

KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem

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To identify genes targeted by the tobacco KNOX homeodomain protein, *Nicotiana tabacum* homeobox 15 (NTH15), we have generated an inducible system using the human glucocorticoid receptor. In this system, steroid treatment strictly induced NTH15 function and immediately suppressed the expression of a gibberellin (GA) biosynthetic gene encoding GA 20-oxidase (*Ntc12*) and also resulted in a decrease in bioactive GA levels. The repression of *Ntc12* was observed even when indirect effects were blocked by cycloheximide. *NTH15* mRNA was present in corpus cells of the shoot apical meristem (SAM), whereas *Ntc12* mRNA was observed in leaf primordia and rib meristem but not in the corpus. Recombinant NTH15 protein strongly bound to a 5-bp dyadsymmetric sequence, GTGAC, in the first intron of *Ntc12* in vitro. Mutation of this sequence in the *Ntc12* gene abolished the NTH15-dependent suppression of *Ntc12* in the corpus of the SAM. Our results indicate that NTH15 directly represses *Ntc12* expression in the corpus of the wild-type SAM to maintain the indeterminate state of corpus cells. The suppression of NTH15 within cells at the flanks of the SAM permits GA biosynthesis, which promotes organized cell proliferation and consequently induces the determination of cell fate.

[Key Words: DNA binding; gibberellin 20-oxidase; in situ hybridization; KNOX homeodomain protein; shoot apical meristem]

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The shoot apical meristem (SAM) successively produces leaves, internodes, and axillary buds, keeping itself as a collection of indeterminate stem-like cells throughout plant development (Steeves and Sussex 1989). KNOX homeodomain proteins encoded by *knotted1*-like homeobox (*knox*) genes are preferentially accumulated in the indeterminate cells around the SAM, but not in the determinate lateral organs, such as leaves (Jackson et al. 1994; Lincoln et al. 1994; Nishimura et al. 1999; Sentoku et al. 1999). Loss-of-function mutations in the *Arabidopsis* *knox* gene *SHOOTMERISTEMLESS* (*STM*) and the maize *knotted1* (*kn1*) gene result in defects in shoot meristem development or maintenance (Long et al. 1996; Kerstetter et al. 1997). The opposite phenotype, namely, formation of adventitious meristems on leaves, has been observed in transgenic plants that constitutively and ectopically express *knox* genes (Matsuoka et al. 1993;

Sinha et al. 1993; Chuck et al. 1996; Nishimura et al. 2000). On the basis of this evidence, KNOX homeodomain proteins are considered to play a role in the maintenance of the indeterminate properties of cells in the SAM (Reiser et al. 2000); however their direct function is still unresolved.

To understand the mechanism of KNOX homeodomain protein function, it is necessary to characterize genes that are targeted by these proteins. In this study, we attempted to identify a target gene of the tobacco KNOX homeodomain protein, *Nicotiana tabacum* homeobox 15 (NTH15). Previously, we reported that ectopic expression of *NTH15* in transgenic tobacco plants under control of the cauliflower mosaic virus (CaMV) 35S promoter causes alterations in leaf morphology, including formation of adventitious meristems with a drastic decrease in the level of endogenous bioactive gibberellin (GA), GA₁ (Tamaoki et al. 1997a). A similar result has also been reported in transgenic tobacco plants that express a rice *knox* gene, *OSH1* (Kusaba et al. 1998b). The decrease in GA₁ levels is probably caused by the suppression of a GA 20-oxidase gene, *Ntc12*, which

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encodes a key enzyme in GA biosynthesis and is mainly expressed in leaf primordia and young leaves (Kusaba et al. 1998a; Tanaka-Ueguchi et al. 1998). Based on these observations, we hypothesized that ectopic expression of *NTH15* inhibits the expression of *Ntc12* in leaf primordia, resulting in abnormal leaf morphologies in transgenic plants through the reduction in GA₁ levels.

Because a number of physiological changes, such as abnormal leaves with adventitious meristems, dwarfism, and loss of apical dominance, occur in transgenic plants that constitutively express *knox* genes, it has not been clear whether a direct interaction between NTH15 and *Ntc12* expression occurs or not. The levels of endogenous auxin and cytokinin also change in transgenic plants that constitutively express *knox* genes (Tamaoki et al. 1997a; Kusaba et al. 1998b; Frugis et al. 1999; Ori et al. 1999; Hewelt et al. 2000). In addition, many photosynthesis-related genes, which are induced by exogenous cytokinin treatment, are up-regulated in transgenic tobacco plants that express *OSH1* (Tamaoki et al. 1997b). These results highlight the difficulty in identifying the primary effects of KNOX homeodomain protein function in transgenic plants that constitutively express *knox* genes.

Recently, an inducible system was developed for plant cells using the regulatory mechanism of the glucocorticoid receptor (GR; Schena et al. 1991; Lloyd et al. 1994; Aoyama et al. 1995; Aoyama and Chua 1997). The steroid binding domain of the GR inactivates the function of a neighboring domain in the chimeric protein molecule in the absence of steroid ligand, but the function is

restored in the presence of ligand, dexamethasone (DEX) even in plants (Schena et al. 1991). This system may provide us with an important advantage in understanding the function of KNOX homeodomain proteins.

In this study, we expressed NTH15–GR fusion protein in transgenic tobacco plants. These transformants showed DEX-dependent induction of NTH15 function. Using this inducible system, we found that NTH15 directly suppresses the *Ntc12* expression. Further analyses revealed that the NTH15 binding sequence in the first intron of *Ntc12* acts as *cis*-motif in the SAM. Based on these results, we propose that NTH15 maintains the indeterminate properties of cells in the corpus of the SAM through the direct suppression of *Ntc12* expression and GA biosynthesis.

Results

NTH15 function is inducible by artificial steroid treatment

To artificially regulate NTH15 activity in vivo, the translational stop codon of *NTH15* was removed and replaced with a 278-amino-acid segment of the human GR that contains the ligand-binding domain. The chimeric protein was expressed under control of the CaMV 35S promoter (35S::*NTH15*–GR; Fig. 1A) and was introduced into tobacco plants by *Agrobacterium*-mediated transformation.

All primary transformants (11 independent lines) de-

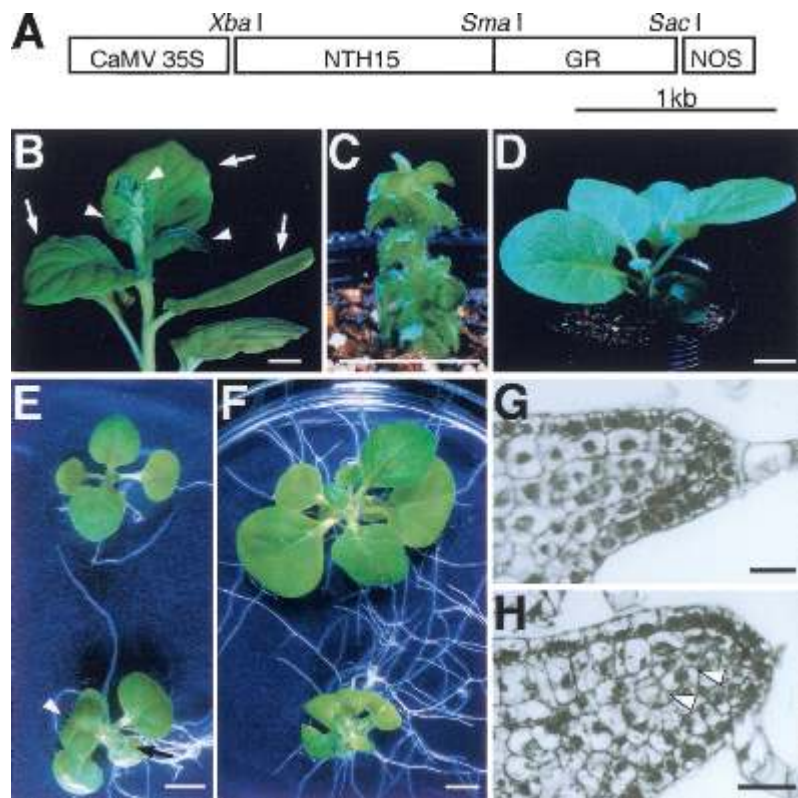


Figure 1. Diagram of *NTH15*–GR fusion construct and represented phenotype. (A) The chimeric gene consists of an in-frame fusion of the entire *NTH15* cDNA and the steroid-binding domain of the human glucocorticoid receptor (GR). This gene was driven by the cauliflower mosaic virus 35S promoter. (B) Transformants carrying the fusion construct produced normal leaves in the absence of dexamethasone (DEX; arrows) and developed severely malformed leaves after a drop of DEX was applied on the shoot apical meristem (arrowheads). (C,D) Transgenic seedlings grown with (C) or without (D) 10 μ M DEX. (E,F) Morphological changes in wild-type (upper) and 35S::*NTH15*–GR transgenic (lower) seedlings at 7 d (E) and 14 d (F) after DEX treatment. Arrowhead and arrow indicate the third and fourth true leaves, respectively. (G,H) Blade initiation in a leaf primordium of wild-type (G) and 35S::*NTH15*–GR transgenic (H) seedlings at 3 d after DEX treatment. Open arrowheads indicate the site of abnormal mesophyll cell division. Bars, 1 cm (B–D); 5 mm (E,F); 20 μ m (G,H).

veloped normal leaves without the steroid (Fig. 1B, arrows), but they produced malformed leaves after treatment with 10 μ M DEX (Fig. 1B, arrowheads). Seeds from self-pollinated transformants were sown with or without 10 μ M DEX and grown for 1 mo. Compared to wild-type seedlings, DEX-treated seedlings were severely dwarfed, and their leaves were very small and thick (Fig. 1C), as found in the severe phenotype of *35S::NTH15* transformants (Tamaoki et al. 1997a). This phenotype was not observed in either *35S::NTH15-GR* seedlings grown in the absence of DEX (Fig. 1D) or in wild-type seedlings treated with the same concentration of DEX (Fig. 1E,F, upper plants), which confirms that NTH15 function was successfully induced in a ligand-dependent manner by DEX treatment.

Abnormal cell divisions result in the aberrant leaf development

To clarify the kinetics of NTH15 action, we followed the process of leaf development after induction of NTH15 function. *35S::NTH15-GR* seedlings were grown without DEX for 14 d and then treated with 10 μ M DEX. At this time, the third true leaf had just reached a visible size. The first indication of abnormal morphology was apparent on the third true leaf; namely, there was a small reduction in the length of midvein and leaf blade was slightly wrinkled (Fig. 1E, arrowhead). The fourth true leaf reached a visible size at 7 d after DEX treatment and was poorly developed, similar to the leaves that had been constitutively treated with 10 μ M DEX (Fig. 1E, arrow). After emergence of the fourth true leaf, gross morphology of *35S::NTH15-GR* seedlings was clearly distinguishable from that of wild-type seedlings (Fig. 1F, cf. lower plant to upper plant).

Because the abnormality in phenotype was first recognized as malformed leaves that emerged from the SAM, we investigated the anatomy of leaf primordia of DEX-treated *35S::NTH15-GR* seedlings. In wild-type tobacco, leaf blade consists of six to seven cell layers that are arranged from the adaxial to abaxial side as: The upper epidermis, the palisade mesophyll, the middle spongy mesophyll, the lower spongy mesophyll, and the lower epidermis (Fig. 1G; McHale 1993). The cell layers are maintained during lateral expansion of leaf blade by a strict pattern of anticlinal divisions. We observed the morphology in leaf primordia every 24 h and found that abnormal cell divisions first became apparent at 3 d after DEX treatment. Although the anticlinal cell divisions were maintained in wild-type leaves (Fig. 1G), abnormal periclinal divisions in the mesophyll cell layers occurred sporadically in leaf blades of *35S::NTH15-GR* seedlings (Fig. 1H). A similar phenomenon has also been observed in *PR1a::OSH1* transgenic tobacco plants, which showed wrinkled and/or lobed leaves (Sato et al. 1996). These abnormal divisions cause the intercalation of cells into the cell layers, which disrupts the lineage of these layers and may result in the formation of abnormally shaped, thicker leaves.

Activation of NTH15 immediately suppresses Ntc12 expression

To follow the changes in endogenous GA biosynthesis, the levels of three GAs were analyzed. The bioactive GA₁ is synthesized from GA₅₃ through GA₄₄, GA₁₉, and GA₂₀. GA 20-oxidase catalyzes the whole series of oxidation reactions at C-20, the steps involved in conversion from GA₅₃ to GA₂₀, and GA 3 β -hydroxylase catalyzes the 3 β -hydroxylation of GA₂₀ to form GA₁ (Hedden and Kamiya 1997). We found that the level of GA₂₀ was drastically decreased within 6 h of DEX treatment (Fig. 2A). GA₁ levels decreased gradually from 6 h to 24 h, whereas the level of GA₅₃ was not significantly changed in either the presence or absence of DEX. These results show that activation of NTH15 interferes within 6 h with a particular step in GA biosynthesis catalyzed by GA 20-oxidase, causing a gradual decrease in bioactive GA₁ levels.

To investigate whether *Ntc12* mRNA levels were also reduced \leq 6 h after DEX treatment, RNA gel blot analysis was performed. The level of *Ntc12* mRNA was drastically reduced from 1 h to 3 h after the treatment (Fig. 2B). In contrast, mRNA levels of *Nty2*, which encodes a GA 3 β -hydroxylase gene (Itoh et al. 1999), remained unchanged until 6 h, after which time the mRNA levels increased, probably because of a feedback up-regulation triggered by the decrease in GA₁ levels (Fig. 2B). These results strongly suggest that the immediate suppression of *Ntc12* within 3 h of DEX treatment caused the change in the level of bioactive GA₁ via a rapid decrease in GA₂₀ levels within 6 h.

The kinetics of the decrease in *Ntc12* mRNA levels was the same even under treatment with α -amanitin, an inhibitor of RNA polymerase II activity (Fig. 2C). This indicates that reduction in the level of *Ntc12* mRNA after DEX treatment reflects the degradation kinetics of *Ntc12* mRNA in vivo. To examine whether the NTH15-dependent suppression of *Ntc12* expression requires prior synthesis of other proteins, *35S::NTH15-GR* seedlings were treated with a protein synthesis inhibitor, cycloheximide (CHX), and changes in *Ntc12* mRNA levels were monitored by RNA gel blot analysis. CHX treatment had no effect on the decrease in *Ntc12* expression (Fig. 2D), but it completely abolished the increase in *Nty2* mRNA levels that occurred in response to DEX treatment (Fig. 2D). The effect of CHX on the incorporation of [³⁵S]methionine confirmed that CHX treatment successfully inhibited protein synthesis (<5%). Taken together, these data indicate that NTH15 directly suppresses the *Ntc12* expression without prior synthesis of any other proteins.

NTH15 binds to the 5' flanking and intron sequences of the Ntc12 gene

The *Ntc12* gene consists of three exons and two introns (Fig. 3A). A 1.9-kb segment of *Ntc12*, which includes 1.0 kb of the 5' flanking region, the first exon, and the entire first intron, is sufficient to achieve correct GUS reporter

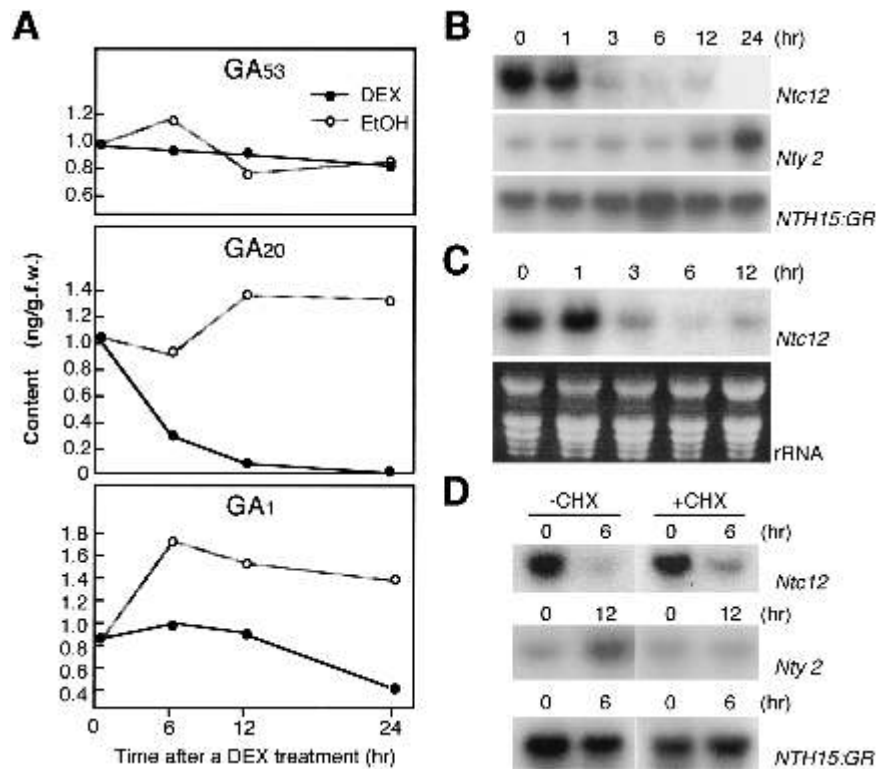


Figure 2. Analysis of the endogenous levels of gibberellins (GAs) and *Ntc12* expression after dexamethasone (DEX) treatment. (A) Changes in the level of endogenous GAs. *35S::NTH15-GR* seedlings were harvested at 0 h, 6 h, 12 h, and 24 h after treatment with (closed circle) or without (open circle) DEX. (B–D) RNA gel blot analysis of *Ntc12* and *Nty2*. Total RNAs (20 μ g) at 0 h, 1 h, 3 h, 6 h, 12 h, and 24 h after DEX treatment without (B) or with (C) α -amanitin were probed with labeled *Ntc12* or *Nty2*. α -Amanitin and DEX were applied at the same time. For blocking of protein synthesis, CHX was treated 1 h before DEX treatment (D). Numbers indicate times after DEX treatment. Expression of *NTH15-GR* (B,D) or rRNA (C) is shown as a loading control.

expression in transgenic tobacco plants (M. Ueguchi-Tanaka, T. Sakamoto, and M. Matsuoka, unpubl.). To investigate the possibility of a direct interaction between NTH15 and the *Ntc12* sequence, this 1.9-kb region was cleaved into seven \sim 350-bp fragments (Fig. 3A) and analyzed with electrophoresis mobility shift assay (EMSA; Ausubel et al. 1995). Recombinant NTH15 protein expressed in *Escherichia coli* cells bound to fragments 1 and 7, resulting in bands with lower mobility than free fragments (Fig. 3B). Bacterial lysate prepared from *E. coli* cells that carry only the vector did not generate complex (data not shown), which indicates that the retardation was specifically caused by an interaction with NTH15. The retarded complex was specifically competed by increasing concentrations of nonlabeled fragment 7 (Fig. 3C). The nonlabeled fragment 7 competed more strongly with the fragment-1 complex than with the fragment-7 complex. This shows that fragment 7, which encompasses the entire sequence of the first intron, has a higher binding affinity with NTH15 than does fragment 1, which is located furthest upstream of the 5' flanking region that we used. The precise NTH15-binding sites in these fragments were identified by DNase I protection assay, which revealed that NTH15 interacted with a sequence TATGTTAC in fragment 1 and with TATGTGAC in fragment 7 (Fig. 4).

To confirm that these binding sequences were specifically recognized by NTH15, DNA sequence-specific binding of NTH15 was analyzed by EMSA using a series of mutagenized probes. Nucleotide positions within the TATGT(G/T)AC element were defined as 1–8 from the 5' end to 3' end, and corresponding mutations were de-

finied as M1–M8 (Fig. 5A). Single nucleotide substitutions at positions 1–3 (M1–M3) had no effect on the affinity of binding with NTH15; however, substitutions at positions 4, 5, 7, and 8 (M4, M5, M7, and M8) dramatically reduced binding to undetectable levels (Fig. 5B). In contrast, substitutions at position 6 led to a reduction in the NTH15-binding affinity, but did not completely eliminate it (Fig. 5B). Competition experiments using four interactive oligonucleotides (wt1, wt2, M6a, and M6c) as probes and nonlabeled wt2 fragment as a competitor revealed that NTH15 preferably interacted with the sequence containing G at position 6 (Fig. 5C). These findings led us to conclude that NTH15 recognizes the 5-bp dyadsymmetric sequence GTNAC at positions 4–8 with a preference for G at position 6.

The NTH15-binding sequence in the first intron of Ntc12 is required for the NTH15-dependent suppression of Ntc12

Because NTH15 binds to two different sites on *Ntc12* in vitro, these two sites may act as *cis*-motives for transcriptional repression of *Ntc12* by NTH15 in vivo. To examine this possibility, we introduced nucleotide substitutions independently at these two sites (Fig. 6A), generated a fusion construct with luciferase (LUC) reporter gene, and then transferred into *35S::NTH15-GR* transformants. LUC luminescence was ubiquitously found at a low level in young leaves and around the SAM grown in the absence of DEX (Fig. 6B, time indicated as 0). This low luminescence activity is consistent with previous observations from RNA gel blot and in situ hybridization

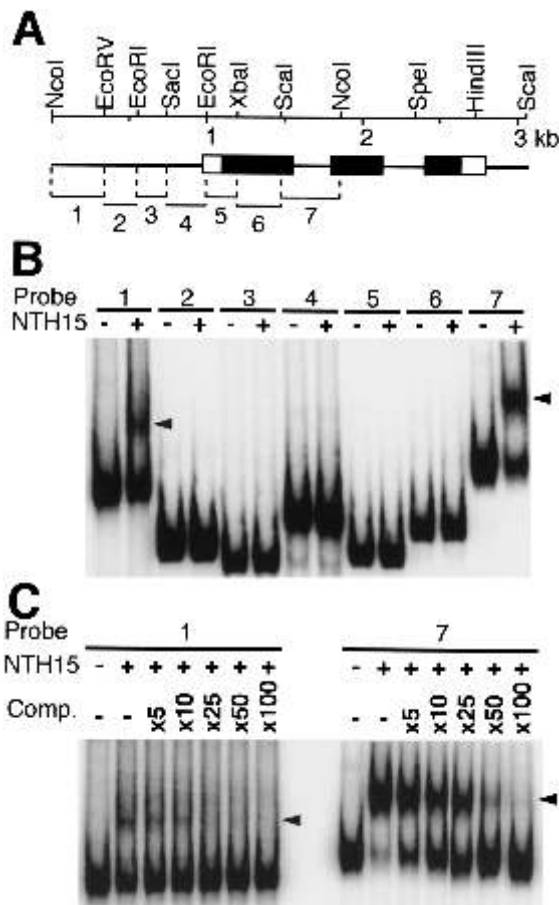


Figure 3. Binding of NTH15 to the 5'-flanking and first intron sequences in the *Ntc12* gene. (A) Genomic structure of *Ntc12*. Lines, open, and closed boxes indicate introns, noncoding, and coding sequences, respectively. Shown at the top is a restriction map of *Ntc12*. Fragments used as probes for electrophoresis mobility shift assay (EMSA) are numbered from 1 to 7. (B) EMSA of the seven probes with recombinant NTH15 protein. DNA fragments were incubated without (-) or with (+) NTH15. Fragments 1 and 7 were shifted by incubation with NTH15 as shown by arrowheads. (C) Fragment 7 binds to NTH15 much more strongly than fragment 1. Binding affinity was estimated using nonlabeled fragment 7 as a competitor, which was added in excess over the probe as indicated (Comp.). The NTH15-DNA complexes are indicated by arrowheads.

analyses of *Ntc12* expression in wild type (Tanaka-Ueguchi et al. 1998). Following DEX treatment, *in vivo* luminescence was steeply decreased in transformants that carry the wild-type promoter (*Ntc12-w.t.*; Fig. 6B), such that LUC activity was reduced to 11.4% of initial levels 6 h after the treatment (Fig. 6D). Similar repression was observed in transformants that contained the mutant promoter at the distal site (*Ntc12-m1*; Fig. 6C,D). However, this suppression of luminescence was not observed in transformants that carry the mutant promoter at either the proximal site or at both sites (*Ntc12-m2* or *Ntc12-m3*, respectively) in which the relative LUC activity remained at 90.2% and 98.3%, respectively (Fig. 6C,D). Thus, the NTH15-binding sequence

TATGTGAC in the first intron of *Ntc12* is essential for the suppression of *Ntc12* expression through the activation of NTH15.

In situ hybridization of the tobacco SAM was performed to confirm that NTH15 suppresses *Ntc12* expression in wild type. *NTH15* mRNA was present in corpus cells, the inner layers of the SAM, but was not seen in the outermost layers (L1), rib meristem or leaf primordia (Fig. 7A), as previously reported by Tamaoki et al. (1997a). In contrast, *Ntc12* transcript was localized to leaf primordia and rib meristem, areas in which *NTH15* mRNA was not detected, and repressed in the corpus of the SAM (Fig. 7B).

We also determined the *in situ* mRNA localization of LUC reporter gene driven by the wild-type and mutant *Ntc12* promoter. As for endogenous *Ntc12* mRNA expression, LUC mRNA was localized to leaf primordia and rib meristem but not in the corpus of the SAM in *Ntc12-w.t.* transformants (Fig. 7C). However, in *Ntc12-*

Fragment 1: CAATATGTTACATGAAATCGT
 |||||
 Fragment 7: AAAGTTATGTTATGTGACGAGGAAA

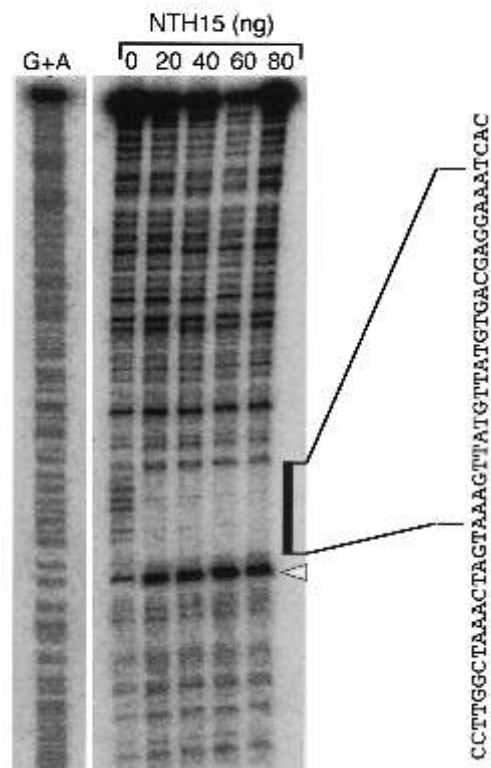


Figure 4. DNase I protection assay of the NTH15-binding sites. An alignment of the regions protected from DNase I digestion by NTH15 in fragments 1 and 7 is shown at top. The reaction of the labeled fragment 7 is presented below. The region protected from DNase I digestion by NTH15 is marked by filled box. Open arrowhead indicates the NTH15-induced DNase I hypersensitive site. The scale to the left shows the reference ladders generated by the A + G sequence reaction of the same DNA fragments performed by the Maxam-Gilbert method.

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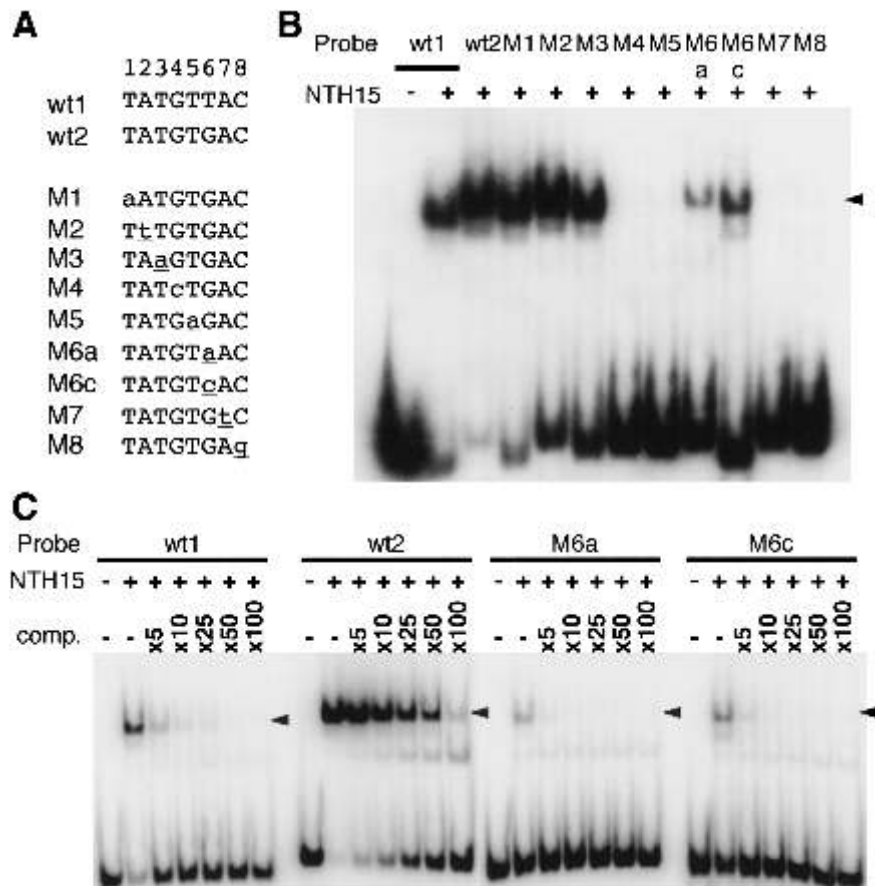


Figure 5. Preference of the NTH15-binding sequence. (A) Core sequences of the NTH15-binding site found in fragments 1 and 7 (wt1 and wt2) and its derivative sequences used for the experiments (M1 to M8). Numbers at the top indicate the nucleotide positions. (B) Electrophoresis mobility shift assay (EMSA) of each DNA fragment. Synthetic oligonucleotides containing the wild-type or mutagenized binding site were used as probes in EMSA. (C) NTH15 preferably interacted with the sequence containing G at position 6. The binding affinity was estimated with non-labeled wt2 as a competitor (Comp.). The NTH15–DNA complexes are indicated by arrowheads.

m2 transformants, *LUC* mRNA was expanded in the corpus of the SAM (Fig. 7D), in which *NTH15* mRNA was detected and *Ntc12* mRNA was not.

Discussion

It has often been reported that ectopic expression of *knox* genes from various plants causes drastic alterations in leaf and flower morphogenesis. However, such phenomena should be the result of a complex combination of direct and/or indirect effects of KNOX homeodomain protein function. Indeed, numerous kinds of genes are up-regulated in transgenic tobacco plants that carry a rice *knox* gene, *OSH1*, and most of them are considered to be induced indirectly by physiological stresses and changes in hormonal levels triggered by *OSH1* expression (Tamaoki et al. 1997b).

To distinguish the direct effects of KNOX homeodomain proteins from the various changes observed in transgenic plants, we generated an artificial inducible system of NTH15 function using the human GR. This induction system has been applied to a number of genes that are involved in plant development, including: The *Arabidopsis* homeobox gene, *Athb1*; the flowering-time gene, *CONSTANS*; the floral homeotic genes, *APETALA3/PISTILLATA*; and the maize transcriptional regulator, *R* (Lloyd et al. 1994; Aoyama et al. 1995; Simon et al. 1996; Robert et al. 1998).

Using this inducible system, we found that the inhibition of GA biosynthesis via the specific suppression of *Ntc12* expression was one of the earliest events caused by the activation of NTH15. The fact that this NTH15-dependent suppression of *Ntc12* expression did not require new protein synthesis indicates that *Ntc12* may be a member of target genes in the genetic systems regulated by NTH15. Indeed, recombinant NTH15 protein bound to the specific intron sequence of *Ntc12* in vitro.

Biochemical studies with EMSA showed that NTH15 recognized the 5-bp dyadsymmetric sequence GTNAC and that it favored G relative to other nucleotides at the third position. In contrast to the typical homeodomain proteins, such as Antennapedia and Ultrabithorax of *Drosophila*, which recognize the core sequence TAAT, TALE-atypical (for three amino acid loop extension between helices I and II) homeodomain proteins interact with a non-TAAT sequence (plant KNOX homeodomain proteins belong to this superclass). For example, the human TALE homeodomain protein, TGIF, binds to the TGTCA core sequence (Bertolino et al. 1995), and the barley KNOX homeodomain protein, Hooded (an ortholog of maize KN1), binds to the core sequence GTCA (Krusell et al. 1997). The NTH15 binding sequence, GTNAC, is very similar to these core sequences but not identical. NTH15 preferentially binds to GTGAC rather than GTCAC, which contains the core sequence of TGIF and Hooded. Conversely, the binding affinity of TGIF is

significantly reduced by a single nucleotide substitution from C to G within its core sequence TGTC A (Bertolino et al. 1995). Taken together, these data indicate that the binding property of NTH15 is slightly different from that of other TALE homeodomain proteins. Such minor differences between TALE homeodomain proteins may enable them to target different genes and consequently cause a unique function of each homeodomain protein.

Analysis of the NTH15-binding sites revealed that the binding sequence in the first intron of *Ntc12* was a functional target of NTH15 for negative regulation of *Ntc12*

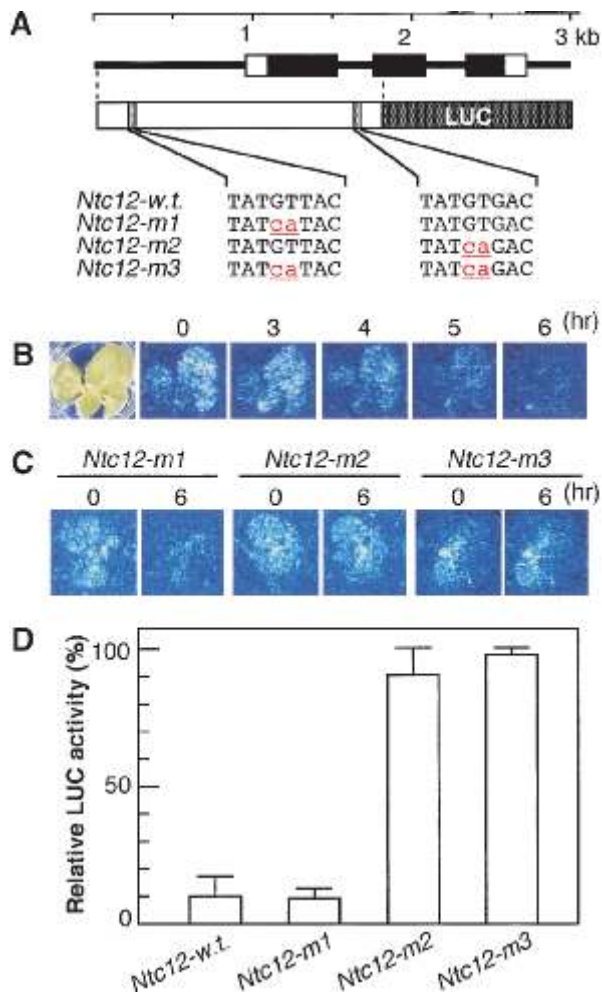


Figure 6. NTH15-dependent repression of *Ntc12* expression and the effect of two-base substitutions on *Ntc12* repression. (A) Schematic structure of the promoter-luciferase (LUC) construct and nucleotide substitutions in the three mutants (*Ntc12-m1* to *Ntc12-m3*) compared to the wild type (*Ntc12-w.t.*). (B) In vivo luminescence of LUC reporter was imaged using a high-sensitivity camera system. *Ntc12-w.t.* seedling was measured at 0 h, 3 h, 4 h, 5 h, and 6 h after dexamethasone (DEX) treatment. The appearance of treated seedling is shown in the left panel. (C) *Ntc12-m1*, *Ntc12-m2*, and *Ntc12-m3* seedlings were measured 0 h and 6 h after DEX treatment. (D) Relative LUC activity 6 h after DEX treatment. The value obtained before DEX treatment was arbitrarily set at 100%. Each data point is the average of five plants.

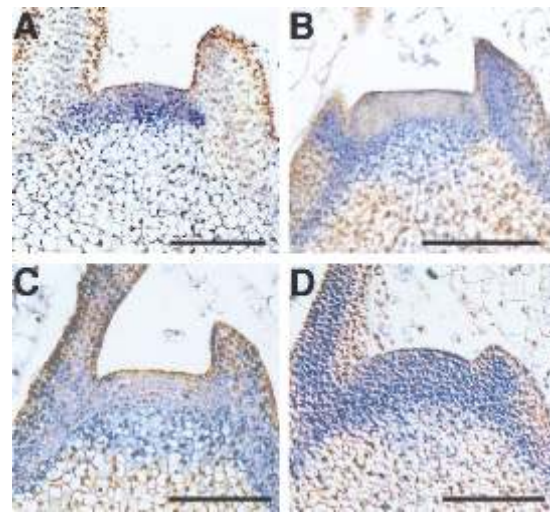


Figure 7. NTH15 directly represses *Ntc12* expression in vivo around the shoot apical meristem (SAM). Probes were labeled with digoxigenin-UTP and the transcript-specific hybridization signal is visible as purple staining. (A,B) In situ hybridization of *NTH15* (A) and *Ntc12* (B) mRNA, respectively, in the wild-type tobacco SAM. (C,D) Localization of luciferase (LUC) reporter gene transcript in the SAM of transformants carrying the wild-type (*Ntc12-w.t.*; C) and mutant promoter-reporter construct (*Ntc12-m2*; D), respectively. Bars, 100 μ m.

expression. Mutation in this sequence abolished the suppression of *Ntc12* by the activation of NTH15 function in vivo. In addition, *NTH15* and *Ntc12* showed an exclusive pattern of in situ mRNA localization in the wild-type SAM. This inverse relationship between *NTH15* and *Ntc12* expression strongly suggests that the suppression of *Ntc12* caused by NTH15 also occurs in the wild-type SAM. This possibility is also supported by the observation that mutation in the NTH15-binding sequence resulted in the expanded expression of *Ntc12* in the corpus of the SAM of *Ntc12-m2* transformants.

It is noteworthy that cells divide randomly in the inner layers of the SAM, where *NTH15* is expressed and *Ntc12* is not. However, the orientation of cell division is regulated in leaf primordia and rib meristem, where *Ntc12* mRNA is present and *NTH15* is suppressed. In addition, activation of NTH15 induces abnormal periclinal divisions in the mesophyll cell layers in leaves, where *Ntc12* and GA biosynthesis are suppressed. Bioactive GA is known to enhance transverse cell division and longitudinal cell expansion by affecting the arrangement of newly deposited cellulose microfibrils in the cell wall (Gunning 1982). Therefore it is possible that NTH15, through the repression of *Ntc12* and GA biosynthesis, plays a regulatory role in maintaining the flexibility of the orientation of cell division to preserve the indeterminate properties of cells in the inner layers of the SAM. We propose that suppression of NTH15 within cells at the flanks of the SAM permits the expression of *Ntc12* and enables them to increase their bioactive GA content, which promotes the organized cell division and consequently induces the determination of cell fate.

Materials and methods

Plasmid constructs

To create NTH15–GR fusion protein, the stop codon of *NTH15* was replaced with *Sma*I site (CCCGGG) by polymerase chain reaction (PCR) as reported previously (Sakamoto et al. 1999). In the same way, we amplified DNA fragments that encode the steroid-binding domain of the human GR (Hollenberg et al. 1985). The chimeric protein sequence was (NTH15 aa 1–342)-Pro-Gly-(GR aa 500–777). NTH15–GR was then cloned between the CaMV 35S promoter and the nopaline synthase (NOS) polyadenylation signal of pBI121 (Fig. 1A; Clontech).

To create a chimeric construct with a reporter gene under control of the promoter of *Ntc12*, 1.0 kb of the 5′-flanking sequence plus the sequences of entire first exon, first intron, and part of second exon of *Ntc12* were introduced at the front of *Luc* cDNA from pSP-luc+ vector (Promega). Nucleotide substitutions were introduced by PCR as previously reported (Sakamoto et al. 1999). The resulting chimeric genes (*Ntc12-w.t.*, *Ntc12-m1*, *Ntc12-m2*, and *Ntc12-m3*) were inserted into the 5′ end of the NOS polyadenylation signal of the hygromycin resistant binary vector pBI-H1 (Ohta et al. 1990).

Plant transformation and growth conditions

Constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv Samsun NN was performed with leaf discs as reported previously (Matsuoka and Sanada 1991). Transgenic plants were selected on media containing 100 mg/L kanamycin or 50 mg/L hygromycin. Antibiotic-resistant plants and seedlings from self-pollinated transformants were adapted to hydroponic growth conditions and grown at 25°C under continuous light.

Chemical treatments

DEX (Wako Pure Chemical Industries) was dissolved in ethanol to 30 mM before use and diluted with growth medium to 10 μM. The same volume of ethanol was added to the control medium. For treatment of whole plants, roots were submerged in growth medium containing 10 μM DEX. To inhibit RNA synthesis, 5 μM α-amanitin (Sigma) was added to growth medium. For inhibition of protein synthesis, seedlings were first soaked in growth medium containing 10 μM CHX (Sigma) for 1 h and then incubated in medium containing 10 μM CHX and 10 μM DEX or 10 μM CHX and ethanol as a control. The effect of CHX on protein synthesis was determined according to the method of Lam et al. (1989).

Histological analysis

Plant material was fixed overnight at 4°C in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated through a graded ethanol series followed by a *t*-butanol series (Sass 1958), and finally embedded in Paraplast Plus (Sherwood Medical). Microtome sections of 7–10 μm in thickness were applied to silan-coated glass slides (Matsunami Glass). The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and dried overnight before staining with hematoxylin.

GA analysis

Whole above-ground portion of 35S::NTH15–GR seedlings was harvested and used for the analysis of endogenous GA levels.

Samples of plant material were extracted with methanol. After addition of the radio-labeled internal standards, methanol extracts were purified as described previously (Nakayama et al. 1989). The purified and methylated samples were then analyzed by GC-SIM with a Hewlett-Packard 5989 B GC-MS system fitted with a DB-1 fused-silica chemically bonded capillary column (J & W Scientific) as described previously (Tanaka-Ueguchi et al. 1998).

RNA gel blot analysis

Total RNA from whole above-ground portion of 35S::NTH15–GR seedlings was extracted and 20 μg of each RNA sample was electrophoresed on a 1.2% agarose gel, then transferred onto Hybond N+ membrane (Amersham Pharmacia Biotech). Hybridization was performed in 5× SSC, 5× Denhardt's solution, 0.5% SDS, 10% dextran sulphate, and 20 mg/L salmon sperm DNA at 65°C for 14 h. The filter was washed in 2× SSC containing 0.1% SDS at 65°C and then further washed in 0.2× SSC containing 0.1% SDS at 65°C.

EMSA and DNase I protection assay

To produce NTH15 protein, the full-length *NTH15* cDNA was inserted in the sense orientation as a translational fusion into the pET-32a expression vector (Novagen) and expressed in BL21 (DE3) *E. coli* cells (Stratagene). The *Ntc12* DNA fragments of ~350 bp were excised with restriction endonucleases, purified on 8% polyacrylamide gels, labeled with [³²P]dATP using Klenow fragment and purified on Sephadex G-50 columns. DNA-binding reactions were performed at 4°C for 30 min in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 500 μM DTT, 10% glycerol, 0.05% IGEPAL CA-630 (Sigma), and 50 mg/L poly(dI-dC) · poly(dI-dC) (Amersham Pharmacia Biotech), and subjected to EMSA with 8% polyacrylamide gels in 0.25 × Tris-borate-EDTA buffer.

For DNase I protection experiment, labeled DNA fragments and NTH15 protein were incubated at 4°C for 30 min in 50 μL of the same solution described above. After incubation, 5 μL of diluent (10 mM MgCl₂, 5 mM CaCl₂) was added, followed immediately by 1 unit of DNase I (Amersham Pharmacia Biotech). After 1 min, reactions were terminated by addition of 150 μL of a stop buffer (192 mM CH₃COONa, 32 mM EDTA, 0.14% SDS, 64 mg/L yeast DNA). Following extraction with 0.2 mL of phenol:chloroform (1:1), nucleic acids were concentrated by ethanol precipitation, electrophoresed in 8% polyacrylamide gels containing 8 M urea, and detected by autoradiography. Reference ladders were generated by the A + G sequence reaction of the same DNA fragments performed by the Maxam-Gilbert method (Maxam and Gilbert 1977).

Luciferase assays

To image LUC luminescence, roots of transgenic seedlings were submerged in a solution of 10 mM potassium luciferin (Bio-Rad Laboratories) for 10 h before DEX treatment. LUC luminescence from transgenic seedlings was visualized using an image-intensifying camera and photon-counting image processors (Hamamatsu Photonic Systems ARGUS-50). Exposure time was 15 min for each assay. Luminescence was quantified from images captured in centroid-processing mode (this records a single pixel of unit depth for each photoelectron detected by the camera) and corrected for background counts from thermal electrons.

In situ hybridization analysis

Plant materials were fixed and embedded as described above. Microtome sections (7–10 μm in thickness) were applied to

glass slides treated with Vectabond (Vector Laboratory). Hybridization with digoxigenin-labeled sense or antisense RNA and immunological detection of the hybridized probe were conducted according to the method described by Kouchi and Hata (1993).

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KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem

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