The Conserved KNOX Domain Mediates Specificity of Tobacco KNOTTED1-Type Homeodomain Proteins

Tomoaki Sakamoto, Asuka Nishimura, Masanori Tamaoki, Masako Kuba, Hiroshi Tanaka, Shuichi Iwahori, and Makoto Matsuoka

Overproduction of the tobacco KNOTTED1-type homeodomain proteins NTH1, NTH15, and NTH23 in transgenic tobacco plants causes mild, severe, and no morphological alterations, respectively. The deduced amino acid sequences of the homeodomains and adjacent ELK domains are highly conserved, and the N-terminal KNOX domains also are moderately conserved. To investigate the contributions of both the conserved and divergent regions to the severity of morphological alterations, we generated chimeric proteins by exchanging different regions of NTH1, NTH15, and NTH23. The severity of the abnormal phenotype was dependent upon the synergistic action of both the N terminus, containing the KNOX domain, and the C terminus, containing the ELK homeodomain. Detailed analysis focusing on the C terminus revealed that the C-terminal half of the ELK domain is more effective in inducing the abnormal phenotypes than are the homeodomains. For the N terminus, severe morphological alterations were induced by exchanging a part of the KNOX domain of NTH1 with the corresponding region of NTH15. This limited region in the KNOX domain of all homeodomain proteins includes a predicted α-helical region, but only that in NTH15 is predicted to form a typical amphipathic structure. We discuss the possibility, based on these results, that the secondary structure of the KNOX domain is important for the induction of abnormal morphology in transgenic tobacco plants.

INTRODUCTION

The homeobox genes were first characterized as regulatory genes that control morphogenesis in Drosophila (reviewed in Gehring, 1987). The products of the genes share a unique structure referred to as the homeodomain. This structure consists of a highly conserved 60–amino acid stretch containing three α helices, which comprise a helix-turn-helix–type DNA binding motif (Desplan et al., 1988; Otting et al., 1990). This motif recognizes and binds to specific DNA sequences, and thus, the products of homeobox genes are believed to regulate the expression of batteries of target genes by acting as transcription factors (Affolter et al., 1990; Hayashi and Scott, 1990; Kissingler et al., 1990; Laughon, 1991).

The first plant homeobox gene to be identified, kn1, was from the maize Knotted1 (Kn1) mutant (Vollbrecht et al., 1991). Many other plant homeobox genes subsequently have been cloned from various species in an effort to address the biological functions of homeobox genes in plants. The KN1-type homeodomain proteins, which are the most intensively investigated plant homeodomain proteins, have been subdivided into two groups, classes I and II (Kerstetter et al., 1994). Class I genes are more similar to the maize kn1 gene, whereas the homeodomains of class II genes are less similar to that of kn1. The class I products share extensive amino acid identity in the homeodomain, and in general, they are strongly expressed around the shoot meristem, moderately to weakly expressed in the embryo and/or or other restricted tissues, and rarely expressed in differentiated organs, such as leaves and roots (Kerstetter et al., 1994). Ecotypic expression of kn1-like class I genes has been reported to cause altered leaf and flower morphology in spontaneous mutants of a number of plant species (Smith et al., 1992; Chen et al., 1997; Parnis et al., 1997) and in transgenic plants (Matsuoka et al., 1993; Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996; Chen et al., 1997; Parnis et al., 1997). The class II genes, which are comparatively less similar to kn1 in their homeodomains, are expressed in most tissues at different levels, depending upon the tissue (Kerstetter et al., 1994). In contrast to the class I genes, overexpression of class II genes in transgenic plants does not cause altered morphology (Tamaoki et al., 1995; Serikawa et al., 1997).

We have isolated six kn1-like homeobox genes from...
tobacco. One of these (NTH23) is categorized as a class II-type gene, and the others (NTH1, NTH9, NTH15, NTH20, and NTH22) represent class I-type genes. The six genes exhibit different expression patterns around the shoot apical meristem (Tamaoki et al., 1997; Nishimura et al., 1999). Interestingly, two of the class I genes, NTH15 and NTH20, cause dramatically altered morphology when overexpressed in tobacco, very similar to the phenotype caused by overexpressing maize kn1 or OSH1 from rice (Kano-Murakami et al., 1993; Sinha et al., 1993; Tamaoki et al., 1997; A. Nishimura, T. Sakamoto, M. Tamaoki, and M. Matsuoka, manuscript in preparation). Overexpression of two other class I genes (NTH1 and NTH9) causes only mild alterations in the phenotype of transgenic plants (A. Nishimura, T. Sakamoto, M. Tamaoki, and M. Matsuoka, manuscript in preparation), whereas overexpression of the class II gene NTH23 does not cause abnormal morphology (Sentoku et al., 1998). The difference in the severity of phenotypes caused by the different tobacco homeobox genes may depend upon their structures and therefore may be a reflection of their functions in wild-type plants.

The six tobacco KN1-type homeodomain proteins have comparatively similar homeodomain sequences. The sequence immediately upstream of the homeodomain, defined as the ELK domain (Vollbrecht et al., 1991; Kerstetter et al., 1994), is also conserved. The ELK domain constitutes a novel form of amphipathic helix (Kerstetter et al., 1994) and can function as a nuclear localization signal (Meisel and Lam, 1996). It was reported recently that a stretch of ~100 amino acids located in the N terminus of various KN1-type homeodomain proteins also is conserved (Bürglin, 1997). The function of this conserved region, termed the KNOX domain, has not been elucidated.

We are interested in determining which part(s) of the homeodomain proteins is important for provoking altered morphologies in transgenic plants. In this study, we have generated 33 chimeric proteins by exchanging various amino acid motifs among NTH1, NTH15, and NTH23. These chimeric proteins were overexpressed in transgenic tobacco plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter. We analyzed their effects on the phenotypic severity of transformants and assessed which part(s) of the homeodomain proteins contributed to the altered morphology of the transgenic plants. We have found, based on these results, that the N-terminal KNOX domain is important for the induction of altered morphology.

RESULTS

Reciprocal Exchanges of N-Terminal Regions among Three Homeodomain Proteins, NTH15, NTH1, and NTH23

In this study, we used the tobacco KN1-type homeodomain proteins NTH1, NTH15, and NTH23 (Figure 1), because overexpression of their encoding genes under the control of the CaMV 35S promoter causes three distinct phenotypes in transgenic plants, that is, mild, severe, and no phenotype, respectively (Figure 2; see below for details of each phenotype). The aligned amino acid sequences of these three homeodomain proteins are shown in Figure 1. The three proteins share well-conserved C-terminal amino acid sequences. This region corresponds to the ELK homeodomain motif. In contrast, the N-terminal sequences of the three proteins are less well conserved. For this reason, we first divided the proteins into two parts: the less conserved N terminus and the well-conserved C terminus. To evaluate the effects of the N-terminal and the conserved C-terminal portions on the abnormal phenotypes of transgenic plants, we reciprocally exchanged these two portions with one another. Nine chimeric genes under the control of the CaMV 35S promoter were constructed and introduced into tobacco (Figure 3). Most of the transformants exhibited abnormal phenotypes, the severity of which depended upon the introduced transgenes.

Figure 1. Comparison of the Deduced Amino Acid Sequences of NTH1, NTH15, and NTH23.

Dashes indicate gaps introduced to facilitate alignment. The ELK homeodomain is indicated by bars beneath the sequences. The KNOX domain sequence reported by Bürglin (1997) is represented by lowercase letters, and the conserved α helix found in the KNOX domain is boxed in black. The junction of domains used for N-terminal reciprocal exchange experiments is indicated by a solid arrow. The three domains in the N-terminal portions are shown by numbered bars above the sequences. The junctions of these domains are indicated by open arrows.
We categorized the severity of the abnormal phenotypes of the transgenic plants into four groups based on leaf and gross morphology (Figure 2). In plants classified as having a wild-type phenotype, we did not observe any abnormalities in leaves or gross morphology (Figures 2A and 2E). Plants exhibiting a mild phenotype exhibited a normal gross morphology but had abnormally shaped leaves (Figures 2B and 2F). Leaf size for plants with a mild phenotype was reduced slightly, and the midvein was curved (Figure 2F). Plants with an intermediate phenotype had severely wrinkled leaves caused by lack of coordination between midvein and leaf blade elongation (Figure 2G). Consequently, the leaves of plants in this category showed a butterfly-like shape with an atrophic midvein. Gross morphology of plants with an intermediate phenotype was not affected severely (Figure 2C). Transformants categorized as having a severe phenotype were severely dwarfed and showed a loss of apical dominance (Figure 2D). The leaves of these plants were very small and thick in comparison to wild-type and other transgenic plants. Midveins and lateral veins of these leaves failed to develop, and the formation of ectopic meristems was observed occasionally on the adaxial side (Figure 2H).

Using these four phenotypic categories, we assessed the phenotype of transgenic tobacco plants containing the various chimeric constructs. Multiple plants transformed with the same chimeric construct did not always have identical phenotypes but sometimes showed a range of phenotypes, which is a phenomenon mainly caused by varying transgene expression levels in individual transformants (Kanomurakami et al., 1993; Sinha et al., 1993; Tamaoki et al., 1997). For instance, 55% of plants transformed with the chimeric gene NTH1/15, which consists of the N terminus of NTH1 and the C terminus of NTH15, exhibited a mild phenotype, whereas 40% had an intermediate phenotype, and the remaining 5% had a wild-type phenotype (Table 1). A similar distribution of abnormal phenotypes also was seen in transgenic plants carrying NTH23/15. Most of the transformants (~85%) carrying NTH15/15 (i.e., the intact NTH15 gene) exhibited a severe phenotype, whereas replacement of the N terminus of NTH15 with that of NTH1 or NTH23 reduced the
effectiveness of the transgene products in provoking a severe phenotype. These results strongly suggest that the N terminus of NTH15 is important for induction of a severe phenotype in transgenic tobacco.

The same conclusion was reached by comparing the phenotypes of plants transformed with NTH15/15, NTH1/15, and NTH23/15. Although most plants carrying NTH1/15 and NTH23/15 had a mild phenotype, 40% of plants carrying NTH15/15 had a severe phenotype, and 30% had an intermediate phenotype. Only 26% of NTH15/15 transformants exhibited a mild phenotype.

The conclusion that the N terminus of NTH15 induces a more severe phenotype in transgenic plants also was confirmed by comparison of NTH15/23, NTH1/23, and NTH23/23. None of the plants transformed with NTH1/23 or NTH23/23 had an abnormal phenotype; therefore, all of these transformants were categorized as having a wild-type phenotype. In contrast, most plants transformed with NTH15/23 had a mild or intermediate phenotype.

It previously has been demonstrated that overexpression of class I homeobox genes causes abnormal phenotypes in transgenic plants or spontaneous mutants but that overexpression of class II genes does not (Tamaoki et al., 1995; Serikawa et al., 1997; Sentoku et al., 1998). However, the above-mentioned results demonstrate that overexpression of NTH15/23, a class II gene (as defined by the presence of a class II C-terminal ELK homeodomain in the portion of the gene product derived from NTH23), also can induce aberrant morphology in transgenic plants. Thus, these results clearly demonstrate that the ability of the homeobox genes to induce an abnormal phenotype is not directly dependent upon the ELK homeodomain but is strongly dependent on the N terminus of the protein product. However, this does not mean that the C terminus, containing the ELK homeodomain, does not affect the induction of an abnormal phenotype. Indeed, when comparing NTH1/15 and NTH1/23, we found that most plants transformed with NTH1/15 (95%) had a mild or intermediate phenotype, whereas plants carrying NTH1/23 did not exhibit any abnormalities. Thus, the structure of the C terminus also affects the phenotypic severity of transgenic plants, as previously reported (Serikawa and Zambryski, 1997).

**Table 1. Morphological Alterations Caused by Exchange of N-Terminal Regions among Three Homeodomain Proteins**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Wild Type</th>
<th>Mild</th>
<th>Intermediate</th>
<th>Severe</th>
<th>Total</th>
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<tbody>
<tr>
<td>NTH15/15</td>
<td>0 (0.0)</td>
<td>2 (3.0)</td>
<td>8 (12.1)</td>
<td>56 (84.9)</td>
<td>66</td>
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</table>

*The total number of independent T1 transgenic plants examined.
*The intact NTH15, NTH1, or NTH23 construct.
*Numbers within parentheses indicate the percentages of T1 transgenic plants in each line exhibiting each phenotype.

**Figure 3. Schematic Representation of Chimeric Proteins Consisting of the N- and C-Terminal Portions of NTH1, NTH15, and NTH23 or the Yeast GAL4 Transactivation Domain.**

The N- and C-terminal halves of the three homeodomain proteins are divided at the N-terminal end of the ELK domain (solid arrow in Figure 1). The first three sets of chimeric proteins, NTH15/15 through NTH23/23, reciprocally exchanged these two portions with one another. Another set of constructs, GAL4/15 through GAL4/23, contain the ELK homeodomains of NTH15, NTH1, and NTH23 and the trans-activating domain of the yeast transcription factor GAL4.

**Replacement of the N-Terminal Portion with the Transactivating Domain of the Yeast Transcription Factor GAL4.**

It is well established that the homeodomains of typical animal homeodomain proteins, such as Antennapedia, are sufficient for binding to specific oligonucleotide sequences. Moreover, the ELK homeodomain of the KN1-type homeodomain proteins is sufficient for nuclear targeting (Meisel and Lam, 1996). These results suggested to us the possibility that a fusion protein containing an ELK homeodomain...
Specificity of KN1-Type Homeodomain Proteins

sufficient for binding to specific DNA sequences and for nuclear targeting, plus a transactivating domain for transcription, might induce an abnormal phenotype when overproduced in transgenic tobacco. To test this possibility, we fused a well-known transactivating domain, the acidic region of the yeast transcription factor GAL4 (residues 768 to 881) (Ma and Ptashne, 1987), in-frame to the N-terminal side of the C terminus of NTH1, NTH15, and NTH23, which contains the ELK homeodomain (Figure 3). The GAL4 acidic domain already has been shown to act as a transactivating domain in tobacco cells (Moore et al., 1998).

Interestingly, none of the transformants carrying this series of fusion genes showed any phenotypic abnormalities (Table 1). RNA gel blot analysis and protein gel blot analysis with an antibody raised against the GAL4 acidic domain revealed that the transgenes were expressed at high levels in most plants showing no phenotype (data not shown). These results demonstrate that exchanging the N terminus of the homeodomain proteins with the transactivating domain of GAL4 causes the transgene products to lose their ability to induce abnormal phenotypes in the transformants. Hence, the N-terminal portions of the homeodomain proteins do not function simply as transactivation domains.

Partial Exchanges of Dissimilar Amino Acids between the ELK Homeodomains of NTH15 and NTH1

As noted earlier, it has been demonstrated that overexpression of the class I homeobox genes causes abnormal phenotypes in transgenic plants, whereas overexpression of the class II genes does not (Tamaoki et al., 1995; Serikawa et al., 1997; Sentoku et al., 1998). These results may indicate that the structure of the ELK homeodomain is important for induction of abnormal phenotypes, because the classification of the proteins as class I or class II depends upon the ELK homeodomain structure. In fact, exchanging the C terminus of NTH15 with that of NTH1 reduced the phenotypic severity (cf. NTH15/15 and NTH15/1 in Table 1). By contrast, replacement of the C-terminal half of NTH1 with that of NTH15 enhanced the phenotypic severity, and some plants showed an intermediate phenotype, which was not found in the NTH1 transformants (cf. NTH1/1 and NTH1/15 in Table 1). These results clearly indicate that the C-terminal portion of homeodomain proteins has some effects on the function of the protein.

To determine which part(s) of the ELK homeodomain induces the different phenotypes, we constructed 14 chimeric genes (CHD for chimeric homeodomain) by replacing divergent amino acids in the ELK homeodomain. Figure 4A provides a comparison of the amino acid sequences of the ELK homeodomains of NTH15 and NTH1. Four divergent regions were identified. Two of these are located in the ELK

Table 2. Effect of Replacement of Specific Regions of the ELK Homeodomains on Phenotypic Severity

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Construct</th>
<th>Wild Type</th>
<th>Mild</th>
<th>Intermediate</th>
<th>Severe</th>
<th>Total</th>
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<tbody>
<tr>
<td>NTH15/15</td>
<td>0 (0.0)</td>
<td>2 (3.0)</td>
<td>8 (12.1)</td>
<td>56 (84.9)</td>
<td>66</td>
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<tr>
<td>NTH1/15</td>
<td>7 (36.8)</td>
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<tr>
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<td>5 (14.7)</td>
<td>28 (82.4)</td>
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<tr>
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<td>2 (11.8)</td>
<td>14 (82.4)</td>
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</table>

*The number of independent T1 transgenic plants examined.

Numbers within parentheses indicate the percentages of T1 transgenic plants in each line exhibiting each phenotype.
domain, and the remaining two are in the first and the second helices of the homeodomain. In the first set of constructs, CHD-1 through CHD-4, we substituted one of the four dissimilar regions of NTH15 with the corresponding sequence from NTH1. In the second set of four constructs, CHD-5 through CHD-8, we substituted one of the four divergent regions of NTH1 with that of NTH15. In the last set, CHD-9 through CHD-14, we substituted two regions of NTH1 with the corresponding sequences from NTH15 (Figure 4B).

More than 80% of the transgenic plants carrying CHD-1, CHD-3, or CHD-4 exhibited a severe phenotype (Table 2). However, in the case of CHD-2, approximately half of the transformants had a severe phenotype, and the remainder exhibited an intermediate phenotype (Table 2), indicating that the C-terminal half of the ELK domain contributes to the induction of the severe phenotype. On the other hand, partial replacement of the ELK homeodomain of NTH1 with the corresponding NTH15 sequence resulted in a slight increase in the phenotypic severity of most of the transgenic plants, with the exception of plants carrying CHD-5 construct. This lack of effect of the replacement of the N-terminal half of the ELK domain from NTH1 with that from NTH15 indicates that this region is not important for the phenotypic severity of the transgenic plants.

In contrast to the N-terminal half, replacement of the C-terminal half of the ELK domain apparently enhanced phenotypic severity. In plants transformed with the constructs that contained the C-terminal half of the ELK domain from NTH15, that is CHD-6, CHD-9, CHD-12, and CHD-13, the frequency of plants exhibiting the intermediate phenotype was always higher than it was in plants transformed with constructs that did not contain it—CHD-5, CHD-7, CHD-8, CHD-10, and CHD-11 (Table 2).

These results confirm the above-mentioned conclusion, based on the result with CHD-2, that the C-terminal half of the ELK domain is important for induction of an abnormal phenotype in transgenic plants. Replacement of the first or second helix of the homeodomain was less effective than that of the C-terminal half of the ELK domain, but replacement of both helices caused an increase in phenotypic severity, with ~30% of transformants exhibiting an intermediate phenotype (Table 2).

### Exchange of Domains in the N-Terminal Regions of NTH15 with the Corresponding Sequences from NTH1

Because the severity of abnormal morphology of the transgenic plants depended strongly on the sequence of the N terminus (Table 1), functional domains also may exist in this region. By comparing the sequences of NTH1, NTH15, and NTH23 (Figure 1), we identified a conserved amino acid sequence in the KNOX domain, the secondary structure of which was predicted by the SOPMA server (http://www.ibcp.fr/serv_pred.html; Geourjon and Deleage, 1994, 1995) to be a long α helix (Figure 1). Keeping this conserved sequence in mind, we divided the N-terminal regions of NTH15 and NTH1 into three domains (Figure 1) and constructed six chimeric genes (CNT for chimeric N terminus; Figure 5). The first domain is located at the N-terminal side of the conserved α helix and contains several homopolymeric amino acid stretches, which are observed often in KN1-type homeodomain proteins, plus the N-terminal part of the KNOX domain. The second domain consists of the C-terminal part of the KNOX domain and contains the conserved α helix. The third domain is located between the C-terminal side of the KNOX domain and the N-terminal side of the ELK homeo-

### Table 3. Effect of Replacement of Specific Regions of the N-Terminal Portions on Phenotypic Severity

<table>
<thead>
<tr>
<th>Construct</th>
<th>Phenotype</th>
<th>Wild Type</th>
<th>Mild</th>
<th>Intermediate</th>
<th>Severe</th>
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<td></td>
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<tr>
<td>CNT-5</td>
<td>0 (0.0)</td>
<td>6 (25.0)</td>
<td>6 (25.0)</td>
<td>12 (50.0)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>CNT-6</td>
<td>1 (1.9)</td>
<td>29 (54.7)</td>
<td>23 (43.4)</td>
<td>0 (0.0)</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>CNT-1</td>
<td>4 (12.5)</td>
<td>15 (40.9)</td>
<td>7 (21.9)</td>
<td>6 (18.8)</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

*The number of independent T1 transgenic plants examined.

* The intact NTH15 or NTH1 construct (as given in Table 1).

Numbers within parentheses indicate the percentages of T1 transgenic plants in each line exhibiting each phenotype.
domain. In this region, there is no significant similarity between NTH1 and NTH15.

In the first set of constructs, CNT-1 to CNT-3, we substituted each of the three N-terminal domains of NTH15 with the corresponding domain from NTH1 (Figure 5). When CNT-1 or CNT-3 was overexpressed, 79 or 54% of the transformants exhibited a severe phenotype, respectively. By contrast, none of the CNT-2 transformants exhibited a severe phenotype (Table 3). In the second set of three chimeric genes, CNT-4 to CNT-6, two domains of NTH15 were substituted with the corresponding domains of NTH1 (Figure 5). None of the plants transformed with CNT-4 or CNT-6 had a severe phenotype, and >50% of them had a mild phenotype, as was seen with the NTH1/15 transformants. By contrast, half of the CNT-5 transformants exhibited a severe phenotype (Table 3). All three constructs that caused a severe phenotype in transgenic plants contained the second N-terminal domain from NTH15, whereas the remaining three constructs, which did not induce a severe phenotype, contained the NTH1 sequence in this region. These results strongly suggest that the C-terminal part of the KNOX domain, containing the conserved α helix, is essential for induction of the severe phenotype.

To confirm the possibility that the second N-terminal domain defines the functional specificity of homeodomain proteins, we substituted only this domain in NTH1 with that from NTH15 (Figure 5, CNTH1). Although overexpression of NTH1 caused only a mild phenotype (Figure 6A), 19% of the CNTH1 transformants exhibited a severe phenotype (Table 3 and Figures 6B and 6C). In addition, we also occasionally observed the formation of ectopic shoots on the adaxial side of the leaves of CNTH1 transformants, which is a characteristic typical of plants exhibiting the most severe phenotype (Figure 6D).

Expression of Chimeric Proteins Containing the c-Myc Epitope in Transgenic Plants

Differences in the severity of abnormal leaf morphologies caused by the overexpression of each construct could be related to differences in the levels and/or the stability of the various transgene products. To investigate this possibility, we performed RNA dot blot analysis with specific probes containing the 5′ or 3′ noncoding sequences of each of the NTH genes. We randomly selected several plants with strong phenotypes for each construct and tested the expression of the transgene transcript. Almost all of the transgenic plants, including plants expressing a wild-type protein, such as those transformed with NTH1/23, expressed the mRNAs transcribed from the transgenes at similar levels (data not shown). In the case of plants expressing NTH23/23, however, the expression level of the transgene could not be measured accurately because the endogenous NTH23 gene is constitutively expressed in normal leaves (Sentoku et al., 1998).

To further confirm the levels and the stability of the various chimeric proteins in transgenic plants, we performed protein gel blot analysis. Because it was impossible to detect the different chimeric proteins with the same antibody raised against each of the tobacco homeodomain proteins, we independently generated five constructs tagged with the sequence encoding a c-Myc epitope, introduced the constructs into tobacco, and performed protein gel blot analysis with an anti-c-Myc monoclonal antibody. The 30 nucleotides encoding the 10 amino acids of the c-Myc epitope were introduced immediately upstream of the termination signal of five intact or chimeric genes, NTH1, NTH15, NTH23, CNT-2 (NTH15 loss of function), and CNTH1 (NTH1 gain of function). We tested several independent plants exhibiting a phenotype typical for each construct. The immunoreactive proteins were detected at similar levels in extracts isolated from all plants transformed with the constructs, whereas no reactive protein was detected in wild-type plants (Figure 7). These results demonstrate that there is not a significant difference in the expression or stability of the various chimeric proteins in the transgenic plants, and therefore the differences

Figure 6. Severe Phenotype of CNTH1-Transformed Plants.

(A) Typical NTH1/1-transformed plant.
(B) Typical NTH15/15-transformed plant.
(C) Severe phenotype of a CNTH1-transformed plant.
(D) Ectopic shoot formation on the leaves of a CNTH1-transformed plant.
Bars in (A) to (C) = 3 cm; bar in (D) = 5 mm.
in phenotypic severity are caused by the inherent properties of each of the chimeric proteins.

**DISCUSSION**

**The N- and C-Terminal Halves of Homeodomain Proteins Act Synergistically in the Induction of Abnormal Phenotypes in Transgenic Plants**

We have demonstrated by reciprocally exchanging the N- and C-terminal halves of three tobacco homeodomain proteins that the severity of abnormal phenotypes caused by overexpression of these proteins in transgenic plants depends more strongly on the N-terminal half than on the C terminus, which contains the ELK homeodomain. In plants transformed with NTH15/15, NTH1/15, NTH15/1, or NTH1/1, for instance, exchanging the N terminus of NTH1 for that of NTH15 increased the phenotypic severity from mild to severe, whereas exchanging the C terminus of NTH1 for that of NTH15 increased the phenotypic severity from mild to intermediate. Recently, Serikawa and Zambryski (1997) performed similar domain exchange experiments between two Arabidopsis homeodomain proteins, KNAT1 and KNAT3. KNAT1 is a class I-type knl-like homeobox gene, and KNAT3 is a class II-type gene. They reported that all transformants carrying the N terminus of KNAT1 (class I protein) and the C terminus of KNAT3 (class II protein) displayed a wild-type phenotype. By contrast, when the N terminus of KNAT3 (class II protein) was fused to the C terminus of KNAT1 (class I protein), transgenic plants exhibited an abnormal morphology that was very similar to but qualitatively different from the KNAT1 overexpression phenotype. Basing their conclusions on these experiments, Serikawa and Zambryski (1997) found that the specificity of the overexpression phenotype of KNAT1 resides mainly in the C-terminal region, extending upstream to the start of the ELK domain of the KNAT1 protein. In agreement with their findings, all of our transgenic plants expressing NTH1/23, in which the C-terminal half of NTH23 (a class II protein) was fused to the N-terminal half of NTH1 (a class I protein), exhibited a wild-type phenotype.

However, it is noteworthy that when the C terminus of NTH23 was fused to the N terminus of NTH15 (a class I protein), most of the transformants showed some abnormalities. This finding is not consistent with the previous result by Serikawa and Zambryski (1997) and clearly demonstrates that a homeodomain protein containing a class II C-terminal half has the potential to induce an abnormal phenotype when it also contains an appropriate N-terminal half from a class I homeodomain protein, such as NTH15. This does not mean that the C terminus does not affect the severity of abnormal phenotypes of transgenic plants. In fact, the reciprocal exchange experiments clearly indicate that the C termini of NTH15, NTH1, and NTH23 exert strong, medium, and weak influences, respectively, on the abnormal phenotype of transgenic plants.

Basing our findings on these reciprocal exchange experiments, we have predicted the effectiveness of the N- and C-terminal regions of the three homeodomain proteins in inducing abnormal phenotypes, as depicted in Figure 8. As noted earlier, the effectiveness of the C termini of the three proteins descends from NTH15 (strong) through NTH1 (moderate) to NTH23 (weak) (Figure 8). Similarly, the N terminus of NTH15 was most effective among the six N and C termini in inducing an abnormal phenotype (Figure 8). By contrast, no clear difference between the effectiveness of the N termini of NTH1 and NTH23 was seen (Figure 8).

The arrangement of the three C termini, with NTH1 roughly midway between NTH15 and NTH23 (Figure 8), suggests that there is not a discontinuous point of difference in the effectiveness of the ELK homeodomain between the class I (NTH15 and NTH1) and class II (NTH23) proteins. Taken together, Figure 8 clearly demonstrates that the severity of abnormal phenotype is a synergistic function of the combination of N and C termini and that the effect of the N terminus is stronger than that of the C terminus.

We also have demonstrated that the N terminus cannot be substituted by the transactivating domain of the yeast transcription factor GAL4. If the ELK homeodomain itself were sufficient for interacting with its target DNA sequence(s) (Meisel and Lam, 1996), chimeric proteins containing the GAL4 transactivating domain and the ELK
The elongated horizontal triangle indicates the phenotypic severity of transformants. The four phenotypic categories, ranging from wild type to severe, are indicated by double-headed arrows and are arranged as a continuum from right to left. The effectiveness of the different N and C termini in inducing an abnormal phenotype is represented by the positions of open circles on the dashed lines and the top or bottom. The phenotype caused by a particular combination of N and C termini is indicated by the position at which a line connecting the two termini crosses the boldface horizontal line. The final placement of each N- and C-terminal region was determined by the phenotypes presented in Table 1. 15-N, N terminus of NTH15; 1-N, N terminus of NTH1; 23-N, N terminus of NTH23; 15-C, C terminus of NTH15; 1-C, C terminus of NTH1; and 23-C, C terminus of NTH23.

Functional Regions in the ELK Homeodomain

Even though the ELK homeodomains are well conserved between NTH1 and NTH15 (72.9% amino acid identity; Figure 4A), these domains had different effects on the induction of an abnormal phenotype (Table 2). Experiments in which four regions of divergence between NTH1 and NTH15 were exchanged demonstrated that the C-terminal half of the ELK domain had the strongest effect of the four regions in inducing an abnormal phenotype and that the N-terminal half of the ELK domain had the smallest effect. Although the biological role of the ELK domain has not been clarified, this domain may contribute to protein–protein interactions through its amphipathic helical structure (Kerstetter et al., 1994). Based on this hypothesis, our expectation would be that exchange of the C-terminal half of the ELK domain between NTH1 and NTH15 causes an exchange in the affinity of interaction with an accessory protein(s), or interaction with a different protein(s), with consequent induction of different effects on the abnormal phenotypes. Alternatively, differences in ELK domain structure may affect the interaction between the homeodomain and target DNA sequences; that is, the ELK domain of NTH15 may be more effective in stabilizing the interaction with target DNA sequences than that of NTH1, and this difference in affinity between the homeodomain and target DNA sequences may result in a difference in the severity of the abnormal phenotype.

The KNOX Domain Is Important in Determining the Severity of the Abnormal Phenotype

Our results clearly demonstrate that the phenotypic severity depends more strongly on the N terminus than on the C terminus. These results suggested to us the possibility that other functional domains exist in the N terminus. The N termini of NTH1, NTH15, and NTH23 contain several homopolymeric amino acid stretches, which have been predicted to act as transcriptional activation domains (Gerber et al., 1994). However, exchange of the homopolymeric amino acid stretches between NTH1 and NTH15 did not result in any difference in the severity of abnormal morphology (Table 3), indicating that differences in these stretches are not the main cause of differences in phenotypic severity. The stretches may not be important for the induction of abnormal phenotypes, because the structures of the N termini, including the homopolymeric amino acid stretches, are divergent even among KN1-type homeodomain proteins that induce severe abnormal phenotypes (Vollbrecht et al., 1991; Matsuoka et al., 1993; Tamaoki et al., 1997). The reason that the N termini of many KN1-type homeodomain proteins contain such homopolymeric stretches of various amino acids remains unclear.

In contrast to the homopolymeric amino acid stretches, the contribution of a second N-terminal domain, consisting of the C-terminal part of the KNOX domain, to the induction of abnormal morphology was apparent in the exchange experiments. None of the fusion proteins of the CNT series that contained the second domain from NTH1 induced a severe phenotype, whereas all of the CNT proteins containing...
the NTH15 sequence in this domain induced a severe phenotype in more than half of the transformants. Moreover, the replacement of only this domain in NTH1 with that of NTH15 conferred the ability to induce a severe phenotype on NTH1, clearly demonstrating that the C-terminal part of the KNOX domain is an essential factor for the severity of the abnormal phenotype. Therefore, the C-terminal part of the KNOX domain may be an important domain in the homeodomain proteins.

The KNOX domain consists of α helices and is split into two subdomains joined by a flexible linker (Bürglin, 1997). The N-terminal subdomain contains an α helix and corresponds to the N-terminal part of the KNOX domain that was included in the first domain in our exchange experiments. Our results show that the N-terminal subdomain is not important for the induction of an abnormal phenotype. On the other hand, the C-terminal subdomain contains 20 amino acids that also are predicted to form an α helix, which was included in the second domain in our experiments. This α-helical structure is conserved in NTH1, NTH15, NTH23 (Figure 1), and other tobacco KN1-type homeodomain proteins. In the α helix of NTH15, hydrophobic residues are clustered together at one side of the helix to produce an amphipathic structure, whereas in the helices of NTH1 and NTH23, two or three hydrophobic residues are located on the hydrophilic sides; consequently, these helices do not form amphipathic structures (Figure 9). The correlation between formation of an amphipathic structure and induction of severe morphological alterations suggests that the amphipathic structure of the C-terminal KNOX subdomain may be important for the induction of the severe phenotype.

The importance of the α-helical structure in the KNOX domain also has been reported in a member of animal MEIS homeodomain proteins, Prep1 (Berthelsen et al., 1998a). This homeodomain protein belongs to the TALE (for three–amino acid loop extension) superclass, which is characterized by three extra residues in the loop between the first and second helices in comparison to typical homeodomain proteins, such as Antennapedia (Bertolino et al., 1995). The TALE superclass of homeobox genes is present in plants, fungi, and animals (Bürglin, 1997), and all of the plant KNOX genes are also members of this superclass. The plant KNOX and animal MEIS homeodomain proteins share not only a conserved homeodomain with a three–amino acid loop between the first and second helices but also a conserved N-terminal domain, termed the KNOX domain for the plant KNOX family and the MEIS domain for the animal MEIS family (Bürglin, 1997). The mammal MEIS homeodomain protein, Prep1, interacts with another TALE homeodomain protein, Pbx (a member of the PBC protein family), through the MEIS domain (Berthelsen et al., 1998b). This interaction between Prep1 and Pbx requires two consensus sequences in the MEIS domain. These two consensus sequences are predicted to form α helices and correspond to the two subdomains of the KNOX domain in plant KN1-type homeodomain proteins. This structural conservation between the plant KNOX and animal MEIS domains strongly suggests that these domains have a similar biological role(s) and that the KNOX domains may be important for interactions with other proteins, probably transcription factors. Based on this speculation, we can conclude that the differences in the severity of altered leaf morphology caused by overexpression of the tobacco KN1-type homeodomain proteins may be mediated by the interaction of different accessory proteins with the different KNOX domains, and these accessory proteins may be the ultimate determinants of phenotypic severity.

This speculation also is supported by a hypothetical model for the evolution of homeodomain proteins (Bürglin, 1998). In this model, the author has assumed a common an-
cestral protein, from which the PBC, MEIS, and KNOX proteins have been derived, because these three types of proteins share the amino acid sequences not only in the TALE homeodomains but also in the conserved N-terminal domains. This virtual protein may form homodimers as well as a typical homeodomain protein forming homodimers or heterodimers. In animals, this protein is predicted to have duplicated to give rise to the PBC and MEIS proteins, and these proteins are presumed to have become specialized partners for particular groups of typical homeodomain proteins (reviewed in Mann and Chan, 1996; Shen et al., 1997) or have retained the ability to interact with PBC and MEIS proteins (Chang et al., 1997; Knoepfler et al., 1997). This interaction is mediated by the conserved N-terminal domain. As occurs in the plant kingdom, a similar regulatory network may exist, and the KNOX proteins may be able to form homodimers or heterodimers and to interact with other proteins containing the typical homeodomain, such as the homeodomain leucine zipper proteins (Bürglin, 1998).

Currently, it is not known whether plant homeodomain proteins act as monomers, homodimers, heterodimers, or as complexes with other cofactors. However, direct interaction between rice KN1-type homeodomain proteins has been demonstrated using the yeast two-hybrid system, and the KNOX domains are essential for this interaction (Y. Sato and M. Matsuoka, unpublished observations). This finding supports the above-mentioned speculation that the KNOX domain is important for interactions with other proteins. Identification and characterization of proteins that interact with the tobacco KN1-type homeodomain proteins through the KNOX domain are important and essential steps in elucidating the biological functions of the KN1-type homeodomain proteins. Identifying DNA binding and nuclear localization abilities of the KNOX homeodomain proteins will also be essential for confirming the function of these proteins as transcription factors.

METHODS

Plasmid Constructs

All chimeric genes generated in this study were introduced between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) polyadenylation signal of pBI121 (Clonetech Co., Palo Alto, CA).

For reciprocal exchange of the N-terminal portions between NTH1, NTH15, and NTH23, the homeodomain proteins were divided into N- and C-terminal halves at the N-terminal side of the ELK domain (see Figure 1). Three homeodomain proteins share invariant amino acids, Glu-Leu, at this site. Because the SacI restriction site (GAGCTC) encodes Glu-Leu, we designed primers with a synthetic SacI site for amplifying regions of the tobacco homeobox genes encoding the N- and C-terminal halves of the NTH proteins. For example, to amplify the region encoding the C-terminal half of NTH15, we made the following primer: 5′-GGAGCTCTTGAATTCAGGCGGCTTG-3′ (the SacI site is underlined, and the NTH15 sequence is in boldface). This primer corresponds to the NTH15 sequence encoding Glu-Leu-Lys-Gly-Glu-Leu-Leu, with two nucleotide exchanges (the NTH15 sequence encoding Glu-Leu is GAACCTG, whereas the primer sequence was GAGCTC) to allow a SacI site at the N-terminal side of the ELK domain to be introduced. Using this primer and the M13 reverse primer, we amplified the region encoding the C-terminal half of NTH15 from a full-length NTH15 cDNA in pUC19. In the same manner, we amplified DNA fragments encoding the N- and C-terminal halves of NTH1, NTH15, and NTH23 and cloned them into pUC19. The polymerase chain reaction (PCR) products were sequenced to confirm that no nucleotide substitutions had occurred during amplification, and they were subcloned into pBI121 in combination with fragments encoding the C- or N-terminal portions of each of the three NTH proteins (Figure 3).

The transactivating domain of the yeast transcription factor GAL4 (residues 768 to 881) was amplified by PCR, using primers with SacI linkers for in-frame fusion with the C-terminal halves of the homeodomain proteins. Sequences of the PCR products were confirmed, and the products were inserted into pBI121 with each of the three NTH C-terminal halves (Figure 3).

To exchange dissimilar amino acids in the ELK homeodomains of NTH15 and NTH1 (see Figure 4A), we performed site-specific in vitro mutagenesis by using PCR. For each construct, we designed two primers with specific nucleotide exchanges targeted toward the region to be mutagenized. For construction of the chimeric homeodomain construct CHD-2, for example, we made reverse and forward primers that shared complementary sequences encoding the NTH1 region to be exchanged (5′-CACCTTCTTCGCCAATCGTC-3′ and 5′-CTTGAGGAAGATTTTGGAAGAGGAGAAAGGC-3′; the NTH15 sequence is underlined, and the NTH1 sequence is in boldface: the NTH1 sequences of the two primers overlap and complement one another). The N-terminal portion of CHD-2 was amplified by using a full-length NTH15 cDNA in pUC19 as a template with the M13-20 primer plus the reverse primer shown above. The C-terminal portion of CHD-2 was amplified with the forward primer shown above plus the M13 reverse primer. The two resulting PCR products were mixed and reamplified using M13-20 and M13 reverse primers. Sequences of the PCR products were confirmed, and the products were ligated into pBI121.

For exchange of N-terminal domains, the N-terminal portions of NTH1 and NTH15 were subdivided into three domains separated by conserved amino acids, Gly-Ala-Pro and Ile-Glu (see Figure 1). We designed eight PCR primers for amplification of the regions encoding the three subdomains of NTH1 and NTH15. The nine-nucleotide sequence GGGGCCCCG encodes Gly-Ala-Pro and contains an ApaI sequence encoding Glu–Leu–Lys–Glu–Leu–Glu–Leu–Lys–Glu, with two nucleotide exchanges (the primer sequence was CAACAATTCCTTTCTC-AAGCTGCTCAAGTGAAACCGCCTTTG-3′ and 5′-TGAGCAGCT-CTTGAAGAAACCGCTTTTGAAGAGGAGAAAAAGGC-3′; the NTH15 sequence is underlined, and the NTH1 sequence is in boldface). The transactivating domain of the yeast transcription factor GAL4 (residues 768 to 881) was amplified by PCR, using primers with SacI linkers for in-frame fusion with the C-terminal halves of the homeodomain proteins. Sequences of the PCR products were confirmed, and the products were inserted into pBI121 with each of the three NTH C-terminal halves (Figure 3).

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Primers containing a Clai site (ATCGAG) also were designed for amplifying fragments encoding the internal and C-terminal domains of NTH1 and NTH15. Introduction of the Clai site required a change of glutamic acid to glycine at the Ile-Glu site. This change is not likely to alter the function of NTH1 or NTH15, as evidenced by the similar morphology of plants transformed with authentic NTH1 or NTH15 compared with plants containing reconstructed NTH1 or NTH15 with the Glu-to-Gly change (data not shown). The synthetic restriction sites were introduced by PCR as described above. The six PCR products, corresponding to the three domains of NTH1 and NTH15, were cloned into pCRII (Invitrogen, Carlsbad, CA), and their sequences were confirmed.

The fragments were ligated in the correct order in all possible
combinations with the C-terminal portion of NTH15 in pBI121 (see Figure 5). In addition, the internal domain from NTH15 was combined with the N- and C-terminal domains of NTH1 and ligated upstream of the fragment encoding the C-terminal half of NTH1 to produce CNTH1. The N-terminal domains were excised with XbaI (derived from the multiple cloning site of the cDNAclone) and Apal (derived from the primers), whereas the internal domains were excised with Apal (derived from the primers) and HindIII (derived from the cloning site of the pCRII vector). These fragments were cloned into pBlue- 
script II SK+ (Stratagene, La Jolla, CA) by three-fragment ligation. The fusions of N-terminal and internal domains were excised with XbaI and Clal (derived from the primers), and the C-terminal domains were excised with Clal (derived from the primers) and SacI (at the invariant amino acid residues Glu-Leu; see above). These fragments also were cloned into pBlue- 
script II SK+ by three-fragment ligation. The resulting chimeric N-terminal portions were excised with XbaI and SacI and then inserted into pBI121 with the C-terminal half of NTH15 or NTH1.

To examine the expression levels of the homeodomain proteins, we introduced the c-Myc epitope tag at the end of five reconstructed or chimeric proteins, NTH1, NTH15, NTH23, chimeric N terminus CNNT-2 (Figure 5), and CNTH1 (Figure 5). The stop codonts of these genes were replaced with SmaI sites by PCR as described above. A short DNA fragment, including the sequence 5′′-CCCtGGGAA- 
CCtAACTCtACtCTCAAGAGGtCTGtTGAGAGtCTC-3′ (SmaI and SacI sites are underlined; the stop codon is in boldface), which encodes the amino acids of the c-Myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn), was introduced between the SmaI site at the C-terminal end of the cDNAs and the SacI site at the 5′ end of the NOS sequence of pBI121.

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 previously reported (Matsuoka and Sanada, 1991). Trans- 

genic plants were selected on media containing 100 mg L-1 kanamy- 
cin. Kanamycin-resistant plants were transplanted to soil and grown in 


c. 116- and B-8 hr-dark cycle.

Protein Extractions and Protein Gel Blot Analysis

Total leaf proteins were extracted from leaf tissue of 30 independent transgenic plants by grinding with an equal volume of 2 × sample buffer (1 × sample buffer is 80 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 0.01% [w/v] bromphenol blue, and 0.02% [w/v] β-mercaptoethanol) and quantitated by using Bradford protein assay reagents (Bio-Rad). After boiling for 3 min, protein samples (20 μg) were subjected to SDS-PAGE and then transferred to Immobilon-P membranes (Millipore Co., Bedford, MA) by semidy blotting. Blots were blocked for 1 hr in TBS (20 mM Tris-HCl pH 7.6, and 137 mM NaCl) with 5% (w/v) non-fat dry milk. The anti-c-Myc antibody (Invit- 
rogen) 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% [w/v] Tween 20). Goat anti-mouse IgG horse- 
radish peroxidase-conjugated secondary antibody (Jackson Immuno- 
Research Laboratories, West Grove, PA) was diluted to 1:10,000 and incubated for 1 hr at room temperature, followed by four washes for 15 min with TBS-T. ECL-Plus chemiluminescent reagents (Amer- 
sham Pharmacia Biotech, Buckinghamshire, UK) were used for de- 
tection.

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The Conserved KNOX Domain Mediates Specificity of Tobacco KNOTTED1-Type Homeodomain Proteins
Tomoaki Sakamoto, Asuka Nishimura, Masanori Tamaoki, Masako Kuba, Hiroshi Tanaka, Shuichi Iwahori and Makoto Matsuoka

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