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Ectopic Expression of the Arabidopsis Transcriptional Activator Athb-1 Alters Leaf Cell Fate in Tobacco

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The *Arabidopsis thaliana Athb-1* is a homeobox gene of unknown function. By analogy with homeobox genes of other organisms, its gene product, Athb-1, is most likely a transcription factor involved in developmental processes. We constructed a series of *Athb-1*-derived genes to examine the roles of Athb-1 in transcriptional regulation and plant development. Athb-1 was found to transactivate a promoter linked to a specific DNA binding site by transient expression assays. In transgenic tobacco plants, overexpression of Athb-1 or its chimeric derivatives with heterologous transactivating domains of the yeast transcription factor GAL4 or herpes simplex virus transcription factor VP16 conferred deetiolated phenotypes in the dark, including cotyledon expansion, true leaf development, and an inhibition of hypocotyl elongation. Expression of *Athb-1* or the two chimeric derivatives also affected the development of palisade parenchyma under normal growth conditions, resulting in light green sectors in leaves and cotyledons, whereas other organs in the transgenic plants remained normal. Both developmental phenotypes were induced by glucocorticoid in transgenic plants expressing a chimeric transcription factor comprising the Athb-1 DNA binding domain, the VP16 transactivating domain, and the glucocorticoid receptor domain. Plants with severe inducible phenotypes showed additional abnormality in cotyledon expansion. Our results suggest that Athb-1 is a transcription activator involved in leaf development.

INTRODUCTION

Transcription factors have frequently been studied for their involvement in cell proliferation, cell differentiation, organ development, and ontogenesis because transcription is thought to be a major point by which biological processes are regulated. Among the various transcription factors, those with similar structures are often involved in similar biological processes. The proteins encoded by homeobox genes are such a class of transcription factors (for reviews, see Gehring, 1987; Kenyon, 1994). The homeobox was originally identified as a region of sequence similarity shared by several genes that when mutated caused homeotic transformations in Drosophila development (Gehring, 1987). The homeobox sequence encodes a 61–amino acid sequence referred to as the homeodomain, which is responsible for sequence-specific DNA binding (for reviews, see Hayashi and Scott, 1990; Gehring et al., 1994). Homeodomain proteins have been identified in many animal species and found to play key roles in developmental processes, including cell specification and pattern formation.

Plant homeobox genes have been cloned from various species, including maize (Vollbrecht et al., 1991; Bellmann and Werr, 1992; Kerstetter et al., 1994), Arabidopsis (Ruberti et al., 1991; Lincoln et al., 1994; Rerie et al., 1994; Quaedvlieg et al., 1995), rice (Matsuoka et al., 1993), and parsley (Korfhage et al., 1994). Their existence in animals as well as in plants indicates that the homeodomain is a common DNA binding structure in eukaryotes. By analogy with homeobox genes of animals, those of plants are expected to encode transcription factors involved in developmental regulation, and some evidence has accumulated to support this view, for example, the development of a trichome (Rerie et al., 1994), the shoot (Smith et al., 1992), and the whole plant (Schena et al., 1993). The functions of the majority of the plant homeobox genes, however, remain largely unknown. With the exception of *knotted1* (*kn1*; Smith et al., 1992) and *glabra2 (gl2*; Rerie et al., 1994), none has been linked to mutant phenotypes.

Arabidopsis thaliana Athb-1 is a homeobox gene whose function is unknown. This gene was cloned from an Arabidopsis cDNA library by sequence homology in the homeobox region (Ruberti et al., 1991). Unlike the animal homeodomain, that of Athb-1 is flanked by a leucine zipper motif at its C terminus, and this structural unit (HD-Zip) has been demonstrated to bind

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DNA as a homodimer in a sequence-specific manner in vitro (Sessa et al., 1993). A number of genes encoding this unit have been identified in Arabidopsis, and they are thought to constitute a large gene family (Ruberti et al., 1991; Mattsson et al., 1992; Schena and Davis, 1992, 1994; Carabelli et al., 1993; Sessa et al., 1994; Söderman et al., 1994; Baima et al., 1995).

In this study, a series of *Athb-1*–derived genes, including chimeric genes containing sequences encoding activation domains of other transcription factors, were constructed and assayed by transient expression and in a transgenic tobacco system. The binding site–specific transactivating function of Athb-1 was demonstrated in transient expression experiments. In transgenic experiments, the overexpression of the DNA binding function of Athb-1 combined with a transactivating function resulted in deetiolated phenotypes in the dark and led to a defect in the development of palisade parenchyma in the light. Moreover, we constructed a system in which these phenotypes could be induced by glucocorticoid by taking advantage of the regulatory mechanism of the glucocorticoid receptor (Picard et al., 1988). Our results suggest that Athb-1 can affect leaf developmental processes.

RESULTS

Transient Assay for the Transactivating Function of Athb-1

The HD-Zip domain of Athb-1 has been demonstrated to bind DNA as a homodimer in a sequence-specific manner in vitro (Sessa et al., 1993). However, it is not known whether Athb-1 can activate transcription. To examine this possibility, the entire *Athb-1* coding sequence transcribed from the −343 cauliflower mosaic virus 35S promoter (*total-Athb1*; Figure 1) was used as the transacting gene in particle bombardment experiments with tobacco leaves. Plasmids carrying the luciferase (*LUC*) reporter gene (de Wet et al., 1987) with the Athb-1 binding sequence (Sessa et al., 1993) or its mutant sequence placed 5′ of the −46 cauliflower mosaic virus 35S promoter were used as reporter plasmids. As negative controls, the *Athb-1* coding sequence was replaced with either the same DNA sequence cloned in reverse orientation relative to the promoter (*anti-Athb1*; Figure 1) or the coding sequence of the *Athb-1* homeodomain–leucine zipper domain (*HDZip1*; Figure 1). Plasmids carrying the transacting genes also contained a β-glucuronidase (*GUS*) gene (Jefferson et al., 1987) transcribed from the −343 cauliflower mosaic virus 35S promoter, and its activity was used as an internal control.

Each transacting plasmid was cobombarded with a reporter plasmid into tobacco leaves. Twenty-four hours after bombardment, the leaves were assayed for LUC and GUS activities. LUC activity was normalized with respect to GUS activity, and the normalized value was considered to represent the strength of the transacting function of each chimeric transcription factor. The results are shown in Figure 2. *Total-Athb1* displayed strong transcriptional activation from the promoter with the Athb-1 binding sequence, whereas no clear transactivation was detected with the mutant binding sequence. Both *anti-Athb1* and *HDZip1* were inactive with either the wild-type or mutant binding sites. Taken together, these results indicate that Athb-1 can transactivate plant promoters in vivo in a binding site–specific manner.

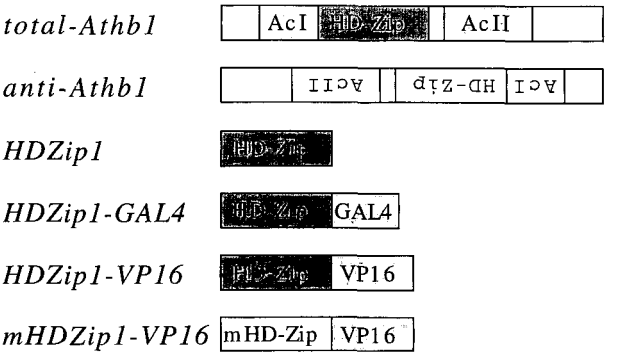

Figure 1. Structures of Transgenes Used To Investigate Athb-1 Functions.

The constructs designated *total-Athb1* and *anti-Athb1* contain the *Athb-1* cDNA fragment in normal and reverse orientations relative to the promoter, respectively. AcI (amino acids 40 to 63) and AcII (amino acids 211 to 243) are regions in Athb-1 rich in acidic amino acids, and HD-Zip (amino acids 61 to 170) contains the DNA binding domain (Ruberti et al., 1991; Sessa et al., 1993). The upside labeling of these regions in *anti-Athb1* indicates the antisense orientation of the coding sequence. The construct *HDZip1* contains the DNA sequences encoding the HD-Zip domain only. *HDZip1–GAL4* and *HDZip1–VP16* encode chimeric transcription factors in which the HD-Zip domain is fused in frame to the transactivating domain of the yeast transcription factor GAL4 and that of the herpes simplex virus transcription factor VP16, respectively. Construct *mHDZip1–VP16* encodes the same protein that is encoded by construct *HDZip1–VP16*, except for a set of mutations in the homeodomain region that abolished the DNA binding function. All of the constructs were transcribed from the cauliflower mosaic virus 35S promoter and flanked at the 3′ end by the poly(A) addition sequence of pea *rbcS-E9*.

Phenotypes of Transgenic Plants under Normal Growth Conditions

To examine the role of Athb-1 in plant development, *total-Athb1*, *anti-Athb1*, and *HDZip1* were introduced into tobacco using Agrobacterium-mediated transformation. More than 20 independent primary transgenic plants for each construct were regenerated in the presence of kanamycin. Detailed analysis of the transgenic plants was performed with T_2 progeny. T_2 plants derived from 10 independent primary transgenic tobacco lines for each transgene were analyzed.

Whereas all lines of transgenic plants carrying *anti-Athb1* or *HDZip1* appeared normal, plants derived from two independent transgenic lines with *total-Athb1* had light green sectors in their cotyledons but not in true leaves (Figure 3B). The analysis of 30 to 40 seeds for each line showed that the light green sector phenotype cosegregated with kanamycin resistance. Eight other *total-Athb1* transgenic lines displayed normal leaves and cotyledons.

The low penetrance of the phenotype caused by overexpression of *total-Athb1* could be due to regulation of its protein product in vivo. For example, the expression level of Athb-1 could be low due to instability or to an insufficiently strong transactivating function. To explore these possibilities, we examined the effects of heterologous activation domains on transient expression. *HDZip1–GAL4* and *HDZip1–VP16* encode chimeric transcription factors in which the HD-Zip domain was fused in frame to the transactivating domain of the yeast transcription factor GAL4 (Ma and Ptashne, 1987) and that of the herpes simplex virus transcription factor VP16 (Triezenberg et al., 1988), respectively (Figure 1). As a negative control, we constructed

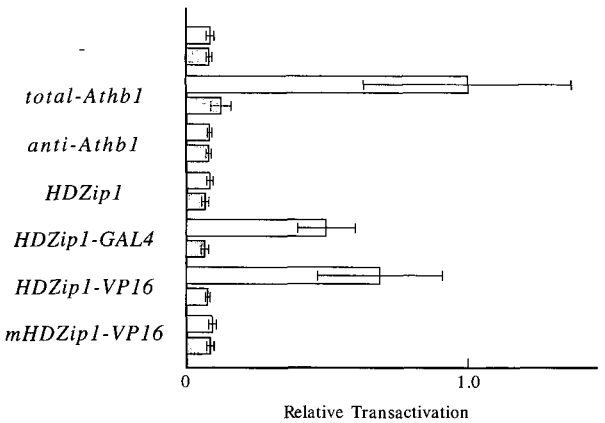

Figure 2. Transient Expression Assays for Transactivation.

A plasmid carrying a transacting gene and a reporter plasmid carrying a *LUC* gene containing at its promoter either six copies of the Athb-1 binding sequence (5′-CAATTATTG-3′) or its mutant sequence (5′-CAATTGTTG-3′) were cobombarded into tobacco leaves. The plasmids of the transacting genes also carried a *GUS* gene driven by the cauliflower mosaic virus 35S promoter, which was used as an internal control. Twenty-four hours after bombardment, the leaves were assayed for LUC and GUS activities. LUC activity was normalized with respect to GUS activity for each bombardment experiment. The average values of four independent experiments were calculated for each combination of transacting and reporter genes. The transactivation value obtained with the combination of construct *total-Athb1* and the wild-type Athb-1 binding sequence was arbitrarily set as 1. The transacting genes used in this experiment are shown at left. The experiments performed without a transacting gene are indicated with a (–). At right, the relative transactivation levels of the various constructs are shown. Open boxes indicate values obtained with the wild-type Athb-1 binding sequence; shaded boxes indicate those obtained with the mutant sequence. Bars indicate standard deviations.

mHDZip1–VP16, which has the same structure as *HDZip1–VP16* except for point mutations in the recognition helix of the homeodomain (Figure 1). The mutated HDZip1 domain has been shown to lack DNA binding activity in vitro (data not shown). Figure 2 shows that HDZip1–GAL4 and HDZip1–VP16 could also activate transcription in a sequence-specific manner, indicating that the HD-Zip domain itself is sufficient to recognize the binding site in vivo. The transactivation obtained with HDZip1–GAL4 was weaker than that of HDZip1–VP16, and both were less active than Athb-1. As expected, no transactivation was observed with the mutant mHDZip1–VP16.

The constructs *HDZip1–GAL4*, *HDZip1–VP16*, and *mHDZip1–VP16* were transferred into tobacco, and at least 10 independent transgenic lines were analyzed for each construct in the presence of kanamycin. Figure 3 shows the phenotypes of young T_2 plants 14 days after sowing on agar medium under normal growth conditions. Although the severity of the phenotype varied among different transgenic lines, all transgenic lines of T_2 plants carrying *HDZip1–GAL4* (Figures 3C and 3D) and those with *HDZip1–VP16* (Figures 3E, 3F, and 3H) displayed light green sectors not only in their cotyledons but also in true leaves. Although the ratio of light green areas to normal areas varied from plant to plant, *HDZip1–GAL4* appeared to cause a less severe phenotype than that obtained with *HDZip–VP16*. The analysis of 30 to 40 seeds for each line showed that the light green sector phenotype cosegregated with kanamycin resistance. Transgenic plants carrying *mHDZip1–VP16* were normal in appearance (data not shown), like wild-type plants (Figures 3A and 3G).

Light Microscopic Analysis of Transgenic Plants

To investigate the internal structure of the light green sectors, cross-sections were prepared from cotyledons and leaves of T_2 transgenic plants. Figure 4 shows that the palisade parenchyma normally found in a wild-type cotyledon (Figure 4A) and leaf (Figure 4B) was not present in the light green part of variegated cotyledons (Figures 4E and 4G) and leaves (Figures 4F and 4H) of *HDZip1–GAL4* and *HDZip1–VP16* plants. Instead, this part was occupied by cells similar in shape to the spongy mesophyll cells. On the other hand, the palisade parenchyma was normal in the dark green parts of variegated cotyledons and leaves. The number of cell layers was the same between the dark green and the light green parts of leaves and cotyledons. Therefore, we concluded that the replacement of palisade parenchyma cells by spongy mesophyll cells caused the light green coloration possibly because a palisade parenchyma cell contains more chloroplasts than a spongy mesophyll cell. The same structural alteration was seen in light green sectors of cotyledons of the *total-Athb1* plants (Figure 4C). No structural abnormality was observed in leaves of the *total-Athb1* plants (Figure 4D) or in cotyledons and leaves of transgenic plants containing other transgenes, namely, *anti-Athb1*, *HDZip1*, and *mHDZip1–VP16* (data not shown).

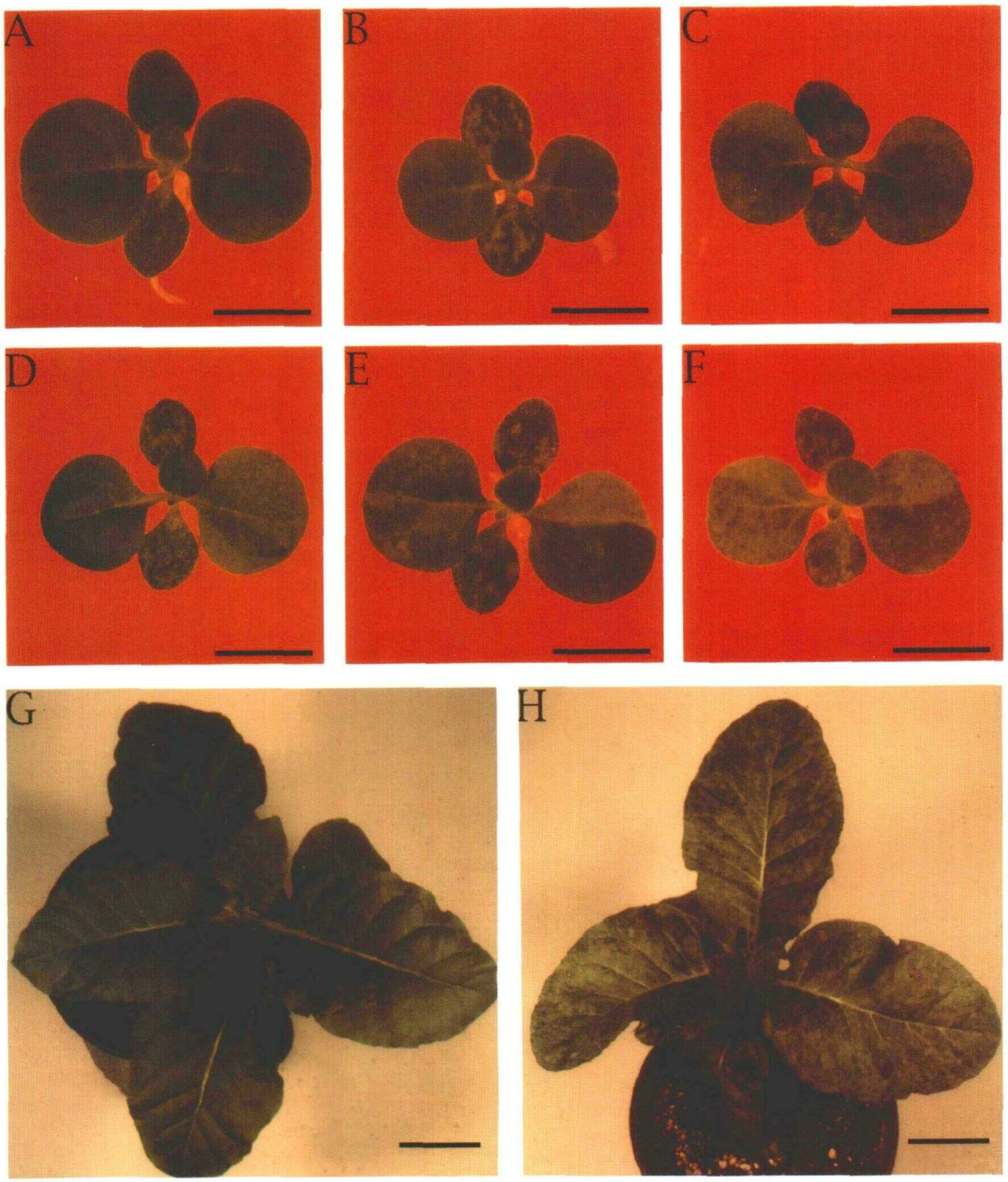

Figure 3. Phenotypes of Young and Mature Transgenic Tobacco Plants under Normal Growth Conditions.

T_2 seeds were sowed on agar medium and grown for 14 days under normal growth conditions. A mature wild-type plant and a T_2 transgenic plant with *HDZip1–VP16* are shown in **(G)** and **(H)**, respectively.

(A) Wild-type SR1.

(B) *Total-Athb1*.

(C) and **(D)** *HDZip1–GAL4*.

(E) and **(F)** *HDZip1–VP16*.

(G) Wild-type SR1.

(H) *HDZip1–VP16*.

Bars = 5 mm in **(A)** to **(F)** and 50 mm in **(G)** and **(H)**.

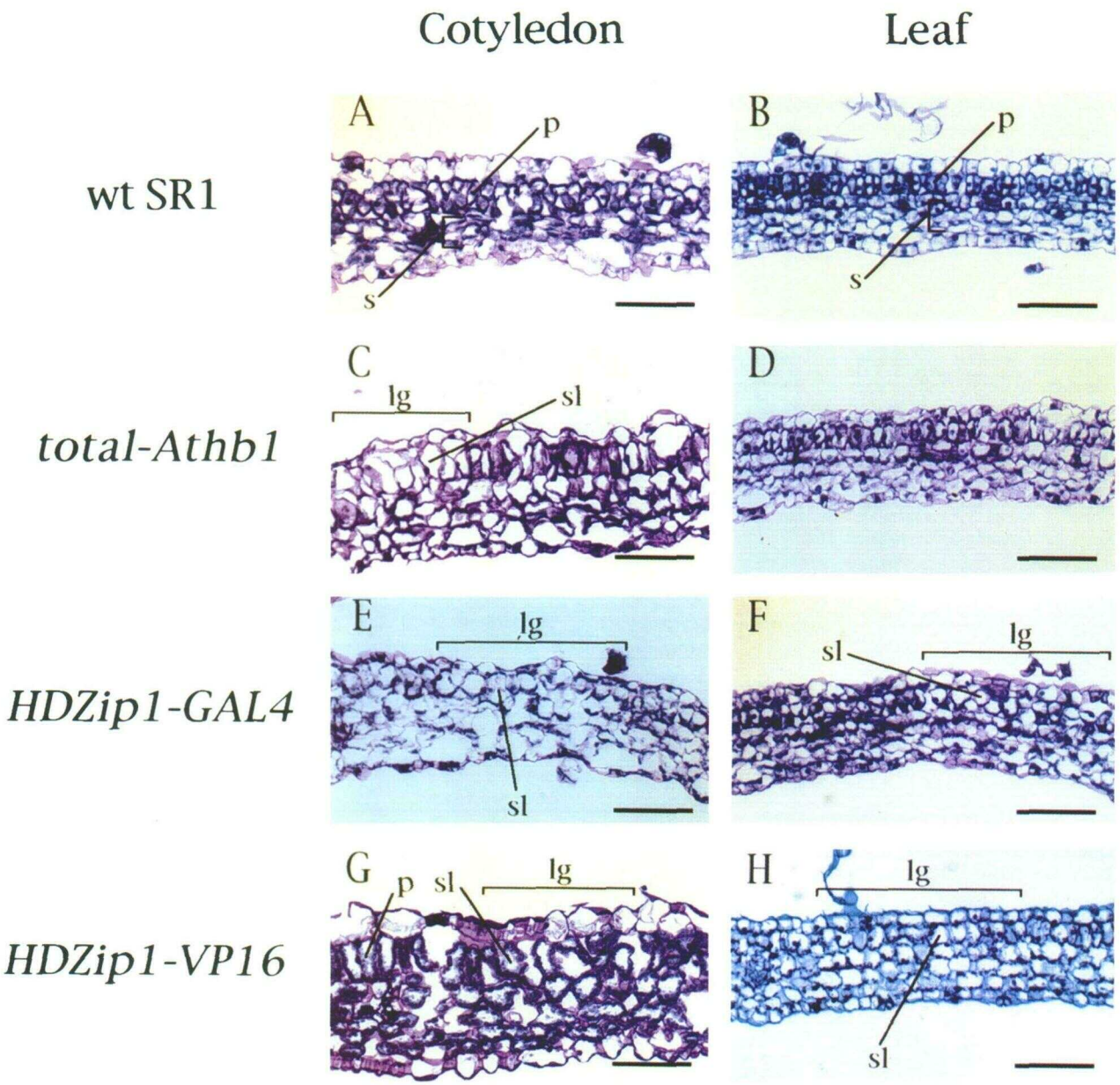

Figure 4. Structural Analysis of Cotyledons and Leaves of Transgenic Plants.

T_2 plants were grown on agar medium under normal growth conditions, and cross-sections were prepared from cotyledons and young leaves. Cotyledons were collected from plants 14 days after sowing, whereas young leaves were collected 14 to 30 days after sowing. Bright-field microscopy was used to show transverse sections of cotyledons in **(A)**, **(C)**, **(E)**, and **(G)** and leaves in **(B)**, **(D)**, **(F)**, and **(H)**. In **(A)** and **(B)**, p and s indicate a palisade cell layer and spongy mesophyll cell layers, respectively. In **(C)**, **(E)**, **(F)**, **(G)**, and **(H)**, light green parts in cotyledons and leaves are indicated by lg, and typical spongylike cells in the upper L2 layer are indicated by sl. In **(G)**, a palisade cell with a normal shape is indicated with a p. wt, wild type.

(A) and **(B)** Wild-type SR1.

(C) and **(D)** *total-Athb1*.

(E) and **(F)** *HDZip1–GAL4*.

(G) and **(H)** *HDZip1–VP16*.

Bars = 0.1 mm.

Analysis of Transgene Expression Levels

Expression levels of transgenes were examined by RNA gel blot hybridizations. Total RNA was prepared from two or three mature leaves of each transgenic plant, and a DNA fragment encoding the HD-Zip region of the *Athb-1* cDNA was used as a probe. As a control, the expression level of the gene encoding the β subunit of the mitochondrial ATPase (Boutry and Chua, 1985) was determined with the same RNA preparations. Figure 5 shows the results of the RNA analysis, along with the severity of the palisade defect in each plant.

The expression level of the transgenes varied with different T_2 transgenic lines. The expression levels of *HDZip1–VP16* (Figure 5, lanes 11 to 16) and *HDZip1–GAL4* (lanes 8 to 10) correlated well with the severity of the palisade defect in each series of lines carrying the same transgene. This result strongly suggests that the phenotype was caused by the expression of the transgenes. Moreover, we also found that *HDZip1–VP16* caused a more severe phenotypic defect than *HDZip1–GAL4* when comparing transgenic plants with the same transgene expression level (compare lanes 9 and 14 or 10 and 15 in Figure 5). With respect to T_2 transgenic plants with *total-Athb1*, expression of the transgene was higher in the lines showing a phenotype in the cotyledons (Figure 5, lane 3) than in those without a phenotype (lane 2). The expression of *anti-Athb1* (lanes 4 and 5), *HDZip1* (lanes 6 and 7), and *mHDZip1–VP16* (lanes 17 and 18) could be confirmed at least at the mRNA level. These results exclude the possibility that the transgenic plants did not express the transgenes.

Phenotypes of Transgenic Plants in the Dark

The most obvious phenotype observed under normal growth conditions was a defect in palisade parenchyma, indicating that the ectopic expression of Athb-1 affects leaf development. To obtain more information on how Athb-1 might affect leaf development, transgenic seedlings were grown in the dark because leaf development is regulated by light. T_2 seeds of 10 transgenic lines for each construct were germinated on agar medium and placed in complete darkness for 20 days.

Wild-type seedlings in the dark had long hypocotyls and unopened cotyledons (Figure 6A). The same morphology was seen in all transgenic seedlings with *anti-Athb1*, *HDZip1*, and *mHDZip1–VP16* (data not shown). On the other hand, seedlings from two of 10 independent lines carrying *total-Athb1* (Figure 6B), two of 10 lines for *HDZip1–GAL4* (Figure 6C), and four of 10 lines for *HDZip1–VP16* (Figure 6D) showed a deetiolated phenotype, including cotyledon expansion and true leaf development. Inhibition of hypocotyl elongation was also observed in plants with *HDZip1–GAL4* (Figure 6C) and *HDZip1–VP16* (Figure 6D). Transgenic lines with the deetiolation phenotype corresponded to those showing the most severe phenotypes of light green sectors under normal light

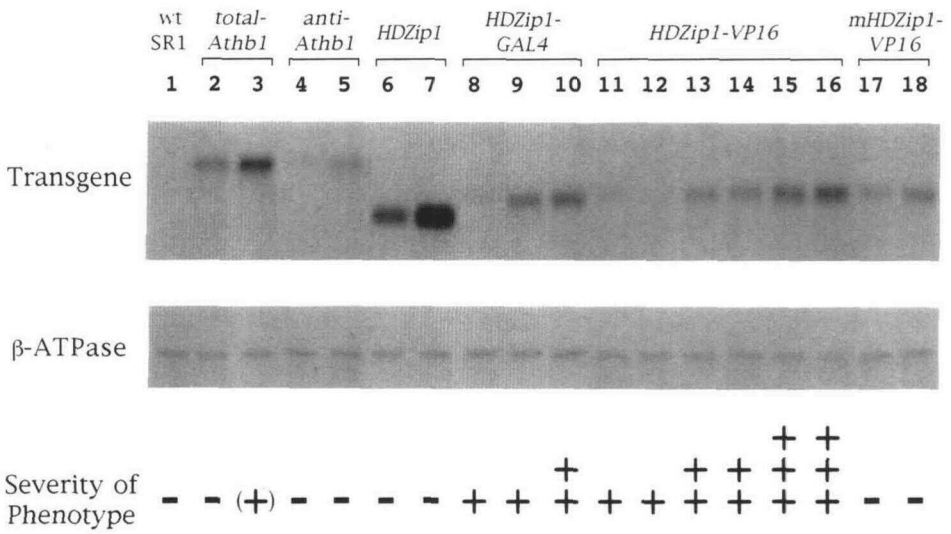

Figure 5. RNA Gel Blot Analysis of Transgene Transcripts.

Total RNA was isolated from two or three mature leaves of each T_2 transgenic plant. A cDNA fragment encoding the HD-Zip region of *Athb-1* and a cDNA fragment of the β subunit of the mitochondrial ATPase were used as probes in RNA gel blot hybridizations. Severity in the phenotype of light green sectors for each transgenic plant is indicated at bottom. Lane 1 corresponds to wild-type SR1; lanes 2 and 3, independent lines of T_2 transgenic plants with *total-Athb1*; lanes 4 and 5, *anti-Athb1*; lanes 6 and 7, *HDZip1*; lanes 8 to 10, *HDZip1–GAL4*; lanes 11 to 16, *HDZip1–VP16*; and lanes 17 and 18, *mHDZip1–VP16*. The severity of the phenotype was determined by the light green–colored area as a percentage of the total mature leaf area and is expressed semiquantitatively as – (0%), + (<30%), ++ (30 to 50%), and +++ (>50%). The (+) in lane 3 indicates that light green sectors were observed in cotyledons only. wt, wild type.

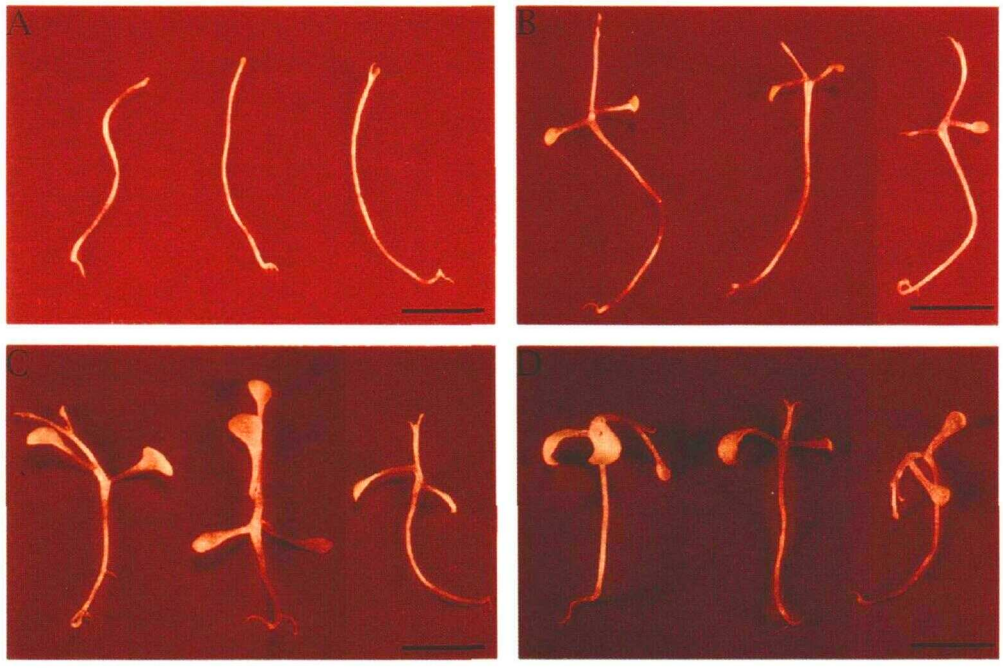

Figure 6. Phenotypes of Transgenic Etiolated Seedlings.

T_2 seeds were sown on agar medium and grown in the dark for 20 days. Expansion of cotyledons and emergence of true leaves in transgenic etiolated seedlings are shown.

(A) Wild-type SR1.

(B) *Total-Athb1.*

(C) *HDZip1–GAL4.*

(D) *HDZip1–VP16.*

Bars = 1 cm.

conditions for *HDZip1–VP16* and *HDZip1–GAL4* and to those with the variegated phenotype in cotyledons for *total-Athb1.*

An Inducible System Using the Regulatory Mechanism of the Glucocorticoid Receptor

From the results given above, it became clear that the DNA binding function of Athb-1 in combination with a transactivating function caused the palisade parenchyma defect and deetiolation in the dark. However, it was difficult to ascertain the developmental stage at which the phenotype developed. To approach this problem, we placed the transactivating function of HDZip1–VP16 under the control of the regulatory mechanism of the glucocorticoid receptor (Picard et al., 1988).

The glucocorticoid receptor domain has been shown to regulate the function of a neighboring domain in the same protein molecule. We fused a DNA fragment encoding the glucocorticoid receptor domain to the 3′ end of a DNA fragment encoding HDZip1–VP16; the resulting chimeric gene was named *HDZip1–VP16–GR* (Figure 7) and was introduced into tobacco. Seventeen independent primary transgenic plants were generated, and all of them developed leaves with normal coloration (data not shown). The induction experiment was done with T_2 generations of all the lines. Seeds were sown on agar medium containing 10 μM dexamethasone (DEX), a synthetic glucocorticoid, and grown for 14 days in the light. Most of the DEX-treated plants showed the same phenotype as that of *HDZip1–VP16* plants or a more severe phenotype (Figures 8F and 8G), whereas all of the plants kept on the medium without DEX were normal (Figures 8B and 8C). The plants with a severe phenotype showed a greater expansion of their cotyledons (Figure 8G) as compared with untreated, control plants (Figure 8C). The DEX treatment had no effect on cotyledons and

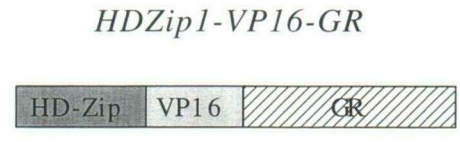

Figure 7. Structure of the Chimeric Gene Encoding an Athb-1 Derivative with a Transactivating Function Inducible by Glucocorticoid.

The chimeric gene is composed of in-frame fusions of DNA fragments encoding the HD-Zip domain of Athb-1 (HDZip1), the transactivating domain of the herpes simplex virus transcription factor VP16 (VP16), and the receptor domain of the glucocorticoid receptor (GR).

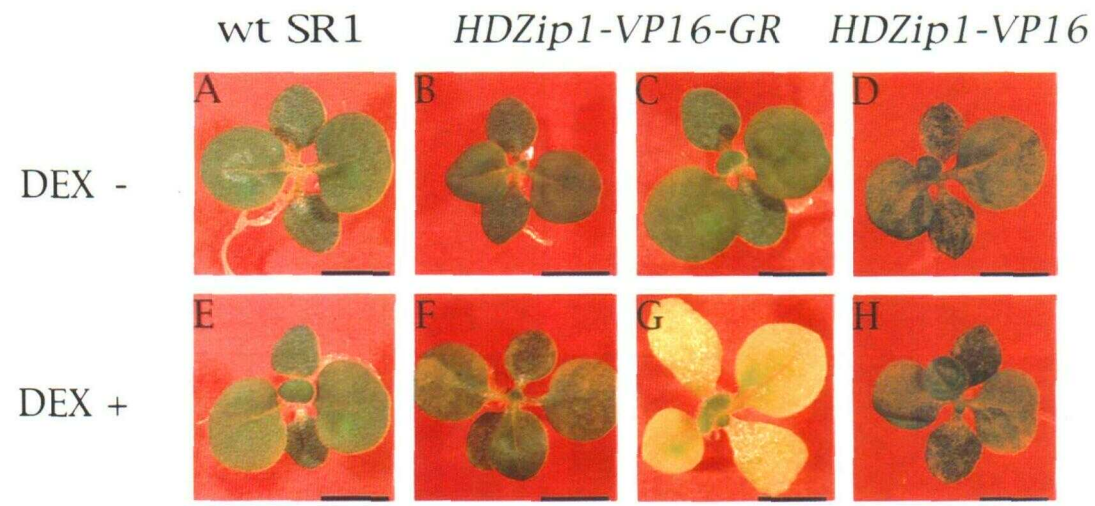

Figure 8. Induction of Developmental Defects in the Leaf Palisade Parenchyma of Transgenic Tobacco Plants with *HDZip1–VP16–GR*.

T_2 seeds were sown on agar medium with or without 10 μM DEX and grown for 14 days under normal growth conditions. Plants shown in **(A)** to **(D)** were grown without DEX; plants shown in **(E)** to **(H)** were grown with DEX. wt, wild type.

(A) Wild-type SR1.
(B) and **(C)** *HDZip1–VP16–GR*.
(D) *HDZip1–VP16*.
(E) Wild-type SR1.
(F) and **(G)** *HDZip1–VP16–GR*.
(H) *HDZip1–VP16*.
Bars = 5 mm.

leaves of the wild-type plants (Figures 8A and 8E) or *HDZip1–VP16* plants (Figures 8D and 8H).

The induction experiment was also done with plants grown under dark conditions. T_2 seeds of all of the lines were sown on agar medium with or without DEX and kept in the dark for 20 days. On the medium with DEX, deetiolated seedlings were found in several independent lines. These seedlings showed cotyledon expansion, true leaf development, and inhibition of hypocotyl elongation (Figure 9E). By contrast, all of the seedlings on the medium without DEX were completely etiolated (Figure 9B). Also in this case, lines showing the DEX-inducible deetiolation in the dark corresponded to those having a relatively severe palisade defect induced by DEX in the light. Again, the DEX treatment had no effect on the wild-type plants (Figures 9A and 9D) or the *HDZip1–VP16* plants (Figures 9C and 9F). From these results, we concluded that HDZip1–VP16–GR displayed the same function as HDZip1–VP16 only in the presence of DEX and that the phenotypes caused by the chimeric transcription factors could be induced after embryogenesis.

DISCUSSION

Athb-1 Is a Transcriptional Activator

Although Athb-1 has been demonstrated previously to be a DNA binding protein in vitro (Sessa et al., 1993), it was not known whether this factor functions as a repressor or an activator in vivo. Here, we show by in vivo transient expression assays that Athb-1 can activate promoters containing its binding site. The level of the activation was higher than that of chimeric factors HDZip1–GAL4 and HDZip1–VP16. The result suggests that Athb-1 has a relatively strong transactivating function because the VP16 domain is known to have a strong transactivating function in animal and fungal systems (Sadowski et al., 1988; Cousens et al., 1989). Although several plant genes encoding homeodomain factors have been cloned (for a review, see Langdale, 1994) and at least one was found to bind specific DNA sequences (Korfhage et al., 1994), none has been demonstrated to have repressor or activator functions in vivo. The fact that Athb-1 is a transcriptional activator is important for the interpretation of the transgenic phenotypes discussed later.

The Athb-1 HD-Zip domain, combined with a transactivating domain of GAL4 or VP16, could activate transcription specifically from a promoter with six copies of the DNA sequence (5′-CAATTTATTG-3′), which has been shown to be an Athb-1 binding sequence in vitro (Sessa et al., 1993). The result demonstrated that the binding site can also be recognized by the Athb-1 HD-Zip domain in vivo. The activation level obtained with the combination of *total-Athb1* and six copies of the mutant sequence (5′-CAATTGTTG-3′) was slightly higher than that of negative controls, suggesting that the HD-Zip domain might bind to the mutant sequence with a very low affinity.

Defect in Palisade Parenchyma Cell Differentiation

Under normal growth conditions, expression of chimeric genes encoding both DNA binding and transactivating functions (*total-Athb1*, *HDZip1–VP16*, and *HDZip1–GAL4*) caused a defect in palisade parenchyma formation. Neither the DNA binding domain alone (*HDZip1*) nor the transactivating domain alone (*mHDZip1–VP16*) would suffice, indicating that the morphological change was likely due to the transcriptional activation of downstream genes whose promoters contain Athb-1-binding sites. When expressed at a similar level, *HDZip1–VP16* was found to cause a more severe defect than that caused by *HDZip1–GAL4*. This result correlates with the observation that the VP16 transactivating domain was stronger than the GAL4 transactivating domain in transient expression experiments.

Anatomical analysis showed that in the light green parts of the cotyledon and leaf, palisade parenchyma cells were replaced by cells resembling spongy mesophyll cells. Nevertheless, the number of cell layers remained the same between the dark green and light green parts of the leaf or cotyledon. These results suggest that the palisade defect occurred during differentiation but not proliferation of the L2 cells. This hypothesis is compatible with lineage information obtained from periclinal chimeras, which indicated that both the upper palisade and the lower spongy mesophyll cells are derived from the L2 layer of the apical meristem (reviewed in Poethig, 1989). However, because the middle spongy mesophyll cells are derived from the L3 layer, we cannot exclude the possibility that there was an inhibition of L2 cell proliferation that was compensated by additional divisions of L3 cells.

It should be pointed out that ectopic expression of the maize KN1 or the Arabidopsis KNAT1 in transgenic tobacco resulted in changes of leaf shape (Sinha et al., 1993; Lincoln et al., 1994). In *KN1* transgenic lines that exhibited a severe phenotype, conversion of palisade to spongy parenchyma cells was seen (Sinha et al., 1993), similar to what we have observed in this work. It has been suggested that ectopic expression of KN1 or KNAT1 in developing leaves, where they are not normally expressed, may alter growth patterns or the timing of cell differentiation within the leaf blade (Smith et al., 1992; Lincoln et al., 1994). An alteration in the pattern of cell division in KN1 and KNAT1 transgenic tobacco plants might also sporadically occur during the formation of leaf primordia. Thus, as observed in *HDZip1–GAL4* and *HDZip1–VP16* transgenic plants, it is not possible to distinguish between an alteration of palisade cell differentiation and a proliferation of L3-derived cells in the upper subepidermal layer.

In our case, further understanding of the palisade defect was provided by the phenotype-induction experiments with DEX. The palisade defect in cotyledons could be induced by

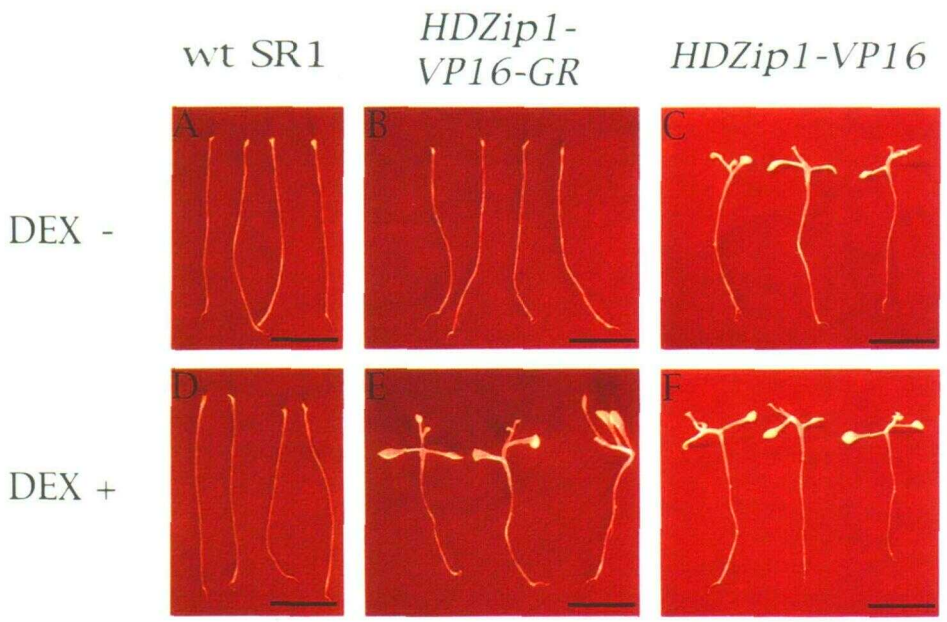

Figure 9. Induction of Deetiolated Phenotypes in the Dark in Transgenic Plants with *HDZip1–VP16–GR*.

T_2 seeds were sown on agar medium with (+) or without (–) 10 μM DEX and grown for 20 days in complete darkness. The same transgenic line carrying *HDZip1–VP16–GR* shown in Figures 8C and 8G was used for these experiments.
(A) and **(D)** Wild-type SR1.
(B) and **(E)** *HDZip1–VP16–GR*.
(C) and **(F)** *HDZip1–VP16*.
Bars = 1 cm.

treating *HDZip1–VP16–GR* seedlings with DEX for 14 days after sowing. Because cotyledons have been formed during embryogenesis, we can conclude that at least in this organ the palisade defect occurred after all the cell layers have been laid down. The phenotype-induction experiments with DEX were also performed in expanding leaves of 2-week-old *HDZip1–VP16–GR* plants. After the induction, only the regions sprayed with DEX showed the palisade defect (data not shown). Taking into consideration all the observations, it is likely that ectopic activation of genes downstream of Athb-1 affects the development of palisade parenchyma cells at the level of cell differentiation.

Ectopic expression of Athb-1 also resulted in the palisade defect in *total-Athb1* transgenic tobacco, but this was confined to cotyledons of plants that expressed the transgene at a high level (Figure 3). This result appears inconsistent with the observation that Athb-1 displayed a stronger transactivation than the chimeric transcription factors in transient assays. Moreover, we have recently found that *Athb-1* is expressed in the Arabidopsis leaf and that the expression level increases with leaf expansion (S. Lucchetti, unpublished data). These observations suggest that under normal conditions, the transactivating function of Athb-1 may be subjected to negative regulation that is not manifested under transient expression conditions. Alternatively, Athb-1 could be less stable than the chimeric factors in transgenic tobacco plants.

Transgenic plants expressing *anti-Athb1* do not exhibit any obvious phenotype. The ineffectiveness of the antisense strategy could be due to either genetic redundancy or insufficient sequence homology between Athb-1 and its tobacco counterpart. The Athb-1 derivative HDZip1 may be expected to function as a dominant-negative mutant because it retains the DNA binding and dimerization domain but is devoid of a transactivating function. Nevertheless, transgenic plants expressing the *HDZip1* transgene also show no apparent phenotype. One possibility is that HDZip1 may not be able to compete effectively with the tobacco Athb-1 counterpart for binding to target promoters. Alternatively, HDZip1 may not be stable in vivo. These possibilities remain to be investigated.

Deetiolated Phenotypes in the Dark

A deetiolation phenotype similar to those seen in the Arabidopsis photomorphogenetic mutants *det* (for deetiolated; Chory et al., 1989) and *cop* (for constitutive photomorphogenic; Deng et al., 1991) was conferred by overexpression of Athb-1 itself or the two chimeric derivatives. The deetiolation phenotype shared by our transgenic tobacco plants and the Arabidopsis mutants includes cotyledon expansion, true leaf development, and the inhibition of hypocotyl elongation (for reviews, see Chory, 1992; Kendrick and Kronenberg, 1994). It is known that dark-grown Arabidopsis *det* and *cop* mutants express genes that are normally expressed only in light-grown plants (for reviews, see Mullet, 1988; Gilmartin et al., 1990). Our results suggest that Athb-1 and the two chimeric derivatives might counteract the negative regulatory effects of the *det* and *cop* gene products in etiolated seedlings and activate some of the downstream genes directly or indirectly. The morphology of the deetiolation itself, however, can also be induced by cytokinins (Chory et al., 1994) or by shaking plants in liquid cultures (Rédei et al., 1974). Molecular markers of photomorphogenesis in addition to the deetiolation morphology should be examined to ascertain how closely the deetiolated phenotype uncovered in this study is linked to light signal transduction pathways (Neuhaus et al., 1993; Bowler et al., 1994). Nevertheless, it is clear that overexpression of Athb-1 or the chimeric derivatives can cause true leaf development in the dark. Because ectopic expression of KN1 and KNAT1 alters leaf development (Lincoln et al., 1994), it would be interesting to see whether transgenic tobacco seedlings overexpressing these genes would also show a deetiolated phenotype in the dark.

Induction of the Transgene Function Using the Glucocorticoid Receptor Domain

We have used the glucocorticoid receptor domain to control the function of HDZip1–VP16. In transgenic plants carrying *HDZip1–VP16–GR*, the phenotypes observed in *HDZip1–VP16* plants could be successfully induced by DEX, whereas the same inducer had no effect on wild-type or *HDZip1–VP16* plants. From these results, we can conclude that the phenotypes are directly linked to the functions of the transgene rather than due to, for example, possible genetic differences between wild-type and *HDZip1–VP16* plants resulting from transgene insertion. Using the DEX-inducible system, we also found that both of the developmental phenotypes, the palisade defect and the deetiolation in the dark, could develop after embryogenesis.

Inducible systems possess the advantage that they allow the study of phenotypes that are normally too toxic to plants when expressed constitutively. In this study, such a severe phenotype was observed only in the *HDZip1–VP16–GR* transgenic plants under induced condition. Cotyledons of the transgenic seedlings seemed to have no palisade parenchyma at all, and they showed abnormal expansion. The latter also suggests that Athb-1 is likely involved in the regulation of leaf and cotyledon expansion. However, no abnormal expansion of true leaves in transgenic plants was seen, possibly because the severe defect in their palisade tissue rendered the growth rate of the whole plant slower than normal.

Concerning the regulatory mechanism of steroid receptor domains, it is thought that in the absence of a ligand, the domain represses, via steric hindrance, the function of a neighboring domain by the formation of a complex including the heat shock protein HSP90; ligand binding results in the dissociation of the complex, thereby derepressing the function (Picard, 1993). This use of a steroid receptor domain to regulate gene expression in transgenic plants has been previously reported by Lloyd et al. (1994), who showed that trichome development in Arabidopsis could be successfully controlled

by a chimeric protein comprising the glucocorticoid receptor domain and the maize transcriptional regulator R. However, the construction of such a chimeric transcription factor whose activity is tightly regulated by the glucocorticoid receptor domain is not always easy and achievable in every case. Tight regulation appears to be critically dependent on the intramolecular structure of the chimeric protein, especially the relative position between the glucocorticoid receptor domain and the domain whose function is to be regulated. In this regard, HDZip1–VP16–GR appears to have a structure conducive for regulation. The combined VP16–GR domain was fused to the HD-Zip domain at the C-terminal end of the leucine zipper. We expect that the VP16–GR domain might be used to regulate successfully the transactivating function of not only HD-Zip proteins but also of other transcription factors in plant systems.

Conclusions and Perspectives

Our transient expression experiments demonstrated that Athb-1 functions as a transcriptional activator in a binding site–specific manner in vivo. In transgenic experiments, we observed three kinds of phenotypes caused by overexpression of Athb-1 or its chimeric derivatives: deetiolated phenotypes in the dark, conversion of palisade to spongy parenchyma cells, and abnormal expansion of cotyledons. All three phenotypes are related to leaf development. It is likely that the three phenotypes described here were caused by an ectopic activation of target genes containing specific *cis* elements recognized by the HD-Zip domain of Athb-1. From these results, we can surmise that the target genes of Athb-1 are closely linked to leaf development. However, it is unclear whether all of the phenotypes are caused by ectopic expression of the same set of genes or of different genes. This issue can be resolved only by the identification of the target genes of Athb-1, which will provide important information for further understanding the transcriptional network underlying leaf developmental processes.

METHODS

DNA Constructs

A derivative of pMON721 (Monsanto Corp., St. Louis, MO) was used as the vector for our transgenic work. The *Arabidopsis thaliana Athb-1*–derived genes were preceded by the −343 to +1 region of the cauliflower mosaic virus 35S promoter (Odell et al., 1985) and flanked by the poly(A) addition sequence of pea ribulose bisphosphate carboxylase small subunit *rbcS-E9* (Coruzzi et al., 1984). The same DNA constructs were used as the transactivating constructs in particle bombardment experiments. pGEM (Promega) was used as a vector for the reporter constructs. To construct promoters with the Athb-1 binding sequence or the mutant sequence, six copies of the Athb-1 binding sequence (5′-CAATTATTG-3′) or a mutant sequence (5′-CAATTGTTG-3′) were fused to the 5′ end of the −46 to +1 region of the 35S promoter, respectively. DNA fragments encoding the sequences of GAL4, VP16, and the glucocorticoid receptor domain were kindly provided by M. Ptashne (Harvard University, Cambridge, MA), S.J. Triezenberg (Michigan State University, East Lansing, MI), and K.R. Yamamoto (University of California at San Francisco), respectively. DNA fragments encoding an isolated domain were obtained by the polymerase chain reaction using primers of the appropriate sequences for in-frame cloning. Each domain contained the region corresponding to the amino acid numbers as follows: the Athb-1 HD-Zip domain (61 to 170; Ruberti et al., 1991), the GAL4 acidic domain (148 to 196; Laughon and Gesteland, 1984), the VP16 acidic domain (413 to 490; Dalrymple et al., 1985), and the glucocorticoid receptor domain (519 to 795; Miesfeld et al., 1986). The sequence Trp-Phe-Gln-Asn (amino acids 112 to 115 in Athb-1) was substituted with Ala-Glu-Phe-Leu in mHDZip1–VP16 by site-directed mutagenesis.

Transient Expression Assay

Distal or proximal halves of mature tobacco leaves (~15 cm in length) were bombarded using a Biolistic PDS 1000/HE particle delivery system (Bio-Rad). Each sample was bombarded with 0.5 mg of 1 μm gold particles coated with a DNA mixture including 0.1 and 0.5 μg of transactivating and reporter plasmids, respectively. The initial pressure of bombardment was 1100 psi, and the traveling distance of particles to the leaf was 10 cm. Bombarded leaves were incubated at 27°C for 24 hr under luminescence light. After incubation, total cell extracts prepared from the leaves were assayed for their specific activities of luciferase (LUC) and β-glucuronidase (GUS), as described by Millar et al. (1992) and Jefferson et al. (1987), respectively. Each relative transactivation level was calculated by normalizing LUC activity with respect to GUS activity.

Transgenic Plants

Constructed plasmids were introduced into *Agrobacterium tumefaciens* ABI (Monsanto Corp.), which was used to transform leaf discs of *Nicotiana tabacum* cv SR1 as described by Horsch et al. (1988). Primary transgenic plants were allowed to self-fertilize, and T_2 seeds were collected. Transgenic progeny were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.8% agar, and 100 μg/mL kanamycin. After selection, young plants were transferred to soil and maintained at 27°C under a 16-hr-light and 8-hr-dark cycle. Under normal growth conditions, seedlings were grown on Murashige and Skoog medium, 3% sucrose, 0.8% agar, and 100 μg/mL kanamycin and maintained at 27°C with a 16-hr-light and 8-hr-dark cycle. For wild-type plants, kanamycin was omitted from the agar medium. Plants were grown in the dark by wrapping plastic growth containers with several layers of aluminum foil.

Other Methods

Cross-sections of leaves and cotyledons were prepared and stained as described by Natarella and Sink (1971). Total RNA isolation and RNA gel blot hybridizations were performed as described previously (Nagy et al., 1988). A stock solution of 30 mM dexamethasone (DEX; Sigma) in ethanol was prepared before use, and appropriate dilutions were added to the agar medium. The same volume of ethanol was added to each negative control medium.

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