The *HAT4* gene of *Arabidopsis* encodes a developmental regulator

Mark Schena,¹ Alan M. Lloyd, and Ronald W. Davis

Department of Biochemistry, Beckman Center, Stanford University Medical Center, Stanford, California 94305-5307 USA

The *HAT4* gene from the plant *Arabidopsis thaliana* encodes a homeo domain protein that contains a leucine zipper motif. Homeo domain–leucine zipper (HD–Zip) proteins have not been found in animal systems, suggesting that *HAT4* may define a new family of transcription factors that regulate higher plant development. To explore this possibility, functional studies of *HAT4* were carried out in yeast and in transgenic plants. Point mutants of *HAT4* isolated in yeast define functionally critical residues within the HD–Zip domain, many of which correspond to highly conserved positions in known homeo domains and leucine zippers. Transgenic plants bearing constructs that alter *HAT4* expression exhibit a series of interesting developmental phenotypes, including changes in morphology and developmental rate. Thus, the *HAT4* gene of *Arabidopsis* encodes an HD–Zip protein that functions as a novel developmental regulator.

[Key Words: *HAT4*; homeo box; leucine zipper; *Arabidopsis*; transcription factor]

Received October 28, 1992; revised version accepted December 22, 1992.

Certain regulatory genes encode proteins that regulate development (for reviews, see Hayashi and Scott 1990; Weintraub et al. 1991; Lewis 1992). In the case of genes that appear to function as master switches (Weintraub et al. 1991), ectopic expression of a given regulator can be sufficient to activate a complex developmental pathway such as the gratuitous formation of a specific cell or tissue type (for review, see McGinnis and Krumlauf 1992). One striking example comes from studies of muscle cell differentiation, in which constitutive expression of the *myoD* cDNA results in conversion of differentiated cell types such as fibroblasts into muscle cells (Davis et al. 1987). A second example derives from work on *Drosophila* neurogenesis. Ectopic expression of the *single-minded* gene can convert cells of the lateral central nervous system (CNS) into midline cells (Nambu et al. 1991). A third example is observed in gain-of-function *Antennapedia* mutations, in which the fly antenna is homeotically transformed into a leg (for reviews, see Gehring 1987; Kaufman et al. 1990). It is important to note that a large fraction of developmental regulatory genes in animal systems encode transcription factors (for reviews, see Hayashi and Scott 1990; Weintraub et al. 1991; McGinnis and Krumlauf 1992).

Though less is known about developmental regulation in higher plants, recent evidence indicates that transcription factors also play a central role in plant development. In flower development, for example, a family of transcription factors that contain a conserved DNA-binding motif known as the MADS box (Ma et al. 1991) are required. One floral regulatory protein, the product of the

agamous gene, is involved in determining stamen and carpel development in *Arabidopsis* (Yanofsky et al. 1990). Development of another structure in *Arabidopsis*, the so-called trichomes or leaf hairs, involves two genetic loci termed *GL1* and *TTG* (Koornneef 1981). The product of the *GL1* gene, which is required to initiate the trichome developmental pathway, encodes a member of the Myb transcription factor family (Oppenheimer et al. 1991). In addition, a Myc homolog encoded by the maize *R* locus (Ludwig et al. 1989) can functionally complement an *Arabidopsis ttg* mutant (Lloyd et al. 1992), indicating that the *TTG* locus probably encodes a transcription factor. In maize, mutations in the homeo box gene *Knotted-1* cause alterations in leaf development (Vollbrecht et al. 1991).

In an attempt to identify new developmental regulatory genes in *Arabidopsis*, the isolation and characterization of homeo box genes was recently undertaken (Schena and Davis 1992). Two observations suggested a priori that the products of homeo box genes (i.e., homeo domain proteins) are good candidates for developmental regulators in *Arabidopsis*. First, considerable evidence indicates that mechanisms of transcriptional control have been highly conserved in eukaryotic organisms (for reviews, see Mitchell and Tjian 1989; Schena 1989; Struhl 1989; Ptashne and Gann 1990; Guarente and Bermingham-McDonogh 1992), including higher plants (Ma et al. 1988; Schena et al. 1991b; Katagiri and Chua 1992). Thus, the role of the homeo domain as a regulatory domain in animal systems (Gehring 1987; Scott et al. 1989; McGinnis and Krumlauf 1992), implied that this motif might also be found in developmental regulatory proteins from plants. Second, it has long been known that

¹Corresponding author.

Arabidopsis morphology changes dramatically in response to specific external stimuli such as changes in light intensity (for review, see Okada and Shimura 1992), indicating that developmental responses of this type may involve linkage of the environment to development via the products of developmental control genes.

Presented here is an analysis of the homeo box gene *HAT4* (homeo box from *Arabidopsis thaliana*), which encodes a member of the *Arabidopsis* homeo domain protein superfamily (Scheda and Davis 1992). *HAT4* was chosen for intensive analysis in part because it contains a novel 90-amino-acid segment termed the homeo domain-leucine zipper (HD-Zip) motif. HD-Zip proteins comprise a subfamily of homeo domain proteins in *Arabidopsis* (Ruberti et al. 1991; Mattsson et al. 1992; Scheda and Davis 1992) and have not yet been identified in animal systems. The apparent uniqueness of HD-Zip proteins to higher plants suggested that these factors might mediate novel developmental pathways in *Arabidopsis*, such as the coupling of development to the environment (Scheda and Davis 1992).

Complementary approaches in yeast and transgenic plants were used to examine *HAT4* function. In the yeast experiments a genetic selection was used to isolate point mutants of *HAT4*. The goal of the yeast experiments was to explore whether the HD-Zip domain contains a functional homeo domain and a leucine zipper, as sequence inspection suggests. The use of yeast to study transcription factors from higher plants is an extension of previous studies showing that basic mechanistic questions regarding mammalian transcription factor function are addressed more rapidly in yeast than in mammalian cells (Scheda and Yamamoto 1988; Scheda et al. 1989). In the second set of experiments, reverse genetics in whole plants was exploited to examine the developmental role of *HAT4*. The basic strategy of the transgenic plant experiments was to use a series of expression vectors to alter the levels of the *HAT4* protein in vivo. It was reasoned that if *HAT4* encodes a developmental regulator, reducing or elevating the levels of the protein should result in phenotypic changes.

Results

Structure of the *HAT4* gene

To determine the structure of the *HAT4* gene, genomic clones were isolated using the polymerase chain reaction (PCR). Sequence analysis of the isolated clones revealed the presence of three introns within the 1.35-kb *HAT4*-coding region, each bordered by nucleotides that closely conform to the AG/GT consensus for *Arabidopsis* splice sites (Fig. 1A). The overall structure of the *HAT4* gene is such that the putative functional domains are located on separate exons; thus, helix 1 of the homeo domain resides on exon 2, helices 2 and 3 share exon 3, and the leucine zipper maps to exon 4 (Fig. 1B).

Yeast slow growth reversal assay

Earlier studies revealed that a variety of eukaryotic

DNA-binding domains fused to acidic activation sequences are toxic in yeast (Scheda 1990; Wilson et al. 1991; Berger et al. 1992). The toxicity of the chimeric proteins, which is manifested as a slow growth phenotype, requires both a functional DNA-binding domain and an intact acidic activation sequence. The slow growth phenotype probably results from binding of the chimeric protein to recognition sites fortuitously present in the yeast genome, followed by the sequestration of essential components of the general transcription apparatus via the acidic activation and DNA-binding domains (Gill and Ptashne 1988; Scheda et al. 1989; Berger et al. 1992).

The dependency of slow growth on DNA binding suggested a new method for analyzing eukaryotic DNA-binding domains of unknown binding specificity, in which mutations that impair function are isolated simply by selecting mutants that reverse the slow growth phenotype in yeast. In this genetic scheme, each of the chimeric proteins also contains a DNA-binding domain from the bacterial LexA protein; thus, mutants that are selectively impaired in the function of the eukaryotic DNA-binding domain retain the capacity to bind to *lexA* DNA and are therefore capable of activating a reporter gene linked to a *lexA* operator. The presence of the LexA domain allows mutants of interest to be rapidly distinguished from those that are compromised in protein solubility or stability.

Expression of *HAT4* in yeast

As a first step toward developing a yeast assay for *HAT4* function, a 390-bp fragment of the *HAT4* cDNA encompassing the HD-Zip domain was inserted between sequences encoding the DNA-binding domain of the bacterial LexA protein and the transcriptional activation domains of yeast GAL4 (Fig. 2A). This chimera was then cloned into a high-copy-number vector and expressed in yeast. High-level expression of the LexA-*HAT4*-GAL4 chimeric protein markedly inhibited the growth of yeast cells relative to those expressing the LexA-GAL4 protein (Fig. 3), indicating that the slow growth phenotype required *HAT4* sequences.

HAT4 mutants isolated in yeast

To generate *HAT4* point mutants, the 390-bp *HAT4* segment was excised selectively from the LexA-*HAT4*-GAL4 molecule, randomly mutagenized using PCR, and reinserted into the expression plasmid. Plasmids were then introduced into *Escherichia coli* to yield $\sim 5 \times 10^4$ independent transformants (Fig. 2B). Plasmids encoding presumptive *HAT4* mutants were cotransformed into yeast cells with a *lexA* reporter plasmid fused to the β -galactosidase (β -gal) gene, and large yeast colonies (slow growth reversal mutants) were selected. The revertants were then secondarily screened for β -gal expression to distinguish mutants of interest from those impaired in solubility or integrity. Yeast clones that displayed high

A

```

ctt gag aaa gtt caa cag aag ATG ATG TTC GAG AAA GAC GAT CTG GGT CTA AGC TTA GGC      ct tat      5
Met Met Phe Glu Lys Asp Asp Leu Gly Leu Ser Leu Gly      65
TTG AAT TTT CCA AAG AAA CAG ATC AAT CTC AAA TCA AAT CCA TCT GTT TCT GTT ACT CCT      13
Leu Asn Phe Pro Lys Lys Gln Ile Asn Leu Lys Ser Asn Pro Ser Val Ser Val Thr Pro      125
TCT TCT TCT TCT TTT GGA TTA TTC AGA AGA TCT TCA TGG AAC GAG AGT TTT ACT TCT TCA      33
Ser Ser Ser Ser Phe Gly Leu Phe Arg Arg Ser Ser Trp Asn Glu Ser Phe Thr Ser Ser      185
G      53
gta ctt cat cgc ttt ctt cgt tcc tct gtt ttt ttt ccc ccg gaa aaa acc tta aag atc      246
tca cct ttt act cta ctt ttc aat ctt atg cat gca g      283

TT CCA AAC TCA GAT TCG TCA CAA AAA GAA ACA AGA ACT TTC ATC CGA GGA ATC GAC GTG      342
Val Pro Asn Ser Asp Ser Ser Gln Lys Glu Thr Arg Thr Phe Ile Arg Gly Ile Asp Val      73
AAC AGA CCA CCG TCT ACA GCG GAA TAC GGC GAC GAA GAC GCT GGA GTA TCT CCA CCA      402
Asn Arg Pro Pro Ser Thr Ala Glu Tyr Gly Asp Glu Asp Ala Gly Val Ser Ser Pro Asn      93
AGT ACA GTC TCA AGC TCT ACA GGG AAA AGA AGC GAG AGA GAA GAA GAC ACA GAT CCA CAA      462
Ser Thr Val Ser Ser Ser Thr Gly Lys Arg Ser Glu Arg Glu Glu Asp Thr Asp Pro Gln      113
GGC TCA AGA GGA ATC AGT GAT GAA GAT GGT GAT AAC TCC AGG AAA AAG CTT AGA CTT      522
Gly Ser Arg Gly Ile Ser Asp Asp Glu Asp Gly Asp Asn Ser Arg Lys Lys Leu Arg Leu      133
TCC AAA GAT CAA TCT GCT ATT CTT GAA GAG ACC TTC AAA GAT CAC AGT ACT CTC AAT CCG      582
Ser Lys Asn Gln Ser Ala Ile Leu Glu Glu Thr Phe Lys Asp His Ser Thr Leu Asn Pro      153

gta att ttt ttt tca ccc gag aaa att tgg ttt tac tgg gga aat aaa tgg cta tga taa      642
att gtg gaa atg aaa att aaa cgc gtg aat gag ata ttc aca tta aaa tga ata tta tta      702
cat gtt gac caa aga aaa aac att act act act att tgt ttc ggt cgc tca taa atg cag      762
ata tta att tat aaa tgg cgt ttt ggt aaa ttt aga ctg aca tca agg tta ttt tca act      822
ttt tta taa cag      834

AAG CAG AAG CAA GCA TTG GCT AAA CAA TTA GGG TTA CGA GCA AGA CAA GTG GAA GTT TGG      894
Lys Gln Lys Gln Ala Leu Ala Lys Gln Leu Gly Leu Arg Ala Arg Gln Val Gln Val Trp      173
TTT CAG AAC AGA CGA GCA AG      914
Phe Gln Asn Arg Arg Ala      179

gta aat taa aaa cac aca aac aaa aga tct ctc tat ttg cct gat tta tca att tta ctt      974
ata agg gta ttt tgg taa atg tgt taa cag      1004

A ACA AAG CTG AAG CAA ACG GAG GTA GAC TGC GAG TTC TTA      1044
Arg Thr Lys Leu Lys Gln Thr Glu Val Asp Cys Glu Phe Leu      193
CGG AGA TGC TGC GAG AAT CTA ACG GAA GAG AAC CGT CGG CTA CAA AAA GAA GTA ACG GAA      1104
Arg Arg Cys Cys Glu Asn Leu Thr Glu Glu Asn Arg Arg Leu Gln Lys Glu Val Thr Glu      213
TTG AGA GTA CTT AAG CTC TCT CCT CAG TTC TAC ATG CAC ATG AGC CCA CCC ACT ACT TTG      1164
Leu Arg Val Leu Lys Leu Ser Pro Gln Phe Tyr Met His Met Ser Pro Pro Thr Thr Leu      233
ACC ATG TGC CCT TCA TGT GAA CAC GTG TCG GTC CCG CCA CCA CCA CCT CAG GCT GCT ACG      1224
Thr Met Cys Pro Ser Cys Glu His Val Ser Val Pro Pro Pro Gln Pro Gln Ala Thr      253
TCA GCG CAC CAC CCG TCG TTG CCG GTC AAT GCG TGG GCT CCT GCT ACG AGG ATA TCT CAC      1284
Ser Ala His His Arg Ser Leu Pro Val Asn Ala Trp Ala Pro Ala Thr Arg Ile Ser His      273
GGC TTG ACT TTT GAC GCT CTT CGT CCT AGG TCC TAA gtc ttt tta ctt gca acc aaa ggg      1344
Gly Leu Thr Phe Asp Ala Leu Arg Pro Arg Ser OCH      284
cat ttt gg      1352

```

B

Figure 1. Structure of the *HAT4* gene. (A) Shown is the nucleotide sequence of the *HAT4* gene and its conceptual translation. Exon sequences are shown in uppercase letters, and intron or untranslated sequences are shown in lower-case letters. Residues corresponding to the three putative α -helices of the homeo domain are underlined, and the four heptadic leucines of the leucine zipper are shown in bold-face type. Numbers in the margin tabulate nucleotides and amino acids, respectively. The sequence of the *HAT4* gene has been submitted to GenBank/EMBL data bases under accession number Z19602. (B) Schematic illustration of the *HAT4* cDNA and gene. The three presumptive homeo domain (HD) α -helices and the heptadic leucines of the putative leucine zipper (Zip) are shown. Rectangular boxes and lines denote coding and noncoding sequences, respectively.

β -gal activity (i.e., expressed intact LexA–HAT4–GAL4 proteins) were characterized further.

A total of 14 mutants that reversed the slow growth phenotype and retained *lexA* DNA-binding activity were isolated using the yeast assay, and in all cases the phenotypes segregated with the *HAT4* segment. Sequence determination of the 14 inserts yielded 13 single mutants (Fig. 2C) and 1 double mutant (data not shown). Of the 13 single mutations, 12 mapped to the *HAT4* homeo domain and 1 to the leucine zipper; notably, 11 of the 13 mutations mapped to residues that are highly conserved in bona fide homeo domains and leucine zippers (Fig. 2C). The remaining two mutations mapped near conserved residues in homeo domain helix 1. β -Gal levels in cells containing the LexA–HAT4–GAL4 mutants were within twofold of the levels observed in yeast cells expressing the LexA–GAL4 protein (Table 1), indicating that the increased growth rate of the yeast cells was not attributable to decreased levels of the mutant proteins.

Transforming whole plants with *HAT4* expression constructs

To investigate the role of the *HAT4* in plant development, a series of expression vectors was constructed (Fig. 4). The vectors each contained the strongly constitutive 35S promoter from the cauliflower mosaic virus fused to one of the following: the *HAT4* cDNA in the antisense

orientation (35S– α HAT4) to reduce the level of HAT4; the developmentally inert β -glucuronidase gene (35S–GUS) as a wild-type control; or the *HAT4* cDNA in the sense orientation (35S–HAT4) to elevate the level of HAT4 (Fig. 4). Transgenic lines transformed with these constructs were selected using kanamycin, and a total of 39 independent first generation (T1) lines were obtained. Seeds from the self-crossed T1 lines were collected, and second generation (T2) plants from 18 of the 39 lines were examined for phenotypic alterations.

Altered *HAT4* expression affects Arabidopsis development

The presence of the expression constructs in the T2 lines was confirmed using PCR. Of the 18 T2 lines examined, 16 exhibited 3 : 1 segregation of the kanamycin resistance marker, indicating that all but 2 of the original T1 lines had acquired copies of the transgene at a single genomic locus (Table 2). Examination of the T2 seedlings revealed interesting developmental phenotypes in nearly all of the transgenic lines. Kanamycin-resistant seedlings of two of the six 35S– α HAT4 lines had shorter hypocotyls (portion of the stem connecting the first two leaves to the roots), larger and more numerous leaves, and a thicker root mass than the wild-type (35S–GUS) controls (Table 2; Fig. 5). Seedlings expressing the 35S–HAT4 construct exhibited roughly reciprocal phenotypes com-

Figure 2. Yeast slow growth reversal assay. (A) The LexAHAT4GAL4 chimeric activator. A fragment of the *HAT4* cDNA encoding the HD-Zip domain was inserted between *lexA* and *GAL4*, yielding a fusion encoding the LexAHAT4GAL4 protein (rectangles). Unique restriction sites bordering the *HAT4* sequence are shown above, and the known or putative functional properties of each domain and the number of residues encoded by each segment are shown below. (B) Schematic illustration of the mutagenesis and selection scheme. The LexAHAT4GAL4 protein causes slow growth in yeast. To isolate mutants impaired in HD-Zip function, the *HAT4* segment was excised, randomly mutagenized using PCR, and reintroduced into the fusion to create a large pool of *HAT4* mutants. The plasmid pool was then cotransformed with a *lexA*-*CYC1*- β -galactosidase reporter plasmid (p1155) into yeast strain BJ2168, and large colonies (i.e., slow growth reversal mutants) that exhibited a β -gal⁺ phenotype were isolated (see Materials and methods). (C) *HAT4* HD-Zip mutants isolated in yeast. Shown is the amino acid sequence of *HAT4* (residues 107–237) encompassing the HD-ZIP motif. Residues of *HAT4* corresponding to positions that are highly conserved in known homeo domains and leucine zippers are shown in boldface type, and the amino acid changes of the *HAT4* mutants are designated by arrows.

pared with the lines expressing the antisense construct. Kanamycin-resistant seedlings of all nine 35S-*HAT4* lines had longer hypocotyls, smaller and fewer leaves, a thinner root mass, and a darker green color than wild-type (35S-GUS) controls (Table 2; Fig. 5).

Figure 3. Expression of the LexAHAT4GAL4 protein in yeast. Yeast cells [BJ2168] transformed with a plasmid (pG-LexAHAT4GAL4) expressing LexAHAT4GAL4 (B) grow more slowly than the same strain transformed with a plasmid (pG-LexAGAL4) expressing LexAGAL4 (A). Both of the expression plasmids are based on pG-1 (Schena et al. 1991a) and thus also contain the glycerol-3-phosphate dehydrogenase (GPD) promoter, the 3-phosphoglycerate kinase (PGK) transcription terminator, the yeast *TRP1* gene, the yeast 2 μ origin of replication, and the bacterial origin of replication and ampicillin resistance gene from pUC18.

The phenotypes of adult T2 plants were similar to seedlings but more exaggerated (Fig. 6). As a result of *HAT4*-dependent changes in morphology and developmental rate, the formation of the first siliques (seed pods) was retarded by ~10% in 35S- α HAT plants and accelerated by approximately twofold in 35S-*HAT4* plants relative to the wild type (Table 2; Fig. 6). Seed production from 35S-*HAT4* plants was routinely 25-fold greater than from wild-type plants of the same age (Fig. 6).

Developmental phenotypes correlate with *HAT4* gene dosage and transcript levels

Third-generation (T3) seedlings derived from self-crossed T2 plants exhibited 3 : 1 or 1 : 0 segregation of kanamycin resistance, as expected for a dominant marker (Table 3). The phenotype of T3 seedlings homozygous for the 35S-*HAT4* transgene was markedly more severe than in the heterozygous T3 seedlings derived from the same parent line (Fig. 7). Crosses of T2 plants homozygous or heterozygous for a given 35S marker to wild-type plants yielded progeny that segregated kanamycin resistance 1 : 0 and 1 : 1, respectively. The altered developmental phenotypes segregated exclusively with the kanamycin marker in >100 seedlings examined from representative lines (data not shown).

The levels of the *HAT4* transcript were assessed in both wild-type plants and in representative transgenic lines by Northern analysis. *HAT4* expression in wild-

Table 1. Point mutants of *HAT4*

Plasmid	Insert	Codon change	Residue change	β-Gal
pG-1	None	N.A.	N.A.	3
pG-LexAGAL4	LexA-GAL4	N.A.	N.A.	3781
pG-LexAHAT4GAL4	LexA-HAT4-GAL4	wild type	wild type	N.D.
pG-T3	LexA-HAT4-GAL4	TCC > TTC	Ser134 > Phe	2048
pG-T51	LexA-HAT4-GAL4	TCT > CCT	Ser138 > Pro	2363
pG-T6	LexA-HAT4-GAL4	CTT > CGT	Leu141 > Arg	2166
pG-T15	LexA-HAT4-GAL4	CTT > CGT	Leu141 > Arg	2159
pG-M25	LexA-HAT4-GAL4	CTT > CCT	Leu141 > Pro	2740
pG-T28	LexA-HAT4-GAL4	CTT > CCT	Leu141 > Pro	2721
pG-T52	LexA-HAT4-GAL4	CTT > CCT	Leu141 > Pro	2461
pG-T72	LexA-HAT4-GAL4	TTC > TCC	Phe145 > Ser	1912
pG-M14	LexA-HAT4-GAL4	TTA > TCA	Leu165 > Ser	1930
pG-M7	LexA-HAT4-GAL4	GTG > GCG	Val170 > Ala	1835
pG-M78	LexA-HAT4-GAL4	GTG > GCG	Val170 > Ala	1582
pG-M15	LexA-HAT4-GAL4	TGG > CGG	Trp173 > Arg	1950
pG-T4	LexA-HAT4-GAL4	CTA > CCA	Leu200 > Pro	1673

Yeast pG-1-based expression plasmids encode LexAHAT4GAL4 mutants that contain single-amino-acid changes in the HAT4 HD-Zip domain (see Fig. 2C). To confirm the integrity of the LexAHAT4GAL4 mutants, expression plasmids were cotransformed with a *lexA-CYC1*-β-gal reporter plasmid (p1155) into yeast strain BJ2168, and transformants were assayed for β-gal activity (see Materials and methods). β-Gal values correspond to the average of triplicate measurements, which varied by <20% and were consistent from day to day. (N.A.) Not applicable; (N.D.) not determined.

type plants was observed during all stages of development and in all tissues examined, although transcript levels were considerably higher in stems and leaves than in flowers (Fig. 8). Examination of the transgenic lines revealed the expected changes in *HAT4* expression; namely, plants expressing the 35S-αHAT4 construct had ~10-fold lower levels of the *HAT4* transcript relative to lines harboring the 35S-GUS construct, whereas those expressing the 35S-HAT4 construct possessed ~10-fold elevated levels of *HAT4* mRNA (Fig. 8).

Potential role of light in *HAT4* function

Arabidopsis morphology adapts profoundly to environ-

Figure 4. Vectors for altering *HAT4* expression in transgenic plants. The intact *HAT4* cDNA in the antisense orientation, the β-glucuronidase (*GUS*) gene, or the *HAT4* cDNA in the sense orientation were inserted downstream of the strongly constitutive 35S promoter from the cauliflower mosaic virus to yield expression vectors 35S-αHAT4, 35S-GUS, and 35S-HAT4, respectively. Plants expressing these constructs are predicted to have the designated *HAT4* protein levels. All plasmids are pBI121 (Clontech) derivatives and thus also contain a transcriptional terminator from the nopaline synthase gene situated 3' of the inserted cDNAs, an RK2 bacterial origin of replication, and a kanamycin resistance gene for selection in bacteria and *A. bidopsis*.

mental stimuli such as changes in light intensity (Fig. 9), suggesting that developmental regulators may play a role in these adaptive responses. To investigate whether any of the transgenic lines with altered *HAT4* expression possess altered light responses, seeds from various transgenic lines were grown in absolute darkness and analyzed for phenotypic alterations. The 35S-αHAT4 seeds germinated normally, giving rise to seedlings that exhibited an etiolated morphology (white, highly elongated, leafless developmental profile) characteristic of wild-type dark-grown plants; in contrast, >50% of the seeds from three independent 35S-HAT4 lines were totally defective in germination (Fig. 10). The germination defect of 35S-HAT4 seeds was light reversible; interestingly, dark treatment of seeds that overproduce *HAT4* yielded plants that were much larger than those derived from untreated seeds, suggesting that darkness and *HAT4* overproduction may act in a synergistic manner to promote plant growth in the light (Fig. 11).

Discussion

It is interesting that the putative functional domains of *HAT4* reside on separate exons. Such a configuration is consistent with the evolutionary model of exon shuffling (Dorit et al. 1990), which would postulate that the *HAT4* gene was assembled during evolution from smaller functional units encoding individual α-helices of the homeo domain (exons 2 and 3) and the leucine zipper coiled coil (exon 4). No compelling information derives from conceptual translation of the first exon of *HAT4*, although exon 1 may encode a transcriptional regulatory domain.

Several lines of genetic evidence suggest that *HAT4* contains a functional HD-Zip domain. First, all of the mutants isolated using the yeast assay map to the 90-amino-acid (270-bp) HD-Zip domain, despite the fact

Table 2. Analysis of second-generation (T2) transgenic *Arabidopsis* lines

T1 line	Vector	Background	T2 Kan ^R /Kan ^S	T2 hypocotyl (mm)	T2 silique (days)	T2 phenotype
1429	35S- α HAT4	Col-O	60 : 24	1.8 $\pm$ 0.2	34 $\pm$ 2.7	short
1431	35S- α HAT4	Col-O	65 : 20	1.9 $\pm$ 0.3	35 $\pm$ 1.9	short
1478	35S- α HAT4	RLD-O	60 : 21	2.7 $\pm$ 0.5	39 $\pm$ 2.7	normal
1479	35S- α HAT4	RLD-O	69 : 24	2.7 $\pm$ 0.4	41 $\pm$ 2.3	normal
1481	35S- α HAT4	RLD-O	59 : 19	2.6 $\pm$ 0.4	38 $\pm$ 1.7	normal
1482	35S- α HAT4	RLD-O	74 : 5	2.9 $\pm$ 0.4	42 $\pm$ 2.0	normal
1419	35S-GUS	RLD-O	71 : 7	2.6 $\pm$ 0.3	40 $\pm$ 2.1	normal
1420	35S-GUS	RLD-O	64 : 23	2.5 $\pm$ 0.5	42 $\pm$ 1.8	normal
1421	35S-GUS	Col-O	61 : 16	2.4 $\pm$ 0.3	30 $\pm$ 1.2	normal
1525	35S-GUS	Col-O	51 : 14	2.5 $\pm$ 0.4	29 $\pm$ 1.3	normal
1423	35S-HAT4	RLD-O	57 : 16	4.2 $\pm$ 1.1	23 $\pm$ 1.2	tall
1425	35S-HAT4	Col-O	73 : 21	6.7 $\pm$ 0.4	16 $\pm$ 2.3	tall
1426	35S-HAT4	Col-O	62 : 22	6.7 $\pm$ 0.4	16 $\pm$ 1.9	tall
1427	35S-HAT4	Col-O	73 : 20	6.9 $\pm$ 0.4	17 $\pm$ 1.6	tall
1468	35S-HAT4	RLD-O	96 : 35	3.5 $\pm$ 0.8	N.D.	tall
1469	35S-HAT4	RLD-O	28 : 11	3.6 $\pm$ 1.1	N.D.	tall
1471	35S-HAT4	RLD-O	97 : 33	4.9 $\pm$ 0.6	24 $\pm$ 1.4	tall
1472	35S-HAT4	RLD-O	121 : 42	5.7 $\pm$ 0.8	22 $\pm$ 2.5	tall
1474	35S-HAT4	Col-O	55 : 27	6.5 $\pm$ 0.9	N.D.	tall

Columbia (Col-O) or RLD (RLD-O) *Arabidopsis* roots were transformed with the designated expression vectors and regenerated into first-generation (T1) plants. Seeds from self-crossed T1 plants were collected, plated on agar, and grown under bright light for 10 days. These second-generation (T2) seedlings were then scored for resistance (Kan^R) or sensitivity (Kan^S) to kanamycin, and hypocotyls from 20 representative kanamycin-resistant seedlings were measured and recorded as standard deviations of the mean. Ten representative 7-day-old kanamycin-resistant seedlings from the same plates were also transferred to soil, and the appearance of the first seed pod (silique) on each plant was recorded and calculated as a standard deviation of the mean. The developmental phenotypes and additional experimental details are described in Results and Materials and methods, respectively. (N.D.) Not determined.

that a 390-bp segment of the *HAT4* cDNA was mutated. Second, 10 of the 12 homeo domain mutations within the 90-amino-acid domain map specifically to residues that are highly conserved in known homeo domains (Scott et al. 1989; Gehring et al. 1990); furthermore, the leucine zipper mutation (Leu200Pro) occurs at a position shown in GCN4 to be sensitive to proline substitution (Hu et al. 1990). The preponderance of mutations in the

homeo domain relative to the leucine zipper is consistent with the compact structural configuration of the homeo domain (Kissinger et al. 1990; Wolberger et al. 1991) relative to the extended conformation of the coiled coil (O'Shea et al. 1989, 1991). The two remaining mutations (Ser134Phe and Ser138Pro) both map to homeo domain helix 1 and may impair HD–Zip function by disrupting the packing or folding of the α -helix.

Figure 6. Effect of altered *HAT4* expression on adult *Arabidopsis* development. Seeds were plated on agar and grown under bright light for 7 days. Representative seedlings were then transferred to soil, and grown under bright light for an additional 28 days (see Materials and methods). Photographs of representative plants were taken and correspond to progeny of the following T1 lines (Table 2): (left) 35S- α HAT4 line 1429; (center) 35S-GUS line 1421; and (right) 35S-HAT4 line 1426. Shown below the photograph is the presumptive HAT4 protein level in each plant.

The spacing between the homeo domain and the leucine zipper of HAT4 is identical to the distance between the basic region and the leucine zipper of CCAAT/enhancer-binding protein (C/EBP) (Landschulz et al. 1988), suggesting that HD-Zip and bZIP proteins may recognize DNA in a similar manner. Our initial modeling studies are consistent with the scissors-grip model (Vinson et al. 1989) for DNA recognition, in which leucine zipper-linked HAT4 monomers utilize bifurcated homeo domains to contact successive major grooves [M. Schena and R.W. Davis, unpub.]. Of the 12 residues of engrailed that directly contact DNA (Kissinger et al. 1990), 10 are identical or conservatively substituted in HAT4, suggesting that these two proteins may bind to an identical or similar dyad-symmetric DNA sequence. The putative leucine zipper of HAT4 contains, in addition to four heptadic leucines, hydrophobic residues at every fourth position relative to the conserved leucines as would be required for interhelical hydrophobic contacts between monomers (Landschulz et al. 1988; O'Shea et al. 1991). One novel feature of the HAT4 leucine zipper is the presence of three cysteine residues clustered near the first

leucine (Leu193) of the zipper, suggesting that HAT4 monomers may form covalent homo- or heterodimers, as has been observed for C/EBP-related proteins (Williams et al. 1991).

Consistent with the developmental role of homeo domain proteins in other organisms (Hayashi and Scott 1990; Lewis 1992), plants with altered *HAT4* expression exhibit interesting developmental phenotypes. Plants expressing a *HAT4* antisense construct are shorter and develop more slowly than normal, whereas those expressing a *HAT4* sense construct are taller and develop more rapidly. One interpretation of these results is that the HAT4 protein normally functions as a master regulator of developmental rate and that reducing or elevating the level of HAT4 slows or accelerates the rate of development, respectively. This proposal would predict that *HAT4* should be widely expressed during *Arabidopsis* development. *HAT4* mRNA is detectable in all tissues examined and during all stages of development; moreover, transgenic plants with reciprocal developmental phenotypes have correspondingly elevated or reduced levels of *HAT4* mRNA relative to the wild-type

Table 3. Analysis of third-generation (T3) transgenic *Arabidopsis* lines

T1 line	Vector	T2 line	T3 Kan ^R /Kan ^S	T3 hypocotyl (mm)	T3 phenotype
1425	35S-HAT4	1425-2.1	115 : 45	8.1 $\pm$ 0.8	tall
1426	35S-HAT4	1426-2.2	106 : 38	8.3 $\pm$ 0.6	tall
1426	35S-HAT4	1426-2.5	69 : 26	8.5 $\pm$ 0.6	tall
1427	35S-HAT4	1427-2.1	81 : 28	8.7 $\pm$ 0.7	tall
1427	35S-HAT4	1427-2.2	118 : 43	9.0 $\pm$ 0.8	tall
1427	35S-HAT4	1427-2.7	119 : 0	8.2 $\pm$ 0.6	tall

Seeds from self-crossed second generation (T2) *Arabidopsis* lines were plated, and the third-generation (T3) seedlings were grown and analyzed as described in the Table 2 footnote and in Materials and methods.

Figure 7. Effect of transgene dosage on seedling development. Seeds were plated on agar and grown for 10 days under bright light (see Materials and methods). Representative seedlings were chosen for the photographs and correspond to progeny of the following T2 lines (Table 3): (A) 35S-HAT4 line 1427-2.2; and (B) 35S-HAT4 line 1427-2.7. Shown below each photograph is the 35S-HAT4 genotype based on the segregation of kanamycin resistance in the T3 lines (Table 3).

plants. Although it is not currently possible to measure the levels of the HAT4 protein in the transgenic lines, comparison of homozygous and heterozygous lines derived from the same parent suggests that less than a two-fold change in the level of the protein is sufficient to alter development significantly.

An alternative explanation for the developmental changes observed in the transgenic plants is that the phenotypes have nothing to do with normal HAT4 function but, rather, are an indirect consequence of altering the expression of the gene. According to this argument, HAT4 overproduction might induce an alternate pathway such as a stress response in *Arabidopsis*, which would lead indirectly to developmental changes. Although this explanation is difficult to exclude, several lines of evidence suggest that this model is unlikely. First, except for an increase in developmental rate, plants that overproduce HAT4 fail to exhibit any of the phenotypes normally associated with stress responses such as elevated anthocyanin production, bleaching, necrosis, or reduced fertility. Second, plants harboring an antisense construct exhibit developmental changes that are reciprocal to those seen in plants transformed with a sense construct, suggesting that the physiological role of the gene is to control development. Third, overproduction of other DNA-binding proteins such as steroid receptors, Myc and Myb homologs, and the FLP and Cre recombination factors in plants fails to elicit any of the phenotypes observed when HAT4 expression is altered (M. Schena, A.M. Lloyd, and R.W. Davis, unpubl.).

Plants with altered HAT4 levels undergo morphological changes reminiscent of those that accompany envi-

Figure 8. HAT4 expression in wild-type and transgenic plants. Samples of total RNA from wild-type (lanes 1–6) or transgenic (lanes 7–11) plants were fractionated by agarose gel electrophoresis, transferred to a hybridization membrane, and probed with the intact (1.2-kb) HAT4 cDNA (Schena and Davis 1992) radiolabeled by random priming. RNA was isolated from wild-type plants at various times after germination (lanes 1–3), or from various adult (6-week) tissues (lanes 4–6). The transgenic lines (Table 2), propagated under kanamycin selection, are as follows: 35S-αHAT4 line 1429 (lane 7), 35S-GUS line 1421 (lane 8), 35S-HAT4 line 1425 (lane 9), 35S-HAT4 line 1426 (lane 10), and 35S-HAT4 line 1427 (lane 11). The position of the HAT4 transcript is indicated (arrow), as are the positions of the ethidium-stained rRNAs (25S and 18S). Additional experimental details are provided in Materials and methods.

Figure 9. Effect of light intensity on *Arabidopsis* seedling development. Seeds were plated on agar and grown for 10 days under either bright light (A) or total darkness (B) (see Materials and methods). Representative seedlings were chosen for the photographs and correspond to progeny of the 35S-GUS T1 line 1421 (Table 2).

Figure 10. Effect of altered *HAT4* expression on germination. Seeds were plated on agar and grown for 10 days in total darkness (see Materials and methods). Representative seedlings were chosen for the photographs and correspond to seeds from the following T1 lines (Table 2): (A) 35S- α HAT4 line 1431; (B) 35S-GUS line 1421; and (C) 35S-HAT4 line 1425. Given below each photograph is the presumptive HAT4 protein level in each line.

ronmental stimuli such as signaling by light (Okada and Shimura 1992) and touch (Braam and Davis 1990). The coupling of growth to the environment in plants is conceptually similar to adaptive responses in other nonmotile organisms such as pseudohyphal growth in *Saccharomyces cerevisiae* (Gimeno et al. 1992). The etiolation response of higher plants, for example, is analogous to the polarized growth of diploid yeast cells; plants subjected to darkness elongate to reach sunlight, just as yeast cells extend pseudohyphal filaments to forage for a richer nitrogen source (Gimeno et al. 1992; Gimeno and Fink 1992). Sessile organisms such as higher plants and yeast are thus able to respond to nutritional shortages by altering their growth patterns, suggesting that highly sophisticated signaling pathways link the environment to development in plants and fungi. The fact that the HD-Zip motif has been found in higher plants but not in animals is consistent with the proposal that this motif may be selectively utilized by plants for developmental

plasticity (Schna and Davis 1992), a capacity not typically required in animal systems. It may be that homo- or heterodimer interactions of HD-Zip proteins impart on higher plants the capacity to parlay small fluctuations in environmental conditions into complex and reversible developmental changes.

Overproduction of HAT4 results in a germination defect in the dark, suggesting a possible link between HAT4 and light signaling. Two additional observations are also consistent with the possible cross-talk between light signaling and HAT4 function. First, dark treatment of seeds and HAT4 overproduction have synergistic effects on the plant growth. Second, plants with elevated HAT4 levels are darker green than wild-type plants, suggesting that chlorophyll production has been stimulated. Perhaps plants with elevated levels of HAT4 perceive that the light intensity has been reduced and hence, respond to transgenic elevations of HAT4 by increasing chlorophyll production. Mutants of *Arabidopsis* with al-

Figure 11. Light reversibility of the germination defect. Seeds from the 35S-HAT4 line 1425 (Table 2) were grown on agar under the following conditions: (A) 10 days of total darkness; (B) 10 days of total darkness followed by 3 days of bright light; (C) 10 days of total darkness followed by 10 days of bright light; or (D) 10 days of bright light (see Materials and methods). Photographs of representative seedlings were taken. The number of days grown in total darkness or bright light is given below each photograph.

tered growth and light responses (Okada and Shimura 1992) should aid in determining the mechanism of HAT4 action; moreover, the direct isolation of HAT4 target genes using molecular strategies should provide considerable insight into this signaling pathway. Given that both flowering and germination depend on a number of other parameters, including the levels of plant hormones, it will also be interesting to test whether substances such as gibberellins, auxins, or cytokinins are altered in the transgenic lines.

The capacity to accelerate the rate of *Arabidopsis* development using a single gene suggests possible commercial uses for plant developmental regulatory genes. Overexpression of homeo box genes may provide a general means by which to modify crop plant development. It is interesting to note that overexpression of *HAT4* in tobacco leads to phenotypic changes that mimic some of the phenotypes observed in *Arabidopsis* (M. Schena, A.M. Lloyd, and R.W. Davis, unpubl.). It will be interesting to investigate whether HAT4 or HAT4 homologs exert a similar effect on the development of other commercially important plants, a possibility that can be tested using direct transfer of the *HAT4* cDNA or related clones. Given the existence of a large family of homeo box genes, it may ultimately be possible to custom design commercial plants to meet specific agricultural and biotechnological needs.

Materials and methods

Genomic sequence determination

Total genomic DNA was isolated (Richards 1988) from *Arabidopsis* (Columbia) seedlings, purified by cesium chloride ultracentrifugation, and subjected to PCR amplification using primers derived from the sequence of the *HAT4* cDNA (Schena and Davis 1992). Five separate reactions containing oligonucleotides PAN17 and PAN31 were prepared and amplified as described (Sambrook et al. 1989). The 1.35-kb reaction products were then cleaved with *Xba*I and *Sac*I, cloned into Bluescript II KS⁺ (Stratagene), and sequenced using an automated sequencer (Applied Biosystems, Inc).

The sequences of the HAT4 oligonucleotide primers are as follows. PAN17: 5'-GGGGGGTCTAGACTTATCTTGAGAAAGTTCAA-3' (position 1 in Fig. 1) and PAN31: 5'-GGGGGGGAGCTCCCAAATGCCCTTTGGTTGC-3' (position 1333 in Fig. 1).

Plasmid constructions

The yeast expression plasmid pG-LexAGAL4 was constructed by excising the *lexAGAL4* sequence from p1027 (Brent and Ptashne 1985) with *Hind*III, adding *Bam*HI sites to both the 5' and 3' ends, and inserting the modified *lexAGAL4* fragment into the *Bam*HI site of pG-1 (Schena et al. 1991a). The 5' *Bam*HI site in *lexAGAL4*, introduced using PCR oligonucleotides PAN22 and PAN23, is located 10 bp upstream of the *lexA* start codon (Horii et al. 1981). The 3' *Bam*HI site in *lexAGAL4* was introduced by end-fill of a *Hind*III site located ~240 bp downstream of the *GAL4* stop codon (Laughon and Gesteland 1984) and attachment of a synthetic *Bam*HI linker. The junction between *lexA* (residues 1–87) and *GAL4* (residues 74–881) contains a unique *Xho*I site (Brent and Ptashne 1985). Yeast expres-

sion plasmid pG-LexAHAT4GAL4 was made by converting the *Xho*I site in pG-LexAGAL4 into a *Sac*I site, using PCR oligonucleotides PAN22 and PAN23, and inserting a 390-bp fragment of the *HAT4* cDNA (encoding residues 107–237) generated by PCR with PAN24 and PAN20, followed by cleavage with *Sac*I and *Xho*I. Plasmid sizes are ~10 kb. [When requesting yeast expression plasmids from the Davis laboratory, please use the following nomenclature: pNN486 (pG-LexAGAL4) and pNN487 (pG-LexAHAT4GAL4).]

The plant expression vectors p35S-HAT4 and p35S- α HAT4 were constructed by excising the β -glucuronidase (*GUS*) gene from p35S-GUS (pBI121, Clontech) using *Xba*I and *Sac*I, and inserting *HAT4* cDNA sequences (Schena and Davis 1992) in either the sense (p35S-HAT4) or antisense (p35S- α HAT4) orientation. In p35S-HAT4, the *Xba*I site was introduced 26 bp upstream of the *HAT4* start codon using PCR oligonucleotides PAN17 and PAN18; the *Sac*I site was introduced ~150 bp downstream of the *HAT4* translation stop codon by subcloning the cDNA into pUC18, followed by excision with *Sac*I. In p35S- α HAT4, the insert is identical to the one present in p35S-HAT4 except that it resides in the opposite orientation; *Sac*I and *Xba*I sites were added to *HAT4* by subcloning into Bluescript II KS⁺ (Stratagene) and pUC18, followed by excision with *Sac*I and *Xba*I, respectively. Plasmid sizes are ~13 kb. [When requesting plant expression plasmids from the Davis laboratory, please use the following nomenclature: pNN488 (p35S- α HAT4), pNN489 (p35S-GUS), and pNN490 (p35S-HAT4).]

The sequences of the PCR oligonucleotide primers are as follows. PAN22: 5'-GGGGGGGATCCAGGGGGCGGAATGAAGC-3' (upstream of *lexA* AUG); PAN23: 5'-GCGCGCGAGCTCAGGTTACCGGCAGCCACACG-3' (*lexA* codon 87); PAN24: 5'-GGGGGGGAGCTCGAAGAAGACACAGATCCACAA-3' (*HAT4* codon 107); PAN20: 5'-GCGCGCTCGAGGCGACATGGTCAAAGTAG-3' (*HAT4* codon 237); PAN18: 5'-CGTTCATGAAGATCTTCTGA-3' (position 147 in Fig. 1).

PCR mutagenesis

The 390-bp *HAT4* segment of pG-LexAHAT4GAL4 was excised using *Sac*I and *Xho*I, inserted into Bluescript II KS⁺, and mutagenized by PCR amplification with T3/T7 or M13forward/M13 reverse primer pairs. Six 50- μ l reactions containing 10 ng of supercoiled DNA, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM dNTPs, 50 pmoles of each primer, and 5 units of *Taq* polymerase (Promega) were overlaid with 100 μ l of mineral oil and incubated in a thermal cycler (Perkin Elmer Cetus) programmed as follows: 1 cycle, 94°C for 5 min; 25 cycles, 94°C for 1 min, 50°C for 2 min, 72°C for 1 min; 1 cycle, 72°C for 10 min. Reaction products were digested with *Sac*I and *Xho*I, fractionated on low-melt agarose, ligated into *Sac*I/*Xho*I-linearized pG-LexAHAT4GAL4 vectors, and transformed into *E. coli* by electroporation. Plasmids derived from the three T3/T7 primer reactions or from the three M13 forward/M13 reverse primer reactions yielded 2×10^4 bacterial transformants (T pool) and 3×10^4 transformants (M pool), respectively. Plasmid DNA from the T pool and M pool transformants was purified using Qiagen columns as recommended by the manufacturer.

Yeast slow growth reversal assay

Competent cells of yeast strain BJ2168 (MATa, *pep4-3*, *prc1-407*, *prb1-1122*, *ura3-52*, *trp1*, *leu2*), grown using standard media (Sherman 1991), were prepared using the lithium acetate procedure (Ito et al. 1983) except that the carrier DNA was denatured to increase the transformation efficiency (Schiestl

and Gietz 1989). Aliquots of the mutagenized pG–LexAHAT4–GAL4 plasmid pools (T and M) were cotransformed with a *lexA*–CYC1- β -galactosidase reporter plasmid p1155 (Brent and Ptashne 1985) into competent BJ2168 cells and plated on minimal agar lacking uracil and tryptophan at a density of 250 transformants per 10-cm plate. A total of 5000 transformants yielded 26 colonies that exhibited a slow growth reversal/ β -gal⁺ phenotype. DNA from 13 of the 26 yeast clones was isolated (Schena et al. 1989), and pG–LexAHAT4GAL4 plasmids were recovered by transformation into *E. coli*. Inserts from each of the 13 plasmids were liberated by digestion with *Xho*I and *Sac*I, inserted into Bluescript II KS⁺, and sequenced using an automated sequencer. The inserts derived from the T pool (T3, 4, 6, 15, 28, 51, 52, 72) and the M pool (M7, 14, 15, 25, 78) were then reintroduced into pG–LexAHAT4GAL4 lacking the *HAT4* sequence and transformed into yeast strain BJ2168, and β -gal assays were performed using at least three independent transformants (Yocum et al. 1984).

Arabidopsis transformation

Expression plasmids p35S– α HAT4, p35S–GUS and p35S–HAT4 were introduced into *Agrobacterium tumefaciens* strain LBA4404 (Clontech) by electroporation (Nagel et al. 1990). *Arabidopsis* lines (Col-O or RLD-O) were then transformed by *Agrobacterium*-mediated root transformation (Valvekens et al. 1988) and regenerated in the presence of kanamycin to select for stable integration of the expression plasmids. Regenerants were moved to soil ~8 weeks after transformation, and seed from 18 independent lines was collected ~4 weeks later.

Arabidopsis growth and phenotypic analysis

Seeds were surface sterilized (two treatments for 10 min with 20% bleach, 0.05% Triton X-100) and plated aseptically in 10 cm $\times$ 2.5-cm petri dishes (Nunc) containing 25 ml of agar (pH 5.8) consisting of 1 $\times$ MS salts (GIBCO), 0.7% Bacto-agar, 2% sucrose, 1 $\times$ vitamins (1.3 μ g/ml of nicotinic acid, 0.25 μ g/ml of thiamine, 1.0 μ g/ml of pyridoxine, and 0.25 μ g/ml of pantothenic acid), and 50 μ g/ml of kanamycin. Dishes were sealed with parafilm, incubated in growth chambers at 22°C under continuous bright light (3.2 $\times$ 10³ lux), and scored for kanamycin resistance or hypocotyl length after 10 days. Photographs of representative 10-day-old kanamycin resistant seedlings were taken using Ultra 50 color film (Agfa) in a Zeiss Stemi SV6 photomicroscope set at eightfold magnification. Dark experiments were performed exactly as described above except that dishes containing sterile seeds were wrapped in a double layer of aluminum foil and incubated in a dark growth chamber. DNA was isolated from T2 seedlings derived from each of the T1 lines and assayed for the presence of a given expression vector using the PCR oligonucleotide PAN36, together with PAN19 (35S– α HAT4), PAN37 (35S–GUS), or PAN20 (35S–HAT4).

After 7 days of growth on kanamycin plates, seedlings were transferred from petri dishes to 2.5-cm pots containing Sunshine Mix potting soil (Fisons Horticulture, Inc.). Plants were grown under continuous bright light (4.8 $\times$ 10³ lux) at 24°C, watered regularly using Peters 20-20-20 soluble plant food (Grace-Sierra Horticultural Products Company) at 200 mg/liter, and scored for the formation of siliques by visual inspection. Photographs of representative 28-day-old plants were taken using an Olympus OM-2 camera equipped with a 50-mm Zuiko lens and Ultra 50 color film (Agfa).

The sequences of the PCR oligonucleotide primers are as follows: PAN36: 5′-GATGTGATATCTCCACTGAC-3′ (35S promoter); PAN19: 5′-GGGGGGCTCGAGAAGAAGACACAGA-

TCCA-3′ (*HAT4* antisense); and PAN37: 5′-ACGCTGCGATG-GATCCCGGC-3′ (GUS gene).

RNA analysis

Total RNA was isolated and analyzed as described previously (Schena and Davis 1992), with certain modifications. Pulverized tissue was homogenized in a buffer containing equal volumes of phenol/chloroform/isoamyl alcohol [50 : 50 : 1 (vol/vol)] and yeast RNA extraction buffer (150 mM NaCl, 5 mM EDTA, 4% SDS, 50 mM Tris-Cl at pH 7.5) and precipitated with ethanol. The RNA was resuspended in TE buffer and subjected to two additional rounds of phenol extraction and ethanol precipitation.

Acknowledgments

We thank Michael Campbell, Anna Astromoff, Matt Scott, and Grace Yen for critical comments on the manuscript, and Daphne Preuss, Chris Lee, and Keith Yamamoto for helpful insights. We also thank members of the PAN Facility at Stanford University for oligonucleotide synthesis and DNA sequencing. This work was supported by grants from the National Science Foundation (DMR-91060-11) and the National Institutes of Health (8 R37 HG00198-17) to R.W.D.; M.S. holds a National Science Foundation Postdoctoral Fellowship in Plant Biology.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Berger, S.L., B. Piña, N. Silverman, G.A. Marcus, J. Agapite, J.L. Regier, S.J. Triezenberg, and L. Guarente. 1992. Genetic isolation of ADA2: A potential transcriptional adaptor required for function of certain acidic activation domains. *Cell* **70**: 251–265.
- Braam, J. and R.W. Davis. 1990. Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell* **60**: 357–364.
- Brent, R. and M. Ptashne. 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* **43**: 729–736.
- Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**: 987–1000.
- Dorit, R.L., L. Schoenbach, and W. Gilbert. 1990. How big is the universe of exons? *Science* **250**: 1377–1382.
- Gehring, W.J. 1987. Homeo boxes in the study of development. *Science* **236**: 1245–1252.
- Gehring, W.J., M. Müller, M. Affolter, A. Percival-Smith, M. Billeter, Y.Q. Qian, G. Otting, and K. Wüthrich. 1990. The structure of the homeodomain and its functional implications. *Trends Genet.* **6**: 323–329.
- Gill, G. and M. Ptashne. 1988. Negative effect of the transcriptional activator GAL4. *Nature* **334**: 721–724.
- Gimeno, C.J., and G.R. Fink. 1992. The logic of cell division in the life cycle of yeast. *Science* **257**: 626.
- Gimeno, C.J., P.O. Ljungdahl, C.A. Styles, and G.R. Fink. 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: Regulation by starvation and *RAS*. *Cell* **68**: 1077–1090.
- Guarente, L. and O. Bermingham-McDonogh. 1992. Conservation and evolution of transcriptional mechanisms in eukary-

- otes. *Trends. Genet.* **8**: 27–32.
- Hayashi, S. and M.P. Scott. 1990. What determines the specificity of action of *Drosophila* homeodomain proteins? *Cell* **63**: 883–894.
- Horii, T., T. Ogawa, and H. Ogawa. 1981. Nucleotide sequence of the *lexA* gene of *E. coli*. *Cell* **23**: 689–697.
- Hu, J.C., E.K. O'Shea, P.S. Kim, and R.T. Sauer. 1990. Sequence requirements for coiled-coils: Analysis with λ repressor-GCN4 leucine zipper fusions. *Science* **250**: 1400–1403.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **53**: 163–168.
- Katagiri, F. and N.-H. Chua. 1992. Plant transcription factors: Present knowledge and future challenges. *Trends. Genet.* **8**: 22–27.
- Kaufman, T.C., M.A. Seeger, and G. Olsen. 1990. Molecular and genetic organization of the antennapedia gene complex of *Drosophila melanogaster*. *Adv. Genet.* **27**: 309–362.
- Kissinger, C.R., B. Liu, E. Martin-Blanco, T.B. Kornberg, and C.O. Pabo. 1990. Crystal structure of an engrailed homeodomain-DNA complex at 2.8 Å resolution: A framework for understanding homeodomain-DNA interactions. *Cell* **63**: 579–590.
- Koornneef, M. 1981. The complex syndrome of *ttg* mutants. *Arabidopsis Inf. Serv.* **18**: 45–51.
- Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* **240**: 1759–1764.
- Laughon, A. and R.F. Gesteland. 1984. Primary structure of the *Saccharomyces cerevisiae* GAL4 gene. *Mol. Cell. Biol.* **4**: 260–267.
- Lewis, E.B. 1992. Clusters of master control genes regulate the development of higher organisms. *J. Am. Med. Assoc.* **267**: 1524–1531.
- Lloyd, A.M., V. Walbot, and R.W. Davis. 1992. *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1*. *Science* **258**: 1773–1775.
- Ludwig, S.R., L.F. Habera, S.L. Dellaporta, and S.R. Wessler. 1989. *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. *Proc. Natl. Acad. Sci.* **86**: 7092–7096.
- Ma, J., E. Przibilla, J. Hu, L. Bogorad, and M. Ptashne. 1988. Yeast activators stimulate plant gene expression. *Nature* **334**: 631–633.
- Ma, H., M.F. Yanofsky, and E.M. Meyerowitz. 1991. *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes & Dev.* **5**: 484–495.
- Mattsson, J., E. Söderman, M. Svensson, C. Borkird, and P. Engström. 1992. A new homeobox-leucine zipper gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* **18**: 1019–1022.
- McGinnis, W. and R. Krumlauf. 1992. Homeobox genes and axial patterning. *Cell* **68**: 283–302.
- Mitchell, P.J. and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**: 371–378.
- Nagel, R., A. Elliot, A. Masel, R.G. Birch, and J.M. Manners. 1990. Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *FEMS Microbiol. Lett.* **67**: 325–328.
- Nambu, J.R., J.O. Lewis, K.A. Wharton, Jr., and S.T. Crews. 1991. The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**: 1157–1167.
- Okada, K. and Y. Shimura. 1992. Aspects of recent developments in mutational studies of plant signaling pathways. *Cell* **70**: 369–372.
- Oppenheimer, D.G., P.L. Herman, S. Sivakumaran, J. Esch, and M.D. Marks. 1991. A *myb* gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. *Cell* **67**: 483–493.
- O'Shea, E.K., R. Rutkowski, and P.S. Kim. 1989. Evidence that the leucine zipper is a coiled coil. *Science* **243**: 538–542.
- O'Shea, E.K., J.D. Klemm, P.S. Kim, and T. Alber. 1991. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* **254**: 539–544.
- Ptashne, M. and A.A.F. Gann. 1990. Activators and targets. *Nature* **346**: 329–331.
- Richards, E. 1988. Preparation of genomic DNA from plant tissue. In *Current protocols in molecular biology* (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, and J.A. Smith), pp. 2.3.1–3. Greene/Wiley-Interscience, New York.
- Ruberti, I., G. Sessa, S. Lucchetti, and G. Morelli. 1991. A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. *EMBO J.* **10**: 1787–1791.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Scheda, M. 1989. The evolutionary conservation of eukaryotic gene transcription. *Experientia* **45**: 972–983.
- Scheda, M. 1990. "Mammalian glucocorticoid receptor action in yeast." Ph.D. thesis, University of California at San Francisco, San Francisco, CA.
- Scheda, M. and R.W. Davis. 1992. HD-Zip proteins: Members of an *Arabidopsis* homeodomain protein superfamily. *Proc. Natl. Acad. Sci.* **89**: 3894–3898.
- Scheda, M. and K.R. Yamamoto. 1988. Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. *Science* **241**: 965–967.
- Scheda, M., L.P. Freedman, and K.R. Yamamoto. 1989. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. *Genes & Dev.* **3**: 1590–1601.
- Scheda, M., D. Picard, and K.R. Yamamoto. 1991a. Vectors for constitutive and inducible gene expression in yeast. *Methods Enzymol.* **194**: 389–398.
- Scheda, M., A.M. Lloyd, and R.W. Davis. 1991b. A steroid-inducible gene expression system for plant cells. *Proc. Natl. Acad. Sci.* **88**: 10421–10425.
- Schiestl, R.H. and R.D. Gietz. 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**: 339–346.
- Scott, M.P., J.W. Tamkun, and G.W. Hartzell, III. 1989. The structure and function of the homeodomain. *Biochim. Biophys. Acta* **989**: 25–48.
- Sherman, F. 1991. Getting started with yeast. *Methods Enzymol.* **194**: 3–21.
- Struhl, K. 1989. Helix-turn-helix, zinc-finger, and leucine-zipper motifs for eukaryotic transcriptional regulatory proteins. *Trend. Biochem. Sci.* **14**: 137–140.
- Valvekens, D., M. Van Montagu, and M. Van Lijsebettens. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci.* **85**: 5536–5540.
- Vinson, C.R., P.B. Sigler, and S.L. McKnight. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246**: 911–916.
- Vollbrecht, E., B. Veit, N. Sinha, and S. Hake. 1991. The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature* **350**: 241–243.
- Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R.

- Benezra, T.K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. Lassar. 1991. The *myoD* gene family: Nodal point during specification of the muscle cell lineage. *Science* **251**: 761–766.
- Williams, S.C., C.A. Cantwell, and P.F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes & Dev.* **5**: 1553–1567.
- Wilson, T.E., T.J. Fahrner, M. Johnston, and J. Milbrandt. 1991. Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* **252**: 1296–1300.
- Wolberger, C., A.K. Vershon, B. Liu, A.D. Johnson, and C.O. Pabo. 1991. Crystal structure of a MAT α 2 homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. *Cell* **67**: 517–528.
- Yanofsky, M.F., H. Ma, J.L. Bowman, G.N. Drews, K.A. Feldmann, and E.M. Meyerowitz. 1990. The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature* **346**: 35–39.
- Yocum, R.R., S. Hanley, R. West, Jr., and M. Ptashne. 1984. Use of *lacZ* fusions to delimit regulatory elements of the inducible divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4**: 1985–1998.

The HAT4 gene of Arabidopsis encodes a developmental regulator.

M Schena, A M Lloyd and R W Davis

Genes Dev. 1993, **7**:

Access the most recent version at doi:[10.1101/gad.7.3.367](https://doi.org/10.1101/gad.7.3.367)

References

This article cites 56 articles, 22 of which can be accessed free at:
<http://genesdev.cshlp.org/content/7/3/367.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).