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**Original Paper**

# DNA-binding and transcriptional activation properties of tobacco *NIC2*-locus ERF189 and related transcription factors

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**Abstract** In tobacco (*Nicotiana tabacum*), several transcription factors belonging to the IXa group of APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) cluster at the regulatory *NIC2* locus for the leaf nicotine level, and directly activate structural genes required for nicotine biosynthesis and transport. Solanaceous *Atropa* and *Hyoscyamus* plants synthesize medicinal tropane alkaloids by utilizing enzymes that partially overlap with the nicotine pathway. We tested whether the tobacco *NIC2*-locus ERF189 binds to potential ERF binding sites in the promoters of various structural genes involved in biosynthesis of nicotine and tropane alkaloids by an electrophoresis mobility shift assay. ERF189 bound four new GCC-box-like sequences in three nicotine structural genes, but did not recognize other candidate binding sites. This binding preference was used to optimize the consensus binding sequence of ERF189 as 5′-(A/C)GC(A/C)NNCC(A/T)-3′. Five group IXa ERFs (tobacco ERF189, tobacco ERF163, *Catharanthus* ORCA3, *Arabidopsis* AtERF13, and *Arabidopsis* AtERF1) were examined whether they bind to the ERF189-recognition site in the promoter of the tobacco putrescine *N*-methyltransferase gene in vitro, and transactivate the promoter in a transient expression assay using cultured tobacco cells. The more the protein sequences were similar to ERF189, the more effective these ERFs were in these functional assays. The transient expression assay also identified transactivation domains in an N-terminal acidic region of ERF189, and in a C-terminal Ser-rich region of AtERF13.

**Key words:** Alkaloids, ERF transcription factors, jasmonate, nicotine, tobacco.

Transcription of multiple structural genes involved in a metabolic pathway is often coordinated by specific transcription factors, which recognize distinct *cis*-elements in the 5′-upstream promoter regions of the structural genes through sequence-specific DNA binding. When DNA-binding preference of a given transcription factor is determined by using degenerate oligonucleotides, putative its potential binding sites can be predicted in many uncharacterized promoters, which may be verified (or rejected) in subsequent experiments (Moyround et al. 2011). Defining the direct connections between target *cis*-elements and their cognate transcription factors is an important first step to figure out an entire architecture of regulatory networks (regulons) (Palaniswamy et al. 2006). Secondary (or specialized) metabolism is frequently subjected to dynamic regulation by environmental and developmental cues. To achieve rapid induction and fine tuning of a multi-step secondary metabolite pathway, master transcription factors often orchestrate expression of a set of enzyme and transport genes involved at the transcription level. Increasing numbers of transcription factors are being identified for plant secondary metabolism as key regulators that mediate environmental and developmental signals to activate distinct sets of secondary products (Grotewold 2008).

Nicotine constitutes a predominant alkaloid in tobacco leaves, and displays potent toxicity against gazing insects. Insect herbivory on tobacco leaves triggers a jasmonate-mediated signaling cascade, which results in increased formation of nicotine in the roots and subsequent transport of nicotine to the aerial parts (Shoji et al. 2008; Shoji and Hashimoto 2011c). Jasmonate- or herbivory-induced tobacco genes include structural genes encoding enzymes and transporters involved in nicotine biosynthesis and accumulation, such as ornithine decarboxylase (*ODC*; Imanishi et al. 1998), putrescine *N*-methyltransferase (*PMT*; Hibi et al. 1994), qunolinate

Abbreviations: ADC, arginine decarboxylase; AP2, APETALA2; CaMV35S, cauliflower mosaic virus 35S; EMSA, electrophoresis mobility shift assay; ERF, ETHYLENE RESPONSE FACTOR; GUS, β-glucuronidase; H6H, hyoscyamine 6β-hydroxylase; MATE, multidrug and toxic compound extrusion; NIC, NICOTINE, ODC, ornithine decarboxylase; ORCA, OCTADECANOID-RESPONSIVE *Catharanthus* AP2; PMT, putrescine *N*-methyltransferase; QPT, qunolinate phosphoribosyltransferase; TESS, Transcriptional Element Search Software; TR-I, tropinone reductase-I

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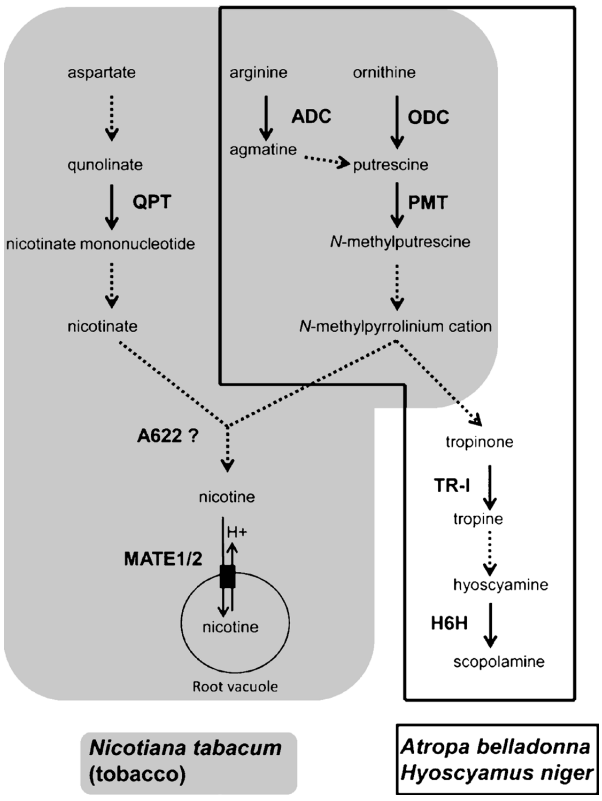


Figure 1. Biosynthetic pathways of nicotine and tropane alkaloids. Simplified biosynthetic pathways and the enzymes involved for nicotine and tropane alkaloids (hyoscyamine and scopolamine). Steps undefined or including multiple reactions are shown with broken arrows. Both types of pyrrolidine alkaloids share an early biosynthetic steps in common, starting from orthinine and up to *N*-methylpyrrolinium cation. The nicotine pathway in tobacco is shaded, while the scopolamine pathway in *Atropa* and *Hyoscyamus* is boxed. A622, a PIP-family oxidoreductase, is involved in a late step of nicotine biosynthesis (Kajikawa et al. 2009). MATE1 and MATE2 are the Multidrug-And-Toxic-compound-Extrusion-type transporters implicated in uptake of nicotine into vacuoles in tobacco roots (Shoji et al. 2009). ODC, ornithine decarboxylase; ADC, arginine decarboxylase; PMT, putrescine *N*-methyltransferase; QPT, qunolinate phosphoribosyltransferase; TR-I, tropinone reductase-I; and H6H, hyoscyamine 6$\beta$-hydroxylase.

phosphoribosyltransferase (*QPT*; Sinclair et al. 2000), a PIP-family oxidoreductase *A622* (De Boer et al. 2009; Hibi et al. 1994; Kajikawa et al. 2009) and tonoplast-localized multidrug and toxic compound extrusion (MATE)-type transporters *MATE1* and *MATE2* (Shoji et al. 2009) (Figure 1). These tobacco genes are expressed in restricted cell types in the roots, and are coordinately elicited in space and time by jasmonate (Shoji and Hashimoto 2011a).

Two genetic loci *NICOTINE1* and *NICOTINE2* (*NIC1* and *NIC2*) specifically control nicotine levels in tobacco plants, and the mutant alleles *nic1* and *nic2* have been used to confer a low-nicotine trait in commercial tobacco cultivars (Hibi et al. 1994; Legg and Collins 1971). We recently found that several highly homologous jasmonate-responsive transcription factors of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family, which form a distinct clade within the group IXa subfamily, are clustered at the *NIC2* locus and at least seven such *ERF* genes, called the *NIC2*-locus *ERF*s, are deleted in *nic2* mutants (Shoji et al. 2010). Decreased expression or dominant suppression of the *NIC2*-locus *ERF*s resulted in considerably lower transcript levels of nicotine biosynthesis genes and of nicotine levels in transgenic *Nicotiana* roots, whereas their overexpression gave the opposite effects (Shoji et al. 2010; Todd et al. 2011). Moreover, these *ERF* genes directly activated the transcription of *PMT* and *QPT* by recognizing GCC-box-like elements residing in their promoters (De Boer et al. 2011; Shoji et al. 2010; Shoji and Hashimoto 2011b). Interestingly, the tobacco *NIC2*-locus ERFs are closely related to ORCA3 (OCTADECANOID-RESPONSIVE *Catharanthus* AP2) from *Catharanthus roseus*, which positively controls a part of the jasmonate-inducible terpenoid indole alkaloid pathway (van der Fits and Memelink 2000), implying that the NIC2/ORCA3 ERF subfamily was recruited independently to regulate jasmonate-inducible secondary metabolism in distinct plant lineages

The Solanaceae includes not only nicotine-producing *Nicotiana* genus but also several medicinal plants that synthesize tropane alkaloids, such as hyoscyamine and scopolamine (Hashimoto and Yamada 1994). Biosynthetic pathway of tropane alkaloids is comprised of two sub-pathways (Figure 1); the early subpathway starts from ornithine and ends with *N*-methylpyrrolinium cation, and is shared with nicotine biosynthesis, including two enzymes (ODC and PMT), while the late pathway from tropinone to scopolamine is specific to tropane alkaloids, and includes tropinone reductase-I (TR-I) and hyoscyamine 6$\beta$-hydroxylase (H6H). Although it is not well established whether herbivory or jasmonates increases formation of tropane alkaloids and expression levels of the biosynthetic genes involved, NIC2/ORCA3-type ERF genes might control biosynthesis of tropane alkaloids in solanaceous medicinal plants.

In this study, we elaborated ERF189-binding sites in several structural genes for nicotine biosynthesis and transport, but tobacco ERF189 did not bind the promoters of the enzyme genes for tropane alkaloids. We also show that two NIC2/ORCA3-type ERFs from non-Solanaceae species (ORCA3 and *Arabidopsis* AtERF13) activate the tobacco *PMT* promoter by recognizing an ERF189-binding site, indicating functional conservation among the NIC2/ORCA3-clade ERFs from different species.

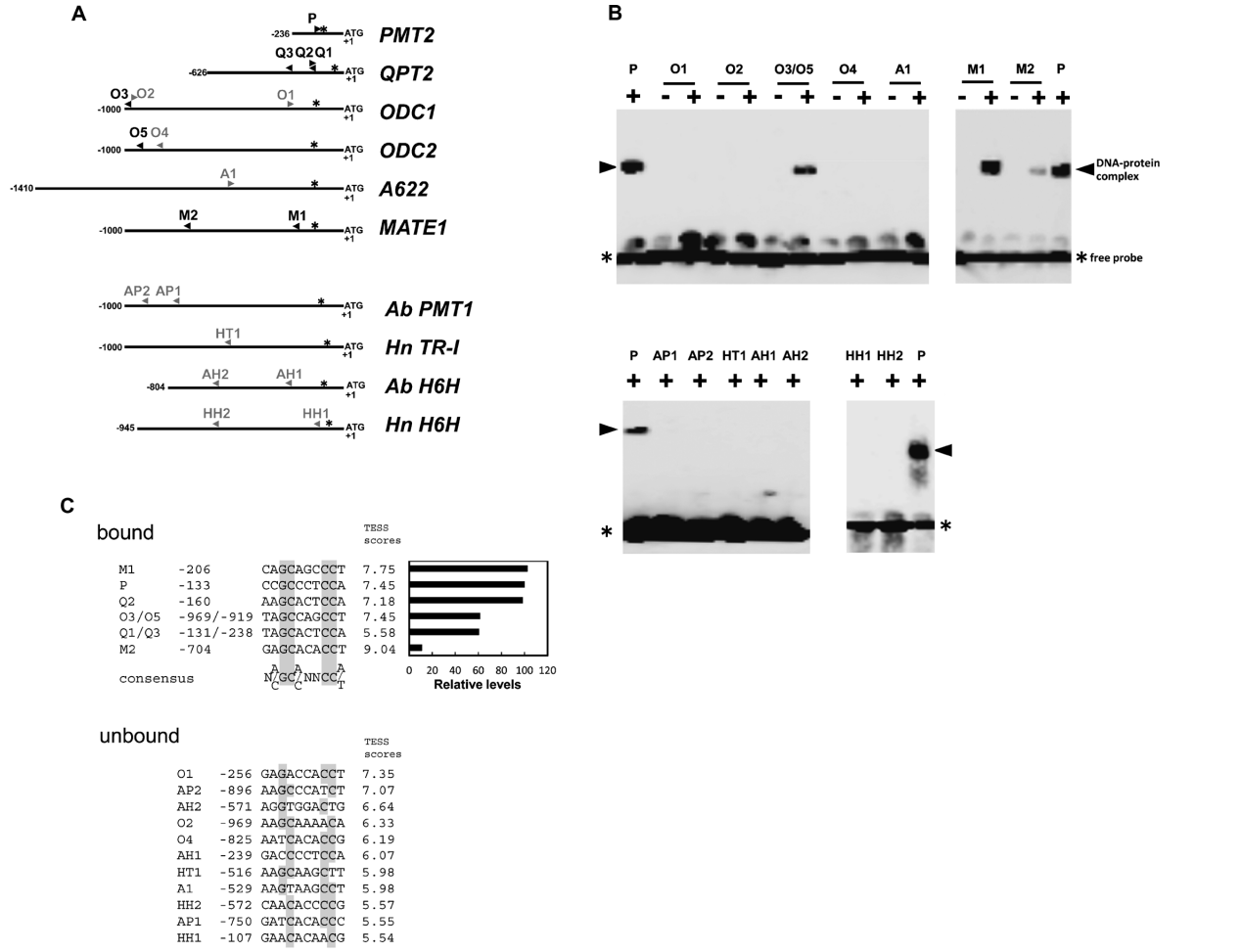


Figure 2. *In vitro* binding of recombinant ERF189 to candidate ERF189-binding sites. (A) Schematic representation of proximal promoter regions. For *PMT2* and *QPT2*, four experimentally verified ERF189-binding sites (P, Q1, Q2, and Q3) are indicated. For other genes, candidate ERF189-binding sites, which scored higher than 5.5 by TESS, are shown. Confirmed and rejected ERF189-binding sites, from the results of Figure 2B, are shown in black and gray arrowheads, respectively. Directions of the arrowheads reflect the orientation of the sequences relative to the P site in *PMT2*. Asterisks indicate predicted TATA boxes. (B) EMSA analysis of complex formation between ERF189 and the oligonucleotide probes containing the candidate 10-bp binding sites flanked by random spacer sequences (see Materials and methods). Candidate binding site sequences were examined with the probe P (*PMT2*) as a reference in each blot. Probes were incubated with protein extracts from empty vector control (−) or recombinant ERF189 (+). (C) Binding strength of candidate ERF189-binding sites. The sequences are shown with their positions relative to the translation initiation ATG, TESS scores, and relative binding activities (with the activity of the probe P as 100). The sequences unbound with ERF189 are listed in the order of TESS scores, while those bound with ERF189 are ordered according to the binding activities. The activity values of the probes Q1/Q3 and Q2 (*QPT2*) were based on the EMSA data reported in Shoji and Hashimoto (2011b). Note that the sequences of Q1 and Q3, and of O3 and O5, are identical.

## Materials and methods

### *Computational prediction of candidate ERF189-binding sites*

Transcriptional Element Search Software (TESS) (http://www.cbil.uppenn.edu./tess) was used to search putative ERF189-binding sites in the query promoter sequences by weight matrix scoring, adapting the binding preference data of recombinant ERF189 at a core 10-bp sequence of the GCC-box-like element in the *PMT2* promoter (Shoji and Hashimoto 2011b). The promoter sequences can be found in the GenBank/EMBL databases under the following accession numbers: AB004323 (*PMT2*), AJ748263 (*QPT2*), AB031066 (*ODC1*), AF233849 (*ODC2*), AB071165 (*A622*), AB286963 (*MATE1*), AF127240 (*ADC1*), AF127241 (*ADC2*), AB018573 (*AbPMT1*), AB026544 (*HnTR-I*), AB017153 (*AbH6H*), and D26583 (*HnH6H*).

### *Electrophoresis Mobility Shift Assay (EMSA)*

The pET32-based expression vectors for ERF189 and ERF163 have been described previously (Shoji et al. 2010). Full-length coding sequences of *AtERF1* (At4g17500) and *AtERF13* (At2g44840) from *Arabidopsis*, and *ORCA3* (GenBank/EMBL accession number EU072424) from *C. roseus*, were cloned in pET32b (Novagen) at the *Eco*RI and *Hin*dIII sites for *AtERF1*, and at the *Bam*HI and *Sac*I sites for *AtERF13* and *ORCA3* to express recombinant ERF proteins fused in tandem to thioredoxin, an S-tag, and a His-tag at their N-terminal ends. Recombinant protein was expressed in *Escherichia coli* BL21

Star (DE3) (Novagen), affinity-purified, quantified, and stained with Coomassie Brilliant Blue R250 after separation on a 12% SDS-PAGE gel (Shoji et al. 2010). Sense oligonucleotides contain the 10-base sequences shown in Figure 2C, which are flanked by 5′-NNNNNNNN-3′ and 5′-NNNNCCTCGG-3′, where N represents any nucleotides. Antisense oligonucleotide (5′-ACACCGAGG-3′) was biotin-labeled, and was annealed to the sense oligonucleotides to generate double-stranded probes (Shoji et al. 2010). The DNA-protein binding assay, gel separation, and detection of the DNA-protein complexes have been described by Shoji et al. (2010). The biotin-labeled DNA probes (20 fmol) and the purified recombinant proteins (2 $\mu$g) were used for each binding reaction.

#### *Transient transactivation assay*

The pBI221-based plasmids for *PMT2pro236-β-glucuronidase* (*GUS*), which contained a *PMT2* promoter fragment (−236 to −1; numbered from the first ATG), its mutant version *PMT2pro236m4-GUS*, *35S::ERF189*, and *35S::ERF163*, both of which contained the cauliflower mosaic virus 35S (CaMV35S) promoter, has been described (Shoji et al. 2010). Full-length coding regions of *AtERF1*, *AtERF13*, and *ORCA3*, were inserted into the *Bgl*II and *Sac*I sites for *AtERF1*, and at the *Bam*HI and *Sac*I sites for *AtERF13* and *ORCA3*, to generate *35S::AtERF1*, *35S::AtERF13*, and *35S::CrORCA3*. To truncate the *ERF* sequences at appropriate sites as shown in Figure 6, PCR-based site-directed mutagenesis (Hemsley et al. 1998) using a high-fidelity Prime Star Max DNA polymerase (Takara) was done with relevant *ERF* effector constructs as templates. Sequence information of the primers used is available upon request. The pBI221-LUC vector harboring firefly luciferase (LUC) under the control of the CaMV35S promoter was co-transformed as an internal standard. Particle bombardment and following measurements of GUS and LUC activities in extracts of the bombarded tobacco BY-2 cells have been described (Shoji et al. 2010).

## Results

### *ERF189-binding sites in the promoters of structural genes involved in biosynthesis of nicotine and tropane alkaloids*

*In vitro* binding assay by electrophoresis mobility shift assay (EMSA) and degenerate oligonucleotides was used to define the binding-site preference of ERF189 as 5′-(A/C)GC(A/C)(A/C)NCC-3′ (Shoji and Hashimoto 2011b). We also found functional ERF189-binding sites in the *N. sylvetris PMT2* promoter (P in Figure 2A; Shoji et al. 2000) and in the *N. tabacum QPT2* promoter (Q1, Q2, and Q3 in Figure 2A; Shoji and Hashimoto 2011b). To examine whether other cloned promoter regions of the structural genes for biosynthesis of nicotine and tropane alkaloids bind ERF189, we searched such available promoter sequences for potential ERF189-binding sites with Transcription Element Search Software (TESS), by using a weight matrix based on the published ERF189-binding preference (Shoji and Hashimoto 2011b). Basically, 5′-upstream regions of 1 kb, or shorter if not available, from the translation initiation ATG were used as queries. When promoter regions have been functionally characterized, minimal functional promoters were used for the search in some cases. To enhance detection sensitivity, a relatively moderate cutoff score of 5.5 was employed. The computer-assisted search predicted three candidates sites (O1, O2, and O3) for tobacco *ODC1* (Imanishi et al. 1998), two sites (O4, and O5) for tobacco *ODC2* (Xu et al. 2004), one (A1) for tobacco *A622* (Shoji et al. 2002), and two (M1 and M2) for tobacco *MATE1* (Shoji et al. 2009). The enzyme genes of tropane alkaloids contained in their promoter regions two potential binding sites (AP1, and AP2) for *Atropa belladonna PMT1* (Suzuki et al. 1999b), one site (HT1) for *Hyoscyamus niger TR-I* (Nakajima et al. 1999), two sites (AH1, and AH2) for *A. belladonna H6H* (Suzuki et al. 1999a), and two sites (HH1, and HH2) for *H. niger H6H* (Kanegae et al. 1994) (Figure 2A). Promoters of two tobacco *arginine decarboxylase* genes (*ADC1,* and *ADC2*) did not contain potential ERF189-binding sites (data not shown), in agreement with the fact that these genes are not regulated by the *NIC* loci (Shoji et al. 2010).

We next tested whether ERF189 indeed recognizes the predicted binding sites by EMSA (Figure 2B). Predicted 10-bp binding sites (Figure 2A) were flanked with random sequence stretches, and were appended at one end with a universal adaptor sequence for fluorescence labeling. Since O3 in *ODC1* and O5 in *ODC2* are identical in sequence, a common probe, named O3/O5, was used. DNA-ERF189 complexes were observed as retarded bands for probes O3/O5, M1, and M2, while no clear complexes were detectable for other probes. An established ERF189-binding site in the *N. sylvestris PMT2* promoter (P in Figure 2A) was used as a reference to measure the intensities of the DNA-ERF189 complexes (Figure 2C). ERF189 bound to the M1 site as efficiently as the reference P site and the Q2 site in the *QPT2* promoter (Shoji and Hashimoto 2011b), moderately to the O3/O5 site at a level comparable with the Q1/Q3 site in the *QPT2* promoter (Shoji and Hashimoto 2011b), and weakly to the M2 site. A jasmonate-inducible 1.4-kb promoter of *A622* (Shoji et al. 2002) contained only one candidate ERF189-binding site (A1), but this site was not bound with ERF189 (Figure 2B). We did not detect any binding activities of ERF189 to the seven predicted binding sites in the promoters for the enzyme genes involved in biosynthesis of tropane alkaloids (Figure 2B).

### *ORCA3 and AtERF13 recognize the ERF189-binding site in the PMT2 promoter*

Group IXa ERFs of tobacco are classified into clade 1 and clade 2, which includes tobacco *NIC2*-locus ERFs

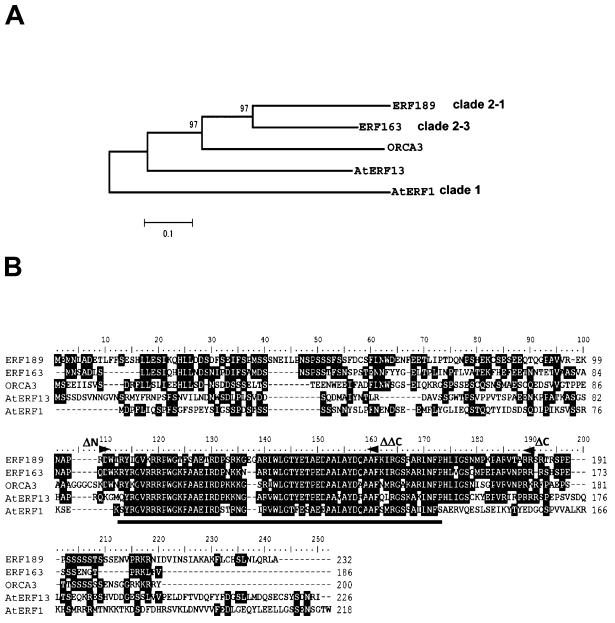


Figure 3. Sequence alignment and phylogenetic analysis of ERF189, ERF163, ORCA3, AtERF1, and AtERF13. (A) Phylogenetic tree based on the alignment of full-length ERF protein sequences. The tree is generated using MEGA4 software (Tamura et al. 2007) with the neighbor-joining algorithm. Bootstrap values are indicated at branch nodes, and the scale bar indicates the number of amino acid substitutions per site. (B) Multiple sequence alignment. Full-length amino acid sequences were aligned with ClustalW (Thompson et al. 1994). Residues identical in at lease two sequences are shaded in black, and dashes indicate gaps introduced to maximize the alignment. The AP2/ERF DNA-binding domain is underlined. Positions relevant for the truncated *ERF* constructs shown in Figure 6 are marked with arrowheads.

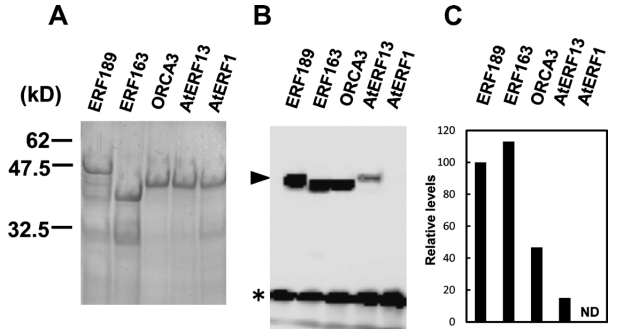


Figure 4. *In vitro* binding of recombinant ERF proteins to the ERF189-binding site in the *PMT2* promoter. (A) Purity of recombinant ERF proteins was analyzed by separation on a 12% SDS-PAGE gel, and subsequent staining with Coomassie Brilliant Blue. The molecular mass of marker proteins is indicated on the left in kilodaltons (kD). (B) Electrophoresis mobility shift assay of purified ERF proteins. The arrowheads indicate ERF-probe complexes, while the asterisks show un-bound probe. In left two lanes, bands of free probes were positioned a little leftward relative to those of complexes. (C) Relative abundance of the complexes. ND, not detected.

and can be further subdivided into three subclades, clade 2-1, 2-2, and 2-3 (Shoji et al. 2010). We had shown that five tobacco clade 2 ERFs (ERF189, ERF115, ERF179, ERF163, and ERF91) bind to a GCC-box-like element in the *PMT2* promoter at comparative efficiency, but a clade 1 ERF (ERF32) does not (Shoji et al. 2010). Although *C. roseus* ORCA3 and *Arabidopsis* AtERF13 belong to the group IXa, it is controversial whether they can be classified in clade 1 or clade 2 merely on the basis of a phylogenetic relationship (Figure 3A).

To characterize the DNA-binding preference of ORCA3 and AtERF13, recombinant proteins of ERF189 (clade 2-1), ERF163 (clade 2-3), ORCA3, AtERF13, and AtERF1 (clade 1) were purified (Figure 4A), and an electrophoresis mobility shift assay (EMSA) was carried out with the proteins and the probe P representing an ERF189-binding site in the *PMT2* promoter (Figure 4B). AtERF1 did not recognize the probe, in agreement with the fact that the ERF189-binding consensus is significantly diverged from the canonical AtERF1 binding sequence of 5′-AGCCGCC-3′ (Allen et al. 1998). In constast, ERF189, ERF163, ORCA3, and AtERF13 formed DNA-protein complexes (Figure 4B). By comparing the intensities of the complexes, we estimate that binding efficiency of ORCA3 is comparable to (or somewhat less strong than) those of ERF189 and ERF163, whereas AtERF13 binds much weakly to the probe (Figure 4B).

### *ORCA3 and AtERF13 transactivate the PMT2 promoter*

We next examined whether the five IXa ERFs studied for their DNA binding activity in Figure 4 were examined for their transactivation ability *in vivo*. A 0.2-kb *PMT2* promoter-driven GUS reporter (*PMT2pro236-GUS*), a CaMV35S promoter-driven luciferase reference, and a CaMV35S promoter-driven *ERF* effector (*35S::ERF189*, *35S::ERF163*, *35S::ORCA3*, *35S::AtERF13*, or *35S::AtERF1*) were co-delivered into tobacco cultured cells by particle bombardment to express the constructs transiently. After the GUS activity in the cell extracts were normalized to the reference luciferase activity, relative induction folds compared to the empty vector-transformed control were calculated. Robust induction of the *PMT2* promoter was observed when ERF189, ERF163, ORCA3, or AtERF13 was expressed (Figure 5A). ERF189 was the most effective activator (11-fold induction) of the *PMT2* promoter, followed by ERF163 (7-fold), ORCA3 (4.5-fold), and AtERF13 (4-fold). AtERF1 was ineffective. When the *PMT2* promoter was mutated at the single ERF189-binding site (Shoji et al. 2010), we did not observe any promoter induction by these effectors (Figure 5B), indicating that the characterized ERF189-binding site mediates the transactivation effect.

### *Transactivation domains in IXa ERFs*

ERF189, ERF163, ORCA3, and AtERF13 contain a

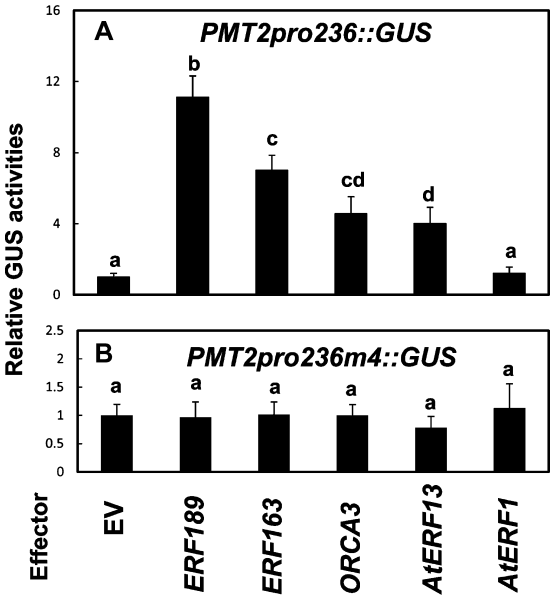


Figure 5. Transactivation of *PMT2* promoters with ERF effectors. Cultured tobacco BY-2 cells were bombarded with a combination of a GUS-expressing reporter plasmid, a luciferase-expressing reference plasmid, and either an ERF-expressing effector plasmid or an empty plasmid (EV). Reporter *GUS* gene was driven either by the wild-type *PMT2* promoter (*PMT2pro236-GUS*), or by a *PMT2* promoter in which the ERF189-binding site was mutated (*PMT2pro236m4-GUS*). GUS activity in the cell extracts is shown relative to the luciferase activity. Error bars indicate the SD for three independent biological replicates. Significant differences among the effectors were determined at $P<0.05$ by one-way ANOVA, followed by the Tukey-Kramer test, and are indicated by different letters.

central DNA-binding domain (Figure 3B), an N-terminal acidic domain rich in Ser, Glu, and Asp, and a short Ser-rich stretch at the C-terminal region (Figure 6; van der Fits and Memelink 2001; Shoji et al. 2010). To localize the regions that affect the transactivation activity, various truncated ERF variants were tested in the transient transactivation assay using cultured tobacco cells (Figure 6). Removal of the C-terminal Ser-rich stretches in ERF189, ERF163, and ORCA3 (ERF189ΔC, ERF163ΔC, and ORCAΔC) did not markedly affect the transactivation ability, whereas the corresponding Ser-rich stretch of AtERF13 (AtERFΔC) was required for activation of the *PMT2* promoter. In ERF189, deletion of a part of the DNA-binding domain (ERF189ΔΔC) or of the N-terminal acidic domain (ERF189ΔN) abolished the transactivation effect.

## Discussion

Using a steroid receptor-based gene inducible system in the presence of a protein synthesis inhibitor, we had previously shown that a tobacco *NIC2*-locus ERF, ERF189, directly activates an entire set of the structural genes involved in nicotine biosynthesis and transport in tobacco (Shoji et al. 2010). These direct targets of ERF189 include *PMT*, *QPT*, *ODC*, *A622*, and *MATE1/2*. The ERF189-binding sites in the promoters of *PMT2* and *QPT2* have been extensively characterized previously (Shoji et al. 2010; Shoji and Hashimoto 2011b), and this study identified new ERF189-binding sites, two elements of the identical core sequences (O3/O5) in *ODC1* and *ODC2*, and two element (M1 and M2) in MATE1. Absence of the ERF189-binding sites in the available *ADC* promoter sequences is consistent with the fact that *ADC* is not regulated by the *NIC2*-locus *ERF* genes (Shoji et al. 2010; Shoji and Hashimoto 2011a). On the other hand, it is puzzling that we did not find any ERF189-binding sites in the jasmonate-regulated promoter region of the *ERF189*-regulated *A622* gene (Figure 2A). The parameter setting used in Transcription Element Search Software might not have been adequate to detect elusive binding sites with low hit scores. Three redundant ERF189-binding sites are shown to confer incremental activation to the *QPT2* promoter (Shoji and Hashimoto 2011b), indicating that several low-affinity binding sites in one promoter may function as positive regulatory elements. Alternatively, jasmonate-induction of the 1.4-kb promoter region (Shoji et al. 2002) might not be ERF189-dependent, and ERF189-binding site(s) might be located further upstream of the characterized promoter region. Chromatin immunoprecipitation assay may be useful to define the elusive ERF189-binding site(s) in the *A622* promoter.

A total of eight ERF189-binding sites identified previously (Shoji et al. 2010; Shoji and Hashimoto 2011b) and in this study conform to an improved consensus sequence, 5′-(A/C)GC(A/C)NNCC(A/T)-3′, in which the underlined four bases are absolutely required for the ERF189 recognition (Figure 2C). The eleven potential ERF189-binding sites predicted by the TESS program, even though they showed moderate or high TESS score, did not bind ERF189 *in vitro*, and had one or more mismatches in these four invariant bases (Figure 2B). The finding in this study will be incorporated into the current TESS program to improve the prediction capability for functional ERF189-binding sites in un-characterized promoters involved in the nicotine regulon.

Biosynthesis of nicotine and tropane alkaloids shares an early part of the pathways (Figure 1), and has evolved in related genera of the Solanaceae (Hashimoto and Yamada 1994). Since tomato and potato, which both belong to the Solanaceae, contain clustered *NIC2*-like loci in their genome sequences (The Potato Genome Sequencing Consortium 2011, The International Tomato Genome Sequencing Consortium), jasmonate-inducible *ERF189*-like transcription factor genes seem to have evolved early during the formation of the Solanaceae, and may well be present in the genera, *Atropa* and *Hyoscyamus*, which synthesize tropane alkaloids. We

may expect that *Atropa* and *Hyoscyamus* utilize ERF189-like transcription factors to regulate structural genes for tropane alkaloid biosynthesis, in response to herbivory and by way of jasmonate signaling. However, it has been controversial whether tropane alkaloid formation is upregulated by herbivory or jasmonates. Although *PMT* expression was reported to be induced by jasmonate treatment in two tropane alkaloid-producing species, *Anisodus acutangulus* (Kai et al. 2008) and *Hyoscyamus niger* (Zhang et al. 2002), jasmonate treatment was ineffective in increasing *PMT* expression in *A. belladonna* (Suzuki et al. 1999b). Formation of hyoscyamine and scopolamine either increased by jasmonate treatment in *H. niger* (Zhang et al. 2002) or did not in *A. belladonna* (Suzuki et al. 1999b), *Datura wrightii* (Hare and Walling 2006), and *H. muticus* (Biondi et al. 2000). We did not observe reproducible induction of other genes involved in alkaloid biosynthesis (e.g., *TR-I* and *H6H*) to be induced by such treatment in *A. belladonna* and *H. niger* (our unpublished results). Apparent absence of the ERF189-binding sites in the functional promoter regions of the enzyme genes involved in tropane alkaloid biosynthesis (Figure 2) is in line with the reports in which alkaloid accumulation and expression of the pathway enzyme genes are not increased after jasmonate treatment to the plants.

The group IXa ERFs have been subdivided into clade 1 and clade 2, among which only clade 2 tobacco ERFs can activate nicotine biosynthesis (Shoji et al. 2010). Tobacco ERF32 (Shoji et al. 2010) and AtERF1 (this study) belong to clade 1, do not recognize the ERF189-binding site in the *PMT2* promoter, and do not activate the promoter in a transient expression assay. Since *Catharanthus* ORCA3 and *Arabidopsis* AtERF13 are not strictly classified within the clade 2 tobacco ERFs (Figure 3A), we compared their properties with ERF189. ORCA3 and AtERF13 indeed recognized the ERF189-binding site (Figure 4), and activated the promoter containing the cognate binding site (Figure 5), although less efficiently compared to ERF189. These results indicate that ERF189, ORCA3, and AtERF13 have similar but distinct DNA-binding preference.

The N-terminal acidic domain in ERF189 was shown in this study to be required for activation of its target promoter, in accordance with the acidic activation domain found in ORCA3 (van der Fits and Memelink 2001). The N-terminal acidic regions in the IXa-subfamily ERFs thus appear to act as the activation domain of the transcription factors. Previously, the C-terminal Ser-rich stretch in ORCA3 was reported to act inhibitory on the activation of the strictosidine synthase promoter in *C. roseus* cells (van der Fits and Memelink 2001). In contrast, we did not find such an inhibitory effect of the Ser-rich stretches in ORCA3, ERF189, and ERF163, when analyzed on the activation of the *PMT2* promoter in tobacco cells (Figure 6). The reason for this discrepancy is not clear, but might be attributable to the differences in the promoters and the transient expression systems used.

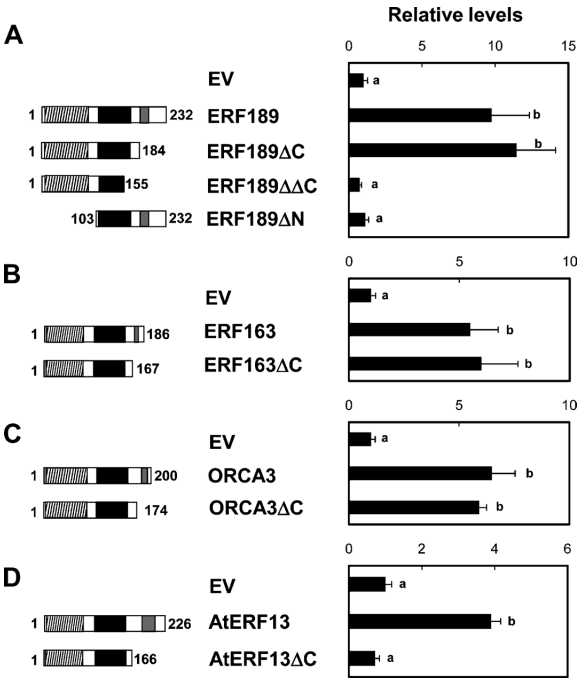


Figure 6. Transactivation of a *PMT2* promoter with ERF variants. Diagrams show the positions of the conserved AP2/ERF DNA-binding domain (black), the Ser/Asp/Glu-rich acidic region (striped), and the short Ser-stretch (gray). The *PMT2pro236-GUS* reporter plasmid was delivered by particle bombardment into cultured tobacco BY-2 cells, together with an effector plasmid expressing an ERF and a luciferase-expressing reference plasmid. GUS activities in the cell extracts are divided by the luciferase activities, and are shown as relative values against those of EV-transformed cells (EV). The bars indicate the SD from three biological replicates. Significant differences among the effectors were determined at $P<0.05$ by one-way ANOVA, followed by the Tukey-Kramer test, and are indicated by different letters.

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