The HAT4 gene of Arabidopsis encodes a developmental regulator

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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]

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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 Baltimore-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

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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 (Schena 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; Schena 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 (Schena 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 (Schena and Yamamoto 1988; Schena 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 [Schena 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; Schena 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 ~5 x 10⁴ 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
pressing the LexA-GAL4 protein (Table 1), indicating 35S promoter from the cauliflower mosaic virus fused to within twofold of the levels observed in yeast cells ex-

attributable to decreased levels of the mutant proteins.

that the increased growth rate of the yeast cells was not

cells containing the LexA-HAT4-GAL4 mutants were

in bona fide homeo domains and leucine zippers (Fig. 2C) and 1 double mutant (data not shown). Of

mutations mapped to residues that are highly conserved

segment. Sequence

of two of the six 35S--HAT4 lines had shorter hypoco-

all of the transgenic lines. Kanamycin-resistant seedlings

equalization of the T2 seedlings

lines had acquired copies of the transgene at a single

of the 18 T2 lines examined,

were examined for phenotypic alterations.

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 express-
ing 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–αHAT4) to reduce the level of HAT4; the developmentally inert β-glucoronidase 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–αHAT4 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-

[Image of nucleotide sequence and gene expression diagram]
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).

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-etHAT 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          | wild type    | wild type      | N.D.  |
| pG-LexAHAT4GAL4  | LexA–HAT4–GAL4     | TCC > TTC    | Ser134 > Phe   | 2048  |
| pG-T3            | LexA–HAT4–GAL4     | TCT > CCT    | Ser138 > Pro   | 2363  |
| pG-T51           | LexA–HAT4–GAL4     | CTT > CCT    | Leu141 > Arg   | 2159  |
| pG-T6            | LexA–HAT4–GAL4     | CTT > CCT    | Leu141 > Arg   | 2740  |
| pG-T72           | LexA–HAT4–GAL4     | TCC > TTC    | Phe145 > Ser   | 1912  |
| pG-M25           | LexA–HAT4–GAL4     | TTA > TCA    | Leu165 > Ser   | 1930  |
| pG-M7            | LexA–HAT4–GAL4     | GTG > GCG    | Val170 > Ala   | 1835  |
| pG-M14           | LexA–HAT4–GAL4     | GTG > GCG    | Val170 > Ala   | 1582  |
| pG-M15           | LexA–HAT4–GAL4     | TGG > CGG    | Trp173 > Arg   | 1950  |
| pG-M15           | LexA–HAT4–GAL4     | CTA > CCA    | Leu200 > Pro   | 1673  |

Yeast pG-l-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-CYCl-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.

Arabidopsis morphology adapts profoundly to environmental 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  | T2  | T2  | T2      | T2    |
|---------|-----------|------------|-----|-----|-----|---------|-------|
|         |           |            | Kan^R/Kan^S | hypocotyl (mm) | silique (days) | phenotype |
| 1429    | 35S-aHAT4  | Col-O      | 60:24 | 1.8 ± 0.2 | 34 ± 2.7 | short |
| 1431    | 35S-aHAT4  | Col-O      | 65:20 | 1.9 ± 0.3 | 35 ± 1.9 | short |
| 1478    | 35S-aHAT4  | RLD-O      | 60:21 | 2.7 ± 0.5 | 39 ± 2.7 | normal |
| 1479    | 35S-aHAT4  | RLD-O      | 69:24 | 2.7 ± 0.4 | 41 ± 2.3 | normal |
| 1481    | 35S-aHAT4  | RLD-O      | 59:19 | 2.6 ± 0.4 | 38 ± 1.7 | normal |
| 1482    | 35S-aHAT4  | RLD-O      | 74:5  | 2.9 ± 0.4 | 42 ± 2.0 | normal |
| 1419    | 35S-GUS    | RLD-O      | 71:7  | 2.6 ± 0.3 | 40 ± 2.1 | normal |
| 1420    | 35S-GUS    | RLD-O      | 64:23 | 2.5 ± 0.5 | 42 ± 1.8 | normal |
| 1421    | 35S-GUS    | Col-O      | 61:16 | 2.4 ± 0.3 | 30 ± 1.2 | normal |
| 1425    | 35S-GUS    | Col-O      | 51:14 | 2.5 ± 0.4 | 29 ± 1.3 | normal |
| 1423    | 35S-HAT4   | RLD-O      | 57:16 | 4.2 ± 1.1 | 23 ± 1.2 | tall   |
| 1425    | 35S-HAT4   | Col-O      | 73:21 | 6.7 ± 0.4 | 16 ± 2.3 | tall   |
| 1426    | 35S-HAT4   | Col-O      | 62:22 | 6.7 ± 0.4 | 16 ± 1.9 | tall   |
| 1427    | 35S-HAT4   | Col-O      | 73:20 | 6.9 ± 0.4 | 17 ± 1.6 | tall   |
| 1468    | 35S-HAT4   | RLD-O      | 96:35 | 3.5 ± 0.8 | N.D.     | tall   |
| 1469    | 35S-HAT4   | RLD-O      | 28:11 | 3.6 ± 1.1 | N.D.     | tall   |
| 1471    | 35S-HAT4   | RLD-O      | 97:33 | 4.9 ± 0.6 | 24 ± 1.4 | tall   |
| 1472    | 35S-HAT4   | RLD-O      | 121:42| 5.7 ± 0.8 | 22 ± 2.5 | tall   |
| 1474    | 35S-HAT4   | Col-O      | 55:27 | 6.5 ± 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--aHAT4 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 heptadric 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 ± 0.8 | tall |
| 1426    | 35S--HAT4 | 1426-2.2 | 106:38 | 8.3 ± 0.6 | tall |
| 1426    | 35S--HAT4 | 1426-2.5 | 69:26 | 8.5 ± 0.6 | tall |
| 1427    | 35S--HAT4 | 1427-2.1 | 81:28 | 8.7 ± 0.7 | tall |
| 1427    | 35S--HAT4 | 1427-2.2 | 118:43 | 9.0 ± 0.8 | tall |
| 1427    | 35S--HAT4 | 1427-2.7 | 119:0 | 8.2 ± 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.

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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), propagates under kanamycin selection, are as follows: 35S–oHAT4 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].
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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-aHAT4 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.

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 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. 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 XbaI and SacI, 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'-GGGGGGGATCCAGGGGGCGGAATGA-3' (position 1333 in Fig. 1) and 3' ends, and inserting the modified lexAGAL4 fragment into the BamHI site of pG-LexAGAL4, introduced using PCR oligonucleotides PAN22 and PAN23, is located 10 bp upstream of the lexA start codon (Horii et al. 1981). The 3' BamHI site in lexAGAL4 was introduced by end-fill of a HindIII site located ~240 bp downstream of the GAL4 stop codon (Laughon and Gesteland 1984) and attachment of a synthetic BamHI linker. The junction between lexA [residues 1-87] and GAL4 [residues 74-881] contains a unique XhoI site (Brent and Ptashne 1985). Yeast expression vectors p3SS-HAT4 and p3SS-aHAT4 were constructed by excising the β-glucuronidase [GUS] gene from p3SS-GUS (pBl121, Clontech) using XbaI and SacI, and inserting HAT4 cDNA sequences (Schena and Davis 1992) in either the sense [p3SS-HAT4] or antisense [p3SS-aHAT4] orientation. In p3SS-HAT4, the XbaI site was introduced 26 bp upstream of the HAT4 start codon using PCR oligonucleotides PAN17 and PAN18, the SacI site was introduced ~150 bp downstream of the HAT4 translation stop codon by subcloning the cDNA into pUC18, followed by excision with SacI. In the sequence of p3SS-aHAT4, the insert is identical to the one present in p3SS-HAT4 except that it resides in the opposite orientation; SacI and XbaI sites were added to HAT4 by subcloning into Bluescript II KS+ [Stratagene] and pUC18, followed by excision with SacI and XhoI, respectively. Plasmid sizes are ~13 kb. [When requesting plant expression plasmids from the Davis laboratory, please use the following nomenclature: pNN486 (pG-LexAGAL4) and pNN487 (pG-LexAHAT4GAL4).]

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The sequences of the PCR oligonucleotide primers are as follows. PAN22: 5'GGGGGGGATCCAGGGGGCGGAATGA-3' (upstream of lexA AUG); PAN23: 5'GGGGGGGGA-

PCR mutagenesis

The 390-bp HAT4 segment of pG-LexAHAT4GAL4 was excised using SacI and XhoI, inserted into Bluescript II KS+, and mutagenized by PCR amplification with T3/T7 or M13forward/M13 reverse primer pairs. Six 50-μl 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 pmol probes of each primer, and 5 units of Taq polymerase (Promega) were overlaid with 100 μl 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 SacI and XhoI, fractionated on low-melt agarose, ligated into SacI/XhoI-linearized p3SS--GUS, 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 × 10⁸ bacterial transformants [T pool] and 3 × 10⁷ 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, prcl-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...
Agrobacterium-mediated photomicroscope set at eightfold magnification. Dark experi-
ments were performed exactly as described above except that DNA sequences of the PCR oligonucleotide primers are as fol-

doctors. DNA from each of the 26 yeasts cloned was isolated (Schenk et al. 1989), and pCG-LexAHAT4-GAL4 plasmids were

Arabidopsis transformation

Expression plasmids p35S–αHAT4, p35S–GUS and p35S–HAT4 were introduced into Agrobacterium tumefaciens strain LBA4404 (Clontech) by electroporation (Nagel et al. 1990). Ar-

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 × 2.5 cm petri dishes (Nunc) containing 25 ml of agar (pH 5.8) consisting of 1× MS salts (GIBCO), 0.7% Bacto-agar, 2% sucrose, 1× vitamins [1.3 μg/ml of nicotinic acid, 0.25 μg/ml of thiamine, 1.0 μg/ml of pyridoxine, and 0.25 μg/ml of pan-
tothenic acid], and 50 μg/ml of kanamycin. Dishes were sealed with parafilm, incubated in growth chambers at 22°C under continuous bright light [3.2 × 10^3 lux], and scored for kanamy-
cin 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 experi-
ments 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 primers PANC36, together with PAN19 (35S–αHAT4), 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 Sun-

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