Control of auxin-regulated root development by the *Arabidopsis thaliana* **SHY2/IAA3** gene

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SUMMARY

The plant hormone auxin controls many aspects of development and acts in part by inducing expression of various genes. *Arabidopsis thaliana* semidominant *shy2* (short hypocotyl) mutations cause leaf formation in dark-grown plants, suggesting that SHY2 has an important role in regulating development. Here we show that the *SHY2* gene encodes *IAA3*, a previously known member of the *Aux/IAA* family of auxin-induced genes. Dominant *shy2* mutations cause amino acid changes in domain II, conserved among all members of this family. We isolated loss-of-function *shy2* alleles including a putative null

mutation. Gain-of-function and loss-of-function *shy2* mutations affect auxin-dependent root growth, lateral root formation, and timing of gravitropism, indicating that *SHY2/IAA3* regulates multiple auxin responses in roots. The phenotypes suggest that *SHY2/IAA3* may activate some auxin responses and repress others. Models invoking tissue-specificity, feedback regulation, or control of auxin transport may explain these results.

Key words: auxin, SHY2, IAA3, *Arabidopsis thaliana*, root development

INTRODUCTION

Plant hormones are central endogenous regulators of many aspects of plant growth and development. Auxin, one of the most extensively studied hormones, regulates cell division, cell elongation, cell differentiation, and patterning (Sachs, 1991; Thimann, 1977). In the intact plant, auxin can inhibit root elongation, promote hypocotyl and stem elongation, mediate root and shoot gravitropism, promote lateral root and adventitious root formation, promote root hair formation, and maintain apical dominance (Estelle and Klee, 1994; Hobbie, 1998). A variety of studies suggest that auxin is also involved in patterning events such as vascular tissue differentiation and phyllotaxy (Sachs, 1991).

Genetic studies in *Arabidopsis thaliana* have yielded at least three classes of mutants with auxin-related phenotypes. One class of mutations affect genes involved in activation of the ubiquitin related protein Rub (such as *axr1*), or its putative downstream effectors *tir1* and *sar1* (Cernac et al., 1997; del Pozo et al., 1998; Ruegger et al., 1998). A second class of mutations affect auxin transport. For example, *AUX1* encodes an putative auxin uptake carrier and *EIR1* encodes a putative auxin efflux carrier (Bennett et al., 1996; Luschnig et al., 1998; Yamamoto and Yamamoto, 1998). Several other loci may also fall in this class, such as *pinoid*, *pin-formed*, *rcn1*, *pis1*, *tir3* and *lop1* (Bennett et al., 1995; Carland and McHale, 1996; Fujita and Syono, 1997; Garbers et al., 1996; Okada et al., 1991; Ruegger et al., 1997). A third class of mutations affect auxin-inducible genes or transcription

factors that regulate such genes, such as *axr3* and *monopteros*.

Several gene families induced rapidly and specifically by auxin have been identified in various plant species (Abel and Theologis, 1996). The *Aux/IAA* gene family has at least 20 members in *Arabidopsis thaliana* (Abel et al., 1995; Kim et al., 1997). The kinetics of induction by auxin are different among these various genes, and some are induced faster than others. For example, induction of *IAA3* and *IAA6* mRNA begins within 5 minutes of auxin treatment and peaks after 10 minutes (Abel et al., 1995). Pea PS-*IAA4* and PS-*IAA6* proteins are localized to the nucleus and are very short-lived, having half-lives of 6–8 minutes, which suggests that the proteins play transient regulatory roles in response to auxin (Abel et al., 1994). The predicted proteins contain four conserved domains. Domains I, II and IV contain typical NLS motifs (Abel and Theologis, 1995). Domain III is predicted to form a $\beta\alpha\alpha$ structure, which is similar to the DNA-binding domain from certain prokaryotic repressors. However, there is no evidence that IAA proteins bind DNA directly. Domains III and IV are required for homo- and hetero-dimerization of IAA proteins (Kim et al., 1997; Ulmasov et al., 1997b).

ARF (Auxin Response Factor) proteins are transcription factors that can bind to auxin response elements present in promoters of numerous auxin-regulated genes, including those of many *IAA* genes (Abel and Theologis, 1996; Guilfoyle et al., 1998a,b; Ulmasov et al., 1997a). There are at least 10 members of this family in *Arabidopsis thaliana*, and most of these (except for ARF3) have C-terminal domains homologous

to domains III and IV of the IAA proteins (Guilfoyle et al., 1998b; Kim et al., 1997). ARF1 binds DNA through its N-terminal VP1-like motif, and its domain III- and IV-like C-terminal domain can interact with IAA proteins in the yeast two-hybrid system (Ulmasov et al., 1997b).

Mutants in several *IAA* and *ARF* genes have provided functional evidence that they are important in mediating vascular tissue differentiation, patterning and other auxin responses. Semidominant *axr3* mutations cause increased apical dominance, reduced root elongation, increased adventitious root formation, auxin-resistant root growth, and upward curling of the leaf edges (Leyser et al., 1996). *AXR3* has been cloned and shown to be *IAA17*, a member of *Aux/IAA* gene family (Rouse et al., 1998). *monopteros* (*mp*) mutants are defective in auxin transport, vascular strand differentiation and apical-basal axis formation in early embryos (Przemeck et al., 1996). *ettin* mutants have defects in defining floral organ number and patterning in the gynoecium (Sessions et al., 1997). The *MONOPTEROS* and *ETTIN* genes encode *ARF5* and *ARF3*, respectively (Hardtke and Berleth, 1998; Sessions et al., 1997).

Semidominant *shy2* mutations were identified in screens for suppressors of the long hypocotyl phenotype of phytochrome-deficient *hy2* or *phyB* mutants (Kim et al., 1996; Reed et al., 1998). *shy2-2* seedlings have short hypocotyls both in the dark and in the light, have curled-up leaves in the light, and flower early (Reed et al., 1998). *shy2-2* mutants can also make leaves in the dark. *shy2-3* mutants have less severe phenotypes, for example expanded cotyledons without extensive leaf formation in the dark (Reed et al., 1998). Similarly, the *shy2-1* mutant, isolated as a suppressor of a *hy2* mutation, has curled-up leaves in the light, and can make true leaves, inflorescence stems and flowers in the dark (Kim et al., 1996, 1998). In addition, light-induced *CAB* and *PSBA* genes are expressed in dark-grown *shy2-1* seedlings (Kim et al., 1998).

In this work, we report that *shy2* mutations fall in *IAA3*, a member of the *Aux/IAA* gene family. We have isolated loss-of-function alleles of *SHY2/IAA3* and characterized phenotypes of both gain-of-function and loss-of-function *shy2* mutants. Our data indicate that *SHY2/IAA3* plays a central role in auxin regulation of root growth, in gravitropism, and in lateral root formation.

MATERIALS AND METHODS

Mapping

We crossed *shy2-2* (in Landsberg *erecta*) with Columbia wild type, and selected F₂ plants of wild-type phenotype for analysis. Genomic DNA from individual F₂ plants was isolated and amplified with primers for various PCR-based CAPS markers including PVV4, NCCI, 0846A and IGF20G19 (Konieczny and Ausubel, 1993; Rouse et al., 1998), then assayed for Landsberg *erecta* and Columbia-specific polymorphisms.

PCR sequencing

We amplified the *SHY2/IAA3* gene from *shy2* mutants. The primers were 5'-AAA ACT GCA GAC ACT CAG CTT CCT CGA AGC-3' and 5'-CCG GGA TCC GGA GTA ATC CAA CCT CGA CC-3', and were designed based on the complete DNA sequence of the *SHY2/IAA3* gene in GenBank from ecotype Columbia (*Pst*I site and *Bam*HI site are underlined). PCR reaction mixtures contained 100 ng

genomic DNA, 0.5 µM of each primer, 0.5 unit of *Taq* polymerase, 0.2 mM dNTP, 1.5 mM MgCl₂, and reaction buffer (20 mM Tris-HCl, pH 4.8, 50 mM KCl), and were incubated in a thermocycler for 34 cycles following hot start. Each cycle consisted of 94°C (45 seconds), 56°C (1 minute), 72°C (2 minutes) followed by 5 minutes at 72°C. For *shy2-2*, the amplified genomic DNA fragments were cloned into pBluescript SK⁻ (*Pst*I/*Bam*HI) by standard restriction enzyme digestion and ligation protocols and sequenced by standard methods. For *shy2-3*, *shy2-21*, *shy2-22*, *shy2-23* and *shy2-24*, we amplified *SHY2/IAA3* in 10 individual reactions and mixed them together, then purified the mixture by running through a Sephadex G50 column to remove primers and small nucleotides. The purified PCR product was sequenced directly by standard methods.

Generation of transgenic plants

A 3.5 kb fragment of genomic DNA including the *SHY2/IAA3* coding sequence and 2.2 kb of upstream DNA was amplified from *shy2-2* DNA using the primers 5'-AGT GCT ATA ATC AAC CAG CG-3' and 5'-CCG GGA TCC GGA GTA ATC CAA CCT CGA CC-3' (underlined is the *Bam*HI site) and cloned into pPZP211 vector (*Pst*I/*Eco*RI) (Hajdukiewicz et al., 1994). (The methods for PCR amplification and cloning were as described above except that the annealing temperature was 60°C instead of 56°C.) The plasmid was introduced into *Agrobacterium* strain GV3101 (Koncz and Schell, 1986) by electroporation. Wild-type plants (Landsberg *erecta*) were inoculated with this strain by vacuum infiltration (Bechtold et al., 1993). Transformed seedlings were identified by selection on 50 µg/ml kanamycin.

Mutagenesis and suppressor screen

We incubated 1800 *shy2-2* M₁ seeds overnight in 0.07% ethyl methane sulfonate (EMS) and 1800 seeds in 0.2% ethyl methane sulfonate, and then rinsed them several times with water. We collected M₂ progeny in 10 batches from 350 M₁ plants per batch. We screened 70,000 M₂ seedlings on MS/SUC/agar plates under red light for those with a long hypocotyl. To detect the starting *shy2-2* mutation, we PCR amplified the *SHY2/IAA3* gene from genomic DNA, and assayed for the presence (wild-type allele) or absence (*shy2-2* mutant allele) of a *Hae*III site. Mutants from different batches were assumed to be independent. PCR products of the *SHY2/IAA3* gene from the intragenic suppressor alleles were sequenced as described above.

RT-PCR

RNA was isolated as described by Chory et al. (1991). Complementary DNA synthesis was performed in a reaction consisting of 13.3 U/µl reverse transcriptase, 1× reverse transcription buffer (50 mM Tris-HCl, 8 mM MgCl₂, 10 mM dithiothreitol), 0.5 mM dNTP mix, 1 U/µl RNase inhibitor, and 1.5 µM oligo(dT) primer. RNA (10 µg) in 19 µl was denatured at 68°C for 3 minutes, then the reaction buffer was added and the mixture incubated at 42°C for 1 hour. 3 µl of the products were then used for PCR after denaturation for 10 minutes at 68°C. PCR methods were as described above. The primers were 5'-CAG AGC TGA GGC TGG GAT TAC-3' and 5'-CGG GAT CCC GTA CAC CAC AGC CTA AAC-3' (the *Bam*HI site is underlined). We found that these primers amplified a fragment of *IAA4* cDNA as well as the *SHY2/IAA3* cDNA. Therefore, the PCR products were cloned into pBluescript SK⁻ (Stratagene, La Jolla, CA), and a *SHY2/IAA3*-specific clone was identified and sequenced.

Seedling growth experiments

Seeds were surface sterilized and plated on MS/agar plates (1× Murashige and Skoog salts, 0.8% phytagar [Gibco BRL], 1× Gamborg's B5 vitamin mix) with or without 2% sucrose, and stored at 4°C overnight to induce germination. The plates were grown at 23°C under a mixture of fluorescent and incandescent bulbs at an intensity of 100-200 µmoles/m² second. For dark experiments, seeds

were induced to germinate in the light for 12 hours after cold treatment, and then transferred to total darkness.

For hypocotyl and root growth rate experiments, seedlings were grown on vertically oriented MS/SUC/agar plates under white light or in the dark. Digital images of the plates were taken daily after germination with a CCD camera and the hypocotyl and root lengths were measured using image analysis software (NIH image, Bethesda, MD). The root growth rates of all genotypes varied quantitatively from experiment to experiment, presumably as a result of variable light, humidity, temperature, or other aspects of the growth conditions, and representative root growth experiments are shown. For the dark experiment, one plate was used for each time point. For measurement of cotyledon sizes, seedlings were grown on horizontally oriented plates in white light for 10 days, and the cotyledons were excised and flattened under a cover slip. The cotyledon sizes were then measured using a microscope equipped with a camera lucida.

Lateral and adventitious root formation

For measurement of lateral root formation, seedlings were grown on vertically oriented MS/SUC/agar plates under white light for 3 weeks, and the number of lateral roots was counted under a dissecting microscope. For measurement of adventitious root formation, the seeds were surface sterilized and plated on MS/SUC/agar plates, stored at 4°C for 3 days, and grown horizontally under white light for 1 day at 23°C. Then the plates were placed vertically in the dark for 5 days, moved to white light again, and set horizontally for 18 days. Adventitious roots were counted under a dissecting microscope.

Gravitropism

Seedlings for the gravitropic angle experiment were grown under different fluence rates of red light on vertically oriented plates as described previously (Reed et al., 1998). After 5 days growth, images were taken with a CCD camera, and angles between the growing direction of hypocotyls and the opposite direction of gravity (hypocotyl gravitropism), and angles between the growing direction of roots and the direction of gravity (root gravitropism) were measured using image analysis software (NIH image). For the gravitropic curvature experiment, seedlings were grown on vertically oriented MS/SUC/agar plates in the dark for 48 hours and then were rotated through 90° in the darkness. For measurement of hypocotyl and root gravitropic curvature, we measured growth angles only of seedlings for which the orientation of hypocotyls and roots before re-orientation was less than 20° from vertical. The angles between the opposite direction of gravity or the direction of gravity after re-orientation and the growing direction of hypocotyls or roots at each time was measured as the curvature. One plate was used for each time point.

Wavy root examination

Seedlings were grown on MS/SUC/1.5% agar plates set in a vertical position for 3 days. Then the plate was tilted to 45° and the seedlings were grown for 3 days and photographed.

Root growth response to exogenous hormones

Seedlings were grown on vertically oriented MS/SUC/agar plates for 5 days under white light at 23°C to allow the roots to grow along the surface. Then 20 seedlings per treatment were transferred to new plates supplemented with various concentrations of hormones (IAA, 2,4-D, BA, ABA, or NPA). The positions of root tips of all seedlings were marked. After 3 days, the amount of new root growth was measured.

RESULTS

Cloning of *SHY2*

Previous work showed that *SHY2* maps to chromosome 1 between markers PVV4 and *nga63*. We mapped the gene more precisely using two polymorphic CAPS markers in this region

developed by Rouse et al. (1998). These data established that *SHY2* is north of 0846A, close to IGF20G19 (Fig. 1). A sequenced BAC clone, IGF19P19, extends 16kb into this region and contains two adjacent *Aux/IAA* genes, *IAA3* and *IAA17* (Fig. 1). *AXR3* also maps to this region and it has been demonstrated to be *IAA17* (Rouse et al., 1998).

shy2 and *axr3* mutants share several phenotypes, such as short hypocotyls, curled-up leaves and leaf formation in darkness. However, unlike *shy2* mutants, *axr3* mutants have few shoot branches and highly agravitropic roots that lack root hairs (Leyser et al., 1996). These observations suggested that *shy2* mutations might affect the other *Aux/IAA* gene family member in this region, *IAA3*. We demonstrated this by sequencing the *IAA3* gene from *shy2* mutants. *shy2-2* has a single nucleotide mutation predicted to change the proline at amino acid position 69 to serine (Fig. 2). *shy2-3* has a single nucleotide change predicted to change the glycine at amino acid position 67 to glutamine (Fig. 2). Both of these mutations affect amino acid residues in domain II, a highly conserved domain among the *Aux/IAA* proteins. Semidominant *axr3-1* and *axr3-3* mutations affect amino acids in the corresponding domain of *IAA17*, suggesting that this domain has a similar regulatory function in both proteins (Rouse et al., 1998).

We transformed the *SHY2/IAA3* gene together with about 2.2 kb of DNA upstream of the open reading frame from *shy2-2* mutant genomic DNA into wild-type (Landsberg *erecta*) plants. 14 out of 15 T1 transformants resembled *shy2-2* plants in having upcurled leaves and short hypocotyls (Fig. 3P,Q). These data show that the point mutation in *shy2-2* is sufficient to confer the characteristic *shy2* phenotype, and suggest that 2.2 kb of upstream sequence is sufficient to direct normal expression. Together with the finding that *shy2-2* and *shy2-3* are semidominant missense mutations, this result suggests that these mutations cause gain of function.

Loss-of-function alleles of *SHY2/IAA3*

We isolated four potential loss-of-function alleles of *SHY2/IAA3* by mutagenizing *shy2-2* with EMS and screening for seedlings with long hypocotyls in red light. F₁ plants from backcrosses of each suppressor to *shy2-2* plants resembled heterozygous *shy2-2* plants, indicating that the new mutations are recessive to *shy2-2*. F₁ plants from crosses to wild type

Fig. 1. Cloning the *SHY2* gene. The position of the *SHY2* locus is shown relative to chromosome one CAPS markers PVV4, NCCI, 0846A and IGF20G19 end. Numbers beneath the line indicate frequencies of recombinants between *SHY2* and each marker. The position and transcription direction (arrows) of *AXR3/IAA17* and *SHY2/IAA3* are also shown.

Fig. 2. Sequences of *shy2* mutations. Three exons (boxes) and two introns (lines) of the *SHY2/IAA3* gene are shown. Black boxes indicate the open reading frame. Positions of *shy2* mutations and corresponding amino acid changes are indicated. *shy2-22* retains the *shy2-2* mutation and has aberrant splicing causing a reading frame shift in the third exon. The altered amino acid sequence is indicated. *shy2-21* is identical to *shy2-22*, and *shy2-23* is identical to *shy2-24*.

appeared similar to wild type, as did all of the F₂ plants from self-fertilization of the F₁ plants. These data showed that the suppressor mutations are closely linked to the *shy2-2* mutation. We sequenced the *SHY2/IAA3* gene from the four tall mutants, and found that all four retained the *shy2-2* mutation and also had an additional point mutation at another position in the gene. Accordingly, we refer to these alleles as *shy2-21*, *shy2-22*, *shy2-23* and *shy2-24*.

shy2-21 and *shy2-22* have identical mutations that change the consensus 3' splice site AG of the second intron to AA (Fig. 2). We cloned and sequenced a RT-PCR product of the *SHY2/IAA3* gene from *shy2-22* seedlings and found that the mutation causes aberrant splicing. Splicing of the second intron uses the mutated A in the last position of the second intron and the G in the first position of the third exon as an alternative 'consensus' AG splicing site. This altered splicing shifts the reading frame of the third exon, thereby replacing the last 24 amino acids at the C-terminus (including half of conserved domain IV) with 8 amino acids (Fig. 2).

shy2-23 and *shy2-24* have identical mutations that introduce a stop codon at amino acid position 61, 8 amino acids upstream of the codon mutated in *shy2-2* (Fig. 2). Since the *shy2-21*, *shy2-22*, *shy2-23* and *shy2-24* alleles each have mutations that truncate the protein (all four alleles) and/or replace a domain (*shy2-21* and *shy2-22*), they are probably loss-of-function alleles. As the *shy2-23* and *shy2-24* mutations truncate the protein by two thirds and confer more extreme phenotypes than the *shy2-21* and *shy2-22* mutations (see below), *shy2-23* and *shy2-24* may be null alleles. *shy2-21* and *shy2-22* are more

complex, in that they appear to have a partial loss-of-function mutation and a gain-of-function mutation within the same gene.

Shoot morphology of *shy2* mutants

Whereas gain-of-function *shy2* mutations caused dramatic shoot phenotypes, loss-of-function mutations had more subtle effects. Light-grown *shy2-2* and *shy2-3* seedlings had larger cotyledons and shorter hypocotyls than wild type seedlings after 10 days of growth, and their cotyledons curled up (Table 1; Fig. 3B; Reed et al., 1998). *shy2-2* and *shy2-3* adult plants were extremely dwarfed with curled-up leaves (Fig. 3F,G). As described previously for hypocotyl elongation, the adult phenotype conferred by *shy2-2* was semidominant (Fig. 3H). In contrast, *shy2-22* and *shy2-24* seedlings did not have strong phenotypes in the shoot. The hypocotyls of *shy2-22* and *shy2-24* seedlings grew slightly faster than wild type at early times, but slowed resulting in similar final hypocotyl lengths to wild-type seedlings (Fig. 3A,C,D, 4A). Cotyledons of *shy2-22*, but not *shy2-24*, seedlings were slightly larger than wild-type cotyledons (Table 1). In contrast to the upcurled cotyledons and leaves of *shy2-2* and *shy2-3* plants, those of *shy2-22* and *shy2-24* plants appeared normal (Fig. 3C,D) and adult *shy2-22* and *shy2-24* plants appeared similar to wild-type plants (Fig. 3I,J).

One-week old dark-grown wild-type seedlings elongate their hypocotyls, have unexpanded cotyledons, retain an apical hook, and fail to develop leaves (Fig. 3K). In contrast, *shy2-2* seedlings had short hypocotyls, expanded cotyledons, opened apical hooks, and true leaf primordia (Fig. 3L; Reed et al.,

Table 1. Measurement of *shy2* mutants

	<i>SHY2</i>	<i>shy2-2</i>	<i>shy2-3</i>	<i>shy2-22</i>	<i>shy2-24</i>
Hypocotyl length (mm)	2.9±0.8	1.3±0.3***	1.7±0.5***	2.9±0.5†	2.8±0.5†
Root length (mm)	25.6±0.9	16.5±3.7***	20.7±2.6***	16.2±6.1***	11.5±4.2***
Cotyledon size (mm ²)	5.6±0.8	6.7±0.4***	7.6±0.8***	7.1±0.9***	6.0±0.4†
% of 21-day dark-grown seedlings having visible leaf primordia	7	100	24	9	22
Lateral roots/seedling	9.1±4.6	2.1±2.1***	ND	14.7±7.4**	16.4±7.9*
Adventitious roots/seedling	2.3±1.7	0.1±0.4***	0.1±0.2***	2.3±1.5†	2.8±2.0†

Hypocotyl length, root length, and cotyledon size for each genotype were measured after 10 days growth in white light. Seedlings for lateral and adventitious root counts were grown as described in Materials and Methods. For each parameter, at least 15 measurements were taken, and the mean and standard deviation are presented. In the adventitious root experiment, we have combined data for the pairs of identical mutants *shy2-21/shy2-22* and *shy2-23/shy2-24*. The measurements of *shy2* mutants were tested for significant difference from wild type by *t*-test. The probabilities that the data would arise from identical populations are indicated: ****P*<0.001; ***P*<0.01; **P*<0.05; †*P*>0.05 (i.e. not significantly different from wild type). ND, not done.

1998). *shy2-3* mutants had phenotypes similar to but less severe than those of *shy2-2* mutants (Fig. 3M; Reed et al., 1998). In contrast, *shy2-22* and *shy2-24* mutants appeared almost identical to wild type (Fig. 3N,O). *shy2-22* and *shy2-24* hypocotyl growth slowed slightly after growing similarly to wild type at early times (Fig. 4B). After 3 weeks of dark growth, all *shy2-2* mutant seedlings formed visible leaf primordia, whereas very few wild type, *shy2-22*, or *shy2-24* seedlings did (Table 1).

Root morphology of *shy2* mutants

Both gain-of-function and loss-of-function *shy2* mutations affected root growth. When grown in the light, all *shy2* mutant seedlings had significantly shorter roots than wild-type seedlings (Table 1). The short root phenotype was most severe in the *shy2-24* mutant, and was reflected in reduced root growth rates compared to wild-type seedlings (Fig. 4C). When grown in the dark, all *shy2* seedlings had average root growth rates similar to those of wild-type seedlings (Fig. 4D).

The *shy2* mutations also affected root branching. When grown on vertically oriented plates in continuous white light for 3 weeks, *shy2-2* mutants made very few lateral roots, whereas the *shy2-22* and *shy2-24* mutants formed more and much longer lateral roots than wild-type seedlings (Table 1, Fig. 5). When grown under conditions that promote adventitious root formation in the hypocotyl, *shy2-2* and *shy2-3* seedlings each made fewer adventitious roots than wild type (Table 1). Although they made excess lateral roots, *shy2-22* and *shy2-24* plants each made similar numbers of adventitious roots to wild type (Table 1).

Auxin response phenotypes

The discovery that *SHY2/IAA3* encodes an auxin-regulated gene suggests that it might play a role in auxin responses. Moreover, alterations in lateral root number and in distribution of seedling growth are consistent with changes in auxin physiology. To investigate directly the effects of *SHY2/IAA3* on auxin responses, we tested

whether *shy2* mutant seedlings could respond to gravity, a known auxin-regulated response; and we measured the effect of exogenous auxin on root growth.

Root and hypocotyl gravitropism

When grown in the dark, hypocotyls of wild-type seedlings grew upward, and roots grew down. Hypocotyls and roots of *shy2-2* mutants grew in more random directions (Fig. 6). In contrast, *shy2-22* and *shy2-24* mutants grew vertically as wild type seedlings did (Fig. 6). Light perceived by phytochromes modulates hypocotyl gravitropism (Gaiser and Lomax, 1993; Liscum and Hangarter, 1993; Robson and Smith, 1996). To assess whether *shy2* mutations altered this response, we measured the angle (relative to vertical) of hypocotyl and root growth under different fluence rates of red light. Wild-type seedlings responded with randomization of the growth direction of hypocotyls. *shy2-2* seedlings did not respond to red light, whereas *shy2-22* and *shy2-24* seedlings responded similarly to wild type (Fig. 6). These data indicate that

Fig. 3. Phenotypes of *shy2* mutants. Seedlings were grown on MS/SUC plates in white light for 7 days (A–D), in white light for 25 days (E–J), or in the dark for 7 days (K–O). Genotypes are (A,E,K) Landsberg *erecta* (wild-type parent), (B,F,L) *shy2-2*, (G,M) *shy2-3*, (H) *SHY2/shy2-2* heterozygous plants, (C,I,N) *shy2-22*, (D,J,O) *shy2-24*. *shy2-21* and *shy2-23* seedlings are similar to *shy2-22* and *shy2-24* seedlings, respectively, and are not shown. (P,Q) 14-day-old and 35-day-old Landsberg *erecta* plants carrying a *shy2-2* transgene.

gravitropic sensing is largely intact in *shy2* loss-of-function mutants.

However, an inductive experiment uncovered altered gravitropic response in *shy2* mutant seedlings. We re-oriented dark-grown seedlings by 90° and then measured the kinetics of hypocotyl and root tip curvature. Hypocotyls and roots of *shy2-2* and *shy2-22* seedlings had a slower response to gravity than those of wild-type seedlings (Fig. 7A,B), although both hypocotyls and roots eventually re-oriented vertically (by 48 hours, data not shown). This result shows that although *shy2-2* seedlings initially grow agravitropically, they retain a correctly oriented response to gravity stimulus. The similarity of the *shy2-22* response to that of *shy2-2* suggests that *shy2-22* retains some of the gain-of-function activity conferred by the *shy2-2* mutation. The putative null mutant *shy2-24* had a contrasting phenotype. Hypocotyls of *shy2-24* seedlings responded similarly to wild-type hypocotyls (Fig. 7A). However, the roots of *shy2-24* seedlings re-oriented more quickly than wild-type roots (Fig. 7B). These results indicate that *SHY2/IAA3* slows root curvature in response to gravity.

Wavy root pattern of *shy2* mutants

These altered root curvature responses were reflected in altered wavy root growth on inclined 1.5% phytagar plates. Wavy root growth is thought to result either from a gravitropism-dependent thigmotropic obstacle-escaping response (Okada and Shimura, 1994), or, in an alternative model, from the combinational effects of circumnutation and gravitropism of the growing root tip (Simmons et al., 1995). As shown in Fig. 8, *shy2-2* showed less wavy growth than wild type, similar to previously reported *wav1-1* and *hy5* mutants (Okada and Shimura, 1990; Oyama et al., 1997). Conversely, *shy2-22* showed increased wavy growth, with each curve encompassing a larger angle than curves in wild-type roots, similar to *wav2-1* mutants (Okada and Shimura, 1990). *shy2-24* (and in some cases *shy2-22*) roots showed an even more pronounced phenotype. They formed less frequent but larger curves which often looped through more than a complete circle. These results confirm that *SHY2/IAA3* affects the extent and/or timing of gravity response.

Hormone sensitivity of root growth

We tested whether the short roots of *shy2* mutants might arise from altered auxin response by measuring root growth in the presence of exogenous auxin. Both *shy2-2* and *shy2-3* are slightly less sensitive than wild type to

the natural auxin indole-3-acetic acid (IAA), by about 2-fold (Fig. 9A). This contrasts with *axr3-1*, which has a much more substantial auxin-resistance in this assay (about 500-fold; Leyser et al., 1996). The *shy2-22* and *shy2-24* mutants behaved unusually in this assay. The short root phenotype of *shy2-22* and *shy2-24* mutants was rescued by low concentrations of exogenous IAA ($1\text{--}3 \times 10^{-8}$ M). Higher concentrations ($>10^{-7}$ M) inhibited root growth, as for wild type and *shy2-2* roots (Fig. 9B). The mutants had qualitatively similar responses to the synthetic auxin 2,4-D (data not shown). These striking results confirm that *shy2* mutants have an altered auxin response.

To assess whether *shy2* affects auxin responses specifically, we tested root growth responses to other hormones. *shy2-2* mutant seedlings were slightly resistant to abscisic acid (ABA; Fig. 9C), the cytokinin benzyladenine (BA; Fig. 9D), the auxin transport inhibitor naphthylphthalamic acid (NPA; Fig. 9E), and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, data not shown). Like IAA, low concentrations of BA ($<10^{-8}$ M) (Fig. 9D) and NPA ($<10^{-7}$ M) (Fig. 9E), but not ABA (Fig. 9C) stimulated root growth of *shy2-22* and *shy2-24*

Fig. 4. Time course of hypocotyl and root elongation. (A,B) Hypocotyl, (C,D) root. Seedlings were grown vertically in white light (A,C) or dark (B,D) on MS/SUC plates. Hypocotyl and root lengths were measured daily. Error bars indicate standard deviations. At least 20 seedlings were measured for each point. Repetitions of the experiment shown in A revealed that *shy2-24* hypocotyls consistently grew faster than wild-type seedling hypocotyls (data not shown).

Fig. 5. Lateral root formation. (A) Wild type (*Landsberg erecta*), (B) *shy2-2*, (C) *shy2-22*, (D) *shy2-24*. Seedlings were grown on vertically oriented plates in white light for 21 days.

mutants. Other auxin-resistant mutants are also resistant to auxin, cytokinin, ABA, and/or auxin transport inhibitor (Hobbie and Estelle, 1995; Leyser et al., 1996; Pickett et al., 1990; Ruegger et al., 1998; Wilson et al., 1990). Our results reinforce the notion that there are significant functional connections among different hormone signalling components.

DISCUSSION

The availability of both gain-of-function and loss-of-function *shy2* alleles allows a complementary analysis of functions for which SHY2/IAA3 is sufficient and/or necessary. The gain-of-function and loss-of-function mutations affect several root phenotypes in opposing senses and reveal that SHY2/IAA3 regulates root system development. Thus, gain-of-function mutations cause reduced lateral root formation, decreased wavy root curvature and slow root re-orientation; whereas loss-of-function mutations cause increased lateral root formation, increased wavy root curvature and accelerated root re-orientation. Auxin is known to stimulate lateral root formation, and to be required for gravitropism. Therefore, these phenotypes are consistent with the interpretation that SHY2/IAA3 is a negative regulator of auxin responses.

Exogenous auxin rescued root growth of loss-of-function mutants suggesting that the morphological phenotypes of the mutants arise from altered response to auxin. However, these data are most consistent with SHY2/IAA3 being a positive regulator of auxin response, rather than a negative regulator as the morphological phenotypes suggest. Thus, loss-of-function mutants may have reduced response to auxin, thereby perceive and respond as if to a lower concentration of auxin than is actually present, and obtain growth stimulation at concentrations of exogenous auxin that normally inhibit wild-type root growth. Consistent with this interpretation, other workers have reported that very low concentrations (10^{-11} – 10^{-10} M) of exogenous IAA can stimulate root growth, and higher concentrations of IAA inhibit root growth (Evans et al., 1994). Gain-of-function *shy2* mutants also had shorter roots than wild type, but rather than stimulating root growth as in loss-of-function mutants, exogenous auxin inhibited root

growth, at slightly higher concentrations than required for wild-type. This slight auxin resistance may indicate that gain-of-function mutant roots have a higher constitutive 'response' than wild type to endogenous auxin and therefore require a higher level of exogenous auxin to show further inhibition of root growth.

Several possible models may reconcile our inference that SHY2/IAA3 is both a positive and negative regulator of auxin responses. One possible interpretation is that SHY2/IAA3 acts differentially in different tissues. For example, SHY2/IAA3 may promote cell enlargement in cotyledons, but inhibit cell enlargement in hypocotyls and roots; and it may promote cell divisions in shoot and root apical meristems leading to leaf formation and primary root elongation, but inhibit pericycle cell divisions thereby preventing lateral root formation. Such tissue-specific effects might arise if SHY2/IAA3 dimerizes with alternate Aux/IAA or ARF proteins present in different

Fig. 6. Growth orientation with respect to gravity of wild-type and *shy2* seedlings grown under different fluence rates of red light. (A) Hypocotyl, (B) root. Seedlings were grown on vertically oriented plates in red light for 5 days. The average angle of hypocotyl or root growth relative to gravity for each genotype is shown. Error bars indicate standard deviations. 25 seedlings were examined for each genotype.

Fig. 7. Time course of gravitropic response of wild-type and *shy2* seedlings. (A) Hypocotyl, (B) root. Dark grown seedlings were rotated by 90° in darkness after 2 days growth. Curvature was measured every 6 hours after re-orientation. Error bars represent standard deviations. At least 20 seedlings were examined for each point.

tissues. However, this model would not explain simply why both gain-of-function and loss-of-function mutants have short roots.

A second possibility is that *SHY2/IAA3* may act differently depending on its level in the cell. For example, weak or transient auxin signalling may induce a low level of *SHY2/IAA3*, sufficient to promote root growth or cotyledon expansion, but stronger or more persistent auxin signalling may induce a higher *SHY2/IAA3* level, inhibiting these responses. In other systems, different concentrations of morphogens or transcription factors can either induce or repress particular genes (Ferguson and Anderson, 1992; Kosman et al., 1991). Consistent with this type of model, many auxin responses are biphasic, and auxin has been proposed to act as a morphogen in tissue patterning (Sachs, 1991; Uggla et al., 1998). Biphasic cellular responses to auxin may depend on the architecture of intracellular feedback control loops, for example, if above a threshold level *SHY2/IAA3* represses its own gene expression or that of other auxin-induced genes. Such feedback loops

Fig. 8. Wavy roots of wild type and *shy2* mutants. (A) Wild type, (B) *shy2-2*, (C) *shy2-22*, (D) *shy2-24*. Seedlings were grown on vertically oriented hard (1.5% phytagar) plates in white light for 3 days, then the plates were tilted to 45° and the seedlings photographed after another 3 days growth.

could also operate over time to give different responses in cells with different histories of auxin perception.

Previous biochemical and molecular studies provide data consistent with models that invoke positive or negative feedback control by *SHY2/IAA3*. Among the *Arabidopsis Aux/IAA* genes characterized in detail by Abel et al. (*IAA1-IAA14*), *SHY2/IAA3* mRNA shows the most transient response to exogenous auxin. Induction begins after 5 minutes of treatment with IAA and reaches 4-fold within 10 minutes. The mRNA level then decreases starting at 30 minutes and reaches starting mRNA levels after 4 hours (Abel et al., 1995). These results suggest that *SHY2/IAA3* induction is a very early step in auxin responses, and that later steps re-establish repression of the *SHY2/IAA3* gene. Since *Aux/IAA* proteins and ARF proteins can heterodimerize (Kim et al., 1997; Ulmasov et al., 1997b), *SHY2/IAA3* may dimerize with particular ARFs to regulate expression of *Aux/IAA* genes. In mediating auxin responses, *SHY2/IAA3* might turn on other genes, and/or it might turn off genes including itself, thereby forming either positive or negative feedback loops.

A third model is that *shy2* mutations affect auxin transport, and the mutant phenotypes arise secondarily from aberrant distribution of auxin. For example, gain-of-function *shy2* mutants may have higher levels of auxin in cotyledons, resulting in excess expansion. In the *shy2-24* putative null mutant, loss of *SHY2/IAA3* protein may result in increased auxin transport from cotyledons and/or leaves to hypocotyl and root, resulting in a slightly longer hypocotyl, and more lateral roots than wild type. Similarly, auxin distribution within the root tissues may be altered, resulting in increased auxin in elongating cells and pericycle cells (causing faster gravitropism and more lateral root formation) and reduced auxin in the meristem (causing decreased growth and altered response to exogenous auxin). Consistent with this model, hypocotyl elongation in light-grown seedlings requires auxin transport (Jensen et al., 1998), and mutants that are defective in auxin transport have agravitropic root growth (Luschnig et al., 1998; Pickett et al., 1990).

Gravitropism of both roots and shoots arises from

differential growth on opposite sides of the root or stem, and is thought to result from stimulation and/or inhibition of cell elongation by asymmetrically localized auxin (Goldsmith, 1977; Masson, 1995; Pickard, 1985; Thimann, 1977; Young et al., 1990). The altered waviness of *shy2* loss-of-function mutant root growth may result from faster curvature of a gravity-responding root, or from decreased correction on the return wave, resulting in more persistent curving. The former model is consistent with the faster gravitropic response of dark-grown *shy2-24* roots. In either case, the wavy root phenotype reveals that correct timing of auxin responses is important for normal growth. Depending on whether the curvature is caused by excess elongation on the outside of the curving root or too little elongation on the inside, the data may indicate a negative or positive effect of SHY2/IAA3 on elongation. More detailed study of this response may clarify this issue.

These studies of SHY2/IAA3 raise the question of what the other 19 *Arabidopsis* Aux/IAA proteins do, and how their activities may be related to those of SHY2/IAA3. The subtle shoot phenotypes of loss-of-function *shy2* mutants suggest that SHY2/IAA3 may act redundantly in shoots with other proteins, possibly encoded by other *Aux/IAA* genes. Perhaps *AXR3/IAA17* is one such gene, as *axr3* gain-of-function mutants are similar to *shy2-2* in having short hypocotyls, curled-up leaves, and leaf formation in the dark (Leyser et al., 1996). However, *shy2* and *axr3* mutant root phenotypes contrast in several ways. For example, gain-of-function *axr3* mutants have more adventitious roots than wild type, whereas gain-of-function *shy2* mutants have fewer adventitious roots than wild type. These results suggest that their functions may diverge in controlling root growth. It is also possible that one of these genes regulates the other and the regulation is positive in shoots but negative in roots. Further genetic and molecular work may provide more information on the regulatory interactions among SHY2/IAA3, *AXR3/IAA17*, and other *Aux/IAA* genes.

The most prominent phenotypes of loss-of-function *shy2*

Fig. 9. Root growth in the presence of exogenous hormones. Dose-response curves for root growth on IAA (A,B), ABA (C), BA (D), and NPA (E). In A, inhibition of root growth is expressed relative to growth without hormone. B-E show root growth measurements. Error bars are omitted for clarity. Each point represents the mean of 20 measurements, and standard deviations were generally less than 20% of the mean.

mutants occur in light-grown seedlings, suggesting that SHY2/IAA3 may be more important in light than in the dark. This is consistent with suggestions that auxin plays different roles in light- and dark-grown seedlings (Jensen et al., 1998). Moreover, the finding that *shy2* gain-of-function mutants make leaves in the dark suggests that SHY2/IAA3 is normally part

of the morphological program of light-grown plants, and that activation of SHY2/IAA3 by mutation may be sufficient to trigger de-etiolation (Kim et al., 1996, 1998; Reed et al., 1998). In addition to helping unravel auxin regulatory networks, *shy2* mutants will provide useful tools for detailed study of whether SHY2/IAA3 forms a functional link between light and morphological responses.

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