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Function of KNOX Homeodomain Proteins in Plant Development

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Abstract

KNOX homeodomain proteins are encoded by *knotted1*-like homeobox (*knox*) genes that constitute a gene family in plants. Similar to the animal homeodomain proteins, KNOX proteins are considered to be key transcriptional regulators that control the expression of genes involved in plant organogenesis at the shoot apical meristem. Therefore, in order to understand the developmental processes in plants, it is important to elucidate the molecular mechanisms underlying KNOX protein regulation of gene expression. In this review, we discuss the structural features of KNOX proteins and the mechanisms by which they interact with and regulate target gene expression.

Introduction

Through analyses of homeotic mutations, homeobox genes were first characterized as the transcriptional regulatory genes that control morphogenesis in fruit fly, *Drosophila* (Gehring, 1987). Products of these genes share a unique structure known as a homeodomain. The homeodomain consists of a highly conserved 60 amino acid stretch containing three α-helices that form 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 homeodomain proteins are believed to regulate the expression of batteries of target genes by acting as transcriptional factors (Affolter *et al.*, 1990; Hayashi and Scott, 1990; Kissinger *et al.*, 1990; Laughon, 1991). Homeobox genes have been found in various organisms including insects and vertebrates, and may occur in virtually all eucaryotic organisms, from hydra to man (McGinnis *et al.*, 1984; Bürglin *et al.*, 1989; Schummer *et al.*, 1992). It is likely that these genes play a crucial role in controlling the genetic switches used by cells to choose a particular pathway among alternative developmental pathways (Krumlaut, 1994).

Since the first plant homeobox gene, *knotted1*, was identified from the maize *Knotted1* mutant (Vollbrecht *et al.*, 1991), many other homeobox genes have subsequently been isolated from various plant species. In the plant kingdom, homeodomain proteins have been divided into several groups: homeodomain zipper proteins (HD-Zip; Ruberti *et al.*, 1991; Mattsson *et al.*, 1992; Schena and Davis, 1992); homeodomain finger proteins (PHD-finger; Bellmann and Werr, 1992; Schindler *et al.*, 1993; Korfhage *et al.*, 1994); *Arabidopsis* GLABRA2 protein (Rerie *et al.*, 1994); BELL1-like proteins (Quaedvlieg *et al.*, 1995; Reiser *et al.*, 1995); and KNOX proteins (Vollbrecht *et al.*, 1991; Matsuoka *et al.*, 1993).

KNOX proteins are encoded by *knotted1*-like homeobox (*knox*) genes that are preferentially accumulated in the indeterminate cells around the shoot apical meristem (SAM), but not in the determinate lateral organs such as leaves (Jackson *et al.*, 1994; Lincoln *et al.*, 1994; Nishimura *et al.*, 1999; Sentoku *et al.*, 1999). The loss-of-function mutants, *shootmeristemless* (*stm*) in *Arabidopsis* and *knotted1* (*kn1*) in maize, show defects in SAM development or maintenance (Long *et al.*, 1996; Kerstetter *et al.*, 1997). The opposite phenotype, namely, ectopic meristem formation in leaves, has been reported in *knox* transformants that ectopically express *knox* genes (Matsuoka *et al.*, 1993; Sinha *et al.*, 1993; Chuck *et al.*, 1996; Nishimura *et al.*, 2000; Sentoku *et al.*, 2000). On the basis of this evidence, KNOX proteins are considered to play a critical role in the maintenance of the indeterminate properties of cells in the SAM (Reiser *et al.*, 2000), although their direct function is still unresolved.

To understand the function of KNOX proteins in plant development, it is necessary to identify the

genes targeted by KNOX proteins and to characterize the mechanism of the specificity of transcriptional regulation of these genes. In this review, we evaluate the structural features and putative functions of domains in KNOX proteins. We also describe the mechanism of target gene recognition.

Structure of KNOX proteins

All KNOX proteins have a highly conserved atypical homeodomain located near the C-terminus (**Fig. 1**). In addition, three extra amino acids situated between the first and second helices (PYP) are totally conserved in all KNOX proteins. These invariant extra residues also occur in the loop between the first and second helices of several homeodomain proteins from other organisms, although they are not found in the typical homeodomain proteins such as Antennapedia (Bertolino *et al.*, 1995). Based on this unique feature, these proteins have been designated TALE (three amino acid loop extension) homeodomain proteins, and all KNOX proteins are members of this superclass (Bürglin, 1997).

The sequence immediately upstream of the homeodomain, termed the ELK domain (Vollbrecht *et al.*, 1991; Kerstetter *et al.*, 1994), is also conserved (**Fig. 1**). The ELK domain constitutes a novel form of amphipathic helix (Kerstetter *et al.*, 1994), and could function as a nuclear localization signal (Meisel and Lam, 1996). The ELK domain is also considered to act as a protein-protein interaction domain (Vollbrecht *et al.*, 1991).

In addition to the conserved ELK and TALE homeodomains, a stretch of approximately 100 amino acids located at the N-terminus of almost all KNOX proteins is also conserved (**Fig. 1**). This conserved region, known as the KNOX domain, may function in protein-protein interactions (Bürglin, 1997). A relatively smaller and less conserved amino acid motif is located between the KNOX and ELK domains. The function of this conserved region, termed the GSE domain, has not yet been determined.

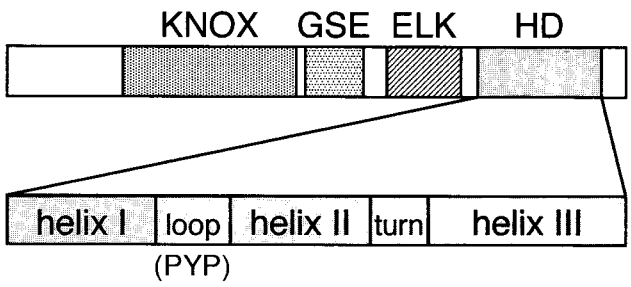

Fig. 1 Schematic representation of KNOX protein structure. Almost all plant KNOX proteins contain a KNOX domain, GSE domain, ELK domain, and TALE homeodomain (HD). The TALE homeodomain consists of three α-helices which comprise a helix-turn-helix type DNA binding motif, and also contains three extra residues (PYP) in the loop between the first and second helices in comparison to the typical homeodomains.

Ectopic expression of KNOX proteins in transgenic plants

KNOX proteins have been subdivided into two groups, class I and class II (Kerstetter *et al.*, 1994). Class I proteins are more similar to the maize KN1 protein, while class II proteins are less similar to KN1. In general, class I genes are strongly expressed around the SAM, moderately-to-weakly expressed in the embryo and other restricted tissues, and are hardly expressed at all in differentiated organs such as leaves and roots (Kerstetter *et al.*, 1994). Ectopic expression of class I genes causes altered leaf and flower morphology in spontaneous mutants of some plant species (Smith *et al.*, 1992; Chen *et al.*, 1997; Parnis *et al.*, 1997) and in transgenic plants, as mentioned above. Class II genes are expressed in most tissues at different levels (Kerstetter *et al.*, 1994). In contrast to class I genes, ectopic expression of class II genes does not cause an altered phenotype in transgenic plants (Tamaoki *et al.*, 1995; Serikawa *et al.*, 1997).

To elucidate the function of *knox* genes in plant development, we isolated six *knox* genes from tobacco and examined the effect of ectopic expression of these genes in transgenic tobacco plants. These six genes show different patterns of expression around the SAM (Tamaoki *et al.*, 1997; Nishimura *et al.*, 1999). Interestingly, two class I genes, *NTH15* and *NTH20*, cause a dramatically altered phenotype in transformants (**Fig. 2 A** and **B**), very similar to the phenotype induced by the expression of *kn1* or *OSH1* from rice (Kano-Murakami *et al.*, 1993; Sinha *et al.*, 1993; Tamaoki *et al.*, 1997; Nishimura *et al.*, 2000). Ectopic expression of two other class I genes (*NTH1* and *NTH9*) causes only minor alterations in morphology (Nishimura *et al.*, 2000) (**Fig. 2C**), whereas over-expression of the class II gene, *NTH23*, does not cause an abnormal phenotype in transgenic plants (Sentoku *et al.*, 1998) (**Fig. 2D**).

Domain exchange analysis of KNOX proteins

The difference in the severity of phenotypes caused by the expression of each of the *knox* genes may depend on their structures and therefore may be

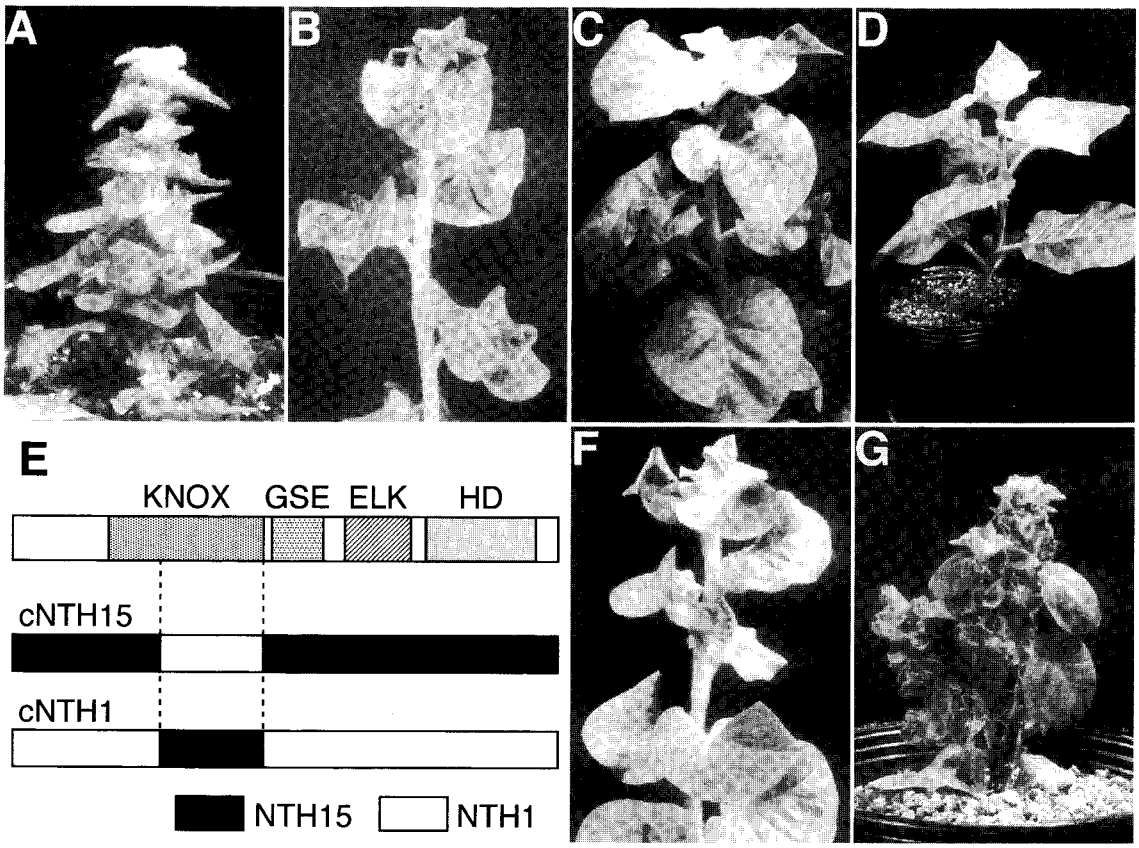

Fig. 2 Phenotypes of transgenic tobacco plants over-expressing KNOX proteins. **A**: Transgenic tobacco plant with a severe phenotype (*35S::NTH15* transformant). **B**: Plant with an intermediate phenotype (*35S::NTH15* transformant). **C**: Plant with a mild phenotype (*35S::NTH1* transformant). **D**: Plant with a phenotype similar to that of the wild-type (*35S::NTH23* transformant). **E**: Schematic representation of chimeric proteins cNTH15 and cNTH1. cNTH15 consists of the entire NTH15 protein, with replacement of the C-terminal half of the KNOX domain by the corresponding NTH1 region. cNTH1 contains the entire NTH1 protein, with replacement of the C-terminal half of the KNOX domain with the corresponding NTH15 region. **F**: Typical cNTH15 transformant with a mild phenotype. **G**: Typical cNTH1 transformant with a severe phenotype.

a reflection of their functions in plant development. To determine which parts of the KNOX proteins are important for an altered morphology in transgenic plants, we generated chimeric proteins by exchanging various amino acid motifs between NTH1, NTH15, and NTH23, and analyzed their effects on the phenotypic severity of transformants (Sakamoto *et al.*, 1999). Experiments involving the exchange of the N- and C-terminal halves of the three proteins show that the severity of abnormal phenotypes depends more strongly on the N-terminal half of KNOX protein than on the C-terminal half. As mentioned above, ectopic expression of NTH15 and NTH1 causes severe and mild phenotypes, respectively (**Fig. 2 A** and **C**). Exchange of the N-terminus of NTH1 with that of NTH15 increases the phenotypic severity from mild to severe (**Fig. 2 A** and **C**), whereas exchange of the C-terminus of NTH1 with that of NTH15 increases the severity from mild to intermediate (**Fig. 2 B** and **C**).

Serikawa and Zambryski (1997) performed similar experiments using two *Arabidopsis* KNOX proteins, KNAT1 and KNAT3. They reported that the chimeric protein containing the N-terminus of KNAT1 (class I) and the C-terminus of KNAT3 (class II) does not induce an abnormal phenotype. However, the chimeric protein containing the N-terminus of KNAT3 (class II) and the C-terminus of KNAT1 (class I) causes an abnormal phenotype which is similar but qualitatively different to the KNAT1 over-expression phenotype. Based on these results, the authors concluded that the specificity of the over-expression phenotype resides mainly in the C-terminal region of KNAT1.

In agreement with this report, we found that all transformants carrying the chimeric protein containing the C-terminus of NTH23 (class II) and the N-terminus of NTH1 (class I) show a normal phenotype. However, it is noteworthy that the chimeric protein containing the C-terminus of

NTH23 and the N-terminus of NTH15 (class I) causes some abnormalities (Sakamoto *et al.*, 1999). This is not consistent with the previous finding by Serikawa and Zambryski (1997) and demonstrates that the chimeric protein containing a class II C-terminus has the potential to induce an abnormal phenotype when it also contains an appropriate N-terminus from a class I protein such as NTH15.

The C-termini of NTH15, NTH1, and NTH23 also exert strong, medium, and weak influences on the abnormal phenotype, respectively (Sakamoto *et al.*, 1999). Taken together, these results indicate that phenotypic severity is determined by a synergistic function of the combination of N- and C-termini, and that the influence of the N-terminus is stronger than that of the C-terminus.

The KNOX domain is important in determining the severity of abnormal phenotype

The transactivating domain of the yeast transcription factor GAL4 cannot substitute for the N-terminus (Sakamoto *et al.*, 1999). If the ELK-homeodomain itself is sufficient for interacting with its target DNA sequence(s) as suggested by Meisel and Lam (1996), chimeric proteins containing the GAL4 transactivating domain and the ELK-homeodomain should transactivate target gene(s) and consequently induce an abnormal phenotype. Our results suggest that the N-terminal half of KNOX protein does not function solely as a transactivation domain but may also contribute to the recognition of target gene(s).

To examine the possibility that other functional domains exist in the N-terminus, we reciprocally exchanged the KNOX domain of NTH15 and NTH1, which was divided into three subdomains. Replacement of the C-terminal half of the KNOX domain of NTH15 with that of NTH1 causes a loss in the induction of a severe phenotype in transformants (**Fig. 2 E** and **F**). However, replacement of this domain in NTH1 with that of NTH15 restores the ability of the transgene products to induce a severe phenotype (**Fig. 2 E** and **G**), clearly demonstrating that the C-terminal portion of the KNOX domain plays an essential role in determining the severity of abnormal phenotype in the transgenic plants (Sakamoto *et al.*, 1999).

The KNOX domain consists of α-helices and is split into two subdomains (Bürglin, 1997). The C-terminal subdomain contains an α-helical structure that is conserved in all tobacco KNOX proteins, however, only that of NTH15 can produce a typical amphipathic structure (Sakamoto *et al.*, 1999). The correlation between the formation of an amphipathic structure and induction of the severe phenotype suggests that the amphipathic structure of the C-terminal half of the KNOX subdomain may be important for induction of the severe phenotype.

The importance of this α-helical structure in the KNOX domain has also been reported in the animal MEIS protein Prep1 (Berthelsen *et al.*, 1998a), which belongs to the TALE superclass. KNOX and MEIS proteins share not only the TALE homeodomain but also a conserved N-terminal domain; the KNOX domain for KNOX family and the MEIS domain for MEIS family (Bürglin, 1997). Prep1 interacts with another TALE protein, Pbx, through the MEIS domain (Berthelsen *et al.*, 1998b). The structural conservation between the KNOX and MEIS domains strongly suggests that these domains have a similar biological role(s), and that the KNOX domains may also be important for interaction with other protein(s), probably transcriptional factor(s). Based on this hypothesis, we consider that the differences in the severity of the abnormal phenotype observed in *knox* transformants may be mediated by the interaction of different accessory proteins with the different KNOX domains, and that these accessory proteins may be the ultimate determinants of phenotypic severity.

KNOX protein directly suppresses the expression of a gibberellin (GA) biosynthetic gene and GA biosynthesis

Recently, an inducible system was established for plant cells using the glucocorticoid receptor (GR) (Schena *et al.*, 1991; Lloyd *et al.*, 1994; Aoyama *et al.*, 1995). The steroid binding domain of the GR contains a regulable inactivation function and this domain can confer hormonal inducibility on a neighboring domain in a chimeric protein molecule even in plants (Schena *et al.*, 1991). In order to artificially regulate NTH15 protein function *in vivo*, we used this system to express a NTH15:GR fusion protein in transgenic tobacco plants (*NTH15:GR*). In these transformants, NTH15 function is successfully induced by exogenous treatment with the steroid ligand dexamethasone (DEX) in a ligand-dependent manner.

In this induction system, the level of GA_{20} drastically decreases within 6 hr of DEX treatment. GA_1 levels decrease gradually from 6 to 24 hr after the treatment, whereas the level of GA_{53} is not significantly changed. Since GA_1 is synthesized from GA_{53} via GA_{20}, and GA 20-oxidase catalyzes the

conversion from GA_{53} to GA_{20} (Hedden and Kamiya, 1997), these results suggest that activation of NTH15 immediately interferes with the steps catalyzed by GA 20-oxidase, causing a gradual decrease in bioactive GA_1 (Sakamoto *et al.*, 2001).

Moreover, the mRNA level of GA 20-oxidase gene, *Ntc12*, is drastically reduced from 1 to 3 hr after DEX treatment. The kinetics of the decrease in *Ntc12* mRNA levels is the same even under treatment with α-amanitin and cycloheximide. These observations indicate that NTH15 directly suppresses *Ntc12* expression without prior synthesis of any other gene products (Sakamoto *et al.*, 2001).

Target sequence of KNOX protein

To further investigate the possibility of a direct interaction between NTH15 and the *Ntc12* sequence, we conducted an electrophoresis mobility shift assay (EMSA). Recombinant NTH15 protein expressed in *E. coli* cells binds to two different sites in the *Ntc12* gene; one is located in the 5' flanking region and the other is in the first intron (**Fig. 3**). The precise NTH15 binding sequences, identified by a DNase I protection assay, are the sequence TATGTTAC in the 5' flanking region and TATGTGAC in the first intron of *Ntc12* (Sakamoto *et al.*, 2001).

Typical homeodomain proteins, such as Antennapedia and Ultrabithorax of *Drosophila*, recognize the core sequence TAAT. In contrast, TALE-atypical homeodomain proteins interact with a non-TAAT sequence. For example, human TGIF protein binds to TGTCA (Bertolino *et al.*, 1995), and the barley Hooded protein binds to GTCA (Krusell *et al.*, 1997). The NTH15 binding sequence TATGT(G/T)AC is similar to these core sequences but not identical. This finding made us question whether the binding sequences of *Ntc12* are specifically recognized by NTH15. To address this question, we analyzed the DNA sequence-specific binding of NTH15 by EMSA using a series of *in vitro* mutagenized probes (Sakamoto *et al.*, 2001) (**Fig. 4**). NTH15 recognizes the 5-bp dyadsymmetric sequence GTNAC in TATGT(G/T)AC, and preferentially binds to GTGAC rather than GTCAC, which contains the core sequence of TGIF and Hooded. Conversely, the binding affinity of TGIF is significantly reduced by a single nucleotide substitution from C to G within its core sequence TGTCA

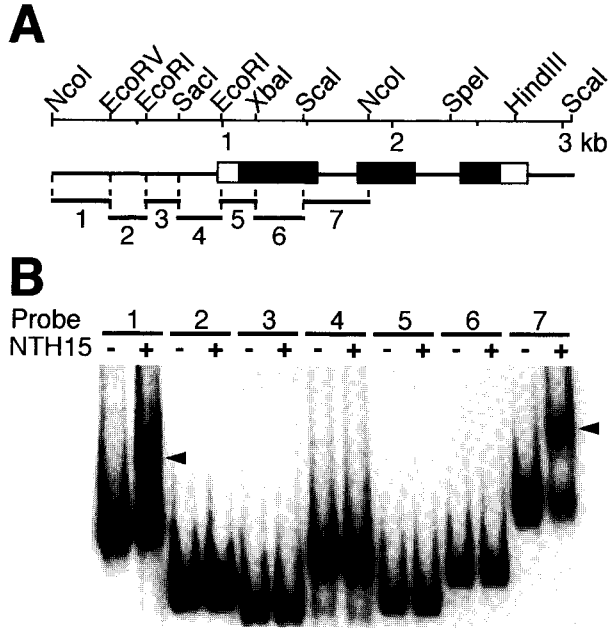

Fig. 3 Binding of NTH15 to the 5' flanking and first intron sequences in the *Ntc12* gene. **A**: Genomic structure of *Ntc12*. Lines, open and closed boxes indicate introns, non-coding and coding sequences, respectively. Shown at the top is a restriction map of *Ntc12*. Fragments used as probes for the EMSA are numbered from 1 to 7. **B**: EMSA of the seven probes with the recombinant NTH15 protein. DNA fragments were incubated without (-) or with (+) NTH15. Fragments 1 and 7 were shifted by incubation with NTH15 as shown by arrowheads.

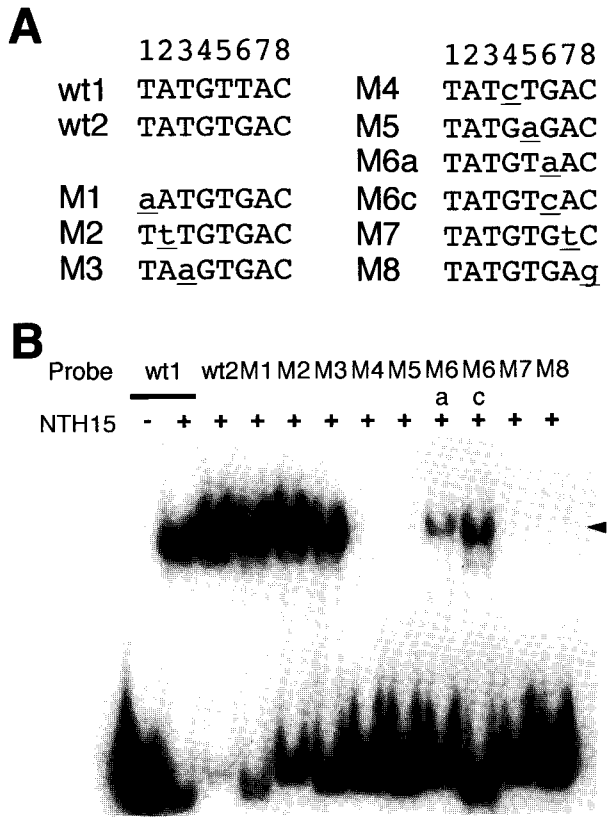

Fig. 4 Preference of the NTH15 binding sequence. **A**: Core sequences of the NTH15 binding site (wt1 and wt2) and its derivative sequences used for the experiments (M1 to M8). Numbers at the top indicate the nucleotide positions. **B**: EMSA of each DNA fragment. Synthetic oligonucleotides containing the wild-type or mutagenized binding site were used as probes in the EMSA.

(Bertolino *et al.*, 1995). These results indicate that the binding property of NTH15 is slightly different from that of other TALE proteins. Such minor differences in the binding properties of TALE proteins may enable them to target different genes and consequently cause a unique function of each protein.

Regulation of the GA 20-oxidase gene by KNOX protein in the tobacco SAM

NTH15 binds to two different sites on *Ntc12 in vitro*; these two sites may act as *cis*-motives for transcriptional suppression of *Ntc12 in vivo*. The binding sequence in the first intron of *Ntc12* is a functional target of NTH15 for negative regulation of *Ntc12* expression *in vivo* (Sakamoto *et al.*, 2001). In addition, *NTH15* and *Ntc12* show an exclusive pattern of *in situ* mRNA localization in the SAM of wild-type tobacco (Tanaka-Ueguchi *et al.*, 1998). The inverse relationship between *NTH15* and *Ntc12* expression strongly suggests that the suppression of *Ntc12* caused by NTH15 also occurs in the SAM. This possibility is also supported by the observation that mutation in the NTH15 binding sequence results in an expanded expression of *Ntc12* in the area where NTH15 is accumulated (Sakamoto *et al.*, 2001).

Conclusion

Although KNOX proteins have been studied extensively over the past 10 years, our understanding of the functions of these proteins is still relatively limited and many interesting questions remain unresolved. For instance, it is not yet known whether plant KNOX proteins act as monomers, homodimers, heterodimers, or as complexes with other cofactors. Recently, a direct interaction between rice KNOX proteins has been demonstrated using the yeast two-hybrid system, and the KNOX domains are essential for this interaction (H. Nagasaki *et al.*, unpublished data). This finding supports the notion that the KNOX domain is important for protein-protein interactions. Identification and characterization of the proteins that interact with KNOX proteins via the KNOX domain will be an essential step in elucidating the biological functions of KNOX proteins.

Recently, a recessive mutation allele of the rice *knox* gene, *OSH15*, was isolated (Sato *et al.*, 1999). Because this *d6* mutation causes the loss-of-function of the OSH15 protein, genes that are differentially expressed between the wild-type and mutant plants are potential candidates for downstream genes of OSH15. Future work in this area will focus on the molecular characterization of the OSH15 protein and its loss-of-function mutant, *d6*, in order to gain an understanding of the role of KNOX proteins in plant development.

Another important issue that needs to be resolved is how *knox* gene expression is regulated in plants. Recent studies have revealed that a myb-like protein in maize, RS2, negatively regulates the expression of a class 1 *knox* gene, *RS1*, and that a loss-of-function mutant of RS2 shows a similar phenotype to that of the *RS1* mutant, which ectopically expresses a *knox* gene in leaves (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999). At present, however, it is unclear whether the RS2 protein can directly interact with the *cis*-acting sequence of the *RS1* gene.

Despite the current uncertainty, we anticipate that further detailed investigations into the regulatory network of KNOX proteins will clarify the molecular mechanisms by which these proteins govern the developmental processes of plants, as has been elucidated in the animal kingdom.

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