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A Mutation in the Arabidopsis *HYL1* Gene Encoding a dsRNA Binding Protein Affects Responses to Abscisic Acid, Auxin, and Cytokinin

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Both physiological and genetic evidence indicate interconnections among plant responses to different hormones. We describe a pleiotropic recessive Arabidopsis transposon insertion mutation, designated *hyponastic leaves* (*hyl1*), that alters the plant's responses to several hormones. The mutant is characterized by shorter stature, delayed flowering, leaf hyponasty, reduced fertility, decreased rate of root growth, and an altered root gravitropic response. It also exhibits less sensitivity to auxin and cytokinin and hypersensitivity to abscisic acid (ABA). The auxin transport inhibitor 2,3,5-triiodobenzoic acid normalizes the mutant phenotype somewhat, whereas another auxin transport inhibitor, *N*-(1-naphthyl)phthalamic acid, exacerbates the phenotype. The gene, designated *HYL1*, encodes a 419–amino acid protein that contains two double-stranded RNA (dsRNA) binding motifs, a nuclear localization motif, and a C-terminal repeat structure suggestive of a protein–protein interaction domain. We present evidence that the *HYL1* gene is ABA-regulated and encodes a nuclear dsRNA binding protein. We hypothesize that the HYL1 protein is a regulatory protein functioning at the transcriptional or post-transcriptional level.

INTRODUCTION

The development, growth, and survival of plants under a wide range of environmental conditions reflect an intricate interplay of physical and chemical conditions with the highly integrated sensing and response networks in plants. The growth habit and physiological properties of plants can differ markedly under different regimes of light, gravity, temperature, humidity, and salinity, among others. Hormones have long been known to be important internal mediating signals in plants, but the components of the underlying cellular machinery are just beginning to be identified and characterized (Trewavas and Malho, 1997; Grill and Himmelbach, 1998; Solano and Ecker, 1998; D'Agostino and Kieber, 1999). The range of proteins involved in receiving, transmitting, and responding to external signals includes receptor-like and other kinds of protein kinases, phosphatases, and transcription factors, as well as enzymes such as thioredoxin and farnesyltransferase, which influence protein structure or localization through mechanisms other than phosphorylation (Mulligan et al., 1997; Becraft, 1998; Bonetta and McCourt, 1998; Hooley, 1998; Bleecker, 1999; Thornton et al., 1999; Hirt, 2000; Urao et al., 2000).

Ample physiological evidence supports the presence of interconnections among plant responses to different environmental stimuli; moreover, evidence is accumulating that certain mutations can simultaneously influence the response to more than one hormone or altered physical parameter (Wilson et al., 1990; Clouse et al., 1996; Nemeth et al., 1998; Ephritikhine et al., 1999a; Beaudoin et al., 2000; Ghassemian et al., 2000). The implication is that individual proteins can be responsible for such interconnections, either transmitting multiple signals or participating in distinct complexes that transmit different signals (Elion, 1998). Proteins can receive information from a small molecule, such as a hormone, and transmit it to a macromolecule, such as another protein, commonly through either a covalent modification, such as phosphorylation, or protein binding (Mulligan et al., 1997; Trewavas and Malho, 1997; Moller and Chua, 1999; Nambara and McCourt, 1999). Proteins can also receive and transmit information at the macromolecular level through protein–protein interactions, phosphorylation or other structural modifications, and protein–nucleic acid interactions. Thus, the concept of a signal transduction "pathway" representing a linear succession of molecules transmitting information is giving way to the concept of an interconnected signaling "network," the protein "nodes" of which are characterized by the number and nature of their interconnections (Trewavas and Malho, 1997; Elion, 1998; Bhalla and Iyengar, 1999; Weng et al., 1999).

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In the present report, we describe an Arabidopsis insertion mutation that alters the plant's responses to several exogenous hormones and present evidence that the mutated gene encodes an abscisic acid (ABA)–regulated nuclear dsRNA binding protein. We develop the hypothesis that the HYPONASTIC LEAVES1 (HYL1) protein is a regulatory protein with transcriptional or post-transcriptional functions.

RESULTS

The *hyl1* Mutation Has Pleiotropic Effects on Growth and Development

The *hyl1* mutant was identified in an Arabidopsis *Dissociation* (*Ds*) insertion mutant collection generated by using a previously described transposon tagging system (Fedoroff and Smith, 1993). Germination of *hyl1* mutant seed is normal, as is cotyledon structure. Seedling, rosette, and cauline leaves of the *hyl1* mutant are narrower than are wild-type leaves and exhibit hyponasty (Figure 1). The hypocotyl elongation rate is reduced in both dark- and light-grown plants, and hypocotyl cells are shorter than those of wild-type plants (Figure 1E). Mature plants attain a stature of <30 cm, whereas wild-type plants reach ~45 cm (Figure 1D). The leaves and flowers of *hyl1* plants are smaller than those of wild-type plants (Figures 1B and 1C), and *hyl1* siliques are both shorter and twisted (not shown). Flowering of the *hyl1* mutant is delayed by ~10 days under short-day conditions, and mutant plants produce more rosette leaves than do wild-type plants. The markedly reduced fertility of the *hyl1* mutant appears to be attributable to the shorter stamen filaments, because fertility is restored upon manual pollination. Mutant plants also exhibit more lateral branching than do wild-type plants, suggesting decreased apical dominance.

The *hyl1* seedling root elongation rate is somewhat slower than that of wild-type plants in both light and dark conditions, and mutant roots exhibit both a reduced gravitropic response and plagiotropic growth (Figure 1A). The roots of wild-type seedlings growing on agar medium showed a curvature of ~70° toward the gravity vector within 10 hr of gravistimulation, whereas the curvature of *hyl1* seedling roots was only ~10° (Figure 2). The root elongation rates were quite similar (5 mm/day for *hyl1* seedlings and 5.5 mm/day for wild-type plants) at the stage at which the measurement was made, indicating that the difference in gravitropic response is not attributable simply to the decreased rate of root elongation in the mutant. In contrast, the *hyl1* mutation does not affect shoot gravitropism. When wild-type Arabidopsis plants are placed in a horizontal position, they curve 90° upward within 90 min in the dark. The curvature rate of *hyl1* mutant stems placed horizontally was similar; thus, the *hyl1* mutation markedly reduces the gravitropic response of the root but not of the shoot.

hyl1 Is a *Ds* Insertion Mutation

To determine whether the *Ds* insertion is responsible for the *hyl1* mutation, we selected and analyzed phenotypic revertants. The progeny of mutant plants homozygous for the *Ds* insertion and containing *Activator* (*Ac*) transposase were examined for wild-type revertants. We obtained 25 putative wild-type revertants and extracted DNA from 10 plants to examine the *Ds* insertion site for a transposon footprint compromising all or part of the 8-bp duplication created upon *Ds* insertion (Pohlman et al., 1984; Schwarz-Sommer et al., 1985). The DNA sequences adjacent to the 5′ and 3′ ends of the *Ds* were amplified by using the thermal asymmetric interlaced (TAIL) polymerase chain reaction (PCR) technique and then were sequenced directly (Liu and Whittier, 1995; Tsugeki et al., 1996). PCR primers corresponding to the genomic DNA sequences adjacent to the 5′ and 3′ ends of the *Ds* were tested for their ability to amplify a fragment of the length expected if the *Ds* had been excised (Tsugeki et al., 1996). A short fragment of the expected size was amplified from eight of the 10 DNA samples. When sequenced, all eight were found to contain a 6-bp footprint at the former insertion site, indicating that phenotypic reversion was caused by excision of *Ds* (Figure 3B). One of the mutant control plant DNAs also supported amplification of a DNA fragment of the length expected for an empty donor site. However, sequencing revealed that this DNA had a 7-bp footprint at the former insertion site, indicating that another mutant allele had been created by a frame-shift when the *Ds* excised. These observations show that the complex mutant phenotype is attributable to mutation in a single gene and that the mutation was caused by insertion of the *Ds* element.

The *hyl1* Mutant Is Hypersensitive to ABA

ABA has an inhibitory effect on seed germination, and Arabidopsis mutations have been identified that both increase and decrease sensitivity to such inhibition (Koornneef et al., 1984; Cutler et al., 1996). We tested the *hyl1* mutant for the effect of exogenous ABA on seed germination. The *hyl1* mutation has no effect on seed germination in the absence of ABA (Figure 4A, left). ABA at 0.5 μM slightly delays germination of wild-type seeds but has a negligible effect on the germination frequency. In contrast, germination of *hyl1* mutant seeds is completely inhibited by 0.5 μM ABA (Figure 4A, right). Moreover, growth of *hyl1* mutant roots is more sensitive to ABA inhibition than are those of wild-type plants (Figure 4B). Several mutants identified by virtue of their altered response to another hormone also show hypersensitivity of seed germination to exogenous ABA. These include *era1-3* (Cutler et al., 1996), *jar1* (Staswick et al., 1992), and *jin4* (Berger et al., 1996). However, root growth of the *era1-3* mutant is unaffected by ABA (P. McCourt, personal communication), suggesting that the *hyl1* mutant is more sensitive to exogenous ABA than is the *era 1-3* mutant.

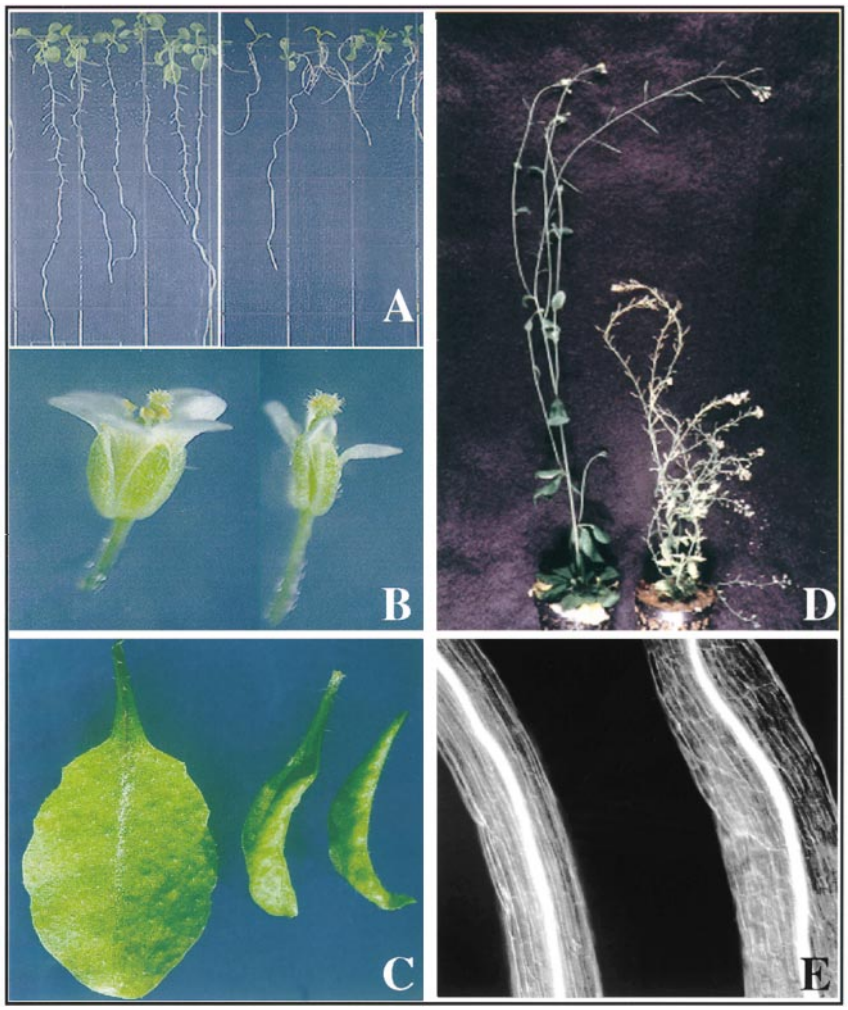


Figure 1. Pleiotropic Phenotype of the Arabidopsis *hyl1* Mutant.

(A) Wild-type (left) and *hyl1* mutant (right) seedlings grown on Murashige and Skoog (MS) plates.
(B) Flowers of the wild-type (left) and *hyl1* mutant (right) plants.
(C) Rosette leaves from three-week-old wild-type (left) and *hyl1* mutant (right two) plants.
(D) Mature wild-type and *hyl1* plants.
(E) Hypocotyls of wild-type (left) and *hyl1* (right) plants.

To examine the transcriptional effects of the *hyl1* mutation on ABA-induced genes, we monitored expression of two ABA-responsive genes, *COR47* and *KIN2*, in mutant and wild-type plants (Gilmour et al., 1992; Kurkela and Borg-Franck, 1992). The steady state amounts of transcripts of both genes increased in response to exogenous ABA in both mutant and wild-type plants (Figure 4C). However, the amounts of uninduced transcripts of both genes were at least three-fold greater in *hyl1* plants than in wild-type plants. Thus, both genes tested appear to be deregulated in the mutant in the absence of exogenous ABA, although they remain ABA-inducible.

The *hyl1* Mutant Exhibits Altered Sensitivity to Auxin and Inhibitors of Auxin Transport

Exogenous auxin inhibits elongation of the primary root in wild-type Arabidopsis plants, and mutants defective in auxin responsiveness do not show normal inhibition (Lincoln et al., 1990). We analyzed the effect of auxin on the growth of *hyl1* and wild-type roots. Exogenous indoleacetic acid (IAA) inhibited the growth of wild-type roots by $>$50% at 1 μM (Figure 5A). In contrast, exogenous IAA stimulated growth of *hyl1* mutant roots at concentrations up to 1 μM, inhibiting elongation only at 5 μM, the highest

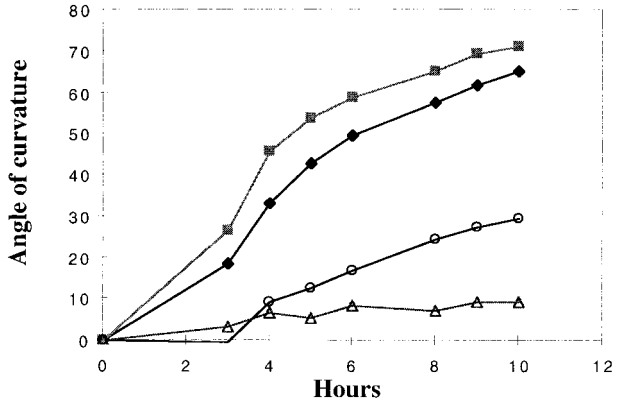


Figure 2. Gravitropic Responses of Wild-Type and *hyl1* Mutant Seedlings.

Seedlings growing on MS medium or MS containing 0.05 μM TIBA were rotated 90° at time 0. Seedlings were aligned on the plates so that all the root tips were perpendicular to the bottom line of the Petri dish. The angle of curvature of the growing root tips was measured at 1- to 2-hr intervals. Filled diamonds, wild-type seedlings on MS medium; filled squares, wild-type seedlings on MS medium + 0.05 μM TIBA; open triangles, *hyl1* seedlings on MS medium; open circles, *hyl1* seedlings on MS medium + 0.05 mM TIBA.

concentration tested. Similarly, wild-type root growth between 5 and 9 days after germination was reduced by 90% by 10 nM 2,4-dichlorophenoxyacetic acid (2,4-D). As noted earlier, *hyl1* mutant roots grow more slowly on agar medium in the absence of 2,4-D than do wild-type roots. However, in the presence of 10 nM 2,4-D, mutant root elongation was reduced by only 50%, indicating that the mutant roots are less sensitive to auxin than are wild-type roots (Figure 5B).

The *hyl1* mutant shows a paradoxical response to inhibitors of auxin transport. The auxin transport inhibitors *N*-(1-naphthyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) are known to inhibit the elongation of wild-type Arabidopsis roots (Katekar and Geissler, 1977). The growth of the *hyl1* mutant roots was inhibited by NPA more than was the growth of wild-type roots were (Figure 5D). At low concentrations, TIBA partially corrected the mutant phenotype of *hyl1* plants with respect to both root growth rate and gravitropic response (Figures 2 and 5C). The chemical structure of TIBA is markedly different from that of NPA, and TIBA and NPA have been suggested to interact with different target proteins (Katekar and Geissler, 1977; Brunn et al., 1992; Fujita and Syono, 1997). The difference between the responses to NPA and to TIBA in the *hyl1* mutant further suggests that the modes of action of the two inhibitors are genetically separable.

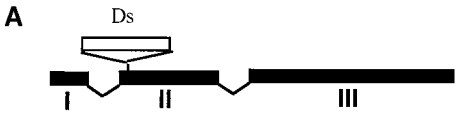
The *hyl1* Mutant Shows Decreased Sensitivity to Cytokinin but Not to Other Growth Hormones

We examined the effect of exogenous 6-benzylaminopurine (BA) on the growth of *hyl1* and wild-type seedlings. The growth of *hyl1* shoots was markedly less inhibited than that of wild-type shoots, whereas the sensitivity of *hyl1* and wild-type roots to increasing concentrations of BA were similar (Figures 6A and 6B). The fresh weight of untreated 10-day-old *hyl1* seedlings is much less than that of wild-type seedlings. However, at BA concentrations >0.01 μM, the fresh weight of *hyl1* shoots substantially exceeded that of wild-type shoots (Figure 6D).

Cytokinins induce ethylene biosynthesis in Arabidopsis, and the ethylene produced in response to low doses of cytokinins is sufficient to induce the triple response in etiolated Arabidopsis seedlings (Vogel et al., 1998). As expected, higher concentrations of cytokinin were required to induce the triple response in etiolated *hyl1* seedlings than in wild-type etiolated seedlings (Figure 6C). In contrast to the results obtained with auxin, cytokinin, and ABA, the growth responses of the *hyl1* mutant to gibberellins, ethylene, brassinosteroids, methyl jasmonate, and salicylic acid were all similar to those of wild-type plants.

Isolation of the *HYL1* cDNA and Characterization of the *HYL1* Gene

As noted earlier, the DNA sequences adjacent to the 5′ and 3′ ends of *Ds* were amplified by TAIL PCR and sequenced directly. The flanking sequences are identical to that of part of an Arabidopsis bacterial artificial chromosome clone



B

Wild type	··· TT	**CCAATTGC**		TA ···
hyl-1	··· TT	**CCAATTGC** (DS)	**CCAATTGC**	TA ···
Revertant	··· TT	**CCAATT***ggc*	***att*GC**	TA ···
hyl-1*	··· TT	**CCAATT***ggc*	***aatt*GC**	TA ···

Figure 3. *Ds* Insertion Site in the *HYL1* Gene.

(A) The exons of the *HYL1* gene are numbered I to III. The *Ds* element insertion site is in the second exon.

(B) The sequence around the *Ds* insertion site is shown for the wild type, the *hyl1* insertion mutant, a wild-type revertant, and the *hyl1** mutant allele created by *Ds* excision. The underlined 8-bp sequence is the target site duplication. The 6- and 7-bp transposon footprints are indicated in italics.

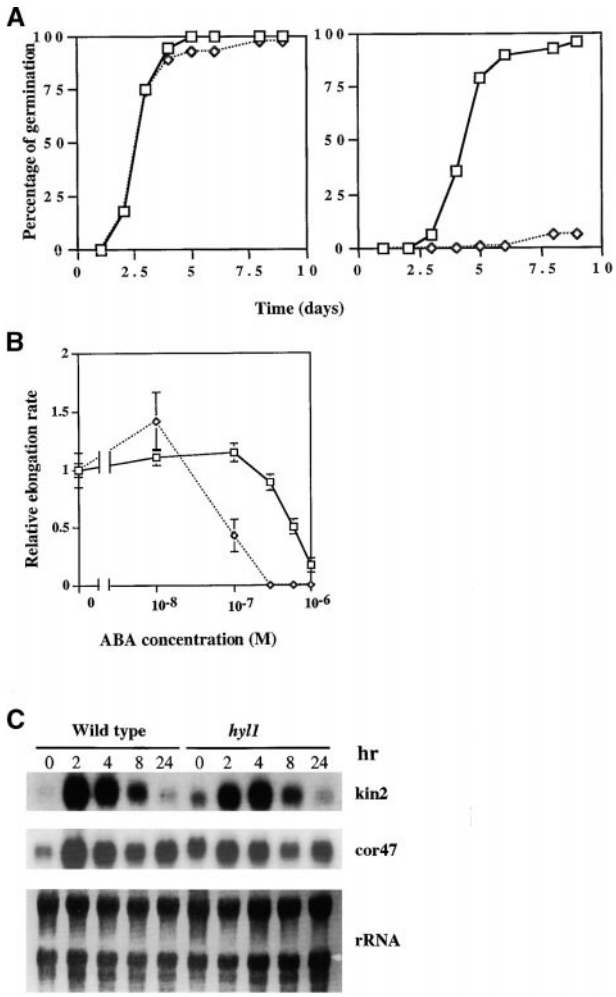


Figure 4. Effects of Exogenous ABA on Wild-Type (open squares) and *hyl1* (open diamonds) Mutant Arabidopsis.

(A) *hyl1* and wild-type seeds were placed on filter paper saturated with either water (left) or 0.5 μM ABA (right), incubated at 4°C for 48 hr, and then transferred to room temperature for germination.

(B) Relative root elongation rates of wild-type Nossen (No-0) and *hyl1* mutant 10-day-old seedlings. Mean values for 100% root elongation were determined on MS medium containing no ABA. Error bars indicate SD.

(C) RNA gel blot analysis of ABA-induced mRNAs in *hyl1* and wild-type plants. Arabidopsis plants were grown on MS plates for 2 weeks, then transferred into 0.1 × MS medium containing 50 μM ABA. Ten micrograms of total cellular RNA isolated from the wild-type and *hyl1* seedlings at the indicated times after treatment with ABA was used in each lane. Probes are indicated for each panel.

(GenBank accession number AC000132, BAC clone F21M12) that has been sequenced and anchored in chromosome 1*s*, close to marker mi443. The sequence in the immediate vicinity of the *Ds* insertion site showed extensive homology with a rice expressed sequence tag cDNA clone (c50188), suggesting that the *Ds* was inserted into an exon. To determine the structure of the *HYL1* gene, we isolated an *HYL1* cDNA clone. A 400-bp TAIL PCR–amplified fragment adjacent to the *Ds* insertion site was used to screen a cDNA library (CD4-7 lambda PRL2, a cDNA library prepared from different tissues and developmental stages). One cDNA of ~1.5 kb was identified among the 400,000 plaques screened, and the cDNA insert was sequenced. The 5′ end of the cDNA has one or more stop codons in reading frames preceding an ATG start codon that initiates an uninterrupted 419–amino acid open reading frame (Figure 7A). RNA gel blot hybridization analysis of RNA isolated from wild-type plants revealed that the *HYL1* cDNA hybridizes to a single major transcript of ~1.5 kb, similar in size to the cDNA insert, suggesting that the cDNA is full-length or nearly full-length (data not shown). Comparing the genomic and cDNA sequences revealed that the *HYL1* gene contains three exons of 25, 263, and 972 bp and two introns of 496 and 403 bp. A comparison of the cDNA sequences with that of the TAIL PCR fragment amplified from the 3′ end of *Ds* showed that the transposon had inserted into the second exon, 31 bp downstream of the putative translation start site (Figure 3A).

The predicted sequence of the HYL1 protein was used to search the GenBank database. The search results showed that the HYL1 protein contains two regions with marked similarities to dsRNA binding motifs (St. Johnston et al., 1992; Bass et al., 1994). The highest scoring segment pairs were those matching the dsRNA binding domains of the human interferon-induced RNA-dependent protein kinase P68, *Escherichia coli* ribonuclease III, and *Xenopus* dsRNA adenosine deaminase (Burd and Dreyfuss, 1994; Kharrat et al., 1995; Brooks et al., 1998). The two dsRNA binding motifs of the HYL1 protein are shown aligned with those of other proteins in Figure 7B. We identified three plant homologs of the HYL1 protein, one of which was the protein encoded by the rice cDNA c26837, which shows 69% identity and 80% similarity with the first dsRNA binding domain in the HYL1 protein. Based on the cDNA sequence, the rice protein contains only one dsRNA binding motif, but whether the cDNA is full length is not known. No homology was found outside of the dsRNA binding domains. We identified two additional putative Arabidopsis genes on chromosome 5 encoding proteins having considerable homology with HYL1 in the dsRNA binding domain (P1 clones MEE6 and AB010072).

As do other dsRNA binding proteins, the predicted HYL1 protein appears to have additional domains. The C-terminal end of the molecule includes six almost perfect repeats of a 28–amino acid sequence. A recent search of the Protein Sequence Databases identified a protein with sequence similarity to the HYL1 protein. Protein F, a fibronectin binding protein from *Streptococcus pyogenes*, contains five repeats

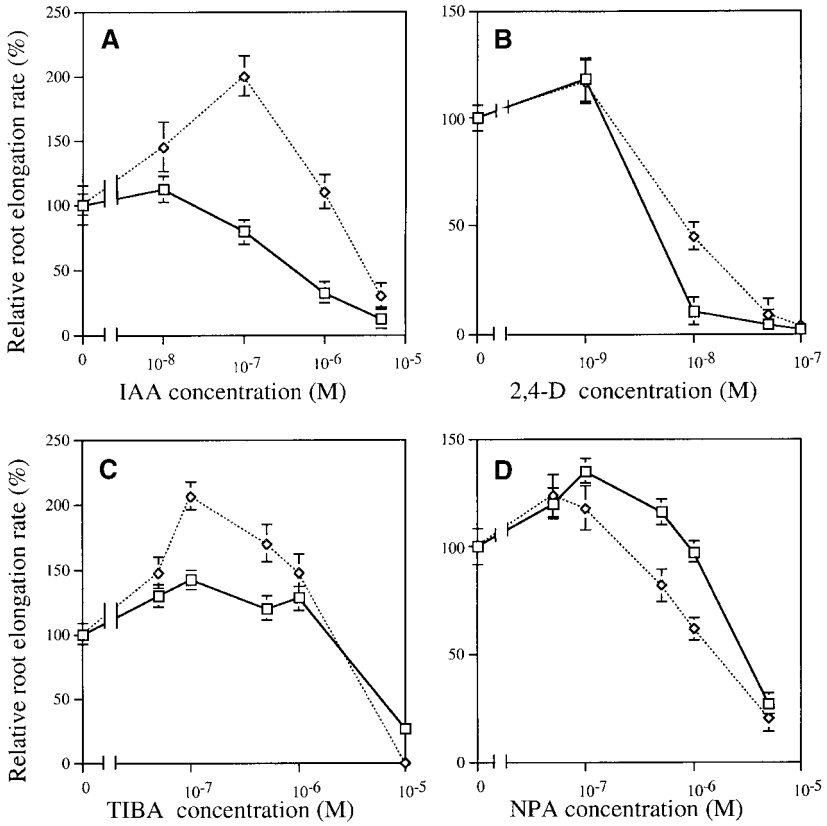


Figure 5. Growth of *hyl1* and Wild-Type Seedlings on Medium Containing Auxin and Auxin Transport Inhibitors.

hyl1 (open diamonds) and wild-type (open squares) seedlings were grown on MS medium supplemented with different concentrations of chemicals; root length was measured 10 days after germination. Root growth is expressed relative to growth on unsupplemented MS medium. Each point represents a test of 20 seedlings.

(A) IAA.

(B) 2,4-D.

(C) TIBA.

(D) NPA.

Error bars indicate SD.

of a 37–amino acid sequence having 30% identity and 48% similarity to the repeat in the HYL1 protein (Hanski and Caparon, 1992; Sela et al., 1993). The HYL1 protein also contains a positively charged region between residues 209 and 222 that resembles a bipartite nuclear localization signal (Varagona et al., 1992). Plant nuclear localization signals generally comprise two regions of basic amino acids separated by ~10 amino acids (Figure 7C). This finding therefore suggests that HYL1 is a nuclear protein.

To determine whether *HYL1* has homologs other than those found in the Arabidopsis sequence database, we performed low-stringency DNA gel blot hybridization experiments, using a cDNA probe. Genomic DNA from the Columbia, Nossen (No-0), and Landsberg *erecta* ecotypes was digested with EcoRI, HindIII, XbaI, and BglII and then probed with the full-length *HYL1* cDNA. A single, strong band was seen in all lanes, as we expected from the absence of internal sites in the gene for these enzymes (data not shown). A single fragment was detected at both high and low stringencies, indicating that the *HYL1* gene is unique in the Arabidopsis genome.

Expression of the *HYL1* Gene

The phenotype of the *hyl1* mutant suggests that the HYL1 protein plays a key role in Arabidopsis development throughout the life cycle of the plant. That is, the mutant has

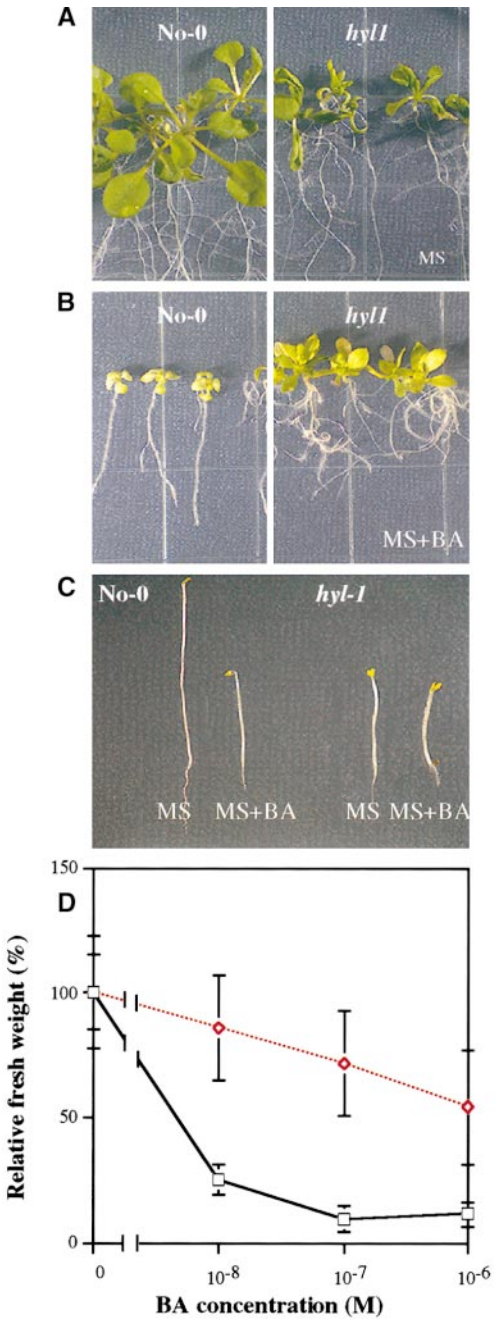


Figure 6. Responses of *hyl1* and Wild-Type Seedlings to Exogenous Cytokinin.

(A) Wild-type (left) and *hyl1* (right) seedlings grown on MS medium.
(B) Wild-type (left) and *hyl1* (right) seedlings grown on MS medium containing 1 μM BA.
(C) Wild-type (left) and *hyl1* (right) seedlings grown in the dark for 3 days on MS medium with or without 0.5 μM BA.
(D) The fresh weight of wild-type (open squares) and *hyl1* (open diamonds) mutant seedlings at 14 days after germination on MS medium containing the indicated concentrations of BA. Shown is the dose–response curve for one representative experiment of three performed. Error bars indicate SD.

abnormal roots, leaves, and flowers, indicating that this gene has a function in most or all tissues. We used gel blot analysis of total RNA isolated from various Arabidopsis tissues to investigate the pattern of *HYL1* gene expression directly. As expected, *HYL1* transcripts were present in all of the tested tissues, including rosette leaves, cauline leaves, stems, roots, and flowers (Figure 8A). Moreover, the abundance of the *HYL1* transcript was similar in different tissues. No hybridization was detected to RNA from *hyl1* plants, indicating that expression of the *HYL1* gene is considerably decreased or even abolished in the *hyl1* mutant (data not shown). Given the site of insertion within the gene, *hyl1* is probably a null mutation.

Because the *hyl1* mutation impairs plant hormone responses, we wanted to determine whether the *hyl1* gene itself is responsive to exogenous hormones. Accordingly, we analyzed *HYL1* gene expression in Arabidopsis seedlings treated with auxin and ABA. Exposure of 2-week-old seedlings to 50 μM ABA for 24 hr decreased the *HYL1* transcript levels to approximately one-third of those in control seedlings, whereas treatment with 2,4-D (0 to 100 μM) had no marked effect on HYL1 expression (Figure 8B). Thus we conclude that the *HYL1* gene is downregulated by ABA.

Nuclear Localization of HYL1 Protein

To gain further insight into the function of the HYL1 protein, we investigated its subcellular location. As noted earlier, no transit or signal sequences have been identified in the HYL1 protein, and it lacks the structural features of a membrane-associated domain. However, the sequence contains a putative nuclear localization signal (Varagona et al., 1992). To determine whether the HYL1 protein is targeted to the nucleus, we fused the coding sequence of the HYL1 protein to that of the *GUS* gene (which encodes β-glucuronidase) expressed from a cauliflower mosaic virus (CaMV) 35S promoter. We introduced the resulting 35S-HYL1-*GUS* construct into onion epidermal cells by particle bombardment (Shieh et al., 1993). For a control, the onion epidermal cells were bombarded with an unmodified *GUS* gene expressed from the same promoter. In cells bombarded with the 35S-*GUS* gene, GUS activity was observed throughout the cell. In contrast, GUS activity was concentrated in the nuclei of all cells bombarded with the 35S-HYL1-*GUS* construct (Figure 9). This result confirms the inference from sequence homology that the HYL1 protein contains a nuclear localization sequence and suggests that the protein functions in the nucleus.

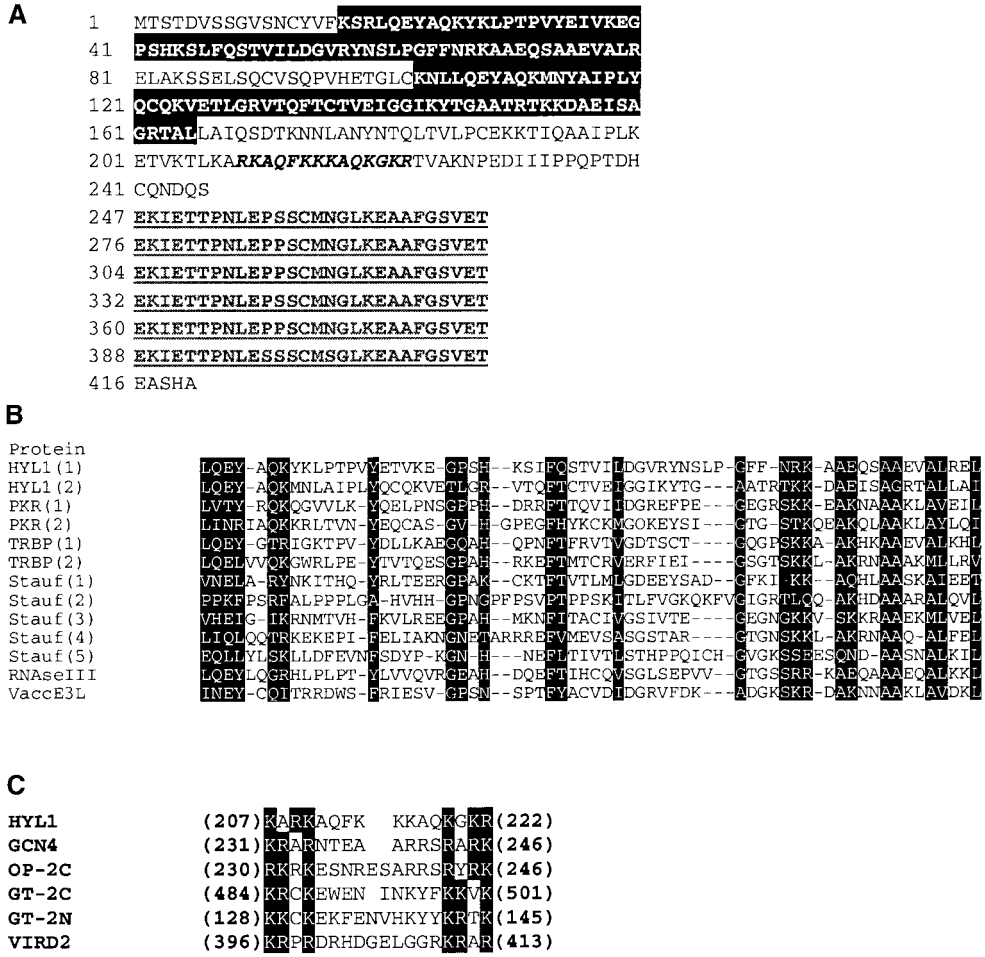


Figure 7. Motifs in the HYL1 Protein Sequence.

(A) Deduced amino acid sequence of the *HYL1* gene. The two putative dsRNA binding motifs are shown in white letters on a black background, the putative nuclear localization motif is indicated in italics, and the six consecutive C-terminal repeats are underlined.
(B) Alignment of the dsRNA binding motifs of HYL1 protein with those of other proteins with dsRNA binding domains. The conserved regions are in white letters on a black background. Dashes were introduced to optimize alignments.
(C) Alignment of the putative bipartite nuclear localization sequence of HYL1 with similar motifs in other proteins. The basic residues at the ends of the motif are shown in white letters on a black background.

HYL1 Protein Binds dsRNA

The results of sequence analyses revealed that the HYL1 protein contains two tandem repeats of a motif characteristic of proteins that bind dsRNA, but not dsDNA. We used the protein encoded by the *HYL1* cDNA to study the nucleic acid–binding properties of the HYL1 protein. To obtain large quantities of the protein, the cDNA was cloned in a pQE vector, which adds a histidine tag to the N terminus of the protein (Makrides, 1996). The fusion protein was purified from *E. coli* by two rounds of ion-exchange chromatography on a nickel–nitrilo triacetic acid (Ni-NTA) column. Protein gel blots showed that in the protein purified under native conditions, the main product was a 30-kD degradation product (data not shown). Because this fragment contains >200 amino acids of the HYL1 N terminus and because the two dsRNA binding motifs are located between amino acids 17 and 165, we reasoned that the truncated protein should contain all of the amino acids necessary for binding to dsRNA.

Mobility shift assays have been used extensively to study the properties of dsRNA binding proteins (Bass et al., 1994; Bevilacqua and Cech, 1996; Bevilacqua et al., 1998). The first part of Figure 10 shows the results of a gel mobility shift

experiment performed under nondenaturing conditions with the truncated HYL1 protein and a ^{32}P-labeled dsRNA fragment of ~100 bp derived from the pBluescript II KS vector. A dsRNA–protein complex was first observed at a protein concentration of 0.1 μM. As the protein concentration increased, all of the labeled dsRNA shifted to a protein-bound form. However, at the same or greater concentrations of protein, no shift was detected when a ssRNA, dsDNA, or ssDNA of the same sequence was used (Figure 10). These results show that HYL1 binds dsRNA preferentially, as suggested by sequence homology with the dsRNA binding motifs of other proteins.

DISCUSSION

The pleiotropic recessive *hyl1* mutation identifies an Arabidopsis gene that is both hormonally regulated itself and involved in auxin, ABA, and cytokinin responses. The pleiotropic developmental phenotype of the mutation suggests defects in the ability to perceive or transmit hormonal information. Thus, the mutant homozygote grows more slowly than does the wild type and shows less ability to respond to gravistimulation. Several of its defects, including reduced fertility, are attributable to decreased relative growth rates. Moreover, the plant shows excessive branching, implying reduced apical dominance. Direct analysis of the responses of the *hyl1* mutant plant to exogenous hormones revealed that the mutation decreases the sensitivity of the plant to inhibition of growth by auxin and cytokinin but also increases its sensitivity to the inhibition of seed germination and root growth by ABA.

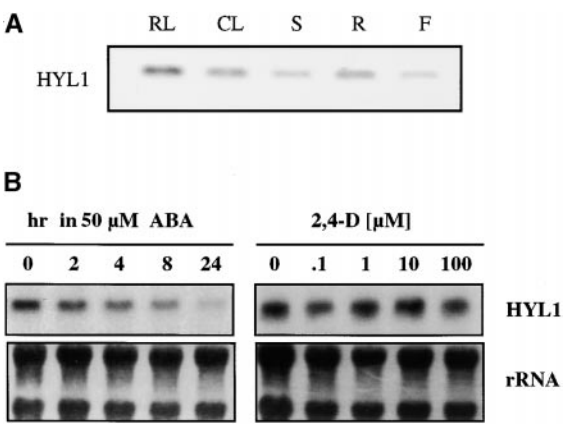


Figure 8. Expression of the *HYL1* Gene.

(A) RNA was isolated from rosette leaves (RL), cauline leaves (CL), inflorescent stems (S), roots (R), and flowers (F); fractionated on a 1.2% formaldehyde–agarose gel; blotted onto nitrocellulose; and probed with a full-length *HYL1* cDNA probe.

(B) RNA was isolated from seedlings 0, 2, 4, 8, or 24 hr after exposure to 50 μM ABA (left) or after 1.5-hr exposure to different concentrations of 2,4-D, ranging from 0.1 to 100 μM. RNA was fractionated and analyzed as described in **(A)**.

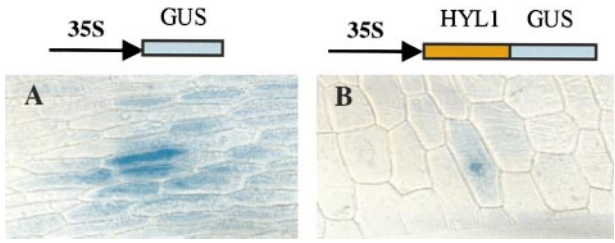


Figure 9. Subcellular Localization of an HYL1–GUS Fusion Protein.

(A) Structure of the *GUS* reporter construct.

(B) Structure of the *HYL1* cDNA–*GUS* fusion construct.

The constructs were introduced into onion epidermal cells by particle bombardment. The cells were stained for GUS activity after 20 hr of incubation at room temperature. The two cells shown are representative of the results obtained in two independent experiments.

The protein encoded by the *HYL1* gene contains a sequence resembling the plant consensus nuclear localization sequence, and an HYL1–GUS fusion protein accumulates in the nucleus, implying that the native protein is targeted to the nucleus. The HYL1 protein has two domains homologous with the dsRNA binding domains of other proteins. We have clearly demonstrated that an N-terminal fragment of the protein expressed in and purified from *E. coli* binds to dsRNA but not to ssRNA or to ss- or dsDNA of the same sequence. Although it remains to be determined whether the dsRNA binding domain of the HYL1 protein is essential for its function, the presence of canonical RNA binding domains and the binding properties of the recombinant protein strongly support the view that the HYL1 protein is a dsRNA binding protein.

Two genes that have previously been shown to be ABA-inducible, the *KIN2* gene coding for a protein similar to type 1 fish antifreeze proteins (Kurkela and Borg-Franck, 1992) and the ABA- and cold-regulated *COR47* gene (Gilmour et al., 1992), exhibit increased basal amounts of transcripts in *hyl1* mutant homozygotes. This suggests that the HYL1 protein is itself a negative transcriptional regulator or is part of a negative regulatory complex. The observation that the *HYL1* gene is itself downregulated by ABA is consistent with such an interpretation. The diminished sensitivity of the *hyl1* mutant to inhibition by high exogenous concentrations of auxins and cytokinins, and the significant stimulatory effects of low concentrations of auxin and of auxin transport inhibitors, suggest that the protein mediates a stimulatory effect of these hormones, either directly or indirectly. Although we do not understand the differential response of the *hyl1* mutant to the auxin transport inhibitors, we note that both NPA and TIBA show a slight but significant stimulation of root

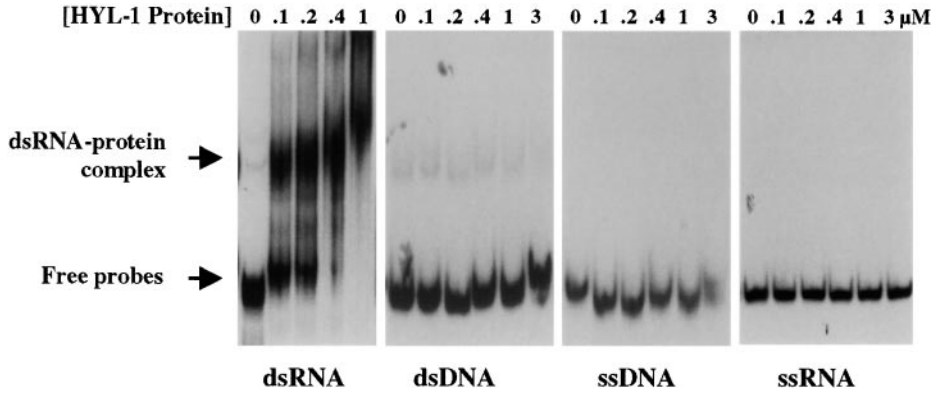


Figure 10. Native Gel Mobility Shift Analysis of HYL1 Protein Binding to Nucleic Acids.

An RNA duplex of ~100 bp was prepared by end-labeling hybridized T7 and T3 transcripts of the pBluescript II KS plasmid. A PCR fragment that includes both T7 and T3 promoters and intervening sequences of the same plasmid was used as dsDNA substrate. ssDNA was produced by denaturing and gel-purifying the same dsDNA fragment. A T7 transcript of the plasmid was used for ssRNA. Approximately the same amount of each test molecule (10 ng) was used for each experiment; the amount of the His-tagged HYL1 protein fragment added is indicated.

growth at very low concentrations, suggesting that inhibition of auxin transport partially compensates for the loss of *HYL1* function.

Growing genetic evidence indicates that a single mutation can affect responses to more than one hormone. Interactions between ABA and such environmental signals as salt, cold, or drought are well documented in Arabidopsis (Ishitani et al., 1997; Zhu et al., 1997). Moreover, antagonisms have been observed between ABA and gibberellic acid (GA) in dormancy and germination (Steber et al., 1998; Wobus and Weber, 1999). ABA was originally identified as an inhibitor of auxin-induced growth of coleoptiles and stems (Davies and Jones, 1991; Taiz and Zeiger, 1991). ABA-deficient and ABA-insensitive mutants rescue the germination of the *ga1* auxotroph and of seeds treated with an inhibitor of GA biosynthesis (Nambara et al., 1992; Steber et al., 1998). Genetic evidence also supports interconnections between ABA and other plant hormones. Thus, for example, the *axr2* mutant was isolated on the basis of its decreased auxin sensitivity, but it is also resistant to ABA (Wilson et al., 1990). The brassinosteroid *bri1* mutant is hypersensitive to ABA (Clouse et al., 1996). The phenotype of the *sax1* mutant in brassinosteroid biosynthesis suggests that brassinosteroids have a negative regulatory effect on ABA and auxin responses and a positive regulatory effect on GA and ethylene responses (Ephritikhine et al., 1999a, 1999b). Two recent reports describe the interactions between ABA and ethylene signaling cascades (Beaudoin et al., 2000; Ghassemian et al., 2000).

Some of the foregoing examples of interconnections among hormonal responses are probably mediated by small intermediary molecules, such as a brassinosteroid or Ca^{2+}. In contrast, the properties of the HYL1 protein suggest that it functions at the macromolecular level. In this regard, it resembles the nuclear WD protein encoded by the Arabidopsis *PRL1* locus (Nemeth et al., 1998). A mutation in that locus increases the sensitivity of the plant to cytokinin, ethylene, ABA, and auxin and confers hypersensitivity to glucose. The observed increase in the basal abundance of transcripts of ABA-inducible genes in the *hyl1* mutant suggests that HYL1 is involved in either the regulation of transcription itself or of mRNA stability, conjectures consonant with its nuclear localization. The HYL1 protein lacks an identifiable DNA binding motif and does not resemble known transcription factors. Although that does not prove that the HYL1 protein is not a transcriptional regulator, it does suggest that the protein is more likely to exert its regulatory effect indirectly, through interactions with other proteins. One possibility is that the HYL1 protein is a coactivator for certain auxin- and cytokinin-inducible genes and a corepressor for certain ABA-inducible genes. Also, it is possible that its molecular effects are similar, despite the apparently opposite phenotypic effects, or that some of its hormonal effects are primary and others secondary.

dsRNA binding proteins have been identified in many organisms, including humans, Drosophila, yeast, *E. coli*, and viruses (Burd and Dreyfuss, 1994). One of the best-characterized dsRNA binding proteins is PKR, a dsRNA-dependent protein kinase. PKR phosphorylates eukaryotic initiation factor 2 when activated by dsRNA. Induced by interferon treatment, PKR mediates the antiviral and antiproliferative effects of interferon; it also has a role in regulating cellular differentiation, stress response, and apoptosis (Clemens, 1997). Staufen, a Drosophila dsRNA binding protein with five dsRNA binding motifs, is required to properly localize *bicoid* and *oskar* mRNAs in the developing oocyte (St. Johnston et al., 1991). A plant protein with an RNA helicase domain and an RNase III–like domain has recently been identified through analysis of an Arabidopsis floral mutant

(Jacobsen et al., 1999). These observations suggest that dsRNA binding proteins play several cellular roles.

Consistent with the diverse functions of proteins with dsRNA binding domains, most have a second functional or catalytic domain. PKR contains a conserved protein kinase domain at its C terminus. dsRNA adenosine deaminase has an adenosine deaminating domain required for conversion of adenosine to inosine. The Drosophila *maleless* protein, on the other hand, carries an RNA helicase domain (Hitti et al., 1998). The HYL1 protein also appears to contain an additional domain. The C terminus of this protein contains six consecutive copies of a 28–amino acid repeat. Using the Smith–Waterman algorithm, we found a weakly homologous repeat in protein F from *S. pyogenes* (24.7% identity and 55% similarity in the 190–amino acid repetitive region). In protein F, however, the domain comprises five repeats compared with six repeats in the HYL1 protein. The repeats are at the C-terminal end of both proteins, with no gaps between the repeated units, and the repeats of both proteins are rich in acidic amino acids (Asp and Glu). Protein F is a fibronectin binding protein, and its repeat region has been shown to be necessary for binding (Hanski and Caparon, 1992; Sela et al., 1993). Using the C terminus of the protein as bait in a yeast two-hybrid system, we were able to identify several interacting proteins (C. Lu and N.V. Fedoroff, unpublished data), which suggests that HYL1 contains a protein–protein interaction domain.

Several recent observations suggest that dsRNAs have regulatory and signaling functions. For example, in a variety of organisms, including nematodes, fruit flies, trypanosomes, hydra, zebrafish, and mice, homologous dsRNA can suppress gene expression selectively and specifically (Fire et al., 1998; Kennerdell and Carthew, 1998; Ngo et al., 1998; Lohmann et al., 1999; Wargelius et al., 1999). And growing, although still circumstantial, evidence suggests that dsRNA mediates both transcriptional and post-transcriptional gene silencing in plants (Vaucheret et al., 1998; Waterhouse et al., 1998; Fire, 1999; Hamilton and Baulcombe, 1999; Jones et al., 1999). Gene silencing can spread systemically in plants and requires an RNA-dependent RNA polymerase (Vaucheret et al., 1998; Voinnet et al., 1998; Dalmay et al., 2000; Mourrain et al., 2000). The recent identification of a plant protein that can carry RNA systemically is consistent with the notion that RNA is a signaling molecule (Xoconostle-Cazares et al., 1999).

Nothing is known about the role of dsRNA in the normal developmental and physiological processes of plants. Although sequence-specificity is not inherent in dsRNA binding proteins (Bevilacqua and Cech, 1996; Bevilacqua et al., 1998), gene silencing is sequence-specific. The properties of the *hyl1* mutant and the protein encoded by the *HYL1* locus suggest the involvement of dsRNA in the hormonal signaling networks that control growth and development in Arabidopsis. Recent studies on the mechanism of dsRNA-induced mRNA destabilization suggest that a multiprotein complex may mediate the sequence-specific destabilization of mRNA by short dsRNAs, which in turn might be enzymatically amplified (Hamilton and Baulcombe, 1999; Tuschl et al., 1999; Grishok et al., 2000; Zamore et al., 2000). Thus, an alternative hypothesis for the role of the HYL1 protein would be its participation in a regulatory mechanism that acts to destabilize transcripts by a dsRNA-dependent mechanism. Further investigation of the molecular interactions between the HYL1 protein and dsRNA, as well as the proteins that interact with HYL1, should provide insight into the role of this gene in development and of dsRNA in hormone signaling.

METHODS

Plant Lines and Growth Conditions

All experiments were performed with *Arabidopsis thaliana* ecotype Nossen. The transposon lines were described by Smith et al. (1997). Plants were grown on Murashige and Skoog (MS) medium (Gibco BRL, Grand Island, NY) containing 1% sucrose. In experiments on sterile medium, seeds were sterilized and grown as described in Lincoln et al. (1990). Indoleacetic acid (IAA), the synthetic auxin 2,4-dichlorophen-oxyacetic acid (2,4-D), abscisic acid (ABA), and the cytokinin 6-benzylaminopurine (BA) were added to the autoclaved medium where indicated. To examine the sensitivity to plant hormones, seedlings were measured ~5 to 10 days after germination. To assess the root gravitropic response, wild-type and mutant seedlings were grown for 5 days on minimal medium (Lincoln et al., 1990). Plants were placed vertically. After 5 days, seedlings were transferred to square Petri plates containing minimal medium. Gravitropic stimulation was achieved by rotating the square Petri plates by 90°. The angle of curvature of the growing root tips was measured at 1-hr intervals.

TAIL-PCR and Footprint Analysis

Total DNA was extracted from leaves of mutant plants for thermal asymmetric interlaced polymerase chain reaction (TAIL PCR) amplification reactions (Dellaporta et al., 1983). TAIL PCR was performed as described by Tsugeki et al. (1996). After tertiary PCR, fragments were separated by gel electrophoresis and purified from gels by using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). Purified fragments were directly sequenced. DNA was extracted from leaves of putative revertants for detection of a transposon footprint. PCR was performed with primers corresponding to the 5′ and 3′ flanking region of the *Ds* insertion site (5′-ATTGGCTTAGCTCACTGGATTTTG and 5′-GGTTTAAAACTGTCTCTCC). Amplified fragments were purified and directly sequenced.

Isolation of Full-Length HYL1 cDNA

A full-length cDNA was isolated from the Arabidopsis CD4-7 lambda PRL2 cDNA library (obtained from the Arabidopsis Biological Resource Center) by using the λZIPLOX selection system (Life Technologies, Inc., Bethesda, MD). Briefly, a genomic fragment was amplified by PCR with the primer pair 5′-ATTGGCTTAGCTCACTGGATTTTG and 5′-GGTTTAAAACTGTCTCTCC and then labeled with

phosphorus-32 by using random primers and following standard procedures. This was used as a probe to screen the cDNA library. The cDNA clones were sequenced by using shotgun cloning and automated sequencing. Database searches for homologous sequences were performed by using the NCBI BLAST programs and the European Molecular Biology Network FDF (Fast Data Finder) tool, which is an implementation of the original Smith–Waterman dynamic programming algorithm.

DNA and RNA Gel Blot Analysis

DNA was extracted from Arabidopsis leaves as described by Dellaporta et al. (1983). DNA digests (500 ng) were fractionated on an 0.8% agarose gel, then transferred to Hybond-N+ membrane (Amersham). The full-length HYL1 cDNA was labeled with [^{32}P]dCTP by random priming and used as a probe.

Total RNA was isolated by using the RNeasy Plant Mini Kit (QIAGEN) from liquid-grown root cultures, rosette leaves, cauline leaves, stems, and flowers of all stages from soil-grown plants ~4 to 6 weeks after germination. To analyze the effects of ABA, seedlings were treated as described previously (Wang et al., 1998). Briefly, 2-week-old seedlings grown in pots were cleared of soil with water, then put into 0.1 × MS medium with 50 μM ABA. Seedling samples were collected after various treatment periods and total RNA was isolated. Approximately 10 μg of total RNA was fractioned in a formaldehyde gel. The HYL1-specific probe was from the cDNA coding region. *KIN2* cDNA was provided by Dr. M.F. Thomashow. DNA probes for *COR47* were amplified by PCR from expressed sequence tag 242B8T7 (Arabidopsis expressed sequence tag library obtained from the Arabidopsis Biological Resource Center). All the DNA probes were labeled with [^{32}P]dCTP. Hybridization was performed at 65°C as previously described (Tsugeki et al., 1996).

Transient Assay in Onion Epidermal Cells

The 35S-HYL1 transcriptional fusion was produced by amplifying the HYL1 coding sequence from the HYL1 cDNA clone with a 5′ T7 primer and the primer 5′-CCATGCCATGGTTGCGTGGCTTGCTTC-TGTCTC-3′ to create a 3′ NcoI site at the 3′ end. The amplified fragment was digested with EcoRI and NcoI and cloned into EcoRI/NcoI-digested plasmid pRTL-GUS, which contains the β-glucuronidase (GUS) coding sequence expressed from a cauliflower mosaic virus (CaMV) 35S promoter. The 35S-HYL1-GUS fusion construct was made by fusing the GUS-encoding gene carried by the plasmid pRTL-GUS to the 3′ end of the *HYL1* gene by inserting the full-length *HYL1* coding sequence into the EcoRI/NcoI site of the 35S-HYL1 plasmid.

Onion epidermal layers were transformed by using biolistic bombardment as described by Varagona et al. (1992). After bombardment, the layers were incubated for 20 hr at room temperature in darkness. GUS activity was detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Carrington, 1995). The subcellular location of the blue precipitate was visualized with a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY).

In Vitro Protein Binding Activity Assay

The *HYL1* cDNA was subcloned into the vector pQE-32 (QIAGEN), designed for overexpression of proteins fused to RGS–His tag. The fusion protein, a 441–amino acid protein, contained all 419 amino acids encoded by the *HYL1* cDNA. The tagged protein was purified by using a Ni–NTA (where NTA is nitrilo triacetic acid) resin according to the manufacturer's protocol under nondenaturing conditions.

Proteins were fractionated by 8% SDS-PAGE and blotted onto PROTRAN membranes (Schleicher and Schuell). The blot was blocked with 3% BSA in Tris-buffered saline (TBS: 10 mM Tris-Cl, pH 7.5, and 150 mM NaCl) and incubated with anti-RGS–His antibody (1:1500 dilution). The blot was then incubated with the secondary antibody (sheep anti–mouse IgG; Amersham-Pharmacia Biotech) conjugated with horseradish peroxidase (1:6000 dilution). After each incubation step with antibodies, the membrane was washed with TBS and TBS-Tween/Triton buffer (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 0.05% [v/v] Tween 20, and 0.2% [v/v] Triton X-100). The ECL-Plus System (Amersham-Pharmacia Biotech) was used for immunodetection according to the manufacturer's instructions.

The binding activity assays were performed as previously described (Bass et al., 1994). A pBluescript II KS vector (Stratagene) containing a short fragment (multiple cloning sites) between the T7 and T3 promoters was used to prepare substrates. The T7 transcript labeled with T4 polynucleotide kinase was used as the ssRNA substrate. To make the dsRNA substrate, T3 transcripts were first kinase-labeled, then excess T7 transcripts were hybridized with labeled T3 transcripts to form dsRNA. The T7/T3 PCR products, native and denatured, were used as dsDNA and ssDNA substrates, respectively.

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