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Ectopic Expression of a Tobacco Homeobox Gene, *NTH15*, Dramatically Alters Leaf Morphology and Hormone Levels in Transgenic Tobacco

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The shoot apical meristem functions to generate the lateral organs of a plant throughout the vegetative and reproductive phases. Homeobox genes play key roles in controlling such developmental programs, but their modes of action have not been well defined. Here we describe isolation and biological functions of a novel tobacco homeobox gene, designated *NTH15* (*Nicotiana tabacum* homeobox 15), from a tobacco shoot apex cDNA library. *NTH15* encodes a polypeptide of 342 amino acids, its homeodomain is very similar to the class 1 KNOTTED-type homeodomains. *NTH15* mRNA is mainly localized in corpus cells in the tobacco shoot apical meristem, but not in tunica layers nor in differentiated lateral organs. The *NTH15* cDNA was fused to the cauliflower mosaic virus 35S promoter and used to generate transgenic tobacco plants. Almost all transgenic tobacco plants showed abnormal leaf and/or flower morphology, and were categorized into three groups depending on severity of the leaf phenotype. In transgenic leaves, drastic decrease of GA_1 and increase of cytokinin were observed, while the levels of other phytohormones were only slightly changed. Taken together, our results suggest *NTH15* is involved in tobacco morphogenesis and abnormal leaf morphology in transgenic plants results from altered hormone levels.

Key words: Homeobox gene — In situ hybridization — Leaf morphogenesis — *Nicotiana tabacum* — Plant hormones — Transgenic plant.

Almost all of the tissues and organs in mature plants are ultimately formed from specialized groups of cells, the shoot and root meristems, which arise at approximately opposite poles during embryogenesis. The shoot apical meristem (SAM) functions to generate the above-ground portions of the plant, which include leaves, stems and flowers, throughout the vegetative and reproductive phases of plant development. The cells of the shoot apex are small, nearly isodiametric, thin-walled and characterized by a high nucleocytoplasmic ratio. In angiosperms, the SAM consists of two regions, tunica and corpus (Schmidt 1924). In general, the tunica consists of one cell layer (L1) in monocots and two cell layers (L1 and L2) in dicots and covers the underlying corpus. This tunica/corpus structure of the SAM is a consequence of differences in the orientation of cell division. Cell divisions in the tunica are ordinarily restricted to the anticlinal plane, thus maintaining the layered arrangement of the tunica. Within the corpus, the plane of cell divisions are less regularly oriented, resulting in a jumbled arrangement of cells. Despite extensive knowledge of the complex structural organization of the SAM, very little is known about the molecular mechanisms responsible for meristem function in processes such as lateral organ formation.

Several dominant mutations have been described that lead to alterations in the determination patterns within the maize leaf blade (Smith and Hake 1992). The most extensively characterized among these is the *KNOTTED1* (*Kn1*) mutation, that results in the formation of pocketed outgrowths (knots) on blade lateral veins (Freeling and Hake 1985). The *Kn1* gene has been cloned by transposon tagging and its cDNA sequence revealed that it encodes a homeodomain protein (Vollbrecht et al. 1991). The homeodomain proteins were first identified as conserved proteins encoded by several genes that control morphogenesis in the fruit fly *Drosophila* (for review, see Gehring 1987). By analogy to the functional roles of animal homeobox genes, plant homeobox genes are thought to play important roles in plant morphogenesis in the SAM. Many homeodomain proteins have been cloned from various plants in efforts to address the biological function(s) of homeobox genes in plant development. Amino acid sequence similarities within the homeodomain or conserved protein motifs outside of the homeodomain have been used to subdivide previously-isolated homeobox genes into related classes. For example, animal homeodomain proteins are roughly grouped into the Antennapedia, Engrailed, Paired, POU and LIM classes (Treisman et al. 1991). In plants, four different types of homeodomain proteins have been described. These include the KNOTTED-type homeodomain proteins, homeodomain zipper proteins (HD-ZIP), plant homeodomain finger proteins (PHD-finger) and the GLABRA2 homeodomain protein from *Arabidopsis* (Kerstetter et al. 1994).

In animal development, the generation of diversity

The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GeneBank Nucleotide Sequence Databases under the accession number AB004785.

and pattern is controlled in part by the functions of homeobox genes (Gurdon 1992). In the case of *Drosophila*, the formation of a gradient in the level of the bicoid protein, which contains a homeodomain, is involved in development of the anterior-posterior axis of the embryo (Johnston and Nüsslein-Volhard 1992), and the sequential activation of *HOM* genes results in morphogenetic segmentation in the larva (Lawrence and Morata 1994). In contrast to animal homeobox genes, the function of most homeobox genes in plant development is, with few exceptions, still unclear. For example, the *STM* gene is known to be required for SAM formation during embryogenesis (Long et al. 1996); *GL2* is necessary for normal trichome formation (Rerie et al. 1994); and *Bell* is involved in ovule development (Reiser et al. 1995). Some genes grouped within the KNOTTED-type gene family are also well characterized. Genes in this group show strong expression around the SAM, and their ectopic expression in spontaneous mutants and in transgenic plants alters leaf and flower morphology (Freeling et al. 1992, Matsuoka et al. 1993, 1995, Jackson et al. 1994, Lincoln et al. 1994, Schneeberger et al. 1995). Based on these observations, it has been proposed that the KNOTTED-type homeobox genes are involved in lateral organ formation from the SAM, but their roles in such processes have not been well defined.

In this paper, we describe the cloning and expression pattern of a novel tobacco homeobox gene, designated *NTH15* (*Nicotiana tabacum* homeobox 15). This gene encodes a homeodomain protein which falls into the KNOTTED-type family of homeodomains and its expression resembles that of other KNOTTED-type genes. We also describe the phenotypes of transgenic tobacco plants expressing *NTH15*. Drastic changes were seen in the levels of several plant hormones in transgenic plants with altered leaf morphogenesis. We discuss the possibility that the product of *NTH15* may regulate the level of plant hormones.

Materials and Methods

Plant growth conditions—Tobacco seeds (*Nicotiana tabacum* cv. Samsun NN) were sterilized in 5% sodium hypochlorite for 5 minutes and germinated on germination medium (Murashige and Skoog salts with 1% sucrose and 0.5% gelangum) under continuous light at 25°C. The seedlings were transplanted to soil and grown at 25°C in a 16 h light–8 h dark cycle.

Screening of cDNA libraries—A cDNA library was constructed from shoot apices of mature plants. Total RNA was extracted from this tissue and poly(A)$^+$ enriched RNA was purified by two passes through an oligo d(T) cellulose (type III, Becton Dickinson Labware) column. The poly(A)$^+$ RNA was used to synthesize double stranded cDNA and cloned into the *Eco*RI site of λgt11 (Stratagene). Screening was performed in 50% Formamide, 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 0.1 mg ml^{-1} salmon sperm DNA at 42°C for 14 h using a homeobox sequence of *OSH1* as a probe (Matsuoka et al. 1993).

Sequence analysis—Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an automated sequencing system (ABI 373A). The cDNA clone was completely sequenced on both strands. Analysis of cDNA and amino acid sequences was carried out using GENETYX computer software (Software Kaihatsu Co., Japan).

RNA gel blot analysis—Total RNA was prepared from various organs and tissues for gel blot analysis. 10 μg of each RNA preparation was transferred to Hybond N membrane (Amersham), and hybridized to a 580 bp *Eco*RI/*Hin*dIII fragment of the *NTH15* cDNA which did not include the homeobox sequence to avoid cross-hybridization with other homeobox genes. Hybridization was performed at 42°C in a solution containing 50% formamide, 5 × SSC, 0.2% SDS, 0.1% N-lauroylsarcosine, and 1% blocking reagent (Boehringer). Filters were washed in 2 × SSC, 0.1% SDS at room temperature and then further washed in 0.2 × SSC, 0.1% SDS at 65°C.

In situ hybridization—Plant material was fixed in 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C, dehydrated through a graded ethanol series followed by a t-butanol series (Sass 1958), and finally embedded in Paraplast Plus (Sherwood Medical). Microtome sections (7–10 μm thick) were mounted on glass slides treated with Vectabond (Vector Labs). Paraffin of mounted sections was removed in xylene, re-hydrated through a graded ethanol series and dried overnight prior to performing in situ hybridization.

In situ hybridization was performed using digoxigenin-labeled sense or antisense RNA produced from the *NTH15* coding region lacking the poly(A)-region. Hybridization and immunological detection of the hybridized probes were performed according to the method of Kouchi and Hata (1993).

Tobacco transformation—A cDNA clone encoding the NTH15 protein was introduced into the *Bam*HI site of pBI121 after deletion of the β-glucronidase gene to produce the 35S-*NTH15* construct. This construct was then introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv. Samsun NN was performed with leaf discs as previously reported (Matsuoka and Sanada 1991). Transgenic plants were selected on media containing 100 mg liter^{-1} of kanamycin.

Plant hormone assays—Leaf material (10 to 20 g) was homogenized in 80% aqueous acetone (4 : 1, v/w) and ^{13}C-IAA and $^{2}H_3$-ABA were added as internal standards. The homogenate was filtered and solid residue was further extracted twice with 80% aqueous acetone (4 : 1, v/w). The extracts were combined and mixed, then divided into two equal parts. One part was used for IAA, ABA and GA analysis, and the other was used for cytokinin analysis. The extract used for IAA, ABA and GA analysis was fractionated by HPLC on the basis of retention times, and each fraction was assayed. The fractions containing IAA or ABA were subjected to GC-MS. The fractions containing GA_1 or GA_4 were assayed by ELISA with specific monoclonal antibodies for GA_1 and GA_4. For cytokinin analysis, samples were also partially purified by HPLC, and the resulting fractions were assayed by ELISA using a monoclonal antibody.

Results

Isolation of a tobacco homeobox gene—A cDNA library, constructed from poly(A)$^+$ RNA extracted from tobacco shoot apices, was screened using the homeobox se-

quence of the rice homeobox gene, *OSH1* (Matsuoka et al. 1993), as a probe. Several independent clones were isolated from this screening. Among these, we focused on characterization of the cDNA clone *NTH15* (*Nicotiana tabacum* homeobox 15) because the predicted homeodomain sequence encoded by this clone was most similar to that of OSH1.

We analyzed the entire nucleotide sequence of the *NTH15* cDNA clone. This clone contained an open reading frame encoding a 342 amino acid polypeptide (Fig. 1). An ATG positioned 129 bp from the 5'-end was considered to be the translation initiation codon because of the presence of an in-frame stop codon (TAA) nine nucleotides upstream. The clone encoded a 64 amino acid conserved homeodomain sequence. This homeodomain sequence was compared with other plant homeodomains (Fig. 2) and showed highest similarity to those of SBH1 from soybean (96% identity, Ma et al. 1994) and STM from *Arabidopsis* (96% identity, Long et al. 1996). Relatively high similarity was also found between NTH15 and other plant homeodomains, including OSH1 from rice (88% identity (Matsuoka et al. 1993), KN1 from maize (88% identity, Vollbrecht et al. 1991), and KNAT1 from *Arabidopsis* (89% identity, Lincoln et al. 1994). Since all these homeobox genes have been categorized in the KNOTTED-type gene class (Kerstetter et al. 1994, Long et al. 1996), *NTH15* was also classified as a KNOTTED-type gene. The cluster of positively-charged amino acids, which is a feature often observed in proteins localized in the nucleus, is just upstream of the first helix of NTH15 homeodomain (Fig. 1, 2A), a feature often observed in proteins localized in the nucleus. Therefore, this cluster in NTH15 may act as a targeting sequence

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AGAGAGGGAAAACAAAAGATAATTACACAGTGTGCGTGTGTGTTGTTTCATACCGAAAAACAAAAGGAACAAAATTAAGACAAGAGGGTT   90
ATAAGAAACTCAGAGAAAGAAAAAGGTAGTAAGAGTTAATGGGGGGGGGTGGTTCTAGTGGAAATACTTCTTCATGTTTAATGGGTTATG  180
                                M  G  G  G  G  S  S  G  N  T  S  S  C  L  M  G  Y  G   18

GAGATGACAACAACAACAATAACAGTGGAAATGCAGCCCTATGTCCTCCTCCTATGATGATGCCTCCTCCTCCTATTAATAATAACAATG  270
  D  D  N  N  N  N  N  S  G  N  A  A  L  C  P  P  P  M  M  M  P  P  P  P  I  N  N  N  N  G   48

GAGAAAGCAGCAATAATATTGGTGGCAACAACAACAACAATATCCTGTTTCTCCCTTTTATGGCCAACAACAACAATAATCCACATGAAG  360
  E  S  S  N  N  I  G  G  N  N  N  N  N  I  L  F  L  P  F  M  A  N  N  N  N  N  P  H  E  D   78

ACGCAAACTGTTCTTCTTCTAGTTCCATCAAGTCTAAGATTATGGCTCATCCTCACTACCCTCGTCTCTTGTCTGCTTATGTCAATTGTC  450
  A  N  C  S  S  S  S  S  S  I  K  S  K  I  M  A  H  P  H  Y  P  R  L  L  S  A  Y  V  N  C  Q  108

AAAAGATAGGAGCTCCGCCGGAAGTGGTGGCAAGGCTAGAGGAAGTATGTGCCACGTCAGCAACAATAGGCCGTAACAGTGGCGGCATTA  540
  K  I  G  A  P  P  E  V  V  A  R  L  E  E  V  C  A  T  S  A  T  I  G  R  N  S  G  G  I  I  138

TCGGAGAAGATCCAGCGCTAGATCAGTTCATGGAAGCTTACTGTGAAATGCTGACAAAATATGAGCAAGAACTTTCAAAACCCTTTAAGG  630
  G  E  D  P  A  L  D  Q  F  M  E  A  Y  C  E  M  L  T  K  Y  E  Q  E  L  S  K  P  F  K  E  168

AAGCCATGGTTTTCCTCTCAAGAATTGAGTGCCAGTTTAAAGCTCTCACTCTTACTTCCTCCTCTGAATCTGTTGCAGCTCTAGGTGAGG  720
  A  M  V  F  L  S  R  I  E  C  Q  F  K  A  L  T  L  T  S  S  S  E  S  V  A  A  L  G  E  A  198

CAATCGATAGAAATGGATCGTCTGAAGAGGAGGTTGATGTGAATAACGGTTTCATCGACCCTCAGGCTGAAGATCAAGAACTGAAAGGTC  810
  I  D  R  N  G  S  S  E  E  E  V  D  V  N  N  G  F  I  D  P  Q  A  E  D  Q  E  L  K  G  Q  228

AATTGCTGCGCAAATACAGTGGTTACTTGGGTAGCCTTAAGCAGGAGTTCATGAAGAAGAGGAAGAAAGGCAAGCTGCCTAAGGAAGCAA  900
  L  L  R  K  Y  S  G  Y  L  G  S  L  K  Q  E  F  M  K  K  R  K  K  G  K  L  P  K  E  A  R  258

GGCAACAACTACTGGACTGGTGGACCAGACATTACAAATGGCCATATCCATCGGAATCCCAGAAGCTGGCACTGGCTGAATCTACAGGAT  990
  Q  Q  L  L  D  W  W  T  R  H  Y  K  W  P  Y  P  S  E  S  Q  K  L  A  L  A  E  S  T  G  L  288

TGGACCAAAAACAAATAAACAACTGGTTTATCAACCAAAGGAAAAGGCACTGGAAACCTTCAGAGGATATGCAGTTTGTGGTAATGGATG 1080
  D  Q  K  Q  I  N  N  W  F  I  N  Q  R  K  R  H  W  K  P  S  E  D  M  Q  F  V  V  M  D  A  318

CTGCTCATCCACATTACTATATGGACAATGTTCTCGGTAATCCTTTTCCAATGGATATTACACCAACTCTCCTCTAAATTCTGGAGAAGG 1170
  A  H  P  H  Y  Y  M  D  N  V  L  G  N  P  F  P  M  D  I  T  P  T  L  L  *               342

CCAATTCCCACTTATGAAGATTAGAGTGATTCTTGAATCTTTAAAATGTGTAGTATTAGATGTATTATCAAGGATATTTTGCATATTACT 1260
CGTATGCATATCTAGTTGTTATTGTAACTTCAAGGCTAAGACCATAAGTACTTGTAGTTCGAGCTATTTCTGTTATGGTTAACTTTATTA 1350
AATTAAATTTACT                                                                               1363
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Fig. 1 Nucleotide and deduced amino acid sequences of *NTH15*. Numbers at right indicate nucleotide (upper) and amino acid (lower) positions. The in-frame stop codon (TAA) located upstream of the first ATG is boxed. A heavy underline indicates the homeodomain. The ELK domain is represented with dashed underline. The glutamine- and proline-rich regions are light-underlined. The putative nuclear localization sequence is marked in boldface type.

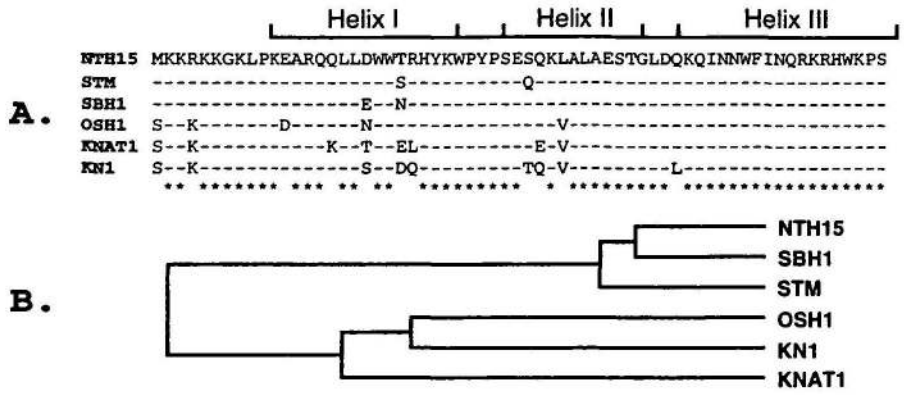

Fig. 2 Comparison of the homeodomains of NTH15 and other KNOTTED-type class 1 plant homeobox proteins. (A) Alignment of the homeodomains of NTH15 and other class 1 plant homeobox proteins. The deduced amino acid sequence of the NTH15 homeodomain is compared with the previously reported sequences of STM (Long et al. 1996), SBH1 (Ma et al. 1994), OSH1 (Matsuoka et al. 1993), KNAT1 (Lincoln et al. 1994) and KN1 (Vollbrecht et al. 1991). Asterisks indicate conserved amino acid residues among these homeodomains. (B) Phylogenetic tree of class1 plant homeodomains. A phylogenetic tree was drawn using the class 1 plant homeodomains that were aligned in (A). The UPGMA (Unweighted Pair Group Method with Arithmetic) tree was calculated using the DNA analysis software Genetyx Mac V. 7.3.

for nuclear localization (Raikhel 1992, Varagona et al. 1992).

The deduced amino acid sequence of NTH15 showed several interesting features outside the homeodomain. First, the flanking ELK (glutamate, leucine, lysine) domain, which is often found in other plant homeodomain proteins (Kerstetter et al. 1994), is also conserved in NTH15. In the ELK motif, hydrophobic residues are repeated at intervals of four and five amino acids and they are predicted to form an amphipathic helix having an important role in protein-protein interactions (Kerstetter et al. 1994). It is also noteworthy that NTH15 contains four aspargine-rich stretches and two proline-rich stretches near the N-terminus (Fig. 1). Both KNAT1 and KNAT2 from *Arabidopsis* also contain aspargine-rich regions at the corresponding locations (Lincoln et al. 1994). Similar homopolymeric amino acid stretches are also conserved in OSH1 (proline and glutamine), KN1 (histidine), and SBH1 (histidine, aspargine, and serine). The biological role of these homopolymeric stretches has not been elucidated. However, it has been suggested that they may be involved in transcriptional activation because polyproline and polyglutamine stretches often act as activation domains in trans-activation factors (Gerber et al. 1994). The presence of these characters in the predicted NTH15 protein indicates that, as found for other homeobox genes, *NTH15* may act as regulatory gene in tobacco.

Expression of NTH15 in various organs—RNA gel blot analysis was performed to elucidate the expression of *NTH15* in various organs in tobacco. Total RNA was extracted from shoot apices, flower buds, mature flowers, stems, roots, young leaves (2 or 3 cm) and expanded leaves (about 15 cm) and probed using a 580 bp fragment of the *NTH15* cDNA. A single strong band was detected in RNA from stems (Fig. 3). The size of this band was approximately 1.6 kb, slightly longer than that of the cDNA clone (1.3 kb). A weak band of the same size was also observed in RNA from shoot apices, flower buds, and flowers but not from roots or leaves. Similar expression patterns were obtained by in situ hybridization (see below), indicating that the *NTH15* gene is expressed in these organs.

In situ localization of the NTH15 expression in tobac-

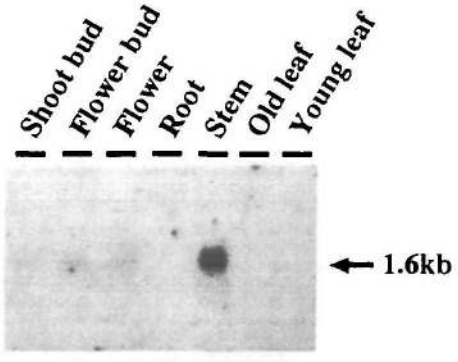

Fig. 3 RNA gel blot analysis of *NTH15* expression in wild-type tobacco. RNA was isolated from plant material enriched in vegetative shoot meristems, flower buds in various developmental stages, mature flowers, roots, vegetative stems, expanded leaves (about 15 cm), and unexpanded leaves (2 or 3 cm). 10 μg of total RNA were loaded per lane. The blot was probed using a 580 bp fragment of the *NTH15* cDNA which did not contain the region encoding the homeodomain. Approximate transcript size is indicated at right.

co tissues—To determine more precisely the spatial pattern of *NTH15* expression in tobacco, in situ hybridization with digoxigenin-labeled RNA probes was performed. The expression of *NTH15* in vegetative SAMs is shown in Figure 4. Control sections, hybridized with a sense-strand RNA probe, showed no signal above background staining (data not shown). Figure 4A shows a near-median longitudinal section through the shoot apex of a mature plant. Purple staining, indicating the presence of *NTH15* mRNA, was observed in the SAM. This staining was not uniform throughout the SAM, but was primarily localized below the two outermost layers of cells (Fig. 4C). The region with the highest level of *NTH15* expression represents the corpus, while the two outer layers of cells, in which little or no expression was observed, represent the tunica (see discussion). At the left side of the summit of the SAM, no *NTH15* transcript was observed even in the corpus region (Fig. 4C). In this region, organized periclinal cell divisions were often observed (arrowheads in Fig. 4C). Such periclinal divisions are the first sign of new leaf formation (Cunninghame and Lyndon 1986) and the next leaf primordium will initiate in this region. Therefore, the onset of leaf formation correlates with the down-regulation of *NTH15* expression (see discussion). Adjacent median and cross sections of the SAM also demonstrate that no signal was detected in leaf primordia although *NTH15* expression was widespread through the SAM (Fig. 4B, D). In other regions of the SAM shown in Figure 4A, strong expression was seen in the procambium and axial meristem, but not in the internal region of the rib or in more developed leaf primordia.

Accumulation of *NTH15* mRNA was also examined in other vegetative tissues. Figure 5A shows the expression of *NTH15* in a stem internode. Punctuate *NTH15* expression was observed at a distance on both sides of the xylem. The position of the primary phloem and xylem in tobacco stems is characteristic of an amphiphloic siphonostele in which the phloem surrounds the xylem both externally and internally (Fahn 1990). The observed pinpoints of *NTH15* expression were localized in companion cells of internal and external phloem. No signal was seen in cross sections of developed leaves or in longitudinal sections of root meristems

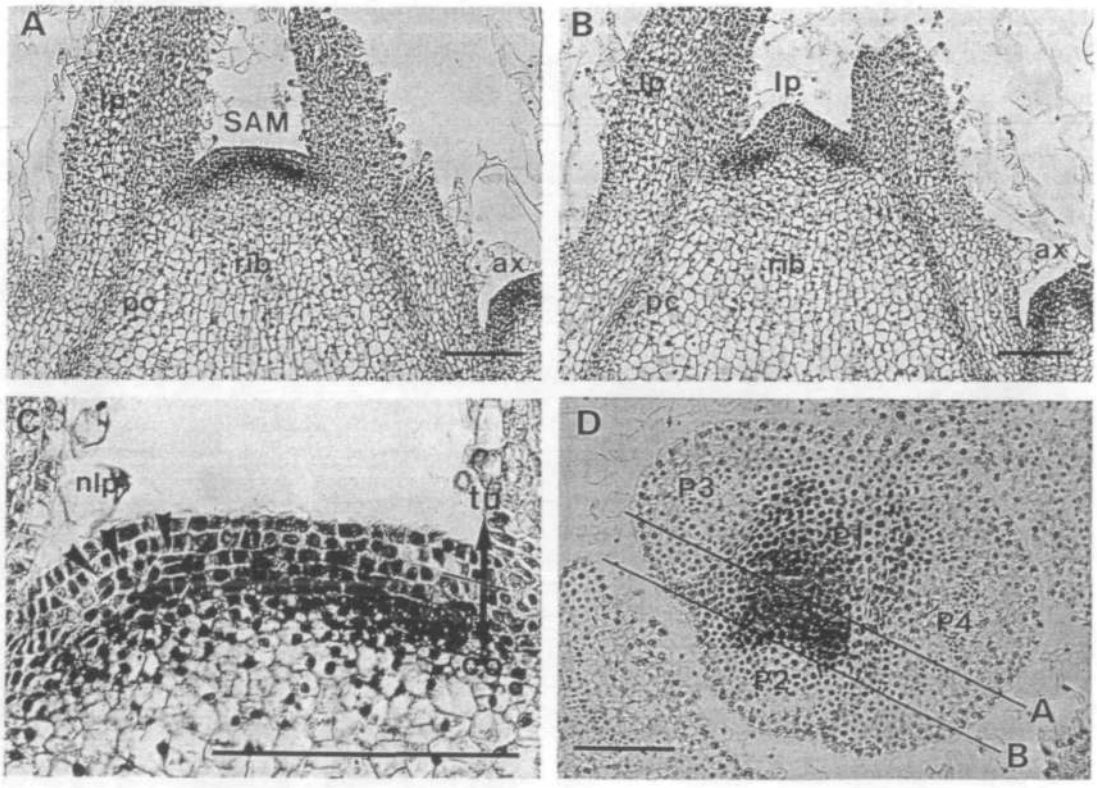

Fig. 4 In situ localization of *NTH15* mRNA in SAMs of wild-type tobacco. All tissues were hybridized with digoxigenin-labeled antisense probes. (A) Median longitudinal section of a vegetative shoot apex from a mature (approximately 60 days old) tobacco plant. (B) Longitudinal section adjacent to (A). This section was taken approximately 70 μm away from the median plane toward the margin of SAM. (C) High magnification of figure (A). Two cell layers of tunica are clearly identified. Arrows indicate periclinal cell divisions. (D) Transverse section of a shoot apex. Four leaf primordia (P1 to P4) are marked. This section passes approximately 20 μm below the summit of the SAM. A and B indicate the lines through which the sections shown in panels (A) and (B) pass. lp, leaf primordium; ax, axillary bud; pc, procambium; SAM, shoot apical meristem; nlp, new leaf primordium. All bars indicate 100 μm.

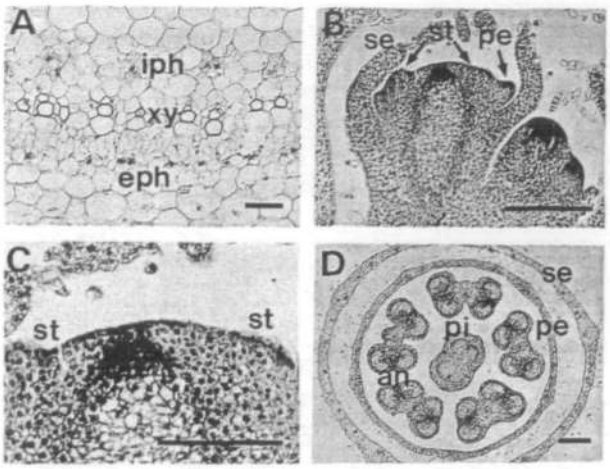

Fig. 5 In situ localization of *NTH15* mRNA in stem and flowers. (A) Cross section of a vegetative stem. (B) Longitudinal section of early stage floral meristems. Very young flower buds are shown at right, and a stage 5 floral primordium is at the center. (C) Higher magnification view of the stage 5 floral primordium shown in (B). (D) Cross section of a developed flower bud. Morphological features of tissues are indicated as follows: xy, xylem; iph, internal phloem; eph, external phloem; se, sepal; pe, petal; st, stamen; an, anther; pi, pistil. Bars, 100 μm in (A), 500 μm in (B), 250 μm in (C), and 1 mm in (D).

(data not shown).

The expression of *NTH15* was also examined throughout floral development. Figure 5B shows the in situ localization of *NTH15* mRNA in early floral meristems. In very early flower buds (right side of Fig. 5B), strong expression of *NTH15* was observed through the floral meristem, but was not detected in regions where floral organs would initiate. The floral meristem shown in the center of Figure 5B roughly corresponds to stage 5 of flower development (Kempin et al. 1993). By stage 5, petal and stamen primordia are apparent (Mandel et al. 1992) and *NTH15* mRNA was specifically localized in the center of the floral meristem, in the region that will later give rise to the carpels (Fig. 5C). A faint signal was also detected in procambial strands, but not in sepal or petal primordia. At a more advanced stage, when the flower formed a mature pistil, *NTH15* expression was restricted to a specific region of anthers (Fig. 5D). Based on the anther developmental stages defined by Goldberg et al. (1993), the anthers seen in this section are between Phase 1 (anther morphology established) and Phase 2 (anthers become functional). More detailed analysis of *NTH15* expression in anthers revealed that strong expression was localized in stomium cells which are involved in dehiscence of the anther and connective cells (data not shown).

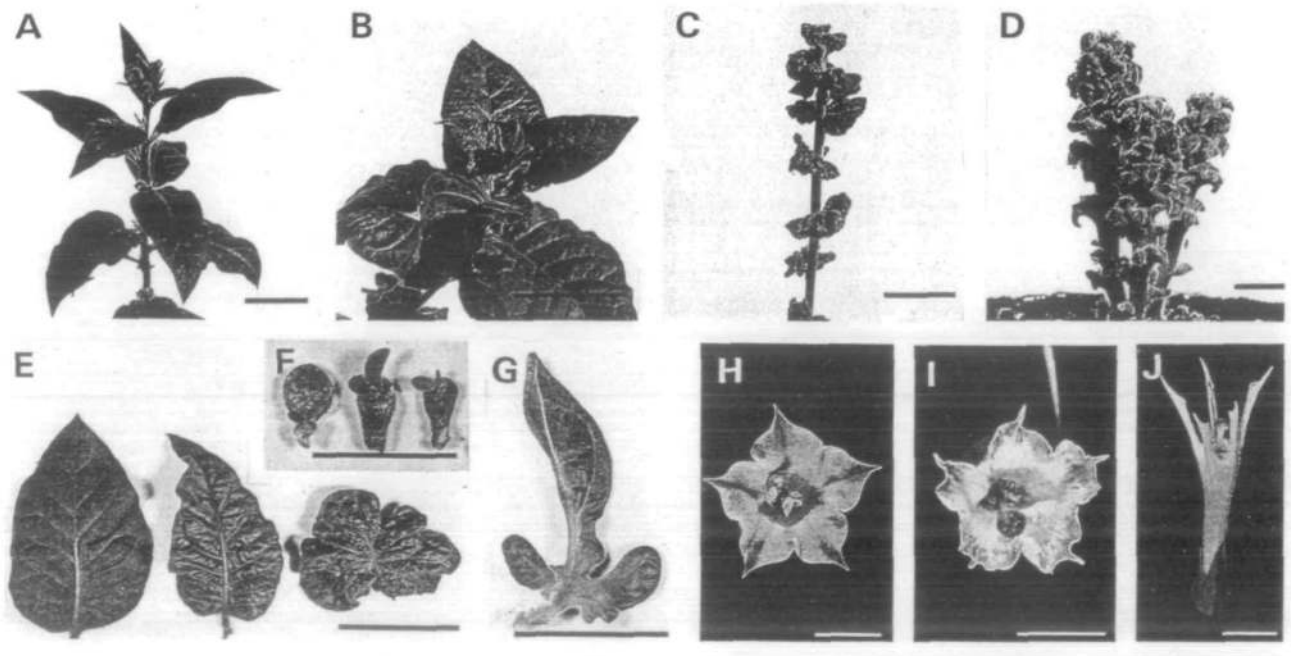

Fig. 6 Phenotypes of transgenic plants carrying the *35S-NTH15* gene. (A) Wild-type tobacco plant in the vegetative stage. (B) A mild-phenotype transgenic plant. (C) A typical intermediate-phenotype transgenic plant. (D) A shooty type of severe-phenotype transgenic plant. (E) Comparison of mature leaves from wild-type (left), mild-phenotype transformant (middle), and intermediate-phenotype transformant (right). (F) Abnormal leaves from severe-phenotype transformants. (G) Mature leaf from a mild-phenotype of *NOS* (nopaline synthetase)-*OSH1* transformed tobacco (Kano-Murakami et al. 1993). (H) Wild-type tobacco flower. (I) Typical flower from an intermediate-phenotype transformant. (J) Dissected flower from a *NOS-OSH1* transformed tobacco plant. Bars, 1 cm (D, F, H, I, J) and 5 cm (A, B, C, E, G).

Ectopic expression of NTH15 causes alterations in the morphogenesis of tobacco leaves—Recent studies have shown that ectopic expression of some KNOTTED-like homeobox genes in leaves causes alterations in morphology (Sinha et al. 1993, Matsuoka et al. 1993, Kano-Murakami et al. 1993). To investigate the biological role of *NTH15* in the development of tobacco, the *NTH15* cDNA was transcriptionally fused to the cauliflower mosaic virus 35S promoter (35S) in the sense orientation and introduced into wild-type tobacco plants. More than forty independent transformants were regenerated in this experiment. Morphological alterations, as shown in Figure 6, were found in almost all transgenic plants. These transformants were divided into three groups ranging from mild to severe on the basis of their leaf phenotypes (Fig. 6E, F). Nine primary transformants in the mild category appeared to have normal growth except for aberrant leaf morphology (Fig. 6B). The size of the leaves in this group was slightly reduced; the midrib was curved, and the shape of the leaf was slightly wavy (Fig. 6E, middle). Nineteen transformants were classified as intermediate; they had severely wrinkled leaves that were thicker than wild-type leaves (Fig. 6C). The elongation of veins of these leaves was inhibited in comparison to the surrounding tissues and no petiole was apparent (Fig. 6E, right). As a result of these alterations, leaves in intermediate-phenotype plants were disk shaped and initiated directly from the stem (Fig. 6C). Thirteen regenerated plants had a severe phenotype; they were quite dwarfed and showed a loss of apical dominance (Fig. 6D). The leaves of these plants were very small and thick in comparison to wild-type and other transgenic plants (Fig. 6E, F). The veins of these leaves could hardly be detected under microscopic examination, and we occasionally observed the formation of ectopic shoots on the adaxial side of the disk shaped leaves.

Morphological alterations were also observed in flowers. On mild phenotype plants, flower morphology was similar to that of wild-type plants (Fig. 6H), whereas flowers on intermediate phenotype plants had waved petals and were smaller than wild-type flowers (Fig. 6I). In addition, anther length was reduced to about half that of pistils. Consequently, spontaneous self-pollination rarely occurred. As pollen grains in these flowers developed normally, we were able to obtain some progeny of this transformant type by artificial pollination. Although plants of the severe phenotype formed tiny flower buds, these did not develop and therefore no progeny of these plants were obtained. Abnormal morphology in mild- and intermediate-phenotype plants was dominantly transmitted to second and third generations and was linked to kanamycin-resistance, indicating that such abnormalities were caused by the introduced gene.

RNA gel blot analysis was performed with total RNA isolated from leaves to determine the relationship between the severity of the phenotype and the expression level of the transgene. *NTH15* mRNA was barely detectable in RNA extracted from wild-type leaves, whereas a single 1.3 kb band, representing the transcript from the transgene, was observed in almost all transgenic plants (Fig. 7). As the 1.3 kb transcript was not the same size as the endogenous *NTH15* transcript (1.6 kb, see Fig. 3), we could easily distinguish the endogenous transcript from that of the transgene by its size. Transgene expression was low (and in some cases could not be detected) in plants exhibiting the mild phenotype, whereas high level transgene expression was observed in plants with intermediate and severe phenotypes.

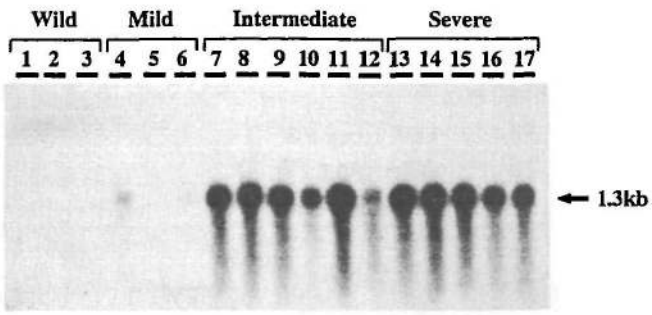

Fig. 7 Transgene expression in leaves of *35S-NTH15* transformants. RNA was isolated from leaves of three independent wild-type and fourteen independent transgenic tobacco plants. Lanes 1–3, wild-type leaves; lane 4, leaves from a mild-phenotype T1 plant; lanes 5 and 6, leaves from mild-phenotype T2 progeny; lanes 7–9, leaves from intermediate-phenotype T1 plant; lanes 10–12, leaves from intermediate-phenotype T2 progeny; lanes 13–17; leaves from severe-phenotype T1 plants. The blot was hybridized with the same probe as that used in Figure 3. The approximate length of the transcript from the transgene is indicated at right. 10 μg of total RNA were loaded to each lane.

Hormonal contents of transgenic tobacco leaves—To obtain further insight into the causes of leaf aberrations in our transgenic tobacco plants, we analyzed endogenous hormone levels present in leaves of these and wild-type plants. We focused on the hormone levels of leaves of plants exhibiting an intermediate phenotype because plants with a mild phenotype showed little difference in hormone levels compared to wild-type plants. Further, we considered that the presence of ectopic shoots on leaves of plants showing a severe phenotype might make it difficult to determine the relationship between hormone levels and altered morphogenesis. This is supported by the observation that, even though IAA levels in leaves of intermediate phenotype plants were reduced relative to wild-type (Table 1), those in leaves of severe phenotype plants were higher than in wild-type (data not shown). This increase could be caused by the presence of ectopic shoots on leaves of the latter plants.

Levels of IAA, ABA, GAs (GA_1 and GA_4) and cytokinins (zeatin and zeatin riboside) were determined in

Table 1 Levels of plant hormons in control and transgenic tobacco

Hormon	Wild-type	Transgenic (% of control)
IAA (ng g FW^{-1})	7.23± 0.07[a]	1.63± 0.03 (23)
ABA (ng g FW^{-1})	10.3 ± 1.14	28.8 ±15.9 (280)
GA_1 (pg g FW^{-1})	1,910 ±70	26.7 ± 4.0 (1.4)
GA_4 (pg g FW^{-1})	149 ± 9.0	113 ±33 (76)
Zeatin (pg g FW^{-1})	7.51± 2.87	45 ± 2.9 (600)
Zeatin riboside (pg g FW^{-1})	375 ±52	72 ± 7.0 (19)

[a] Values are mean±SE.

leaves of transformed and wild-type plants (Table 1). GA levels in transgenic tobacco plants were severely reduced compared to wild-type plants. In this experiment, we analyzed the levels of GA_1 and GA_4 because these forms are known to be strongly active in GA-specific responses in plant development (Graebe 1987). In leaves of wild-type tobacco, the content of GA_1 was more than ten times of that of GA_4, and therefore GA_1 acts as the dominant GA compound for tobacco development. Levels of both GA_1 and GA_4 were reduced in leaves of transformants, but to different degrees. A drastic (approximately 80-fold) decrease in GA_1 was observed in transgenic leaves, while the level of GA_4 showed only a two-fold decrease (Table 1). In contrast to GAs, the level of zeatin (active cytokinin) in leaves of transgenic plants increased by about ten-fold over wild-type and the ratio of zeatin to zeatin riboside (an inactive form) also increased. Although IAA and ABA levels were also altered in leaves of transformants (four-fold decrease and three-fold increase, respectively), the magnitude of the changes was not as great as for GA and cytokinin.

Discussion

NTH15 encodes a homeodomain belonging to the KNOTTED-type class 1 homeodomain family—The *NTH15* gene that we have cloned from tobacco encodes a 342 amino acid protein which contains a homeodomain. Homeodomains represent a highly conserved protein motif that recognizes and binds specific DNA sequences (Gehring et al. 1990). Based on this DNA binding property, homeodomain-containing proteins are believed to act as transcriptional factors to regulate the expression of specific target genes (Affolter et al. 1990).

According to the classification scheme of plant homeodomains proposed by Kerstetter et al. (1994), the homeodomains of plant homeobox genes can be divided into four classes based on their amino acid sequences. The NTH15 homeodomain was most similar to proteins in the KNOTTED-type class. KNOTTED-type homeodomains can be further subdivided into two classes, class 1 and class 2 (Kerstetter et al. 1994). Class 1 genes resemble *KN1*, sharing high amino acid identity in the homeodomain, and are expressed primarily in shoot and floral meristems but not in differentiated organs such as leaves. Class 2 genes show comparatively less similarity to the KN1 homeodomain and have different patterns of expression from that of class 1 genes. Class 2 homeodomains share only about 60% identity with KN1, and they appear to be expressed at varying levels in all organs, including differentiated organs. The homeodomain of NTH15 is 88% identical to that of KN1, and its mRNA is expressed in vegetative and floral meristems but not in leaves. These features indicate that *NTH15* is a class 1 KNOTTED-type gene.

The homeodomain of NTH15 shows the highest sequence similarity to those of STM from *Arabidopsis* (Long et al. 1996) and SBH1 from soybean (Ma et al. 1994). *STM* is one of the most characterized homeobox genes in plants, and the expression of this gene in the shoot apex is required for SAM formation during embryogenesis. The high sequence similarity of these genes leads us to propose that *NTH15* may be a tobacco counterpart of *STM*. However, there are some differences between *NTH15* and *STM*, not only in the primary structure of the predicted protein, but also in the expression pattern within the SAM. For example, homopolymeric amino acid stretches, which are a feature of the N-terminal region of NTH15, are not found in the corresponding region of STM. Major differences are also observed in the mRNA expression patterns in the shoot apex. While *STM* mRNA is localized throughout the SAM of *Arabidopsis* (Long et al. 1996), *NTH15* expression is down-regulated in tunica layers of the tobacco SAM (see below).

These contrasting characteristics of different homeobox genes suggest that *NTH15* is not a functional homologue of *STM* and that it may play different roles in tobacco development.

NTH15 expression in the SAM is similar to other class 1 KNOTTED genes—NTH15 mRNA was detected in both vegetative and floral meristems. In the vegetative meristem, *NTH15* mRNA accumulated in discrete regions of the SAM but not in leaves or leaf primordia (Fig. 4A, B, C, D).

A detailed examination of sections through the vegetative SAM showed that the region lacking *NTH15* expression was localized at the SAM margin, where a high proportion of periclinal cell divisions was found (Fig. 4C). Periclinal cell divisions in the flanking region(s) of the SAM have been described as the first indication of new leaf formation (Esau 1965, Cunninghame and Lyndon 1986). Therefore, a new leaf primordium would be expected to initiate in this region and *NTH15* expression is down-regulated at the predicted position of leaf formation. Detailed observation of *NTH15* expression in the SAM also revealed that *NTH15* mRNA is mainly localized in the cells underlying the two outermost cell layers of the shoot apex (Fig. 4A, C). According to the tunica-corpus concept proposed by Schmidt (1924), the two outermost layers of the tobacco shoot apex comprise the tunica (L1 and L2 layers) and the more deeply seated regions represent the corpus (Poethig and Sussex 1985). Therefore, the cells in which *NTH15* is expressed correspond well to the corpus, with no signal being detected in the two layers of tunica (Fig. 4C). In other words, it is possible to distinguish the corpus and tunica regions of the tobacco SAM by the pattern of *NTH15* expression, even though these regions are not clearly recognizable under normal histological examination.

Expression of *OSH1* in rice and *KN1* in maize has been shown by in situ hybridization to occur mainly in corpus cells and not in the tunica (Jackson et al. 1994, Nemoto, unpublished results). In these cases, however, the genes are down-regulated only in the L1 layer of the SAM. This difference in the number of layers lacking expression of these homeobox genes may be caused by differences in meristem histogenesis. Indeed, monocots generally have only one layer of tunica cells, while dicots have two (Smith et al. 1992). Therefore, absence of or reduced expression of some KNOTTED-type genes in the tunica layer(s) is conserved in both monocot and dicot plants. This similarity of expression pattern in the SAM may indicate that the function of *NTH15* in tobacco development resembles that of *OSH1* and *KN1*. While the biological meaning of the down-regulation of these genes in the tunica layer(s) has not been clarified, detailed examination of *OSH1* expression in rice indicates that the expression of this gene is localized in tissues in which the cells divide randomly. *OSH1* expression is not seen in any tissues where the orientation of cell division is regulated (e.g. leaf primordia, leaf traces in the stem, internodes and the L1 layer of the SAM; Nemoto, unpublished results). Therefore, the expression or suppression of *OSH1* may correspond to the regulation of the orientation of cell division (Sato et al. 1996, Nemoto, unpublished results). According to this prediction, it is possible that the expression of *NTH15* may play a regulatory role in maintaining flexibility of the orientation of cell division.

Smith et al. (1992) have shown that the KN1 protein is present in the L1 tunica layer, even though *KN1* mRNA cannot be detected in this layer. The KN1 protein has been proposed to act as a signal that moves between cells via plasmodesmata (Lucas et al. 1995). This movement of the KN1 protein would presumably be selective since the protein does not move into leaf primordia. Based on this, it is difficult to predict the function of homeobox genes (including *NTH15*) solely from the localization patterns of their mRNA. Studies of the expression of homeodomain proteins in the SAM and/or the mechanism and regulation of movement of these proteins between cells may be necessary to obtain further information concerning the functions of homeobox genes in plant development.

Alterations of leaf morphology in transgenic tobacco plants are mediated by changed hormone levels in leaves— To obtain further insight into the function of *NTH15* in tobacco development, tobacco plants were transformed with a 35S-*NTH15* chimeric construct. In dicot plants, the 35S promoter causes high level expression of genes to which it is linked (Jefferson et al. 1987), resulting in disruption of the regulated temporal and spatial expression of the associated gene. Therefore, transgenic tobacco plants containing the 35S-*NTH15* construct were expected to exhibit morphological alterations in differentiated organs in which *NTH15* would not normally be expressed. All regenerated plants formed aberrant leaves and flowers, indicating that the *NTH15* gene product functions as a morphological regulator in tobacco. Transformants were classified into three groups, mild, intermediate, and severe, depending on the severity of their leaf phenotypes. We found two distinct effects of the transgene on leaf morphogenesis. The primary effect was a reduction in overall leaf size and the second effect was a change in leaf shape. These effects were more pronounced with increasing severity of phenotype. Primary transformants classified as having a mild phenotype formed leaves that were slightly smaller than normal and were somewhat puckered in appearance due to their veins failing to elongate as much as surrounding tissues. Puckering of leaves was more noticeable in intermediate-phenotype transformants. The leaves of these plants were wrinkled and rounded (again caused by inhibited elongation of veins relative to surrounding tissues) and had no petiole. Leaves of severe-phenotype plants were severely reduced in size and occasionally small ectopic shoots initiated on their adaxial surface. Northern hybridization analysis showed that low transgene expression was found in mild phenotype plants, whereas high level expression was observed in both intermediate and severe phenotypes. These results suggest a correlation between the severity of alteration of leaf morphology and the expression level of the transgene. However, it is possible that the expression level of the transgene in expanded leaves may not simply correlate with the phenotype of transgenic plants because a low correlation was observed in intermediate- and severe-

phenotype plants. Indeed, we have previously reported that transgenic tobacco plants expressing *OSH1* under the control of different promoters showed different levels of *OSH1* expression in leaves, even though similar leaf morphological abnormalities were observed (Sato et al. 1996). This report demonstrates that limited *OSH1* expression in young leaf primordia is sufficient to induce severe morphological aberrations of leaves. Taken together, these findings indicate that the phenotypic variation of transgenic plants is the result of both quantitative variation of the expression level and qualitative variation of the expression pattern of the transgene.

Transgenic tobacco plants overexpressing the homeobox genes *OSH1* and *KN1* also show alterations in leaf and flower morphology (Kano-Murakami et al. 1993, Sinha et al. 1993). Although leaf and flower morphology of the rumpled *KN1* phenotype and mild *OSH1* phenotype resemble the intermediate *NTH15* phenotype, midrib and petiole development were never observed in the latter plants, while both were easily identified in leaves of *OSH1* and *KN1* tobacco transformants. Moreover, tobacco plants overexpressing *OSH1* typically form complex-shaped leaves, but this type of leaf were never observed in *NTH15*-transformed tobacco plants (Fig. 6G, Sato et al. 1996). Flower morphology is also different among these transgenic tobacco plants. Flowers with dissected petal lobes are observed in rumpled *KN1* and mild *OSH1* phenotypes (Fig. 6J), but are not seen in any phenotypes of *NTH15*-transformed plants (Fig. 6I). These results strongly suggest that features of the intermediate phenotype shown here are, at least in part, caused by specific effects of *NTH15* overexpression in tobacco leaves. This also indicates that the biological function of *NTH15* does not completely mimic that of *OSH1* and *KN1*.

It is widely accepted that plant hormones regulate plant development and morphogenesis. Consequently, leaf development is also likely to be influenced by hormone levels. Transgenic plants containing the 35S-*NTH15* construct showed not only alteration of their leaf morphology, but also changed hormone levels. In the wrinkled leaves of intermediate-phenotype plants, the most notable changes were a decrease of GA_1 and an increase of cytokinin, although levels of all hormones examined in this study were affected (Table 1). Such changes in hormone levels were also found in leaves of mild- and severe-phenotype plants (data not shown). It is therefore possible that *NTH15* overexpression in transgenic leaves effects some changes in hormone biosynthesis.

Transgenic tobacco plants containing the NOS (nopaline synthetase) promoter-*OSH1* construct formed wrinkled leaves (Kano-Murakami et al. 1993) and exhibited dramatically reduced leaf GA_1 levels (Kusaba, unpublished results). It has been reported that wrinkled leaves occur in tobacco plants treated with inhibitors of GA biosynthesis (Dehio et al. 1993). Further, transgenic tobacco plants constitutively expressing the *rolA* gene from *Agrobacterium rhizogenes* TL-DNA showed a reduction of leaf gibberellic acid content and a corresponding alteration of leaf morphology (Dehio et al. 1993). These observations suggest that the wrinkled leaves seen in intermediate-phenotype *NTH15* transformants are caused, at least in part, by decreased GA levels. Therefore, it may be possible that the abnormal leaf morphology in these plants is not a direct result of *NTH15* overexpression but rather is due to changed gibberellic acid levels. This implies that the product of *NTH15* may regulate the expression of genes encoding enzymes for GA biosynthesis. Our recent results show that expression of the *C20-oxidase* gene, whose products are involved in the GA biosynthetic pathway, is inhibited in stems of NOS-*OSH1* transformed tobacco plants (Kusaba, unpublished results). This finding supports our proposal that plant homeodomain proteins may regulate hormone biosynthesis.

Cytokinin levels were also changed in *NTH15*-transformed plants (Table 1). The alteration of cytokinin levels was observed in leaves of severe-phenotype plants, which formed ectopic shoots on their adaxial side. In these leaves, endogenous cytokinin levels were dramatically increased (data not shown). Similar leaf morphology, including ectopic shoot formation, is also seen in transgenic tobacco plants containing 35S-*OSH1*. Further, cytokinin levels are increased in leaves of these plants (Kusaba, unpublished data). Tobacco plants transformed with the *isopentenyl transferase* gene from the *Agrobacterium* Ti plasmid (a gene that is involved in cytokinin biosynthesis) also form many ectopic shoots on their leaves (Smart et al. 1991, Hagen and Guilfoyle 1992). These observations indicate that ectopic shoot formation observed in severe-phenotype plants might be also caused, in part, by increased cytokinin levels. Taken together, our observations of both GA and cytokinin changes suggest that the NTH15 protein, which is expressed in a specific region of the SAM, may act as a trans-acting factor to regulate the expression of genes involved in hormone metabolism. Further studies are needed to test the possibility that some homeodomain proteins control plant hormone levels by regulating the expression of hormone biosynthetic enzymes.

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