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Over-Expression of Tobacco *knotted1*-Type Class1 Homeobox Genes Alters Various Leaf Morphology

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We compared the phenotypes of transgenic tobacco plants over-expressing various *knotted1*-type class1 homeobox genes. All transformants showed abnormal leaf morphology, with the degree of abnormality depending upon the *Nicotiana tabacum homeobox* (*NTH*) gene that was over-expressed. Tobacco plants over-expressing *NTH1* or *NTH9* showed a relatively weak phenotype, while *NTH15* and *NTH20* over-expressing plants exhibited severe alterations, with occasional ectopic shoot formation on the leaves. Plants over-expressing *NTH22* had a relatively severe phenotype, but did not form any ectopic shoots. These results indicate that all of the *NTH* genes can influence leaf development from the shoot apical meristem, but that the effect varies with the gene. Based on phylogenetic analysis of the *NTH* genes and comparison of the phenotypes of plants over-expressing them, we suggest that the *kn1*-type class1 family can be divided into two subgroups, and that the differences in their ability to induce the abnormal phenotype corresponds to the structures of their conserved domains.

Key words: *kn1*-type homeobox family — KNOX domain — Leaf morphology — Transgenic plant — Tobacco.

Homeobox genes are involved in many important aspects of developmental process of multicellular eukaryotes. Homeobox genes encode a large family of homeodomain proteins that regulate the expression of downstream target genes as transcriptional factors (Affolter et al. 1990, Andrew and Scott 1992). In *Drosophila*, homeodomain-containing homeotic genes play key roles in cellular or regional differentiation during embryogenesis. In plants, the first identified homeobox gene, maize *knotted1* (*kn1*), was isolated from a gain-of function mutation, which alters leaf development. Many different homeobox genes have been isolated from various plants, including maize, rice, barley, *Arabidopsis*, soybean, tomato and tobacco (Vollbrecht et al. 1991, Matsuoka et al. 1993, Müller et al. 1995, Lincoln et al. 1994, Ma et al. 1994, Harven et al. 1996, Tamaoki et al. 1997). According to sequence similarities in their homeodomains, plant homeobox genes can be divided into several families (Chan et al. 1998), including the *kn1* family, the HD-Zip family (Ruberti et al. 1991, Schena and Davis 1992), the glabra2 family (Lu et al. 1996, Rerie et al. 1994), the PHD-finger family (Bellmann and Werr 1992, Korfhage et al. 1994, Schindler et al. 1993) and the BELL1 family (Reiser et al. 1995, Quaedvlieg et al. 1995). Among them, the *kn1* family has been the most extensively characterized. The homeodomains of proteins encoded by this family contain three extra amino acids between helix 1 and helix 2, and are therefore referred to as the TALE (Three Amino acid Loop Extension) superclass (Bürglin 1997). This family also contains another conserved domain, the KNOX domain, located at the N-terminal region of the homeodomain. By comparative analysis of these conserved motifs, this family has been subdivided into two classes, class1 and class2 (Kerstetter et al. 1994). The class1 genes studied are mainly expressed in the shoot apical meristem (SAM), and some loss-of-function mutations affect meristem maintenance and/or formation. Furthermore, all class1 genes analyzed caused dramatic alteration of leaf morphology when ectopically expressed (Hake et al. 1995). These results indicate that the class1 genes play important roles in SAM. In contrast, the class2 genes studied are expressed in most tissues and their ectopic expression does not cause altered morphology (Sentoku et al. 1998).

To elucidate the functions of *kn1*-type class1 homeobox genes in plant developmental processes, we have been isolating *kn1*-type class1 homeobox genes from tobacco, using PCR with degenerate primers. Sequence analysis of nine distinct amplified DNA fragments revealed that the isolated genes could be classified into five groups. Expression analyses indicated that the genes were expressed in different SAM regions (Nishimura et al. 1999). In this study, we examined the effect of ectopic expression of various tobacco class1 homeobox genes on leaf morphogenesis to investigate whether the genes in each group cause similar alterations or not. Based on these studies, we discuss the functional differences among the *kn1*-type class1 homeobox genes and suggest that it may be correlated with the difference of each protein structure.

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Materials and Methods

Protein extraction and western blot analysis—Total leaf proteins were extracted from leaf tissue of twenty independent transgenic plants by grinding with an equal volume of 2× sample buffer (1× concentration: 80 mM Tris-HCl pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 0.01% [w/v] bromphenol blue, 0.02% [w/v]-mercaptoethanol), and quantitated by the Bradford assay. After boiling for 3 min, protein samples (20 μg) were subjected to SDS-PAGE, then transferred to an Immobilon-P membrane (Millipore) by semi-dry blotting. Blots were incubated in a Tris buffer (TBS, 20 mM Tris-HCl pH 7.6, 137 mM NaCl) with 5% (w/v) nonfat dry milk for 1 h for blocking. Anti-c-Myc antibody (Invitrogen) was used at a final dilution of 1 : 500 and incubated overnight at room temperature, followed by three washes for 15 min each in TBS-T (TBS with 0.1% [v/v] Tween 20). The blots were incubated in diluted (1 : 10,000) goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature, followed by four washes for 15 min with TBS-T. ECL-Plus chemiluminescent reagents (Amersham) were used for detection.

Construction of 35S promoter::NTH::c-Myc epitope chimeric genes—To examine the expression levels of the homeodomain proteins, we introduced the c-Myc epitope tag at the 3′ end of *35S promoter::NTH1, 35S promoter::NTH15* and *35S promoter::NTH23*. A *Sma*I site was introduced just downstream of the stop codon of each cDNA by PCR using primers with a *Sma*I linker. A short DNA fragment including the sequence, 5′-CCCGGGGAACAAAAACTCATCTCAGAAGAGGATCTG-**TGA**GAGCTC-3′ (*Sma*I and *Sac*I sites are underlined; the stop codon is in boldface), which encoded the amino acids of the c-Myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-leu-Asn), was introduced between the *Sma*I site at the C-terminal end of the cDNAs and the *Sac*I site at the 5′ end of the nopaline synthetase terminator (*nos*) sequence of pBI121.

Construction of 35S promoter::NTH chimeric genes—A DNA fragment containing the *35S* promoter was excised from pBI121 (Clontech Laboratories, Inc.) at the *Hin*dIII/*Sma*I site and introduced into the same site of pUC18 to produce a plasmid designated p35S. A DNA fragment containing the *nos* sequence was excised from pBI121 at the *Sst*I/*Eco*RI site and introduced into the same site of p35S to produce a plasmid designated p35Snos. Then a DNA fragment containing the *35S* and *nos* sequences was excised from p35Snos at *Hin*dIII/*Eco*RI site and inserted at the site of pBI121 to produce a plasmid designated pBI35Snos. This pBI35Snos was used for four *NTH* over-expression constructs. Full-length *NTH1* cDNA in pBluescriptII was cut with *Eco*RV and *Sac*I and introduced into the *Sma*I/*Sac*I site of pBI35Snos to construct the *35S::NTH1*. The *NTH9* and *NTH22* cDNAs in pBluescriptII were excised at the *Eco*RI (followed by filling-in) plus *Xba*I and *Xba*I/*Eco*V, respectively, then inserted into the *Xba*I/*Sma*I site of pBI35Snos to construct the *35S::NTH9* and *35S::NTH22*. We amplified the *NTH20* coding region in pBluescriptII by PCR using the M13 reverse primer as a 5′ primer and oligo(dT)15 with a *Bam*HI linker as a 3′ primer. The product was cut with *Not*I (followed by filling-in) plus *Sac*I and introduced into the *Sma*I/*Sac*I site of pBI35snos to construct the *35S::NTH20*.

Transformation and regeneration of tobacco—The *35S::NTH* chimeric constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Agrobacterium-mediated transformation of *Nicotiana tabacum*, cv. Samsun NN was performed using leaf discs as reported previously (Matsuoka and Sanada 1991). Transgenic plants were selected on medium containing 100 mg liter^{-1} kanamycin.

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.

RNA gel blot analysis—Total RNA was isolated from the leaves of wild or independent transgenic tobacco plants. Ten micrograms of each RNA preparation was resolved by electrophoresis, transferred to Hybond N$^+$ membrane (Amersham), and probed using the 5′ regions of the various cDNAs. The probes excluded the ELK-homeodomain to avoid cross-hybridization due to the high conservation of this region. Hybridization was performed at 65°C in a solution containing 10% dextran sulfate, 6× SSC, 5× Denhardt's solution, 0.5% SDS and 0.1 mg ml^{-1} salmon sperm DNA. Filters were washed with 2× SSC, 0.1% SDS at room temperature and then further washed in 0.2× SSC, 0.1% SDS at 65°C.

Results

Expression levels of the homeodomain proteins in transgenic plants—We observed previously that tobacco plants over-expressing *NTH1, NTH15* and *NTH23* (a *kn1*-type class2 gene) exhibited mild, severe and no morphological alterations in leaves, respectively (Tamaoki et al. 1997, 1999, Sentoku et al. 1998). To confirm that the differ

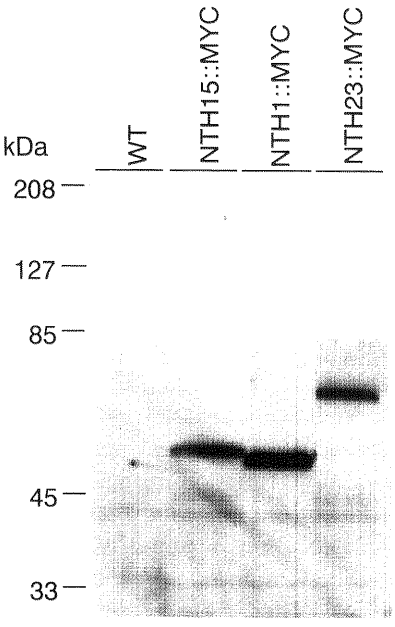

Fig. 1 Immunodetection of homeodomain proteins containing a c-Myc epitope tag. Total protein was extracted from leaves of more than twenty independent transgenic tobacco plants for each construct, and the amount of protein was quantified by Bradford assays. Twenty micrograms of total protein were subjected to SDS-PAGE, electroblotted, and analysed by western blotting using an anti-c-Myc antibody (Invitrogen).

ences in severity of abnormal morphology were not due to differences in expression levels of recombinant homeodomain proteins in transgenic plants, we directly estimated the expression levels of *NTH1, NTH15,* and *NTH23* in the transformants. Because of the difficulty in estimating relative levels of the different NTH proteins using separate antibodies, we produced respective *NTH* proteins including a c-Myc epitope tag for direct quantitation using an anti-c-Myc monoclonal antibody. As shown in Figure 1, similar levels of proteins were detected in transgenic tobacco plants for the three NTH proteins exhibiting morphological alterations of different severity. This result demonstrates that the variation in severity caused by the over-expression of different *NTH* genes was not due to the differences in the level of the protein products. Therefore, the observed differences in the phenotypes of the transformants seem to depend on the functions of the NTH proteins.

Phenotypic alteration of leaves in transgenic tobacco plants over-expressing the various NTH genes—To understand the functions of the various *kn1*-type class1 homeobox genes, we produced transgenic plants transformed with cDNAs representing *NTH1, NTH9, NTH20* or *NTH22* driven by the *CaMV 35S* promoter. Almost all of the resulting transgenic plants exhibited similar abnormal leaf morphology but at different degrees of severity. These phenotypes have also been observed in other *kn1*-type class1 homeobox genes, such as, *NTH15* transformants that we reported previously (Tamaoki et al. 1997), rice *OSH1* or *OSH15* introduced tobacco (Kano-Murakami et al. 1993, Sato et al. 1998) and maize *kn1* introduced tobacco (Sinha et al. 1993). Based on these observations, we interpreted that these phenotypes were reflected in the similar effect of *kn1*-type class1 genes. Therefore we classified the transformants for the five *NTH* genes *NTH1, NTH9, NTH15, NTH20* and *NTH22* into six categories (Table 1): 'normal' (Fig. 2A, F), 'curved' (Fig. 2B, G), 'wrinkled' (Fig. 2C, H), 'butterfly' (Fig. 2D, I), 'dwarf' (Fig. 2E, J) and 'others' (Fig. 3A–C).

Thirteen, out of 20 transformant lines carrying *35S::NTH1* displayed the 'curved' phenotype (Fig. 2B, G and Table1). The other seven were indistinguishable from the non-transformants (Fig. 2A, F), though low levels of *NTH1* transcript were detectable in the normal leaves (data not shown). The abnormality of the 'curved' plants was limited to leaf morphology: Leaves were slightly wrinkled and curved as a result of differential development of right and left sides of the leaf blade. The severity of leaf curvature was somewhat different with the line, and correlated with the expression levels of the transgene (Fig. 4).

Thirty-five out of 48 independent *35S::NTH9* transgenic lines showed an abnormal phenotype, while the remaining thirteen transformants with lower levels of *NTH9* expression in leaves did not show any abnormalities (Fig. 2A, F and Fig. 4). More than half of the abnormal plants showed a typical 'curved' phenotype (Fig. 2B, G),

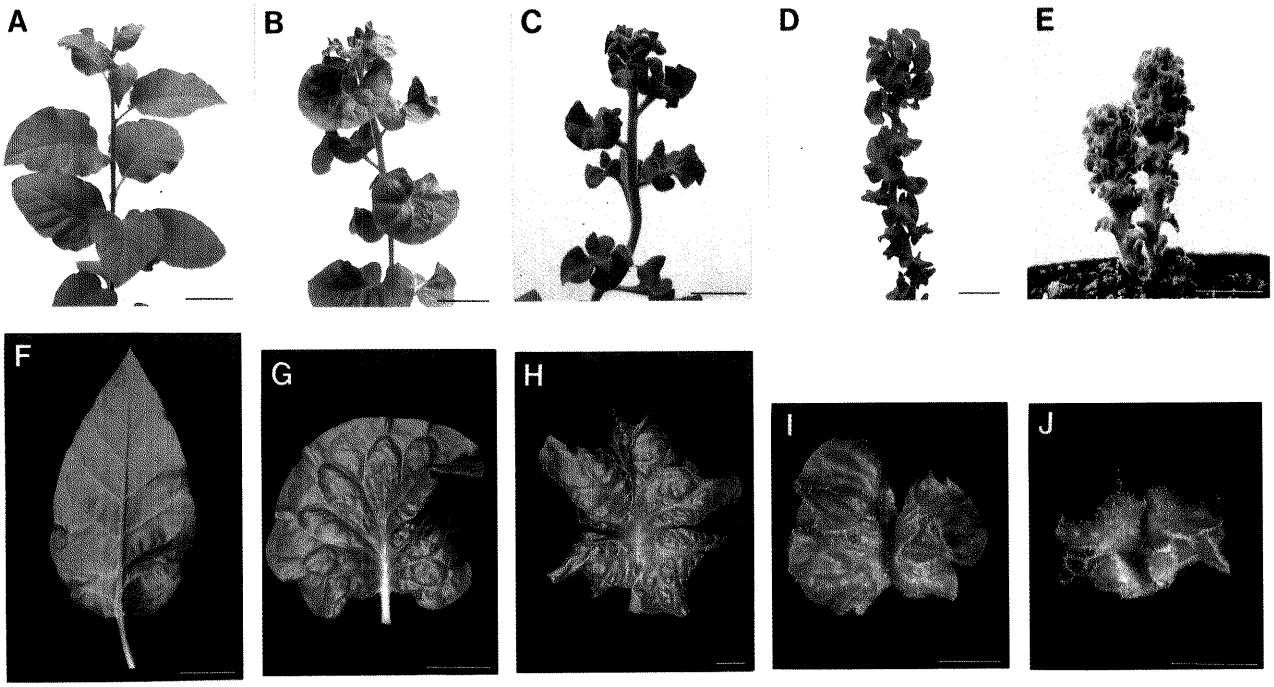

Fig. 2 Phenotypic categories of transgenic plants carrying *35S::NTH* genes. Typical transgenic plants exhibiting normal (A), curved (B), wrinkled (C), butterfly (D) and dwarf (E) phenotypes. Leaves from 'normal' (F), 'curved' (G), 'wrinkled' (H), 'butterfly' (I) and 'dwarf' (J) phenotype plants. Bars represent 5 cm (A, B, C, D, E) and 1 cm (F, G, H, I, J).

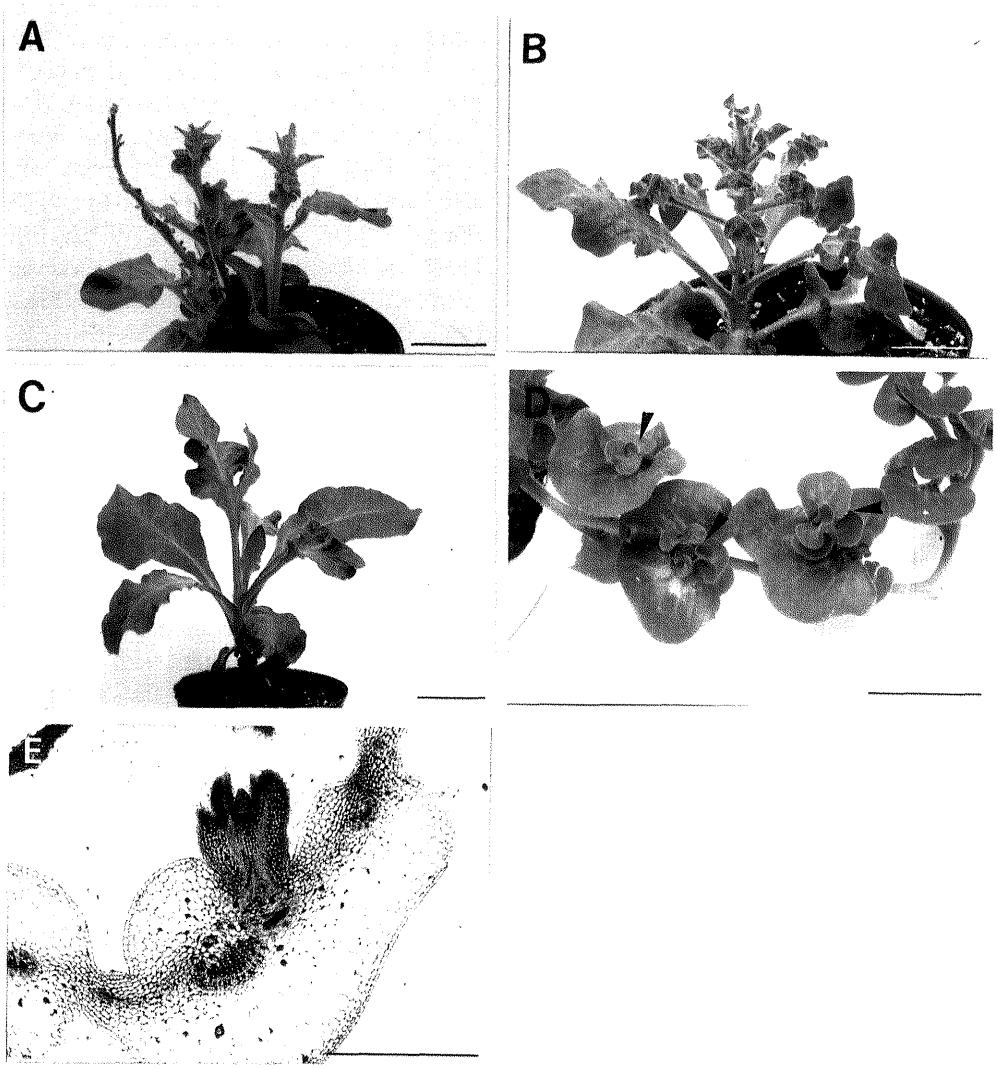

Fig. 3 Specific phenotypes of *35S::NTH20* transformants. (A), (B), (C) Dwarf plants with various leaf morphologies. Several plants lacked apical dominance (A). (D) Ectopic shoots on the leaves of plants with a 'butterfly' phenotype. (E) Longitudinal section of a leaf with ectopic shoots.

whereas the remainder showed a more severe phenotype (Table 1). These plants with the severe phenotype formed leaves with wrinkled laminae and a shortened midrib (Fig. 2C, H).

Table 1 Distribution of each phenotype in transgenic plants carrying *35S::NTH* gene

Construct	Phenetypic categories					
	Normal	Curved	Wrinkled	Butterfly	Dwarf	Others
35S::NTH1	7[a] (35.0)[b]	13 (65.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
35S::NTH9	13 (27.1)	26 (54.2)	9 (18.8)	0 (0.0)	0 (0.0)	0 (0.0)
35S::NTH20	0 (0.0)	2 (4.1)	5 (10.2)	33 (67.3)	4 (8.2)	5 (10.2)
35S::NTH22	3 (12.0)	2 (8.0)	5 (20.0)	12 (48.0)	3 (12.0)	0 (0.0)
35S::NTH15	0 (0.0)	9 (22.0)	0 (0.0)	19 (44.3)	13 (31.7)	0 (0.0)
35S::NTH23	25 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Number of transformants categorized into each phenotype was counted.
[b] Percentage.

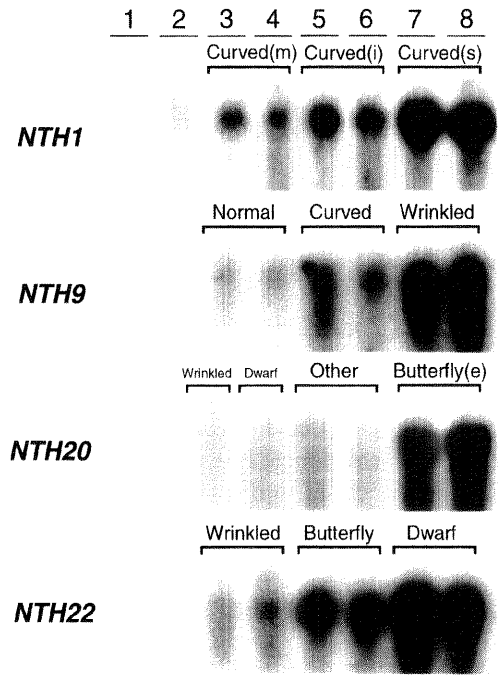

Fig. 4 Transgene expression in leaves of *35S::NTN* transfomants. RNA was isolated from leaves of two independent wild types and one or two independent transgenic tobacco plants from the each category. Lanes 1 and 2, wild type leaves; lanes 3 to 8, transgenic leaves. Curved: leaves with the 'curved' phenotype, (m) mild, (I) intermediate, (s) severe; Normal: leaves with the 'normal' phenotype; Wrinkled: leaves with 'wrinkled' phenotype; Butterfly; leaves with the 'butterfly' phenotype, (e) leaves with ectopic shoots; Dwarf: leaves from 'dwarf' phenotype plants; Others: leaves from other phenotype plants (see text).

Plants with various degrees of malformations were obtained among the *35S::NTH20* transformants. All transformants showed abnormalities in leaf morphology, but with different kinds of abnormalities that could be classified into the five categories (Table 1). Two plants displayed the 'curved' phenotype similar to those of the *NTH1* and *NTH9* transformants (Fig. 2B, G). Five transformants were classified into the 'wrinkled' category, with wrinkled laminae and a shortened midrib (Fig. 2C, H). About 70% of the plants were grouped into the 'butterfly' category (Fig. 2D, I). Leaves of the plants in this category exhibited extremely reduced elongation of midrib, as well as reduced development of lateral vein. As a consequence, leaf size was reduced and gross leaf shape resembled a butterfly. In addition, the leaves in this category developed ectopic shoots on their surface (Fig. 3D, E). More severe phenotypic perturbations were observed in four transformants, which were classified into the 'dwarf' category (Fig. 2E, J). The morphology of these plants was abnormal entirely. Leaves were severely reduced in size and the plants were very short as a result of inhibited internode elongation. Furthermore, apical dominance of the main shoot was lost (Fig. 2E). The remaining five transformants could not be grouped into any phenotype category on the basis of their leaf shape, thus we categorized these plants as 'others'. These plants displayed unique phenotypes as shown in Figure 3A–C. All of these plants were considered to have a severely abnormal phenotype because, in all cases, stem elongation was severely inhibited and leaves were severely malformed. RNA gel blot analysis showed the highest expression in 'butterfly' leaves which included ectopic shoots. Interestingly, the severe phenotypic abnormalities ('wrinkled', 'dwarf' and 'others') could be induced even by low levels of ectopic expression unlike ectopic expression of other *NTH* genes (Fig. 4).

The phenotypes of the *NTH22* transformants could also be classified into the above five categories (Table 1). Half of the transformants belonged to the 'butterfly' category, with leaves having no obvious midrib, as observed in the *NTH20* transformants (Fig. 2D, I). However, in contrast to the *NTH20* transformants, no ectopic shoots were observed on the 'butterfly' leaves of *NTH22* transformants. More than 10% of plants over-expressing *NTH22* had a normal phenotype (Fig. 2A, F), approximately 30% showed a mild or intermediate phenotype (falling in the 'curved' and 'wrinkled' groups; Fig. 2B, G and Fig. 2C, H), and smaller percentages showed a severe phenotype ('dwarf' category; Fig. 2E, J). The ectopic shoot formation has also never been observed on these leaves (data not shown). The ectopic expression level corresponded to the degree of phenotypic abnormality (Fig. 4).

Tobacco plants over-expressing *35S::NTH15* were previously categorized into three groups, 'curved', 'butterfly' and 'dwarf'. About half of these transformants exhibited a 'butterfly' phenotype and 30% showed the dwarf phenotype. The remaining 20% showed a 'curved' phenotype. Ectopic shoots were formed in most 'dwarf' plants (Tamaoki et al. 1997).

These results indicate that the degree of phenotypic abnormality in tobacco plants over-expressing the *NTH* genes depends upon the transgene: *NTH15*≒*NTH20*> *NTH22*>*NTH9*≒*NTH1*. The degree of phenotypic abnormality in the transformants also depended upon the expression level of the introduced transgenes, with a high degree of phenotypic abnormality occurring in plants with higher transgene expression (Fig. 4). This comparison is valid within plants over-expressing a particular *NTH* gene, but does not apply to plants carrying different transgenes. For example, plants expressing *NTH1* or *NTH15* at similar levels exhibited 'curved' and 'dwarf' phenotypes, respectively. In fact, the expression levels of the *NTH* genes were similar in 'curved' plants transformed with *35S::NTH1*, in 'curved' and 'wrinkled' plants transformed with *35S::NTH9*, and in 'butterfly' and 'dwarf' plants transformed with *35S::NTH20, 35S::NTH22, 35S::NTH15* (Fig. 4 and Tamaoki et al. 1997). These results demonstrate that the

severity of morphological abnormality among the transformants depends on the difference of *NTH* transgenes, but not on their expression levels.

Discussion

Since the ectopic expression of some *kn1*-type class1 homeobox genes in transgenic tobacco plants induces similar abnormal leaf morphology (Sinha et al. 1993, Kano-Murakami et al. 1993, Sato et al. 1998, Tamaoki et al. 1997), this gene family is speculated to be involved in lateral organ formation. In this study, we further analyzed transgenic tobacco plants over-expressing five different *kn1*-type class1 homeobox genes from tobacco to examine the functional difference between the genes. Misexpression of any of the *NTH* genes under the control of the 35S promoter led to alter leaf morphology. This indicated that all the class1 genes are involved in leaf formation process from SAM. Interestingly, the degree of phenotypic abnormality in the transgenic plants varied depending upon the introduced *NTH* gene. The most severely abnormal phenotype was seen in plants transformed with *35S::NTH15* or *35S::NTH20*. These plants were severely stunted and formed disk-shaped leaves with numerous ectopic shoots on their surfaces. A similar degree of phenotypic abnormality has also been observed in transgenic tobacco plants over-expressing rice *OSH1* or *OSH15*, maize *kn1* or *Arabidopsis KNAT1* (Kano-Murakami et al. 1993, Sato et al. 1998, Sinha et al. 1993, Hake et al. 1995). The similarity among transgenic plants over-expressing these homeobox genes suggests that the genes from various plants function in a similar manner, at least when ectopically expressed under the control of the 35S promoter in transgenic tobacco plants. This indicates that these genes may affect common target genes when expressed at a high level in tobacco plants. However, in contrast to these genes, neither *NTH1* nor *NTH9* induced severe phenotypic abnormalities when over-expressed under the same conditions. The most severe phenotypic abnormality seen in plants transformed with these genes was 'curved' or 'wrinkled' leaves, even though their transgenes were highly expressed (Fig. 1 and Fig. 4). Such phenotypes under the highly ectopic expression of *kn1*-type class1 genes have not been seen yet. The plants over-expressing *NTH22* exhibited 'butterfly' or 'dwarf' phenotype; however, ectopic shoots have never been observed on their leaves, unlike the *NTH20* or *NTH15* transformants. These differences also suggest that not all of the NTH products may function at the same point in the morphogenesis. It is possible that *NTH15* and *NTH20* target more upstream genes than do other *NTH* genes in the network controlling lateral organ formation. Alternatively, *NTH15* and *NTH20* may be able to interact with a wider range of genes involved in morphogenesis. We previously investigated the expression pattern of *NTH* genes around the SAM, and found a difference in the regional expression for different *NTH* genes (Nishimura et al. 1999). However, we could not find any correlation between these expression patterns and the severity of over-expression phenotypes. For example, *NTH1* and *NTH15* showed overlapping expression in the SAM, but their over-expression phenotypes were quite different. These features may be reflected in the characteristic of each *NTH* gene product.

The results of the phylogenetic analysis of the *NTH* genes (Fig. 5), implied that the degree of phenotypic severity induced by over-expression of the various *NTH* genes might be related to the structure of their products. In agreement with this speculation, the products of *NTH20* and *NTH15*, which induced the most severe phenotypic abnormalities, are closer to one another than to the other *NTH* genes, whereas *NTH1*, which induced the least severe phenotypic abnormality, was distant from *NTH20* and *NTH15* (Fig. 5B). Similarly, all of the homeobox genes from other plants that induce severe phenotypes in transgenic plants, such as *KNAT1, OSH1, OSH15* and *kn1*, fell into the same group as or closely related groups to that including *NTH15* or *NTH20*. Therefore, we can classify the class1 homeobox genes into two subgroups (Fig. 5); one group comprised of *NTH20, NTH15* and other genes from various plants, such as *KNAT1, OSH1, OSH15* and *kn1*, whose overexpression induces a severely abnormal phenotype in transgenic tobacco, with ectopic shoots on the surface of malformed leaves, and another group consisting of *NTH1, NTH9* and *NTH22*, whose overexpression induces a mild or intermediate phenotype without ectopic shoot formation.

Furthermore, a comparison of the phylogenetic tree based on the KNOX domains with that based on the ELK-homeodomain, which is more commonly used in homeodomain protein alignment, indicates that similarity in the KNOX domains is more closely related to the phenotypic severity induced by the class1 genes (Fig. 5 and see Nishimura et al. 1999). This suggests that the ability of the various homeobox genes to induce an abnormal phenotype depends more on the structure of the KNOX domain than on that of the ELK-homeodomain. Recently, domain-swapping experiments between *NTH1* and *NTH15* (Sakamoto et al. 1999), have revealed two domains important for induction of abnormal phenotypes in transgenic tobacco: the ELK-homeodomain and the KNOX domain. The KNOX domain of *NTH15* was found to be essential to necessary for induction of a severely abnormal phenotype: a chimeric protein containing the KNOX domain from *NTH15* and the ELK-homeodomain from *NTH1* induces a severely abnormal phenotype, whereas a chimeric protein containing the KNOX domain from *NTH1* and the ELK-homeodomain from *NTH15* does not.

Recently, Berthelsen et al. (1998) reported that the

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KN1        IKAKIISHPHYYSLLTAYLECNKVGAPPEVSARLTEIAQEVEARQRTALGGLAAA--TEPELDQFMEAYHEMLVKFREELTRPLQEAMEFMRRVESQLNSL
OSH1       ..........S...A...D.Q........A....AV..DL.L.......V.G..--.................Y.............L....T...T.
HvKNOX3    ..........S...A...D.Q............AV..DL.L.........GT.--.................Y.............L....T.....
KNAT1      M.....A....ST..Q...D.Q.I....D.VD.I.AAR.DF....QRSTPSVS.SS-RD..........CD....Y.......I......I..I....SM.
Tkn1       L.....A..QCSN..D..MD.Q........A...SAVR..F.....RS.TDRDVS--KD..........YD....Y..............QKI.A...M.
NTH20      L.....A..QCSN..D..MD.Q........V...SAVR..F.V...DSSTDRDVS--KD..........YD....Y........H...D...KI.T...M.
KNAP1      ......A..Q..N.VE..MD.QR....SD.VP..SVAR..F.....SSGTSRETS--KD..........YD....Y.......I....D....I.T...M.
KNAP2      ......A..Q.SN..E..MD.QR....SD.V...SVAR..F.....SSGTSRETS--KD..........YD....Y.......I....D....I.T...M.
OSH15      .....MA..Q.SA..A...D.Q........LE.-LTATAAKLDA----RPPGRHDA-RD..........CN..G.Y.......ID.....LK......DTI
RS1        .....VA..Q.SA..A...D.Q......D.LE.-LTAMAAKLDA----SAAGRHEP-RD..........CN....Y.......ID.....LK...A..DCI
SBH1       V....MA....HR..A..VN.Q........V...E.ACSSAATMAGGDAAAGSSCIGED.A........C...T.YEQ..SK..K...L.LQ.I.C.FKN.
STM        V....MA....HR..A..VN.Q........V...E.ACSSAA.AAASMGPTG--CLGED.G........C.....YEQ..SK.FK...V.LQ...C.FK..
Tkn2       ..S..MA....HR......N.Q.I......V...E..CATSATMG.SSSSSGGGIIGED.A........C...T.YEQ..SK.FK...V.LS.I.C.FKA.
NTH15      ..S..MA....PR..S..VN.Q.I......V...E.VCATSATIG.---NS-GGIIGED.A........C...T.YEQ..SK.FK...V.LS.I.C.FKA.
POTH1      .....V...Y.PK..N..ID.Q.....AGIVNL.E..-RQQTDF.KPNATSICIGA--D....E...T.CDI.L.YKSD.S..FD..TT.LNKI.M..GN.
NTH22      ....VV...F.PKFVR..ID.Q.......IATV.E..-RQQNDF.KPNATSICIGA--D....E...T.CDI...YKSD.S..FD..TT.LSKI.L..SN.
KNAT2      ..S..A...L.PR..QT.ID.Q.....M.IACI.E..QRENHVYK.DVAPLSCFGA--D....E...T.CDI...YKTD.A..FD..TT.INKI.M..QN.
NTH9       .R...S...L.PK..RT.ID.H.....---.DEIVDMLDNINIVHEND.SRRSNRLSDDS...A...T.CDV.A..KSD.E..FN..TT.LNDI.T..TN.
NTH1       ...Q.AN..L.PN..S...Q.R.....----QEMAS.LE.ISKENHLISS.HNTEIGTD....D...S.CAV.L.YK...SK.FD..TT.LNNI....S..
                 **        *  *   ***                                  ** *** *   * *    *  *  **  *    * *
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B.

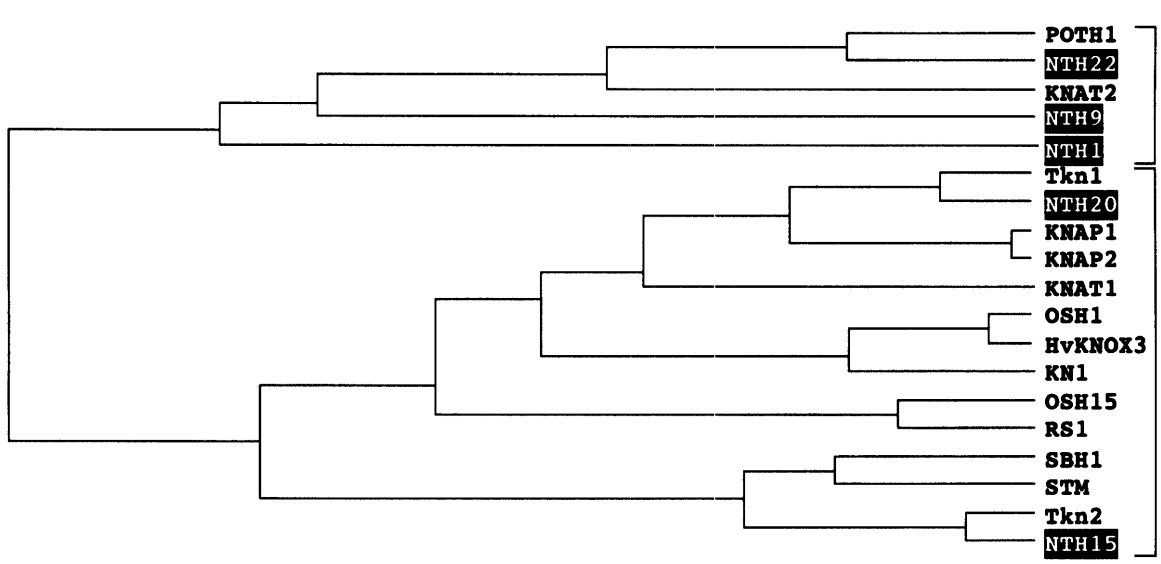

Fig. 5 Comparison of the KNOX domain sequences between NTH and other KNOTTED-type class1 plant homeobox proteins. (A) Alignment of the KNOX domains of the NTH proteins with other plant class1 KNOX domains. Asterisks indicate amino acids conserved among the KNOX domains. (B) Phylogenic tree of the KNOX domains presented in (A). The UPGMA (Unweighted Pair Group Method with Arithmetic) tree was calculated using the DNA analysis software Genetyx Mac V. 7.3.

KNOX domain of a human homeodomain protein, Prep1, is essential for its function. Prep1 belongs to the TALE superclass, the homeodomains of which resemble the plant *kn1*-type homeodomains (approximately 60% identity). This homeodomain protein also contains a sequence resembling the KNOX domain of the *kn1*-type homeodomains, in the upstream region of the homeodomain. The KNOX domain of the human Prep1 homeodomain protein is essential for interaction with another TALE-type homeodomain, Pbx1, and this interaction is essential for binding to their target DNA sequence. By analogy to the human homeodomain protein, the KNOX domains of the *NTH* genes may be important for interactions with other *NTH* homeodomain proteins or other TALE-type homeodomain proteins, to form a trans-acting complex that can stably bind to target DNA sequences. Based on this speculation, the different abilities of the various *NTH* genes to induce abnormal phenotypes may depend upon companion pro-

teins that interact with the KNOX domain of the NTH homeodomain proteins.

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