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-triodobenzoic 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; Bleeker, 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 HY Ponastic 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 Dissocia-
tion (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 (Pohlan 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 (Li 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 era1-3 mutant.
To examine the transcriptional effects of the hy1 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 threefold greater in *hy1* 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 hy1 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 *hy1* 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 *hy1* mutant roots at concentrations up to 1 μM, inhibiting elongation only at 5 μM, the highest
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 (Figure 5B). 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...
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that has been sequenced and anchored in chromosome 1s, 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.
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
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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 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.
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 assay using a 70-mer DNA pool containing a unique stretch of 12 consecutive guanosines (G), which can be used as a DNA or RNA mimic. The protein purified under native conditions was incubated with the DNA pool and analyzed by gel electrophoresis. The shift in gel mobility of the DNA pool upon incubation with the protein indicates the ability of the protein to bind the DNA mimic. The results show that the protein binds to the DNA pool, indicating its ability to bind dsRNA.
experiment performed under nondenaturing conditions with the truncated HYL1 protein and a \(^{32}\)P-labeled dsRNA fragment of \(\sim 100\) bp derived from the pBluescript II KS vector. A dsRNA–protein complex was first observed at a protein concentration of 0.1 \(\mu\)M. 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 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.

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 \(HYL1\) gene 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

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**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 \(\mu\)M ABA (left) or after 1.5-hr exposure to different concentrations of 2,4-D, ranging from 0.1 to 100 \(\mu\)M. 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.
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., 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 hy1 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.

**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.
of mRNA by short dsRNAs, which in turn might be enzymatically amplified (Hamilton and Baulcombe, 1999; Tuschi 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-dichlorophenoxacyclic 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′-ATGGGTAGCTCAGTGGATTTG and 5′-GGTTAAAAACTGTCTCCTCC). 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 ZIPLOX selection system (Life Technologies, Inc., Bethesda, MD). Briefly, a genomic fragment was amplified by PCR with the primer pair 5′-ATGGGTAGCTCAGTGGATTTG and 5′-GGTTAAAAACTGTCTCCTCC 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 (Amer- sham). The full-length HYL1 cDNA was labeled with [32P]dCTP by random priming and used as a probe.

Total RNA was isolated by using the RNasy 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 fractionated in a form-aldehyde 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 242BF17 (Arabidopsis expressed sequence tag library obtained from the Arabidopsis Biological Resource Center). All the DNA probes were labeled with [32P]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′-CCATGCCATGTTGGGCTTGGCTC-TGTCTC-3′ to create a 3′ Ncol site at the 3′ end. The amplified fragment was digested with EcoRI and Ncol and cloned into EcoRI/Ncol-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/Ncol 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 nitrio 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-Pharmaica 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-Pharimacia 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.

ACKNOWLEDGMENTS

We thank Dr. Ryuji Tsugeki for the help with cDNA screening and phenotypic characterization, Maya Olson for revertant screening, and Dr. Michael F. Thomashow for the Kin2 clone. We are also grateful to Drs. Hong Ma, Sarah Assmann, Simon Gilroy, Philip Bevilacqua, Ramamurti Mahalingam, Fuqiang Chen, and Surhali Raina and to Hongchang Cui and Philip Day for their helpful discussion and critical review. This work was supported by National Aeronautics and Space Administration Grant NAG5-3970.

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