 57 amino acids differ from the corresponding sequence in NtMYB4), on protein gel blotting (Figure 6D). Considering the specificity of the antibody, the result of the protein gel blot shows that NtMYB2 protein was induced by wounding or elicitor treatment (Figure 6D). These results indicate that

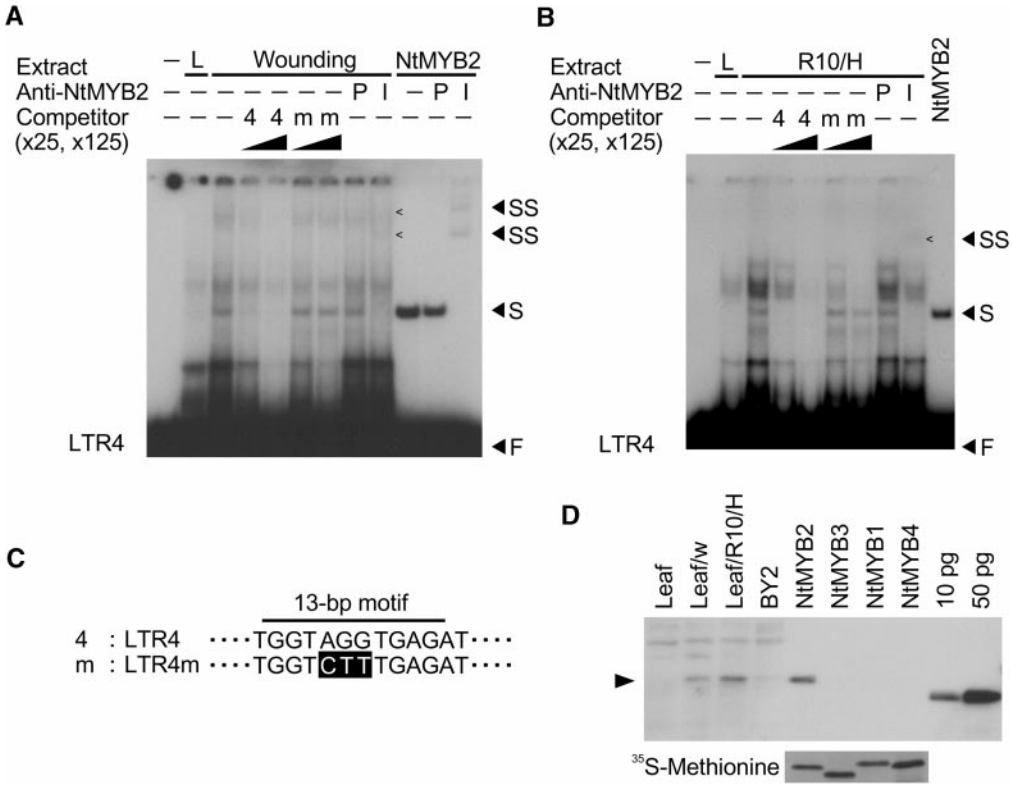

Figure 6. EMSA of the 13-bp Motif Performed with Nuclear Extracts.

(A) EMSA experiments were performed with nuclear extracts or in vitro–translated NtMYB2 (0.1 μL) (NtMYB2). Nuclear extracts were prepared from nontreated leaf (L) or wounded leaf incubated for 1 hr (Wounding), and were incubated with ^{32}P-labeled LTR4 probe (−92 to −37) and 25-fold (×25, left lane under the triangle) or 125-fold (×125, right lane under the triangle) excess amounts of unlabeled wild-type (4) and mutant (m) LTR4 fragment. Preimmune antiserum (1:1000 dilution) (P) or anti-NtMYB2 serum (1:1000 dilution) (I) was added in the binding reaction. Free probe (F), mobility shift (S) by NtMYB2 complex, and the mobility shift (SS and <) by NtMYB2–anti-NtMYB2 antibody complexes are indicated. Dashes indicate the absence of a given component.

(B) EMSA experiments were performed with nuclear extracts prepared from nontreated leaf (L) or leaf treated with elicitor (autocleaved Onozuka cellulase solution) for 1 hr (R10/H). Nuclear extracts were incubated with ^{32}P-labeled LTR4 probe (−92 to −37). Abbreviations as in **(A)**.

(C) Differences in sequences of the wild-type (4) and mutant (m) LTR fragments used as nonlabeled competitors. Highlighted CTT shows the substituted nucleotides.

(D) Protein gel blot analysis was performed with the affinity-purified anti-NtMYB2 antibody (final concentration, 0.66 μg/mL). Nuclear extracts prepared from nontreated leaf (Leaf), wounded leaf (Leaf/w), elicitor-treated leaf (Leaf/R10/H), or BY2 cell (BY2) as well as from in vitro–translated NtMYB2, NtMYB3, NtMYB1, or NtMYB4 (0.1 μL each) were loaded onto the gel. To assure equal loading of in vitro–translated MYB proteins, these proteins were labeled with ^{35}S-methionine and detected by autoradiography. The purified fusion protein (10 or 50 pg) of glutathione *S*-transferase with a part of NtMYB2 (amino acid positions from 174 to 231) was loaded as a quantitative marker. An arrowhead indicates the NtMYB2 protein signals.

NtMYB2 binding activity is induced concomitantly with the appearance of NtMYB2 protein.

NtMYB2 Binding Activity Is Rapidly and Transiently Induced by Wounding through Protein Synthesis

To examine the correlation between the induction of NtMYB2 binding activity and the synthesis of NtMYB2 protein, nuclear extracts prepared at various times after wounding were assayed. The NtMYB2 binding activity was rapidly but transiently induced by wounding (Figure 7A), disappearing by 8 hr after wounding. The protein gel blotted with anti-NtMYB2 antibody shows that the time course of the appearance of NtMYB2 binding activity resembles that for the appearance of NtMYB2 protein (Figure 7B), suggesting that the induction of NtMYB2 binding activity requires the synthesis of NtMYB2 protein by way of RNA induction. To determine whether NtMYB2 is synthesized de novo after wounding, we examined the effect of cycloheximide (CHX), an inhibitor of protein synthesis, on the induction of NtMYB2 binding activity. As shown in Figure 7C (Leaf/W/X), CHX treatment inhibited the induction of NtMYB2 binding activity. The existence of NtMYB2 protein was also examined by using an anti-NtMYB2 antibody. Figure 7D shows that NtMYB2 protein was not induced in wounded leaf nuclei treated with CHX (Leaf/W/X). These results indicate that the induction of NtMYB2 activity is the result of the synthesis of NtMYB2 protein.

Overexpression of NtMYB2 Activates Target Genes in Transgenic Tobacco

We have shown that NtMYB2 can act as a positive regulator in protoplasts. After wounding, *NtMYB2* expression, de novo synthesis of NtMYB2 protein, and NtMYB2 binding activity have been detected, suggesting that NtMYB2 activity is regulated mainly at the transcriptional level. If this is so and if NtMYB2 can work as a positive regulator in plants, then overexpression of NtMYB2 should activate target genes that contain the 13-bp motif and L box in their regulatory region. To test this possibility, transgenic tobacco plants carrying 35S promoter–driven *NtMYB2* cDNA with the 3′- and 5′-UTRs removed were produced. The tobacco plant used for transformation was a transgenic plant carrying nine copies of the 13-bp motif upstream of the *GUS* reporter gene (Takeda et al., 1999). The RNA gel blot analysis of the transgenic lines showed that the amounts of expression of *NtMYB2* and of the *GUS* reporter gene fused to multimeric 13-bp motifs well correlated to each other (Figure 8; NtMYB2 and GUS). Endogenous genes *Tto1* and *PAL*, which have a 13-bp motif and L box in the promoter region, were also activated in *NtMYB2*-overexpressing plants (Figure 8; Tto1, PAL) In contrast, the amount of mRNA for the

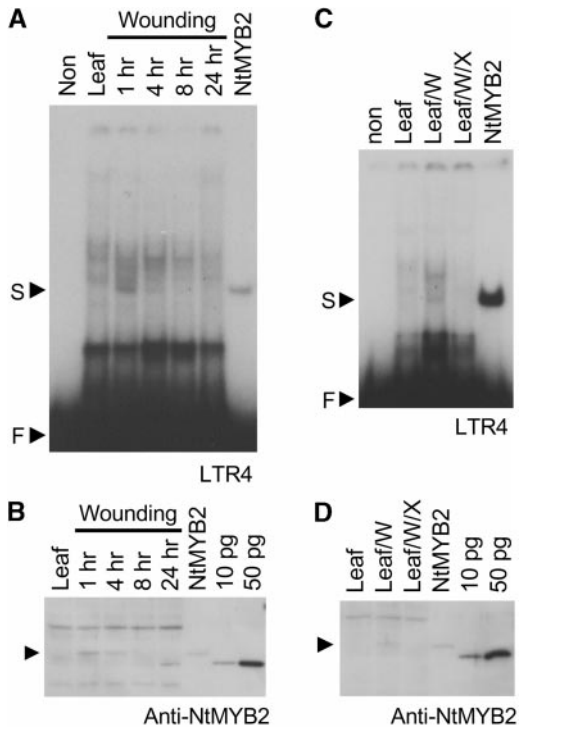

Figure 7. Induction of the NtMYB2 Protein and Its Binding Activity by Wounding.

(A) EMSA was performed without (Non) or with nuclear extracts prepared from nontreated leaf (Leaf) or wounded leaf incubated for 1, 4, 8, and 24 hr by incubating with the ^{32}P-labeled LTR4 probe (−92 to −37). EMSA with in vitro–translated NtMYB2 (0.1 μL) was also performed to show mobility shift patterns specific to each protein. Free probe (F) and the mobility shift by NtMYB2 (S) are indicated.

(B) Protein gel blot analysis was performed with purified NtMYB2-specific antibody. Nuclear extract prepared from nontreated leaf (Leaf), wounded leaf incubated for indicated times (Wounding), and in vitro–translated full-length NtMYB2 (0.1 μL) (NtMYB2) were analyzed together with 10 or 50 pg of the purified fusion protein of glutathione *S*-transferase and a part of NtMYB2. An arrowhead indicates the NtMYB2 protein signals.

(C) Inhibition of induction of NtMYB2 protein synthesis and NtMYB2 DNA binding activity by cycloheximide (X) treatment. EMSA was performed with nuclear extracts prepared from nontreated leaf (Leaf) or wounded leaf incubated for 1 hr in the absence (Leaf/W) or presence of 0.5 mM CHX (Leaf/W/X) by incubating with the ^{32}P-labeled LTR4 probe (−92 to −37). EMSA with in vitro–translated NtMYB2 (NtMYB2) was also performed. Free probe (F) and the mobility shift by NtMYB2 (S) are indicated. The CHX treatment inhibited de novo protein synthesis by 85%.

(D) Protein gel blot analysis was performed with purified NtMYB2-specific antibody. The nuclear extracts used in **(C)**, 0.1 μL of in vitro–translated NtMYB2 (NtMYB2), and the 10 or 50 pg of purified fusion protein of glutamine *S*-transferase with a portion of NtMYB2 were analyzed. An arrowhead indicates the NtMYB2 protein signals.

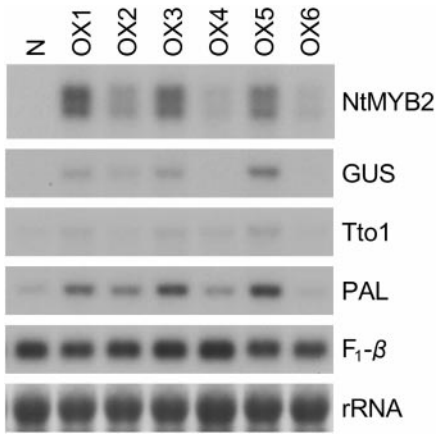

Figure 8. Overexpression of the *NtMYB2* cDNA Driven by the 35S Promoter in Independent Transgenic Tobacco Lines.

Each lane was loaded with 5 μg of total RNA from nontreated transgenic (OX1 to OX6) and control tobacco leaf (N) and was probed with *NtMYB2*, *GUS*, *Tto1*, *PAL*, and F_1-ATP synthase β-subunit (F_1-β)–specific DNA fragments. rRNA was stained with methylene blue.

nuclear-encoded β subunit of mitochondrial F_1-ATP synthase (Boutry and Chua, 1985; Imanishi et al., 2000) remained constant (Figure 8; F_1-β). These results show that NtMYB2 is a positive regulator of genes having a 13-bp motif and L box as a *cis* element.

Phosphatase Inhibitor Induces Both *NtMYB2* Expression and 13-bp Motif–Dependent Gene Expression

As an initial step in understanding the signal transduction pathways involved in wounding-induced expression, the effects of protein synthesis and protein phosphatase inhibitors on the expression of *NtMYB2* and 13-bp motif-dependent reporter gene (*GUS*) expression were examined. Experiments with protein phosphatase inhibitors, such as okadaic acid, a potent inhibitor of protein phosphatase 1 and 2A, suggested that protein phosphorylation positively regulates the signaling of various plant defense responses, such as accumulation of several PR proteins in tobacco (Raz and Fluhr, 1993) or the expression of defense genes encoding PAL, CHS, and a hydroxyproline-rich protein in soybean (Gianfagna and Lawton, 1995). As we have recently found (Takeda et al., 1999), the 13-bp motif–dependent gene expression is induced by treatment with okadaic acid (Figure 9B; Mes + OA). Under the same conditions, the *NtMYB2* RNA (Figure 9B; NtMYB2) was also induced but earlier than the 13-bp motif–dependent gene expression (Figure 9B; GUS). This result suggests that protein phosphorylation positively regulates *NtMYB2* expression and that the induction of the 13-bp motif–dependent GUS expression is mediated by *NtMYB2* expression. Even under control conditions (Figure 9B; Mes), weak induction of both the 13-bp motif–dependent gene expression and *NtMYB2* expression was observed (the signal for NtMYB2 expression is difficult to see in the figure but is clearly detected in the original autoradiograph). This induction must have been caused by wounding stimuli induced during leaf detachment. The patterns of induction of *NtMYB2* and the 13-bp motif–dependent gene expression observed in wounded leaves (Figure 9A; Wounding) were quite similar to those observed in okadaic acid–treated leaves, further supporting the involvement of NtMYB2 in the 13-bp motif–dependent gene expression. Treatment with CHX abolished the induction of the 13-bp motif–dependent gene expression by wounding but did not affect that of *NtMYB2* (Figure 9A; Wounding + CHX). By assuming the involvement of NtMYB2 in the 13-bp motif–dependent gene expression, the inhibition of the 13-bp motif–dependent gene expression by CHX can be readily explained by the inhibition of NtMYB2 protein synthesis. However, CHX treatment rather stimulated the expression of *NtMYB2* RNA. Interestingly, *NtMYB2* RNA, especially the smaller RNA, was also induced by CHX treatment even without wounding (Figure 2C; CHX).

DISCUSSION

The NtMyb2 and Regulation of Retrotransposon *Tto1* and Defense-Related Genes

Deletion analysis of the LTR promoter and gain-of-function analysis based on the reporter gene expression in protoplasts and transgenic plants showed that the 13-bp motif is a sufficient *cis* element responsive to wounding, jasmonate, elicitors, and tissue culture (Takeda et al., 1999). Here, we describe the molecular cloning and characterization of four R2R3-type MYB-related proteins that specifically bind the 13-bp motif. NtMYB2, NtMYB3, and NtMYB4 are newly described MYB-related proteins, and the fourth is identical to the previously reported NtMYB1 (Yang and Klessig, 1996). Previous studies have shown that plant MYB-related proteins regulate different plant-specific processes, such as the biosynthesis of anthocyanin and phlobaphen, trichome formation, and the regulation of epidermis cell shape (reviewed in Martin and Paz-Ares, 1997). In addition, some MYB-related protein mRNAs are induced by dehydration stress (Urao et al., 1993), gibberellic acid (Gubler et al., 1995), and SA (Yang and Klessig, 1996), suggesting that MYB-related proteins play a regulatory role downstream of these stimuli. Recently, the expression of >90 different MYB-related genes of Arabidopsis was examined under >20 different growth conditions (Kranz et al., 1998). That study indicated the potential for involvement of MYB-related genes in diverse regulatory processes. The present work provides evidence that NtMYB2 is a positive regulator of both *Tto1* and a defense-related gene, *PAL*. Considering the conservation

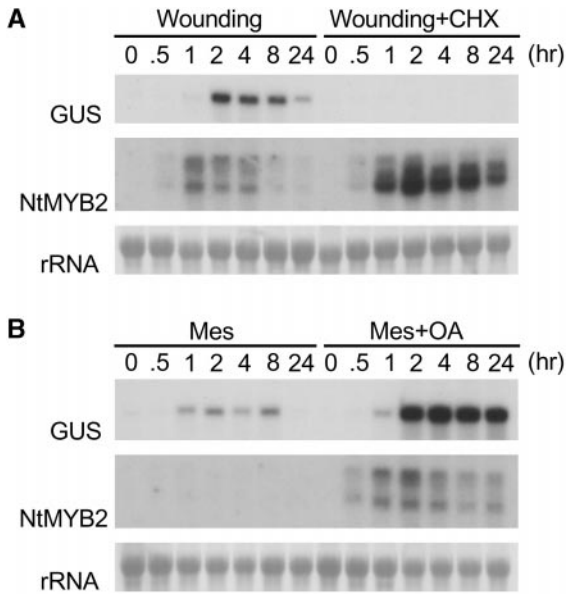

Figure 9. Expression of *NtMYB2* and *GUS* Driven by the Promoter Containing Multiple Copies of the 13-bp Motif in Response to Wounding and Treatment with Inhibitors for Protein Synthesis or Protein Phosphatase.

Transgenic tobacco plants carrying 13AB-LTR(–37)-GUS were used in which nine copies of the 13-bp motif were fused with the minimal LTR promoter–GUS construct (Takeda et al., 1999).

(A) Each lane was loaded with 5 μg of total RNA from wounded leaf incubated in Mes buffer in the absence (Wounding) or presence of 0.5 mM CHX (Wounding+CHX) for 1 hr. The RNA gel blot was hybridized with an *NtMYB2*-specific or *GUS*-specific probe.

(B) Each lane was loaded with 5 μg of total RNA from detached whole leaves for which the petioles had been dipped into Mes buffer in the absence (Mes) or presence of 0.5 μM okadaic acid (Mes+OA) for the times indicated. The RNA gel blot was hybridized with the *NtMYB2*-specific or the *GUS*-specific probe.

of the functional *cis* elements in many defense-related genes (see Introduction), *NtMYB2* probably also plays a role in the regulation of defense-related genes other than *PAL.*

Also as discussed in the Introduction, the functional *cis* elements of *Tto1* and *Tnt1* share the core sequence of the H box. This sequence is presumably the recognition sequence of NtMYB2, because NtMYB2 can bind the box-like sequence of the H box in the *PAL* gene (Figure 5). These findings suggest that NtMYB2 may also regulate the expression of *Tnt1* by binding the functional *cis* element of the latter. Consistent with this idea, the expression pattern of *Tnt1* is similar to that of *Tto1*. For example, expression of *Tto1* and *Tnt1* is induced by wounding and treatment with elicitors. Furthermore, *Tto1* and *Tnt1* are expressed in roots (Pouteau et al., 1991; S. Takeda and H. Hirochika, unpublished data), where NtMYB2 is also expressed (Figure 2B).

Multiple alignment of R2R3-MYB proteins shows that NtMYB2, NtMYB3, NtMYB4, NtMYB1, PhMYB2, AtMYB13, and AtMYB15 form a cluster (Kranz et al., 1998). NtMYB1, for which its RNA was shown to be induced by SA treatment or TMV infection, preferentially bound to the sequence GTTTGGT found in the *PR-1a* promoter (Yang and Klessig, 1996). Much the same as the *PR-1a* gene, *Tto1* expression was also activated by SA treatment or TMV infection (Hirochika and Otsuki, 1995, H. Hirochika, unpublished results). As shown in the present study, NtMYB1 also binds to the 13-bp motif and shows the same affinities as NtMYB2 to a series of 13-bp motif mutants (K. Sugimoto and H. Hirochika, unpublished data). These results suggest the involvement of *NtMYB1* in the expression of *Tto1* in response to SA and TMV infection. *AtMYB13* also was induced by wounding; however, the time course for its induction differs from that for *NtMYB2*. Accumulation of *AtMYB13* mRNA peaked 4 hr after wounding. AtMYB13 could bind to MBSII, which was recognized by PhMYB3 (Solano et al., 1995), but the binding sequence also was different from that for NtMYB2. Ectopic overexpression of AtMYB13 led to a change in the influorescence architecture (Kirik et al., 1998b). Among AtMYBs, AtMYB15 shows the most similarity to NtMYB2 (Kranz et al., 1998), and its RNA is detected in roots, as is *NtMYB2* RNA; however, the function of *AtMYB15* is not known.

NtMYB2 Not Essential for Specific Cooperating Partners

The maize *C1* gene, the first isolated c-*MYB* homolog in plants, requires a cooperating partner, *R* or *B*, which encodes a Myc-like protein with a basic helix-loop-helix (bHLH) motif to regulate the synthesis of anthocyanin (Paz-Ares et al., 1987; Ludwig et al., 1989). As Goff et al. (1992) have shown, the N terminus of C1 protein interacts directly with the N terminus of B protein, which lacks the bHLH domain. Another MYB-related protein, AN2 of petunia. also interacts with a bHLH protein to activate transcription of a target gene (Quattrocchio et al., 1993). Unlike C1 or AN2, some of the MYB-related proteins such as maize P protein and petunia PhMYB3 activate target genes without a partner (Grotewold et al., 1994; Solano et al., 1995). The reason why C1 required the cooperating partner, whereas the closely related P did not, can be explained by the difference in the affinity of the genes to their respective target *cis* elements. As shown in binding site–selection experiments, the DNA binding specificity of C1 is broader than that of P (Sainz et al., 1997). One of the MYB-related proteins, AmMYB305, which is expressed in the flower of snapdragon, activates the *Pv-PAL2* promoter through binding to the AC boxes (Sablowski et al., 1995). Petunia PhMYB3 also activates petunia *CHS-J* promoter in tobacco protoplasts (Solano et al., 1995). NtMYB2 overexpression in protoplasts and transgenic plants also activates 13-bp motif/L box genes, suggesting that NtMYB2 is a member of the latter group, proteins that activate target gene expression without partners. However, the amounts of *Tto1* and the 13-bp

motif–dependent reporter gene expressed in the *NtMYB2*-overexpressing transgenic plants (Figure 8) seem relatively less than those induced by wounding (Figure 9) (Takeda et al., 1998). This may indicate that NtMYB2 must be modulated by wounding to become fully active.

Regulatory Mechanism of *NtMYB2*

Two sizes of *NtMYB2* RNA, 1.2 and 1.5 kb, were induced by wounding. Similarly, two sizes of *P*, *PhMYB2*, and *NtMYB1* RNAs were detected (Grotewold et al., 1991; Avila et al., 1993; Yang and Klessig, 1996). In the case of the *P* gene, the two forms of RNA are produced by alternative splicing (Grotewold et al., 1991); the small *P* RNA encodes a negative regulator that lacks a C-terminal activation domain. The mechanism and function of the production of the two sizes of *PhMYB2* and *NtMYB1* RNAs are not known. Analysis of cDNAs and RNA gel blot analysis (Figure 2D) showed that two sizes of *NtMYB2* RNA were produced by alternative polyadenylation. Because the alternative polyadenylation does not change the coding region, its function is unclear. However, the 3′-UTR truncated by the alternative polyadenylation is AT-rich and can form a large palindrome structure. Transgenic tobacco carrying *NtMYB2* cDNA driven by the 35S promoter also showed two sizes of mRNA. Although *NtMYB2* cDNA introduced into tobacco was partially truncated at the 5′- and 3′-UTR, the putative upstream poly(A) signal (nt 1015 to 1020: AATACA) remained in the NtMYB2 cDNA. If the polyadenylation signals in *NtMYB2* cDNA and *nos* terminator work alternatively, a 0.2-kb difference would be expected (Bevan et al., 1982). Our results (Figure 8) are consistent with this hypothesis.

NtMYB2 activity is regulated mainly at the transcriptional level. Because okadaic acid, a protein phosphatase inhibitor, activates NtMYB2 activity and *NtMYB2* expression itself is not inhibited by CHX treatment, the transcription factor or factors involved in *NtMYB2* expression may be regulated by a post-translational mechanism such as phosphorylation. Recent studies show that various mitogen-activated protein kinase (MAPK) activities are induced by various stimuli in plants. In tobacco, wounding induces 46-kD PMSAPK or WIPK activity; SA, wounding, or elicitor induces SIPK activity; and a bacterial protein elicitor harpin induces 49-kD HAPK activity (Seo et al., 1995; Suzuki and Shinshi, 1995; Usami et al., 1995; Ádám et al., 1997; Zhang and Klessig, 1997, 1998; Zhang et al., 1998). Interestingly, *NtMYB2* RNA was induced under conditions where these MAPKs were activated. For example, PMSAPK activity (Usami et al., 1995) and *NtMYB2* RNA are not induced by treatment with jasmonic acid (data not shown) but are induced by wounding or chitin oligomer treatment (Figure 2). NtMYB2 protein and binding activity are inhibited by CHX treatment, but the *NtMYB2* RNA I is increased by CHX treatment (Figures 2 and 8). CHX treatment enhances or induces MAPK activities in tobacco (Suzuki and Shinshi, 1995; Usami et al., 1995; Ádám et al., 1997; Böger et al., 1997). These observations suggest that the transcription of *NtMYB2* may be regulated by MAPKs, such as PMSAPK, WIPK, and SIPK, or by NTFs (Wilson et al., 1993), possibly through phosphorylation of one or more transcription factors. Further analysis of *NtMYB2* and the *NtMYB2* promoter will contribute to an understanding of the molecular mechanism for activation by stress, particularly the early wound and elicitor response of retrotransposons and defense-related genes.

METHODS

Plant Materials and Treatment of Plant Tissues

Tobacco plants (*Nicotiana tabacum* cv Xanthi nc) were grown at 26°C in a greenhouse. Protoplasts were prepared from BY2 suspension culture as described previously (Hirochika et al., 1996b). Leaves were wounded as follows: a whole leaf on paper towels was wounded by pressing down a cheese grater, after which the leaf was floated on 0.05% Mes/KOH, pH 5.7. For elicitor treatment, a petiole with a whole leaf was dipped into elicitor solutions such as autoclaved (121°C, 20 min) cellulase Onozuka R10 (Yakuruto Biochemicals Co., Tokyo, Japan) and chitin oligomer (Pias Co., Rockford, IL).

Construction of an Activation Domain–Tagged cDNA Library and Screening

Tobacco tissues were frozen and homogenized in liquid nitrogen, and total RNA was extracted using ISOGEN (Nippongene, Tokyo, Japan). Poly(A)$^+$ RNA was purified with an oligo(dT) column (Stratagene). cDNA was synthesized by using the HybriZAP Two-Hybrid cDNA Gigapack Cloning Kit (Stratagene). The resulting activation domain–tagged cDNA library (AD/cDNA) contained 2.3×10^6 independent clones. Phagemid clones were excised from the HybriZAP vector and purified by CsCl gradient centrifugation.

To prepare a yeast reporter strain (YM4271-13H), a construct with six repeats of wild-type 13-bp motif (13OR: CTAGATGGTAGGTGAGAT) at the unique XbaI site upstream of minimal *HIS3* promoter of pHISi-1 (MATCHMAKER One-Hybrid System; Clontech) was integrated at the *URA3* loci of YM4271 (relevant genotype *ura3-52, his3-200*). The phagemid AD/cDNA library was introduced into the reporter strain by electroporation. Approximately 2.6×10^5 transformed cells were plated onto a synthetic medium lacking histidine and uracil and were then incubated for 3 days at 30°C in a liquid medium. The resulting colonies were collected and cultured for 16 hr at 30°C. Protein extracts were prepared from these yeast cultures by vortex-mixing with glass beads in binding buffer (see below, electrophoretic mobility shift assay [EMSA]). One microgram of yeast extracts was analyzed by EMSA with the wild-type (9 mer of AB oligonucleotide CTAGATGGTAGGTGAGATA) or the mutant-type (8 mer of EF oligonucleotide CTAGATGGTCTTTGAGATA) 13-bp motif probe.

DNA Sequencing and Analysis

Phagemid vectors having an AD/cDNA fusion were rescued by transformation of an *Escherichia coli* strain with total DNAs extracted from

yeast strains. EcoRI (in the adopter)-XhoI (derived from the primer sequence for reverse transcription; see below) DNA fragments derived from the phagemid clones were subcloned into EcoRI-XhoI sites of pBlueScript SK+ (pSK-NtMYB2, NtMYB3, and NtMYB4). DNA sequences were determined by using a Kilo Sequence Deletion Kit (Takara, Kyoto, Japan) and a Taq Dideoxy Terminator Cycle Sequencing Kit (ABI, Wellesley, MA). Analyses of primary sequences and alignment of multiple sequences were calculated with the software GENETYX-Win Ver.4.00 (Software Development, Tokyo, Japan).

DNA and RNA Gel Blot Analysis

Using ISOGEN (Nippongene), we extracted total RNAs from tobacco tissues that had been frozen and homogenized in liquid nitrogen. Total DNAs were prepared and their concentration was determined as described by Hirochika (1993). Blotting, preparation of probes, and hybridization were as described previously (Hirochika et al., 1992; Sugimoto et al., 1994). ScaI (nucleotide [nt] 566)-XhoI (end of the cDNA) of *NtMYB2* cDNA was used as a *NtMYB2*-specific probe. HincII (nt 1088)-XhoI fragment of NtMYB2 cDNA was used as a 39-untranslated region (UTR) probe. F1-ATP synthase β-subunit (F1-β)–specific DNA fragment (1st exon) was prepared by the polymerase chain reaction (PCR), using 5′-GGTGATAGGAGAACCAGTAT and 5′-ATGGCTTCTCGGAGGCTTCT from tobacco genomic DNA (*N. tabacum* cv Xanthi nc). β-Glucuronidase (*GUS*) and phenylalanine ammonia lyase (*PAL*)-specific DNA fragments were prepared as described by Takeda et al. (1998).

Preparation of Nuclear Extracts and EMSA

Nuclear extracts were prepared as described by Green et al. (1987), except for the desalting step, from leaves wounded by a cheese grater and incubated for 1 hr or treated with elicitors (1 mg/mL autoclaved [120°C, 20 min] cellulase Onozuka R10) for 1 hr. To retain the NtMYB2 binding activity, the dialysis step of Green et al. was replaced by gel filtration with a Bio-Spin 6 Column (Bio-Rad). Wounding with or without cycloheximide (CHX) treatment was performed as follows: a whole leaf was floated on 0.05% Mes/KOH, pH 5.7, in the presence or absence of 0.5 mM CHX, after which the leaf was wounded by pressing down a cheese grater on the paper towel holding the leaf; the leaves were then floated on the buffers again and incubated for 1 hr. DNA binding reactions were performed in a buffer containing 25 mM Hepes/KOH, pH 7.9, 1 mM EDTA, 50 mM KCl, 10 mM 2-mercaptoethanol, and 10% glycerol. In vitro translation was performed with the TNT Coupled Reticulocyte Lysate System (Promega) and using 1 μg of pSK-NtMYB2 per 50 μL of reaction volume. The translation efficiencies of each NtMYB protein were examined by assaying ^{35}S-methionine incorporation. The 0.2 μL of in vitro translates, 1 μg of yeast total protein, or 5 μg of nuclear extracts was mixed with 0.5 ng of ^{32}P-labeled DNA, 2 μg of poly(dI·dC)·poly(dI·dC), and cold (unlabeled) competitor fragments and then incubated for 20 min at 4°C. To separate free and bound DNA, reactions were loaded on 5% 40:1 w/w polyacrylamide-bisacrylamide gel in 0.5× TGE buffer (1× TGE buffer is 25 mM Tris, 192 mM glycine, and 1 mM EDTA).

Hydroxyl Radical Interference

Hydroxyl radical interference experiments were performed as described by Schickor and Heumann (1994). EcoRV-XhoI and HindIII-HincII fragments of pBC-LTR40W (see next section), asymmetrically labeled with ^{32}P-dCTP by filling in with the Klenow fragment, were used as probes.

Constructs for Transcriptional Activation Assay

Oligonucleotides with sequences the same as a part of the long terminal repeat (LTR; 5′-ATTGCAAATTGATCCTTCTTGGTAGGTGAGATTTGCACTT, corresponding to nt −92 to −52 of LTR promoter) and of the LTR with mutations (5′-ATTGCAAATTGATCCTTCTTGGTCTTTGAGATTTGCACTT) were cloned into an XbaI site of pBlueScript SK+, the end of which had been blunted with the Klenow fragment of DNA polymerase I, resulting in pSK-LTR40W and pSK-LTR40M, respectively. Underline indicates the substituted nucleotides.The sequence of cloned LTR was confirmed by sequencing. The LTR (−92 to −52) fragment was prepared from pSK-LTR40W by cutting with XbaI and filling in and then was cloned into the blunt-ended ClaI site of pBC-SK+, resulted in pBC-LTR40W. Oligonucleotides of wild-type (AB: 5′-ctagaTGGTAGGTGAGATa and 5′-ctagtATCTCACCTACCAt) and mutant-type (EF: 5′-ctagaTGGTCTTTGAGATa and 5′-ctagtATCTCAAAGACCAt) 13-bp motifs were annealed, ligated, and finally digested with SpeI and XbaI to select fragments in which the 13-bp motifs are multimerized in the same orientation. The fragments consisting of eight to nine copies of motifs were prepared by native PAGE and cloned into pBlueScript SK+ that had been digested with SpeI and XbaI, yielding pSK-13AB and pSK-13EF. These multimerized motifs were prepared from each plasmid by SpeI and XbaI digestion and cloned into the XbaI site of pLTR-CAT-5 (Hirochika et al., 1996b), yielding pLTR-CAT5AB and pLTR-CAT5EF. The XbaI-KpnI fragment carrying *NtMYB2* cDNA from pSK-NtMYB2 was ligated with p35S-CAT-N (Hirochika et al., 1996b) and then digested with XbaI and KpnI, resulting in p35S-NtMYB2. p35S-GUS is the same as pBI221 (Clonetech). The *Pv-PAL2* promoter fragment was obtained by PCR amplification with primers PL2-294F (5′-CGTCTATCTTCTATTCCACG, corresponding to nt −294 to +274 of *Pv-PAL2* promoter) and PL2+65R (5′-TAGTGGCGCGTTTGGGTAGG, corresponding to nt +65 to +85). The amplified fragment was digested with RsaI (nt −254) and DraI (nt +55) and cloned between blunt-ended PstI and BamHI sites of p35S-CAT, resulting in pPL2P-CAT-W. The sequence of the L box was changed by using PCR with primers PL2-LmF (5′-TTATACCCAAAGACCAGACAC) and PL2-LmR (5′-GTGTCTGGTCTTTGGGTATAA) and the sequence of the P box was changed with primers PL2-PmF (5′-ATTTCTCCAAACACCCCTTC) and PL2-PmR (5′-GAAGGGGGTGTTTGGAGAAAT), resulting in pPL2P-CAT-Lm and pPL2P-CAT-Pm, respectively. By using pPL2P-CAT-Lm as a template, the sequence of the P box was changed by PCR with primers PL2-PmF and PL2-PmR, resulting in pPL2-CAT-PLm.

Transient Transfection Assays in Tobacco Protoplasts

One milliliter of protoplasts (10^6) prepared from the BY2 cell line was mixed with 5 μg of plasmids and 20 μg of sonicated calf thymus DNA and electroporated using a Gene Pulser (Bio-Rad) electroporation apparatus. After the electroporation, the protoplasts were cultured overnight. Lysis of the protoplasts and the chloramphenicol acetyl transferase (CAT) assay were performed as described by Hirochika et al. (1987).

Plant Transformation

The binary vector for the Agro-infection was derived from pBI-H1 (Akama et al., 1992). The pBI-H1 NPT-II gene from the PmeI site to HindIII was replaced by annealed oligonucleotides (5′-AAACTGAAGGCGGGAAACGACAATCTA-3′ and 5′-AGCTTAGATTGTCGTTTCCCGCCTTCAGTTT-3′), resulting in pBIS-H1. The 35S promoter fragment was excised from p35S-CAT-N (Hirochika et al., 1996b) by using HindIII and BamHI. PCR-amplified *NtMYB2* cDNA from pSK-NtMYB2 (using primers 5′-GTGATTAAAGGATCCATGGTGAGAG-3′ and M13-20 primer 5′-GTAAAACGACGGCCAGT-3′) was digested with BsmI, blunt-ended with Klenow DNA polymerase, and digested with BamHI. The 35S promoter fragment and the *NtMYB2* cDNA fragment were ligated with pBIS-H1 and then digested with SacI, blunt-ended with T4 DNA polymerase, and digested with HindIII, resulting in pBIS-NtMYB2SF. *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986) was transformed with pBIS-NtMYB2SF by electroporation. Transgenic tobacco AB-2, which has nine copies of the 13-bp motif upstream of a reporter *GUS* gene (Takeda et al., 1999), was retransformed by using the leaf-disc method (Horsch et al., 1984).

Preparation of Specific Antibody and Protein Gel Blotting

The *NtMYB2* cDNA fragment from the SalI (nt 673) site to the KpnI (nt 845) site, which had been blunt-ended with T4 DNA polymerase, was inserted into the SalI and blunt-ended NotI site of pGEX-4T3 with the Klenow fragment of DNA polymerase I, yielding in pGEX-NtMYB2M. The glutathione *S*-transferase fusion with NtMYB2 was expressed in an *E. coli* strain (XL1 Blue) and purified on a glutathione–Sepharose affinity column. The NtMYB2 polypeptide was eluted from an affinity column by thrombin digestion and used to immunize rabbits. The *NtMYB2*-specific antibody was purified by antigen–Sepharose. This purified antibody (0.6 mg/mL) was diluted 1500-fold and used as a primary antibody for protein gel blotting in an ECL Detection kit (Amersham). For the gel-shift assay, preimmune serum and immune serum were used.

Assay of Protein Synthesis Inhibition

Inhibition of protein synthesis with CHX was assayed according to the method described by Lam et al. (1989). A 1.5- × 1.0-cm leaf disc was wounded with the cheese grater and then was floated at room temperature for 1 hr on 0.5 mL of 0.05% Mes/KOH, pH 5.7, containing ~10 μCi of ^{35}S-methionine (Amersham; SJ1015) with or without 0.5 mM CHX. The leaf discs were homogenized in 0.25 mL of 50 mM Hepes/KOH, pH 7.5, on ice. Aliquots (10 μL) of the supernatant from each sample were precipitated with trichloroacetic acid, and the total uptake of ^{35}S-methionine was measured by scintillation counting.

Accession Numbers

The DDBJ/EMBL/GenBank accession numbers for NtMYB2, NtMYB3, and NtMYB4 are AB028649, AB028650 and AB028652, respectively. NtMYB2, NtMYB3, and NtMYB4 were registered as LBM1, LBM2, and LBM4, respectively.

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MYB-Related Transcription Factor NtMYB2 Induced by Wounding and Elicitors is a Regulator of the Tobacco Retrotransposon *Tto1* and Defense-Related Genes

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