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Negative autoregulation of the *Arabidopsis* homeobox gene *ATHB-2*

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Summary

The *Arabidopsis* homeobox gene *ATHB-2* is tightly regulated by light signals, and is thought to direct morphological changes during shade avoidance responses. To understand how *ATHB-2* mediates light signals in plant morphogenesis, we investigated its transcriptional network. We constructed a gene encoding a chimeric transcription factor (HD-Zip-2-V-G) that is expected to activate target genes of ATHB-2 in a glucocorticoid-dependent manner. In transgenic *Arabidopsis* plants expressing HD-Zip-2-V-G, glucocorticoid treatment activates the *ATHB-2* gene itself, independent of *de novo* protein synthesis. An *in vitro* DNase I-footprinting experiment showed that recombinant ATHB-2 protein specifically bound to an *ATHB-2* promoter region. These complementary results indicate that ATHB-2 recognizes its own promoter. Consistent with the fact that ATHB-2 itself has been shown to act as a repressor, expression of the endogenous *ATHB-2* gene was repressed in transgenic plants overexpressing an *ATHB-2* transgene. Moreover, target-gene analysis using the HD-Zip-2-V-G suggested that ATHB-2 recognizes other *HD-Zip II* subfamily genes. We conclude that *ATHB-2* has a negative autoregulatory loop and may be involved in a complicated transcriptional network involving paralogous genes, as is the case with animal homeobox genes.

Keywords: induction system, target gene, glucocorticoid receptor, DNase I footprinting, transcriptional network, *HD-Zip* gene.

Introduction

Plants undergo morphogenesis in response to environmental stimuli and developmental programs. These heterogeneous factors are successfully integrated to regulate cell proliferation and morphogenesis. Although plants likely possess an effective, and probably complex, mechanism for this process, they lack highly differentiated organs, such as the central nervous systems of animals. In plants, intracellular and intercellular signal transduction systems, including those for phytohormones, likely play an important part in the mechanism. At the end of the signal transduction, gene expression directing plant morphogenesis is regulated transcriptionally. To understand the mechanism regulating plant morphogenesis at the level of transcription, we studied the *Arabidopsis* transcription factor ATHB-2.

ATHB-2, also known as HAT4, is a transcription factor belonging to the *Arabidopsis* homeodomain-leucine zipper (HD-Zip) protein family (Ruberti *et al.*, 1991; Schena and Davis, 1992). There is evidence that the *ATHB-2* gene is a regulator that processes light signals from phytochromes to alter morphogenesis during shade avoidance responses. First, transcription of *ATHB-2* is strongly upregulated by far-red-rich light, i.e. light with low red:far red (R:FR) ratios (Carabelli *et al.*, 1993); this induces the shade avoidance responses in higher plants (Morelli and Ruberti, 2000; Smith and Whitelam, 1997; Smith, 1995). Analyses using phytochrome mutants have revealed the involvement of phytochromes in this light signal transduction (Carabelli *et al.*, 1996; Steindler *et al.*, 1997). Second, overexpression of *ATHB-2* causes long hypocotyls and reduced leaf expansion (Schena *et al.*, 1993; Steindler *et al.*,

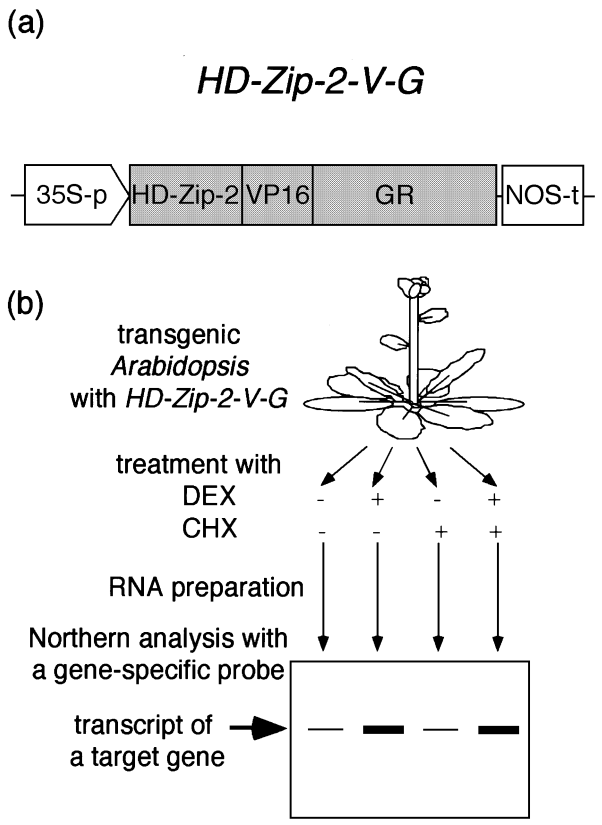


Figure 1. Architecture of the target-gene analysis.
(a) Structure of the *HD-Zip-2-V-G* gene. The coding regions for the HD-Zip-2 domain, the transactivating domain of the herpes viral protein VP16, and the hormone binding domain of the rat GR are indicated as HD-Zip-2, VP16 and GR, respectively. The cauliflower mosaic virus 35S promoter (Odell *et al.*, 1985) and the terminator of the Ti plasmid-encoded nopaline synthase gene (Bevan *et al.*, 1983) are indicated as 35S-p and NOS-t, respectively.
(b) Strategy for the target-gene analysis. Transgenic *Arabidopsis* plants carrying the *HD-Zip-2-V-G* gene were non-treated or treated with DEX and/or CHX. The RNA fractions were prepared and subjected to Northern analysis using a probe specific to a given gene. If the gene was a direct target of HD-Zip-2-V-G, the transcript level was increased by DEX treatments both in the absence and presence of CHX.

1999). These phenotypes occur as a shade avoidance response in dicotyledonous plants (Smith, 1995). Recently, *ATHB-2*-induced and shade-induced elongation of the hypocotyl was found to depend on the auxin transport system (Steindler *et al.*, 1999).

The molecular functions of the ATHB-2 protein have also been studied thoroughly. HD-Zip is a plant-specific domain structure in which a homeodomain is accompanied by a leucine-zipper motif at the C-terminus (Aso *et al.*, 1999; Ruberti *et al.*, 1991; Schena and Davis, 1994). *In vitro* binding experiments showed that the HD-Zip domain of ATHB-2 (HD-Zip-2 domain) is sufficient for sequence-specific DNA binding as a homodimer (5′-CAAT(C/G)ATTG-3′ as the HD-Zip-2 binding consensus sequence; Sessa *et al.*, 1993). In transient expression experiments, ATHB-2 repressed the expression of a reporter gene containing six copies of the HD-Zip-2 binding consensus sequence in its promoter region, while an ATHB-2 derivative with a strong transactivating domain activated the same reporter gene (Steindler *et al.*, 1999). These results indicate that ATHB-2 itself acts as a repressor.

To determine how light signals are processed via *ATHB-2*, we examined the transcriptional network regulated by ATHB-2. To this end, we constructed an ATHB-2-derived transcription factor (HD-Zip-2-V-G; illustrated in Figure 1a; Steindler *et al.*, 1999), consisting of the HD-Zip-2 domain, the transactivating domain of the herpes viral protein VP16 (Triezenberg *et al.*, 1988) and the hormone binding domain (HBD) of the rat glucocorticoid receptor GR (Picard *et al.*, 1988). To identify target genes of transcription factors, a fusion protein with the GR HBD is frequently used *in vivo* (Grandori and Eisenman, 1997; Picard, 1994; Sablowski and Meyerowitz, 1998; Samach *et al.*, 2000; Wagner *et al.*, 1999). Such a fusion protein exhibits its original function, i.e. transcriptional regulation of its target genes, only in the presence of glucocorticoid (Picard, 1994). In our case, although ATHB-2 itself is a repressor (Steindler *et al.*, 1999), we used its transactivator derivative, since we thought that target genes were more easily detected as inducibly upregulated transcripts than as downregulated ones. The inducibility of HD-Zip-2-V-G has been confirmed in transgenic *Arabidopsis* plants (Steindler *et al.*, 1999). Glucocorticoid treatment enhanced radial expansion and inhibited longitudinal elongation in the hypocotyls and petioles of the transgenic seedlings. Since the phenotype is the opposite to that of *ATHB-2* overexpressor plants, it is thought that ATHB-2 and HD-Zip-2-V-G act as a repressor and an inducible transcriptional activator, respectively.

In this report, we performed a target-gene analysis using the HD-Zip-2-V-G system to investigate the interaction of ATHB-2 with its own gene and paralogous genes, since animal homeobox genes are frequently involved in auto-regulatory networks (for review, see Serfling, 1989). The results indicated that *ATHB-2* has a negative autoregulatory loop and is possibly involved in a complicated regulatory network involving *HD-Zip II* subfamily genes.

Results

Target-gene analysis using HD-Zip-2-V-G

An important characteristic of target-gene analyses using GR-fusion transcription factors is that the induction of the protein function does not require *de novo* protein synthesis (Picard, 1994). Hence, transcriptional activation of direct target genes occurs even in the presence of a protein synthesis inhibitor like cycloheximide (CHX). On the other hand, CHX prevents indirect transcriptional activation

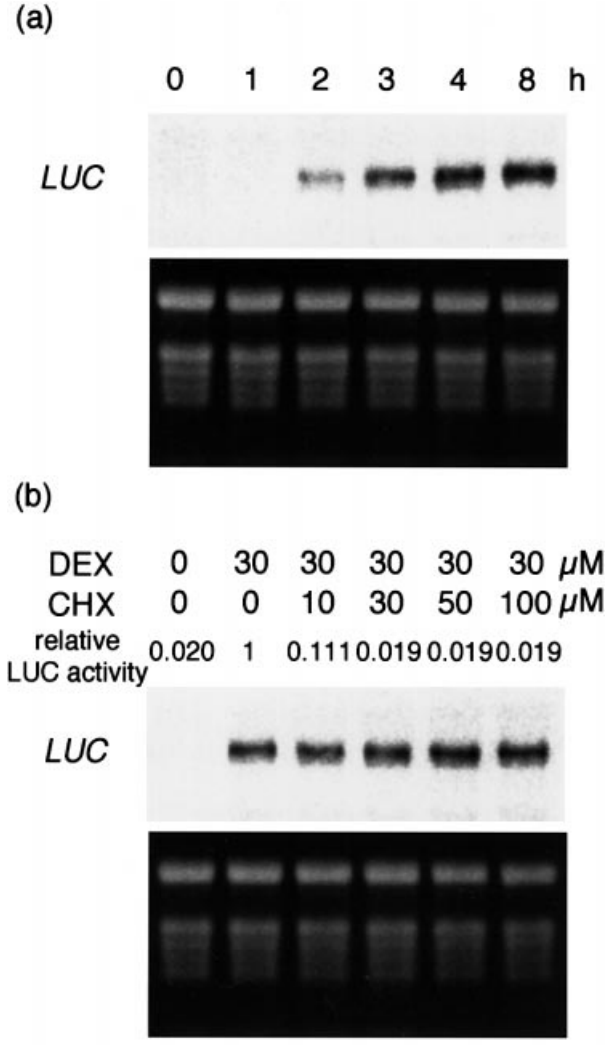


Figure 2. Optimization of chemical treatment.
RNA fractions prepared from transgenic plants carrying the *GVG* gene and the GVG-inducible *LUC* gene were subjected to Northern analysis using a probe specific for the *LUC* gene.
(a) Plants were treated with 30 μM DEX for the period indicated above each lane.
(b) Plants were treated with the concentrations of DEX and CHX indicated above each lane for 3 h. The relative luciferase activity from the same plants is also shown above the lanes. The value obtained at 30 μM DEX and 0 μM CHX was arbitrarily set as 1.
The same RNA fractions electrophoresed on an agarose gel and stained with ethidium bromide are shown below the autoradiograms in (a) and (b).

following the expression of direct target genes. This allows us to ascertain whether HD-Zip-2-V-G activates a given gene directly (illustrated in Figure 1b).

First, we determined the optimal conditions under which dexamethasone (DEX), a glucocorticoid derivative, clearly produced transcriptional induction and CHX completely blocked *de novo* protein synthesis. For this purpose, we used transgenic *Arabidopsis* plants carrying the *GVG* gene and a luciferase reporter gene (*LUC*) that was inducibly activated by GVG (Aoyama and Chua, 1997). Since GVG has the same structure as HD-Zip-2-V-G, except that it has the GAL4 DNA-binding domain instead of the HD-Zip-2 domain, the conditions optimized with GVG were thought to be applicable to HD-Zip-2-V-G.

We treated transgenic plants with an excess concentration of DEX (30 μM; Aoyama, 1998) to achieve rapid, uniform induction (for details, see Experimental procedures). After a 3 h treatment, the induced *LUC* transcript was clearly observed in the Northern analysis, and a weak signal could be detected after 2 h (Figure 2a). We found that 30 μM CHX was sufficient to inhibit the induction of luciferase activity in our system (Figure 2b). In subsequent target-gene analyses, we treated plants for 3 h with 30 μM DEX to induce HD-Zip-2-V-G function, and with 30 μM CHX at same time as DEX to inhibit protein synthesis.

HD-Zip-2-V-G directly recognizes ATHB-2 in vivo

Since many examples of autoregulation have been found in animal homeobox genes (for review, see Serfling, 1989), we first examined the interaction of ATHB-2 with its own gene. Total RNA fractions were prepared from four samples of mature HD-Zip-2-V-G plants, non-treated or treated with DEX, CHX, or DEX and CHX, and subjected to Northern analysis using a DNA probe specific for *ATHB-2*. A probe for *ATHB-1* (Ruberti *et al.*, 1991) was used as a control.

Figure 3(a) shows that DEX treatment did not change the *ATHB-1* transcript levels, regardless of the absence or the presence of CHX. On the other hand, DEX treatment doubled the *ATHB-2* transcript levels in the absence of CHX and increased them by about 10 times in the presence of CHX. This indicates that HD-Zip-2-V-G directly upregulated the transcription of *ATHB-2*. *ATHB-2* transcript levels were higher in CHX-treated plants than in non-treated plants (see Discussion).

To confirm that the HD-Zip-2 domain mediates the upregulation, we performed another control experiment, using transgenic plants carrying the *GVG and* GVG-inducible *LUC* genes. As shown in Figure 3(b), DEX did not change the *ATHB-2* transcript levels, while CHX again tended to increase the levels. On the other hand, DEX treatment clearly upregulated the *LUC* transcript levels, both in the absence and presence of CHX. This clearly indicates that the DNA-binding domain of ATHB-2 mediates the upregulation of *ATHB-2* transcription.

Recombinant ATHB-2 protein binds to specific sequences in the ATHB-2 *promoter region* in vitro

These results suggested that the ATHB-2 protein recognizes its own gene directly. However, it was still possible

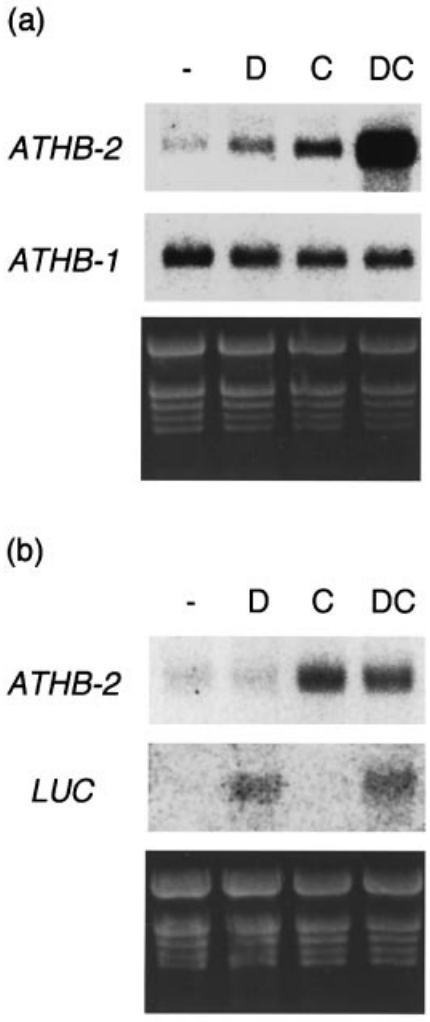


Figure 3. *In vivo* target-gene analysis of the *ATHB-2* gene.
RNA fractions prepared from transgenic plants carrying the *HD-Zip-2-V-G* gene (a) or the *GVG and* GVG-inducible *LUC* genes (b) were subjected to Northern analysis using probes specific for the genes indicated on the left side of each autoradiogram. Lanes -, D, C, and DC indicate RNA fractions prepared from non-treated plants or those treated with DEX, CHX, and DEX and CHX, respectively. The same RNA fractions electrophoresed on an agarose gel and stained with ethidium bromide are shown below the autoradiograms in (a) and (b).

that HD-Zip-2-V-G differed from the authentic ATHB-2 protein with respect to DNA recognition. To confirm the interaction of the full-length ATHB-2 protein with its own gene, we purified recombinant ATHB-2 protein from *Escherichia coli* for use in footprinting experiments (for details, see Experimental procedures). The recombinant protein had the same primary structure as the authentic ATHB-2, except an additional six amino-acid residues at the N terminal.

Figure 4(a) shows the results of a DNase I-footprinting experiment using a DNA fragment encompassing the 200-base pair (bp) upstream region from the transcription start site of *ATHB-2*. The recombinant ATHB-2 strongly protected both the top and bottom strands of the DNA fragment from DNase I at two sites (around positions –75 and –55). This indicates that ATHB-2 can interact with the *ATHB-2* promoter directly. The protected sites contained the sequences 5′-TAATCATTA-3′ and 5′-TAATTATTA-3′ (Figure 4b), which are similar to the HD-Zip-2-binding consensus sequence (see Discussion). Weakly protected sites included the region around position –135, which was protected on both strands. This contained another sequence (5′-TAATCATCT-3′) similar to the binding consensus sequence.

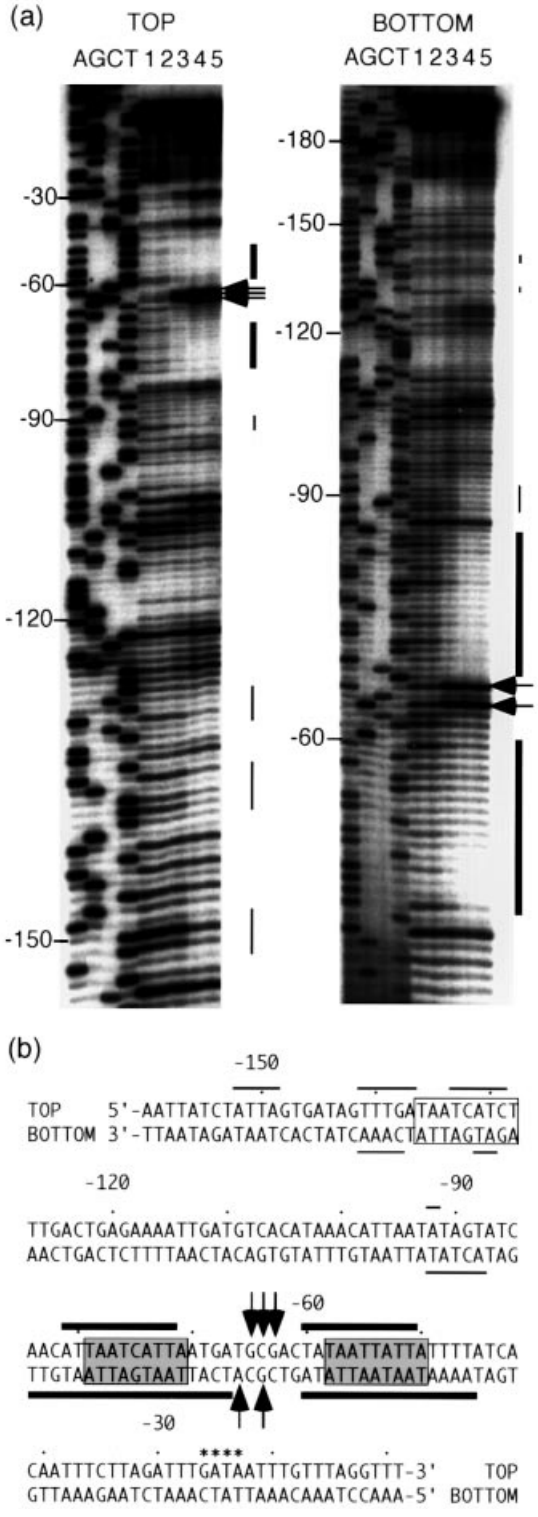
The endogenous ATHB-2 *gene is repressed in transgenic plants overexpressing an* ATHB-2 *transgene*

To obtain *in vivo* evidence that the authentic ATHB-2 protein recognizes its own gene, we examined transcript levels of the endogenous *ATHB-2* gene in transgenic plants overexpressing an *ATHB-2* transgene. The total *ATHB-2* expression from both the 35S promoter-driven transgene and the endogenous gene was determined by Northern analysis using a probe specific to an ATHB-2-coding region (Figure 5a). To determine the endogenous gene transcript level, we performed a semi-quantitative reverse-transcriptase-PCR (RT–PCR) experiment, because probes specific to the non-coding exon regions of the endogenous gene, i.e. endogenous-gene-specific probes, did not give a clear signal in the Northern analysis. The PCR bands corresponding to the endogenous gene transcript were less intense for the *ATHB-2* overexpressor plants than for the wild-type plants (Figure 5b). On the other hand, the band intensities for internal control transcripts from the *TUA4* gene (Kopczak *et al.*, 1992) were comparable between transgenic and wild-type plants (Figure 5b), which is consistent with the result of the Northern analysis (Figure 5a). Since ATHB-2 is thought to act as a repressor, this provided correlative *in vivo* evidence that ATHB-2 recognizes its own gene.

HD-Zip-2-V-G directly recognizes other HD-Zip II *genes*

Next, we examined the interaction of ATHB-2 with other *HD-Zip* genes, using HD-Zip-2-V-G-containing plants. The same Northern analyses described above were repeated with probes specific to various *HD-Zip* genes. As shown in Figure 6, DEX increased the transcript levels from all the *HD-Zip II* genes examined (*ATHB-4*, *HAT1*, *HAT2*, *HAT3*, *HAT9* and *HAT22*; Carabelli *et al.*, 1993; Schena and Davis, 1992; Schena and Davis, 1994), both in the absence and presence of CHX, although the basal and induced levels varied with each gene. This suggests that ATHB-2 recognizes all the *HD-Zip II* genes examined. CHX treatment also increased both the transcript levels and the

magnitude of upregulation by DEX for the *HD-Zip II* genes examined. On the other hand, the DEX treatment did not change the transcript levels of genes belonging to other subfamilies (data not shown). These included *ATHB-3* and *ATHB-5* (subfamily I; Mattsson *et al.*, 1992; Soderman *et al.*, 1994), *ATHB-14* and *IFL1* (subfamily III; Sessa *et al.*, 1998; Zhong and Ye, 1999) and *ATHB-10* (subfamily IV; Di Cristina *et al.*, 1996).



Discussion

A system for target-gene analysis using HD-Zip-2-V-G

The HBD of GR has been used as a *cis*-acting regulatory domain to control plant transcription factors *in vivo* (Aoyama *et al.*, 1995; Lloyd *et al.*, 1994; Sablowski and Meyerowitz, 1998; Simon *et al.*, 1996; Wagner *et al.*, 1999), and contributed to the detection of direct target genes of AP3, LEAFY and CO (Sablowski and Meyerowitz, 1998; Samach *et al.*, 2000; Wagner *et al.*, 1999). We applied the same strategy to detect target genes of ATHB-2. In our case, however, the chimeric transcription factor HD-Zip-2-V-G was used, rather than a simple fusion between ATHB-2 and the GR domain.

Our system had certain advantages and disadvantages to target-gene analysis. First, we added the advantage of the VP16 domain to strongly activate target genes. Second, we detached parts other than the HD-Zip-2 domain to minimize the possibility of post-translational regulation that might interfere with the transactivating function of the chimeric transcription factor. These modifications allowed us to simply detect candidate target genes as strongly induced transcripts. On the other hand, the modifications increased the possibility that the chimeric protein might recognize genes in a manner different from that of the authentic ATHB-2 protein. Such a possibility, however, cannot be excluded even in a simple GR-fusion system; hence, candidates obtained from HBD-fusion systems should always be confirmed by other methods.

Induction systems with artificial regulatory mechanisms such as the GR-domain fusion might confer unexpected side-effects on plants. Regardless of whether such side-

Figure 4. DNase I-footprinting experiment with recombinant ATHB-2 protein and an *ATHB-2* promoter DNA fragment.
(a) Autoradiograms of DNase I footprints of the top (left) and bottom (right) strands of the *ATHB-2* promoter fragment are shown. The approximate amount of recombinant ATHB-2 protein added to the DNA fragment (0.05 pmol) was 0, 1, 2, 4, and 8 pmol for lanes 1–5, respectively. Lanes A, G, C, and T are reference sequence ladders. Strongly and weakly protected sites and the positions of strongly enhanced bands are indicated by thick and thin bars and arrows, respectively. Numbers with a minus sign indicate the nucleotide position upstream from the transcription start site.
(b) Strongly and weakly protected sites and the positions of strongly enhanced bands are indicated along the double stranded sequences of the *ATHB-2* promoter region by thick and thin bars and arrows, respectively. Sequences similar to the HD-Zip-2 binding consensus located in strongly and weakly protected sites are indicated by shaded and open boxes, respectively. Numbers with a minus sign indicate the nucleotide position upstream from the transcription start site. The TATA-box sequence of the promoter is indicated by asterisks.

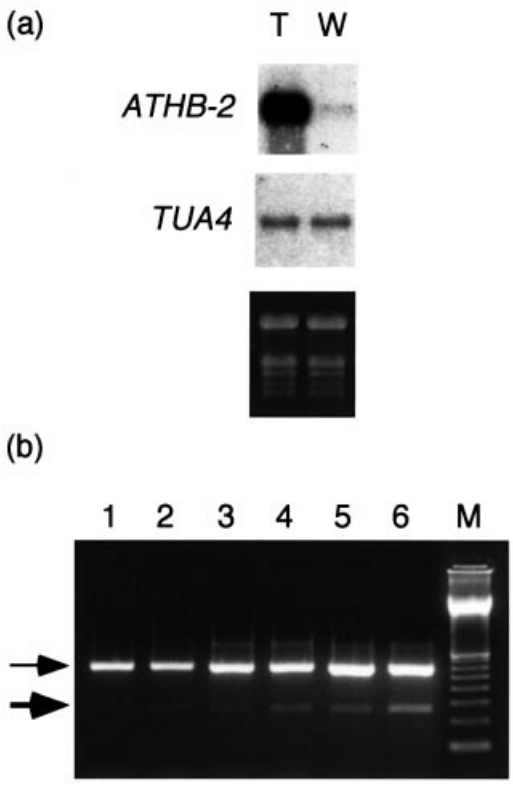


Figure 5. Quantification of *ATHB-2* endogenous gene transcript in transgenic plants overexpressing an *ATHB-2* transgene.
(a) RNA fractions prepared from mature transgenic (lane T) and wild-type (lane W) plants were subjected to Northern analysis with probes specific for the genes indicated on the left side of each autoradiogram. The same RNA fractions electrophoresed on an agarose gel and stained with ethidium bromide are shown below the autoradiograms.
(b) The plants from part (a) were pooled and then used in batch. RNA fractions prepared from mature transgenic (lanes 1, 3 and 5) and wild-type (lanes 2, 4 and 6) plants were subjected to a quantitative RT–PCR experiment using two pairs of PCR primers in the same reaction mixture: one for the *ATHB-2* endogenous gene and the other for the internal control gene *TUA4*. The PCR products were electrophoresed on an agarose gel after 22 (lanes 1 and 2), 24 (lanes 3 and 4), or 26 (lanes 5 and 6) reaction cycles. M indicates the lane containing a 100-bp ladder marker. The positions of the PCR products corresponding to transcripts from the *ATHB-2* endogenous gene (274 bp) and the *TUA4* gene (622 bp) are indicated by thick and thin arrows, respectively.

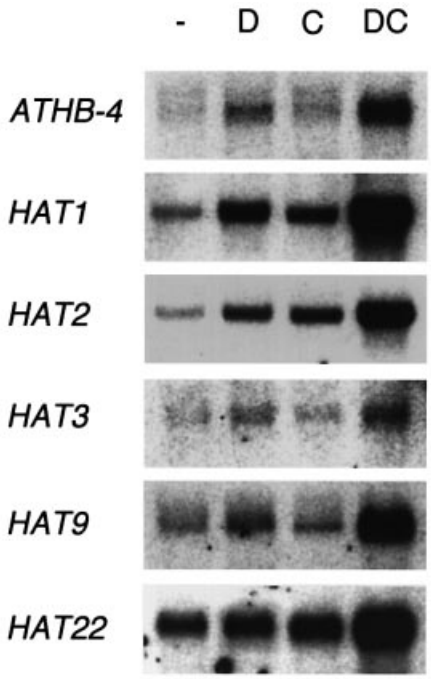


Figure 6. *In vivo* target-gene analysis of the *HD-Zip II* genes.
The same RNA fractions used in Figure 1(a) were subjected to Northern analysis using probes specific to the genes indicated on the left side of each autoradiogram. Lanes -, D, C, and DC indicate RNA fractions prepared from non-treated plants, or those treated with DEX, CHX, and DEX and CHX, respectively.

effects are detectable, it is essential for induction experiments to compare the induced phenomenon with that of an appropriate negative control. In this context, we used transgenic plants carrying the *GVG* gene as a negative control to confirm that the upregulation of *ATHB-2* is not a side-effect.

In vivo *target-gene analyses*

In transgenic plants expressing HD-Zip-2-V-G, transcripts of all the *HD-Zip II* subfamily genes examined, including *ATHB-2* itself, were upregulated by DEX treatment, even in the presence of CHX. This indicated that the DNA-binding domain of ATHB-2 directly recognizes the *HD-Zip II* genes *in vivo*. In our analyses, CHX tended to increase the *HD-Zip II* transcript levels. Translational inhibition by CHX has been shown to influence mRNA stability, although this depends on the gene in question (Green, 1993; Sullivan and Green, 1993). Its effect might be to stabilize transcripts of the *HD-Zip II* genes. DEX also tended to upregulate *HD-Zip II* gene transcript levels more in the presence of CHX than in its absence. Although the effect of CHX on mRNA stability can also explain this, another explanation is possible. Without CHX, HD-Zip-2-V-G competes for target genes with the repressor molecule ATHB-2, whose amount is increased by the function of HD-Zip-2-V-G. This competition does not occur in the presence of CHX since ATHB-2 synthesis is inhibited; hence, the magnitude of the transcriptional activation of the target genes is larger in the presence of CHX.

The observation that the endogenous *ATHB-2* gene was repressed in transgenic plants overexpressing an *ATHB-2* transgene provides *in vivo* supporting evidence for both autoregulation and for the repressive function of ATHB-2. Although all the findings so far indicate that ATHB-2 acts as a repressor, it is still possible that ATHB-2 mediates

transcriptional activation of other genes through interaction with cell-type-specific or condition-specific factors.

The interaction of ATHB-2 with target sequences

The interaction of the ATHB-2 protein with its own gene was confirmed in the *in vitro* DNase I-footprinting experiment. The two sites near the *ATHB-2* promoter were strongly protected by the recombinant ATHB-2 protein. They contain sequences 5′-TAATCATTA-3′ and 5′-TAATAATTA-3′. The HD-Zip-2 domain binds specifically to the sequences 5′-(C/T)AAT(C/G)ATT(G/A)-3′ *in vitro* (Sessa *et al.*, 1993). Moreover, the sequence 5′-CAAT(A/T)ATTG-3′ was recognized by the HD-Zip-2 domain *in vitro* (Sessa *et al.*, 1997), and by ATHB-2 in an *in vivo* transient expression experiment (Steindler *et al.*, 1999). These observations strongly suggest that ATHB-2 recognizes the sequences of the two sites *in vivo*. The similar sequence (5′-CAATCATCT-3′) in the weakly protected site around position –135 is also a possible *in vivo* target. It is, however, unclear to what extent it contributes to the recognition by ATHB-2.

Bands between the two strongly protected sites were enhanced on both strands (Figure 3a; around position –65). Since DNase I is assumed to be a DNA conformation reporter (Drew and Travers, 1984), the enhanced signals might mean torsion caused by the interaction between the two ATHB-2 homodimers. The centers of the two sequences are separated by 21 bp (i.e. just two turns of the DNA helix); hence the homodimers are located in the same face on the DNA, which also suggests that they interact. Bands with weakly reduced intensity seen in relatively short regions are thought to reflect DNA conformational changes caused by binding of the protein to other sites.

We found one or more derivatives of 5′-(C/T)AAT(C/G)ATT(G/A)-3′ with up to one-base mismatch within 500-bp upstream from the translation initiation site for each *HD-Zip II* gene examined. Although the transcription start sites of these *HD-Zip II* genes have not been determined, these sequences might be located near their promoters and be recognized by ATHB-2.

Biological meaning of ATHB-2 *regulation*

Evidence from *in vivo* and *in vitro* experiments indicates that ATHB-2 directly represses its own expression. Autoregulation is prevalent in eukaryotic transcription factor genes, notably in animal homeobox genes (Serfling, 1989). Positive autoregulation of animal homeobox genes has been proposed as a mechanism that establishes and maintains the determined cell state directed by the genes (Bienz and Tremml, 1988; Hiromi and Gehring, 1987; Kuziora and McGinnis, 1988). Floral homeotic genes in plants, including *deficiens* of *Antirrhinum* (Schwarz-Sommer *et al.*, 1992) and *APETALA3* of *Arabidopsis* (Hill *et al.*, 1998; Tilly *et al.*, 1998), have been found to have this type of positive autoregulation, although they encode MADS-box proteins instead of homeodomain proteins. Examples of negative autoregulation have also been reported in animal homeobox genes: *Ultrabithorax* of *Drosophila*, *goosecoid* of *Xenopus*, and *Cdx-2/3* of mouse (Danilov *et al.*, 1998; Irvine *et al.*, 1993; Xu *et al.*, 1999). *ATHB-2* is the first reported case of negative autoregulation in plants.

Negative feedback regulation is used by many organisms as an effective mechanism for stabilizing the quantity of a substance. Assuming that the release of ATHB-2 molecules from the *ATHB-2* promoter is the rate-limiting step for its gene expression, the concentration of ATHB-2 in the nuclei can be tightly controlled within a very narrow range in which the binding equivalence between ATHB-2 and the promoter is maintained. In addition, when the *ATHB-2* gene is upregulated by other factors, negative autoregulation can moderate the gene expression at an appropriate level. Consequently, these effects might perform a fine adjustment of the gene expression in plant morphogenesis. Moreover, this autoregulatory loop consists of the ATHB-2 protein alone, suggesting that the regulation is very rapid. This might contribute to rapid switching off of *ATHB-2* expression when its induction signal stops. Autoregulation of *ATHB-2* might provide both fine and rapid regulation of gene expression during shade avoidance responses.

We examined HD-Zip genes in the target-gene analysis using HD-Zip-2-V-G and found that the HD-Zip-2 domain can also recognize other *HD-Zip II* subfamily genes. This suggests a transcriptional network involving paralogous genes, like the networks found in animal homeobox genes (Beachy *et al.*, 1988; Maconochie *et al.*, 1996; Serfling, 1989). In animals, the competition of different homeodomain proteins for similar target sequences is involved in the complicated transcriptional regulation of homeobox genes (Hoey and Levine, 1988; Maconochie *et al.*, 1996; Serfling, 1989). HD-Zip II proteins might also recognize the same target sequences, since amino-acid sequences are highly conserved in their HD-Zip domains. If this is the case, both autonomous and mutual regulation are to be expected among the *HD-Zip II* genes. Under the shade condition, it might be important that *ATHB-2* represses other *HD-Zip II* genes to exclusively regulate common target genes. Alternatively, other *HD-Zip II* genes might also do this under some other conditions. For further analysis of the transcriptional network among *HD-Zip II* genes, it will be necessary to investigate the expression profile of each gene.

Conclusion

In this study, we performed a series of target-gene analyses for ATHB-2, a homeodomain transcription factor involved in the shade avoidance response. *In vivo* and *in vitro* results indicate that ATHB-2 constitutes a negative autoregulatory loop, and may be involved in a complicated regulatory network involving *HD-Zip II* genes, similar to the networks found in animal homeobox genes. The features of the transcriptional network are still unclear because the downstream target genes whose molecular functions are related to shade avoidance responses are unknown. Using the HD-Zip-2-V-G system, we analysed genes that are possibly involved in shade avoidance responses. They included a photosynthesis-related gene *CAB6*, cell-expansion-related genes *atEXT1* and *EXGT-A1* and auxin transporter genes *ATPIN1* and *EIR1*. So far, however, none of their expression has been found to respond to DEX under the conditions used in this paper (data not shown). To reveal the transcriptional network involving ATHB-2, we are going to combine our GR-induction systems (HD-Zip-2-V-G and ATHB-2-G; Steindler *et al.*, 1999) with a gene-expression-profile analysis using the microarray technology. Such systematic approaches will allow us to address the question of how *ATHB-2* mediates light signals in plant morphogenesis.

Experimental procedures

Plant materials and transgene constructs

Arabidopsis thaliana (ecotype Columbia) was used as the wild-type plant and for producing transgenic plants. The construction of the *HD-Zip-2-V-G* gene was described previously (Steindler *et al.*, 1999). The plasmid containing the *GVG* gene and a GVG-inducible *LUC* gene was constructed by inserting a luciferase-coding sequence at a site downstream from the GVG-inducible promoter in pTA7002 (Aoyama and Chua, 1997; McNellis *et al.*, 1998). The 35S promoter-driven *ATHB-2* gene was constructed by inserting an ATHB-2-coding sequence between the *Sma*I and *Sac*I sites of pBI121 (Datla *et al.*, 1992). *Agrobacterium tumefaciens* strain LBA4404 was transformed with these plasmids. Transgenic *Arabidopsis* plants were obtained by the vacuum infiltration method of Bechtold *et al.* (1993). T3 or T4 plants homozygous with each transgene were used in the experiments. Wild-type and transgenic seeds were germinated on agar medium containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968) and 1% sucrose, and were grown under constant light at 22°C unless otherwise noted.

Chemical treatment of Arabidopsis *plants*

Transgenic *Arabidopsis* plants were germinated on an agar plate and grown for 3 weeks. The plants were then transferred together with the agar onto a plastic mesh with 2–3 mm high spacers attached to the lower side. They were placed in a tall plastic container (PLANTCON®; Flow Laboratories Inc., Mclean, VA, USA) with just enough water to cover the bottom. The top of the plastic container was gradually removed over several days to adapt the plants to open-air conditions and enable them to grow vigorous roots in the water. For chemical treatment, the water was replaced with a solution containing 30 μM DEX (Sigma Chemical Co., St. Louis, Missouri, USA) and/or 30 μM CHX (Sigma). The treatment was continued for 3 h, then the plants were harvested.

Northern and RT–PCR analyses

Total RNA isolation and RNA gel blot hybridizations were performed as described previously (Nagy *et al.*, 1988). The DNA fragments used as probes were chosen so that they were hybridized specifically to each transcript. Their regions were as follows (the position of the first nucleotide of each coding sequence is arbitrarily set as 1): *ATHB-1* : 487–819, *ATHB-2* : 30–388, *ATHB-3* : 238–548, *ATHB-4* : 1–521, *ATHB-5* : 1290–1518, *ATHB-10* : 682–1796, *ATHB-14* : 1381–2549, *HAT1* : 252–637, *HAT2* : 1–469, *HAT3* : 1–509, *HAT9* : 1–381, *HAT22* : 1–240, *IFL1* : 1383–2522, *TUA4* : 707–1328.

RT–PCR analysis for the quantification of the endogenous *ATHB-2* transcript in transgenic plants overexpressing an *ATHB-2* transgene was performed according to the instructions for the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a set of endogenous-gene-specific primers (5′-TCAGTTCTACATGCACATGA-3′, 5′-ACATGTTAACAA-CTACATGC-3′) and a set of primers specific to the internal control gene, *TUA4* (5′-CTTCCTTGACTGCTTCTC-3′,5′-TCATCGTCACCA-CCTTCA-3′) in the same reaction mixture.

Preparation of the recombinant ATHB-2 protein

To prepare recombinant ATHB-2 protein, we constructed a GST-ATHB-2 fusion protein gene. A DNA fragment encoding ATHB-2 was inserted between the *Bam*HI and *Sma*I sites of the plasmid pGEX-6P-1 (Amersham Pharmacia Biotech) in-frame. After removing the GST moiety, the recombinant ATHB-2 protein is expected to have six additional amino-acid residues (Gly-Pro-Leu-Gly-Ser-Pro) at its N terminal.

Escherichia coli cells (strain BL21) harboring the constructed plasmid were grown to 1×10^8 cells ml^{-1} at 30°C in L broth (300 ml) and then treated with isopropyl β-D-thiogalactopyranoside (1 mM) for 4 h. The fusion protein GST-ATHB-2 was purified from the cells with glutathione sepharose 4B (Amersham Pharmacia Biotech) according to the instructions for the RediPack GST Purification Module (Amersham Pharmacia Biotech). Then, the purified fusion protein was digested with PreScission™ protease (20 units; Amersham Pharmacia Biotech) while being dialyzed against PBS buffer [140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3)] supplemented with 300 mM NaCl, 1 mM DTT, and 1% Triton X-100 for 12 h at 4°C. The GST moiety and the protease were removed by passage through a glutathione sepharose 4B column. The eluent was applied onto a HiTrap® heparin column (1 ml; Amersham Pharmacia Biotech) and the recombinant ATHB-2 was eluted with TED buffer [5 mM EDTA, 1 mM dithiothreitol, 50 mM Tris–HCl (pH 8.0)] containing 700 mM NaCl. The approximate concentration of the recombinant protein was estimated on a gel stained with Coomassie brilliant blue after SDS polyacrylamide gel electrophoresis.

DNase I footprinting

Single-5′-end-labeled DNA fragments encompassing the 200 bp region upstream from the transcription start site of *ATHB-2* were made by PCR with the primers 5′-TACGATTAGATTTTGTTCTC-3′ and 5′-AACTCTCTCAAACCTAAAC-3′, one of which was ^{32}P-labeled at the 5′ end. The labeled oligonucleotides were also used to make reference sequence ladders. The labeled DNA fragments (0.05 pmol) were incubated with 0.03–0.25 μg of the recombinant ATHB-2 protein (about 1–8 pmol) or without the protein in 0.1 ml of binding buffer (100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5)) at 22°C for 20 min. Then, they were treated with DNase I (0.1 μg ml^{-1}) at 22°C for 3 min. After phenol extraction and ethanol precipitation, the DNA was electrophoresed on a sequencing gel together with reference ladders.

Other methods

The extraction of luciferase and assays for relative luciferase activity were carried out as described by Millar *et al.* (1992). Autoradiograms were generated and the signal intensities were measured with a Fujix BA100 Bio-Image Analyzer (Fuji Photo Film Co., Minami-ashigara, Japan).

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References

Aoyama, T. (1998) Glucocorticoid-inducible gene expression in plants. In *Inducible Gene Expression in Plants* (Reynolds, P.H.S., ed.). Wallingford, UK: CABI Publishing, pp. 43–59.

Aoyama, T. and Chua, N.-H. (1997) A glucocorticoid-mediated transcriptional induction system for transgenic plants. *Plant J.* **11**, 605–612.

Aoyama, T., Dong, C.-H., Wu, Y., Carabelli, M., Sessa, G., Ruberti, I., Morelli, G. and Chua, N.-H. (1995) Ectopic expression of the Arabidopsis transcription factor Athb-1 alters leaf cell fate in tobacco. *Plant Cell*, **7**, 1773–1785.

Aso, K., Kato, M., Banks, J.A. and Hasebe, M. (1999) Characterization of homeodomain-leucine zipper genes in the fern *Ceratopteris richardii* and the evolution of the homeodomain-leucine zipper gene family in vascular plants. *Mol. Biol. Evol.* **16**, 544–552.

Beachy, P.A., Krasnow, M.A., Gavis, E.R. and Hogness, D.S. (1988) An *Ultrabithorax* protein binds sequences near its own and the *Antennapedia* P1 promoters. *Cell*, **55**, 1069–1081.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis* plants. *C. R. Acad. Sci. Paris. Life Sci.* **316**, 1194–1199.

Bevan, M., Barnes, W.M. and Chilton, M.-D. (1983) Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucl. Acids Res.* **11**, 369–385.

Bienz, M. and Tremml, G. (1988) Domain of *Ultrabithorax* expression in *Droshophila* visceral mesoderm from autoregulation and exclusion. *Nature*, **333**, 576–578.

Carabelli, M., Sessa, G., Baima, S., Morelli, G. and Ruberti, I. (1993) The *Arabidopsis Athb-2* and *Athb-4* genes are strongly induced by far-red-rich light. *Plant J.* **4**, 469–479.

Carabelli, M., Morelli, G., Whitelam, G. and Ruberti, I. (1996) Twilight-zone and canopy shade induction of the *Athb-2* homeobox gene in green plants. *Proc. Natl Acad. Sci. USA*, **93**, 3530–3535.

Danilov, V., Blum, M., Schweickert, A., Campione, M. and Steinbeisser, H. (1998) Negative autoregulation of the organizer-specific homeobox gene *goosecoid*. *J. Biol. Chem.* **273**, 627–635.

Datla, R.S., Hammerlindl, J.K., Panchuk, B., Pelcher, L.E. and Keller, W. (1992) Modified binary plant transformation vectors with the wild-type gene encoding NPTII. *Gene*, **122**, 383–384.

Di Cristina, M., Sessa, G., Dolan, L., Linstead, P., Baima, S., Ruberti, I. and Morelli, G. (1996) The *Arabidopsis* Athb-10 (GLABRA2) is an HD-Zip protein required for regulation of root hair development. *Plant J.* **10**, 393–402.

Drew, H.R. and Travers, A.A. (1984) DNA structure variations in the E. coli tyrT promoter. *Cell*, **37**, 491–502.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50**, 151–158.

Grandori, C. and Eisenman, R.N. (1997) Myc target genes. *Trends Biochem. Sci.* **22**, 177–181.

Green, P.J. (1993) Control of mRNA stability in higher plants. *Plant Physiol.* **102**, 1065–1070.

Hill, T.A., Day, C.D., Zondlo, S.C., Thackeray, A.G. and Irish, V.F. (1998) Discrete spatial and temporal *cis*-acting elements regulate transcription of the *Arabidopsis* floral homeotic gene *APETALA3*. *Development*, **125**, 1711–1721.

Hiromi, Y. and Gehring, W.J. (1987) Regulation of the *Drosophila* segmentation gene *fushi tarazu*. *Cell*, **50**, 963–974.

Hoey, T. and Levine, M. (1988) Divergent homeo box proteins recognize similar DNA sequences in *Drosophila*. *Nature*, **332**, 858–861.

Irvine, K.D., Botas, J., Jha, S., Mann, R.S. and Hogness, D.S. (1993) Negative autoregulation by *Ultrabithorax* controls the level and pattern of its expression. *Development*, **117**, 387–399.

Kopczak, S.D., Haas, N.A., Hussey, P.J., Silflow, C.D. and Snustad, D.P. (1992) The small genome of Arabidopsis contains at least six expressed α-tubulin genes. *Plant Cell*, **4**, 539–547.

Kuziora, M.A. and McGinnis, W. (1988) Autoregulation of a *Drosophila* homeotic selector gene. *Cell*, **55**, 477–485.

Lloyd, A.M., Schena, M., Walbot, V. and Davis, R.W. (1994) Epidermal cell fate determination in *Arabidopsis*: patterns defined by a steroid-inducible regulator. *Science*, **266**, 436–439.

Maconochie, M., Nonchev, S., Morrison, A. and Krumlauf, R. (1996) Paralogous Hox genes: Function and regulation. *Annu. Rev. Genet.* **30**, 529–556.

Mattsson, J., Soderman, E., Svenson, M., Borkird, C. and Engstrom, P. (1992) A new homeobox-leucine Zipper gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* **18**, 1019–1022.

McNellis, T.W., Mudgett, M.B., Li, K., Aoyama, T., Horvath, D., Chua, N.-H. and Staskawicz, B.J. (1998) Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death. *Plant J.* **14**, 247–257.

Millar, A.J., Short, S.R., Chua, N.-H. and Kay, S.A. (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell*, **4**, 1075–1087.

Morelli, G. and Ruberti, I. (2000) Shade avoidance responses. Driving auxin along lateral routes. *Plant Physiol.* **122**, 621–626.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid

growth and bioassays with tobacco tissue. *Physiol. Plant.* **15**, 493–497.

Nagy, F., Kay, S.A. and Chua, N.-H. (1988) Analysis of gene expression in transgenic plant. In *Plant Molecular Biology Manual* (Given, S. and Schilperoort, R., eds). Dordrecht: Kluwer Academic Publishers, pp. 1–12.

Odell, J.T., Nagy, F. and Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature*, **313**, 810–812.

Picard, D. (1994) Regulation of protein function through expression of chimeric proteins. *Curr. Opin. Biotechnol.* **5**, 511–515.

Picard, D., Salser, S.J. and Yamamoto, K.R. (1988) A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell*, **54**, 1073–1080.

Ruberti, I., Sessa, G., Lucchetti, S. and Morelli, G. (1991) A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. *EMBO J.* **10**, 1787–1791.

Sablowski, R.W. and Meyerowitz, E.M. (1998) A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. *Cell*, **92**, 93–103.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. *Science*, **288**, 1613–1616.

Schena, M. and Davis, R.W. (1992) HD-Zip proteins: Members of an *Arabidopsis* homeodomain protein superfamily. *Proc. Natl Acad. Sci. USA*, **89**, 3894–3898.

Schena, M. and Davis, R.W. (1994) Structure of homeobox-leucine zipper genes suggests a model for the evolution of gene families. *Proc. Natl Acad. Sci. USA*, **91**, 8393–8397.

Schena, M., Lloyd, A.M. and Davis, R.W. (1993) The *HAT4* gene of *Arabidopsis* encodes a developmental regulator. *Genes Dev.* **7**, 367–379.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.-E., Saedler, H. and Sommer, H. (1992) Characterization of the *Antirrhinum* floral homeotic MADS-box gene deficiens: Evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* **11**, 251–263.

Serfling, E. (1989) Autoregulation – a common property of eukaryotic transcription factors? *Trends Genet.* **5**, 131–133.

Sessa, G., Morelli, G. and Ruberti, I. (1993) The Athb-1 and -2 HD-Zip domains homodimerize forming complexes of different DNA binding specificities. *EMBO J.* **12**, 3507–3517.

Sessa, G., Morelli, G. and Ruberti, I. (1997) DNA-binding specificity of the homeodomain-leucine zipper domain. *J. Mol. Biol.* **274**, 303–309.

Sessa, G., Steindler, C., Morelli, G. and Ruberti, I. (1998) The *Arabidopsis Athb-8-9* and *-14* genes are members of a small gene family coding for highly related HD-ZIP proteins. *Plant Mol. Biol.* **38**, 609–622.

Simon, R., Igeno, M.I. and Coupland, G. (1996) Activation of floral meristem identity genes in *Arabidopsis*. *Nature*, **384**, 59–62.

Smith, H. (1995) Physiological and ecological function within the phytochrome family. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 289–315.

Smith, H. and Whitelam, G.C. (1997) The shade avoidance syndrome: multiple responses mediated by multiple phytochromes. *Plant Cell Environ.* **20**, 840–844.

Soderman, E., Mattsson, J., Svenson, M., Borkird, C. and Engstrom, P. (1994) Expression pattern of novel genes encoding homeodomain leucine-zipper protein in *Arabidopsis thaliana*. *Plant Mol. Biol.* **26**, 145–154.

Steindler, C., Carabelli, M., Borello, U., Morelli, G. and Ruberti, I. (1997) Phytochrome A, phytochrome B and other phytochrome(s) regulate *ATHB-2* gene expression in etiolated and green *Arabidopsis* plants. *Plant Cell Environ.* **20**, 759–763.

Steindler, C., Matteucci, A., GiSteindler, C., Matteucci, A., Sessa, G., Weimar, T., Ohgishi, M., Aoyama, T., Morelli, G. and Ruberti, I. (1999) Shade avoidance responses are mediated by the ATHB-2 HD-Zip protein, a negative regulator of gene expression. *Development*, **126**, 4235–4245.

Sullivan, M.L. and Green, P.J. (1993) Post-transcriptional regulation of nuclear-encoded genes in higher plants: the roles of mRNA stability and translation. *Plant Mol. Biol.* **23**, 1091–1104.

Tilly, J.J., Allen, D.W. and Jack, T. (1998) The CArG boxes in the promoter of the *Arabidopsis* floral organ identity gene *APETALA3* mediate diverse regulatory effects. *Development*, **125**, 1647–1657.

Triezenberg, S.J., Kingsbury, R.C. and McKnight, S.L. (1988) Functional dissection of VP16, the transactivator of herpes simplex virus immediate early gene expression. *Genes Dev.* **2**, 718–729.

Wagner, D., Sablowski, R.W. and Meyerowitz, E.M. (1999) Transcriptional activation of *APETALA1* by *LEAFY*. *Science*, **285**, 582–584.

Xu, F., Li, H. and Jin, T. (1999) Cell type-specific autoregulation of the *caudal*-related homeobox gene *Cdx*-2/3. *J. Biol. Chem.* **274**, 34310–34316.

Zhong, R. and Ye, Z.H. (1999) *IFL1*, a gene regulating interfascicular fiber differentiation in Arabidopsis, encodes a homeodomain-leucine zipper protein. *Plant Cell*, **11**, 2139–2152.