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Regulation of Both Light- and Auxin-Mediated Development by the *Arabidopsis IAA3/SHY2* Gene

Moon Soo Soh[1], Sung Hyun Hong[1], Byung Chul Kim[2], Igor Vizir[1], Deok Hoon Park[1], Giltsu Choi[3], Moon Young Hong[4], Yong-Yoon Chung[4], Masaki Furuya[2], and Hong Gil Nam[1]*

[1]Department of Life Science and School of Environmental Engineering, Pohang University of Science and Technology, Hyoja Dong, Pohang, Kyungbuk, 790-784, Korea
[2]Hitachi Advanced Research Laboratory, Hatoyama, Saitama, 350-0395, Japan
[3]Kumho Life & Environmental Science Laboratory,1 Oryong Dong, Kwangju 500-712, Korea
[4]Department of Biology, Korea University, Anam Dong, Sungbuk Gu, Seoul, Korea

Light affects plant growth and development throughout the life cycle. However, light signals do not function autonomously but should be integrated with endogenous developmental factors such as the plant hormone auxin to specify correct developmental decisions. We have previously reported that the *Arabidopsis shy2-1D* mutation alters various light responses, including highly photomorphogenic development in darkness. Here we show that the mutation also alters various auxin responses, including constitutive formation of lateral roots and reduced auxin sensitivity in inhibition of hypocotyl and root growth. The mutation is a gain of function mutation occuring in the *IAA3* gene, one of the *Aux/IAA* family genes encoding putative transcription factors of auxin-responsive genes. These results suggest that IAA3/SHY2 may play important roles in both light- and auxin-mediated development. Considering that Aux/IAA proteins and auxin response transcription factors interact with one another, we propose that IAA3/SHY2 may integrate light signals into auxin-mediated developmental responses.

Keywords: *Arabidopsis thaliana*, *Aux/IAA*, auxin, light signaling, *SHY2*

Light, depending on the quantity, quality, direction, and duration, exerts profound effects throughout plant development from germination to flowering (Smith, 1994; Chory et al., 1996). One of the most dramatic effects of the environmental light signals on plant development is observed during seedling development. Dark-grown seedlings undergo skotomorphogenesis, resulting in a long hypocotyl, unopened cotyledons, and an apical hook. In contrast, light-grown seedlings undergo a photomorphogenic program, exhibiting a short hypocotyl, apical hook opening, cotyledon expansion, and development of true leaves (Kendrick and Kronenberg, 1994). Three distinct classes of photoreceptors are involved in mediating informational light signals into the developmental programs of plants. They are the red/far-red absorbing phytochromes, the blue/UV-A absorbing cryptochromes, and the UV-B receptors (Furuya, 1993).

Numerous components in the light signaling pathway have been identified through genetic approaches using the dramatic developmental differences between light- and dark-grown *Arabidopsis* seedlings (Quail et al., 1995; Whitelam and Miller, 1998). Screening of mutants that display reduced photoresponses in light identified mutations in the photoreceptors, in phytochrome chromophore biosynthesis, and in several positively acting elements (Koornneef et al., 1980; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). In contrast, the *det/cop/fus* mutations confer photomorphogenic phenotypes in the absence of light. The recessive nature of these mutations suggested DET/COP/FUS might act as negative regulators of light-signaling. The combined molecular, biochemical, physiological, and genetic analyses of the photomorphogenic mutants provided important insights into the light-signaling mechanism (Deng et al., 1992; Castle and Meinke, 1994; Pepper et al., 1994; Wei et al., 1994; Li et al., 1996). However, the mechanisms transducing the light-signals into a developmental program remain largely undetermined. To specify correct developmental decisions, light signals should be integrated with endogenous developmental factors such as phytohormones (Chory and Li, 1997; Kraepiel and Miginiac, 1997). In fact, light and phytohormones show various interactions; additive, synergistic, and antagonistic interactions (Chory et al., 1994; Kraepiel et al., 1995; Su and Howell, 1995; Weatherwax et al., 1996;Peng and Harberd, 1997; Jensen et al., 1998).

*Corresponding author; fax +82-562-279-2998
e-mail hgn@bric.postech.ac.kr

The knowledge on the molecular nature of the interactions between light and phytohormone signals will be important to understand how light signals control developmental programs in plants.

As a part of our efforts toward better understanding of the light signaling mechanism, we previously identified and characterized a dominant photomorphogenic mutant, *shy2-1D* (Kim et al., 1996; Kim et al., 1998). The *shy2-1D* mutation was originally isolated as a dominant suppressor mutation of *hy2* (Kim et al., 1996), a phytochrome photoreceptor mutation that shows reduced photomorphogenic characters in light (Koornneef et al., 1980). The *shy2-1D* mutation confers exaggerated photomorphogenic phenotypes, such as a very short hypocotyl, when grown in light (Fig. 1a). Furthermore, dark-grown *shy2-1D* seedlings develop highly photomorphogenic characters, including a short hypocotyl, expanded cotyledons, expression of light-inducible genes, partial development of chloroplasts, and formation of true leaves and floral organs (Kim et al., 1998). The mutation also shows genetic interaction with the phytochrome photoreceptors in darkness (Kim et al., 1998). The pleiotropic photomorphogenic phenotypes suggest that SHY2 is critical in light-regulated developmental control.

During the phenotypic characterization of the *shy2-1D* mutant, we found that the gravitropic response of the hypocotyl was severely impaired in *shy2-1D* mutants (Kim et al., 1998). Here, we extended the phenotypic analyses of the *shy2-1D* mutation, showing the mutation alters various auxin responses. Furthermore, we show that *shy2-1D* is a gain-of-function mutation in the *IAA3* gene, a member of the *Aux/IAA* gene family encoding putative transcription factors of auxin-responsive genes. Taken together with our previous analyses of photo-responsive phenotypes of the *shy2-1D* mutation, here we propose that SHY2 may play regulatory roles in both light and auxin signaling. We further discuss a possible role of IAA3/SHY2 in transducing light-signaling into the auxin-mediated developmental processes.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Arabidopsis thaliana ecotype La-O was used throughout the experiment. A triploidic *SHY2/SHY2/shy2-1D* line was obtained by crossing a tetrapolid wild type plant with a homozygotic *shy2-1D* plant. General growth conditions of plants were as described (Kim et al., 1996; Kim et al., 1998).

For measurement of the lateral root formation and root curling responses, seedlings were grown on germination medium (GM) containing Gamborg B5 salts with 2% sucrose, 0.5 g/L 2-(N-Morpholino)ethanesulfonic acid (Gibco), and 0.8% phytagar (Gibco). For examination of the root waving response, seedlings were grown on germination medium with 2% agar. Seedlings under comparison were grown on the same plates.

For phytohormone response assays, seeds were sown on MS plates without sucrose. After cold-treatment, the plates were irradiated with white light (4 W/m^2) for 2 h to promote germination. The plates were then kept in darkness or in light for 4 days.

NMB Mutagenesis and Isolation of the shy2-4D Mutant

Ten batches of 1,000 *Arabidopsis* seeds were incubated in a 0.001% NMB solution as described (Usmanov and Sokhibnazarov, 1975). The M2 seeds were pooled separately from each batch. The *shy2-4D* mutant was isolated from the M2 seedlings first by screening for a mutant that exhibited phenotypes (short hypocotyl and curled leaf) similar to the *shy2-1D* mutant and then examining the dark phenotypes (short hypocotyl and opened cotyledon) of the seedlings.

Transformation of *Arabidopsis*

A DNA fragment containing 2.0 kb of the promoter region and the whole coding region of the *IAA3/SHY2* gene was amplified by PCR using two oligonucleotides, 5'-CTTCTAGAGCTCTGTAGGGCAAGC-3' and 5'-GGGAGTAATCCAATCTAGACCATC-3'. The amplified fragment was digested with XbaI and ligated into the pGA482 vector. The resulting plasmids were introduced into the *Agrobacterium* strain AGL1. Transformation of *Arabidopsis* was performed as described (Bechtold and Pelletier, 1998). The transformed plants were selected on a plate containing 50 mg/L kanamycin. T2 generation seeds were collected individually from each transformant. Homozygous lines were screened at the T3 generation and used for phenotype analysis.

Yeast Two Hybrid Experiment

The Matchmaker two-hybrid system (Clontech, CA) was used. The *PAP1* and *PAP2* genes were fused to

the GAL4 DNA binding domain sequence of the pGBT9 and used as baits. The mutant and wild type *IAA3/SHY2* sequences were fused to the GAL4 activation domain sequence of the pGAD424 vector and used as preys. The genes were cloned into the respective vectors after RT-PCR with primers containing appropriate restriction sites. The primers for the *PAP1* gene were 5′ CGGGATCCTCAGTGCATCATCTTCTCTTG 3′ and 5CGGAATTCATGGAAGGTTGTCCAAGAAAC 3′. The primers for the *PAP2* gene were 5′ CGGAATTCATGTCTGTATCTGTAGCAGCAG 3′ and 5′GAAGATCTCTAGTTCCTGCTTCTGCACTTC 3′. The primers used for the *IAA3/SHY2* gene were 5′ CCTGAATTCCTTGAAGAAATGGATGAGTTTG 3′ and 5′ TTCTGCAGGTTCTTTTGTGTCTCTGTTAG 3′. All of the PCR clones were verified by sequencing. Growth of yeast cells and measurement of the β-galactosidase activity were performed as described (Ni et al., 1998).

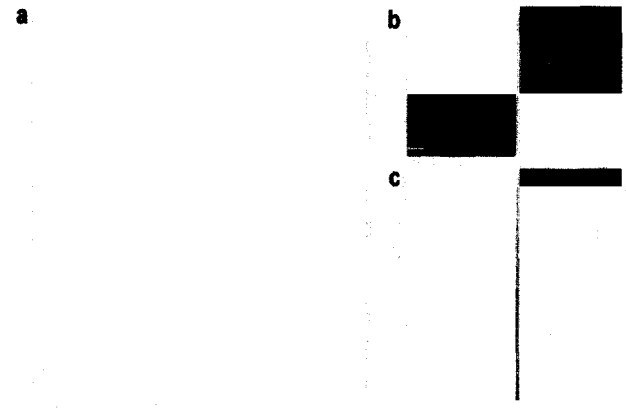

Figure 1. Root phenotypes of the *shy2-1D* mutant. **a.** Lateral root formation in 7 day-old seedlings of wild type (left) and *shy2-1D* (right). **b.** Root curling response in 5 day-old seedlings of wild type (left) and *shy2-1D* (right). Seedlings were grown on an agar (0.8%) plate in the horizontal position. **c.** Root growth pattern of wild type (left) and *shy2-1D* (right) grown on a hard agar (2%) plate. Seedlings were grown on tilted (45°) plates for 4 days. Scale bars, 1 mm.

RESULTS AND DISCUSSION

Altered Auxin Responses in the *shy2-1D* Mutant

The plant hormone auxin plays a major role in shoot gravitropism in addition to controlling many other aspects of plant growth and development (Trewavas, 1992; Chang et al., 1994; Kaufman et al., 1995; Estelle, 1996; Kim and Mulkey, 1997a,b). Interestingly, the *shy2-1D* hypocotyl showed reduced negative gravitropism in darkness (Kim et al., 1998), while wild type hypocotyl showed strong negative gravitropism. The reduced negative gravitropism caused by the *shy2-1D* mutation suggested that the mutation modifies auxin responses. We found alterations of other auxin-related phenotypes in *shy2-1D* seedlings. Lateral root formation is an auxin-regulated response, and exogenous auxin increases lateral root formation in *Arabidopsis* seedlings (Wightman and Thimann, 1980; Boerjan et al., 1995). *shy2-1D* seedlings developed more lateral roots than wild type seedlings in the absence of auxin (Fig. 1a). Wild type seedlings, when grown for 10 days, produced 15 ± 1 and 22 ± 4 lateral roots, respectively, in the absence and in the presence of 0.1 μM α-naphthaleneacetic acid (NAA), an auxin analog. In contrast, under the same conditions, *shy2-1D* seedlings formed 37 ± 1 and 35 ± 2 lateral roots, respectively. The result showed that the mutation confers a constitutive or hypersensitive auxin response in lateral root formation. When wild type seedlings are grown on an agar plate positioned horizontally, their roots enter the agar and exhibit circular root curling on the bottom of the plate (Fig. 1b). Wild type roots also show a wavy growth pattern when grown on a tilted hard agar (2%) surface (Fig. 1c). These responses are auxin-mediated, since the responses can be eliminated by an auxin transport inhibitor, 1-naphthylphthalamic acid and in auxin transport mutants (Fujita and Syono, 1997; Luschnig et al., 1998). Under the same conditions, *shy2-1D* root showed neither the circular root curling phenotype on the bottom of a plate (Fig. 1b) nor the wavy growth pattern on a tilted agar plate (Fig. 1c).

In wild type seedlings, an exogenous auxin causes inhibition of hypocotyl growth (Fig. 2a). In contrast, *shy2-1D* hypocotyl was insensitive to various auxin concentrations. Exogenous indole-3-acetic acid (IAA), a natural auxin, inhibited root growth in the wild type. This response was reduced in the *shy2-1D* mutant (Fig. 2b). We also examined the root growth inhibition response of *shy2-1D* seedlings to two other plant hormones, cytokinin and ethylene. Exogenous addition BA (a synthetic cytokinin) or ACC (an immediate biosynthetic precursor of ethylene) inhibited root growth in wild type plants (Fig. 2, c and d). In contrast to the case of IAA (Fig. 2a), *shy2-1D* seedlings showed a nearly normal inhibition response to BA or ACC. The result showed that *shy2-1D* preferentially altered auxin response at least in the root. Together with our previous reports (Kim et al., 1996; Kim et al., 1998), the results here show that *shy2-1D*

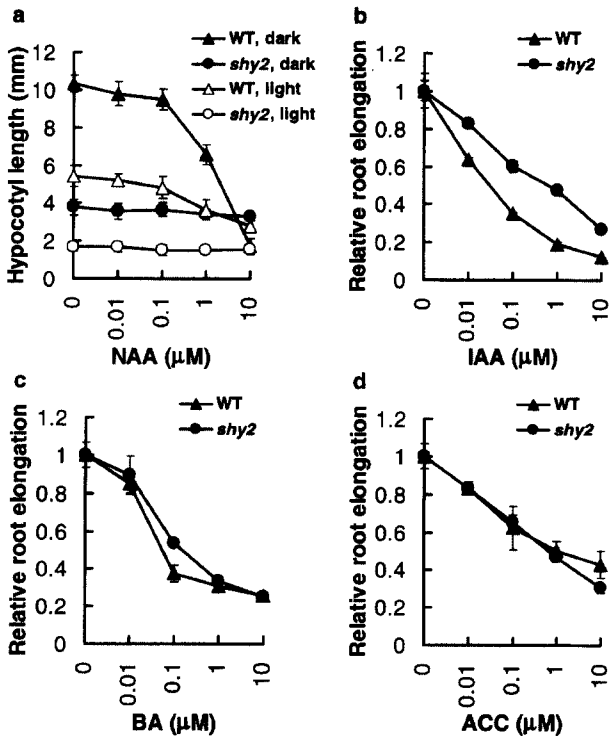

Figure 2. Dose responses of *shy2-1D* seedlings to exogenous phytohormones. **a.** Hypocotyl growth responses of the wild type (WT) and the *shy2-1D* mutant (*shy2*) to NAA in darkness and in blue light (0.7 W/m^2). Seedlings were grown for 4 days. **b-d.** Root growth inhibition response to various hormones. Seedlings of the wild type (WT) and the *shy2-1D* mutant (*shy2*) were grown for 4 days in various concentrations of IAA (b), BA (c), and ACC (d). At least 20 seedlings were sampled for each measurement. The bars indicate standard errors.

causes pleiotropic effects in both the light- and auxin-dependent developmental pathways.

The *SHY2* Gene Encodes an Aux/IAA Protein

The *shy2-1D* mutation was previously located in the middle of the two genetic loci, *PVV4* and *NCC1*, on the chromosome 1 (Kim et al., 1998). The *Arabidopsis* genome database revealed that this genomic region was covered by contiguous BAC and YAC clones (Fig. 3a), and full sequences of many of the clones were available. While searching for a possible candidate gene for *shy2-1D* from the genomic sequences available in this region, we found four *Aux/IAA* genes, *1AA3*, *IAA10*, *IAA12*, and *IAA17* (Fig. 3a). *Aux/IAA* genes are early auxin-inducible and were suggested to play regulatory functions in auxin responses (Abel et al., 1995; Kim et al., 1997; Ulmasov et al., 1997b; Guilfoyle, 1998). Furthermore, a previous report showed that the dominant *axr3* mutations confer hypersensitive auxin responses and are due to point mutations in the *IAA17* gene (Rouse et al., 1998; Fig. 3c). Since the *shy2-1D* mutation alters various auxin responses as dominant mutation like the *axr3* mutations do, one of the *Aux/IAA* genes around the *SHY2* locus was a potential candidate gene for *shy2-1D*. We, thus, compared the sequences of the *IAA3*, *IAA10*, and *IAA12* genes in the *shy2-1D* mutant with those in wild type, after isolating genomic clones corresponding to the coding regions of the three genes by PCR. We found that the *shy2-1D* genome has a point mutation in the *IAA3* gene. The same exact point mutation was found in *shy2-4D* (Fig. 3c), another mutant isolated from a seed pool mutagenized with *N*-nitroso-*N*-methylbiuret (NMB); *shy2-1D* was isolated from a seed pool mutagenized with ethyl methanesulfonate. To further confirm that *IAA3* is the gene for the *shy2-1D* mutation, we generated transgenic *Arabidopsis* lines containing a genomic clone of the mutant *IAA3/SHY2* gene; the genomic clone was composed of the coding sequence and 2.0 kilobases (kb) of the promoter region. In 9 out of 10 transgenic lines, we observed photomorphogenic phenotypes including short hypocotyl and expanded cotyledons in darkness (Fig. 3d), which are reminiscent of the *shy2-1D* mutant. These results showed that *IAA3* is the *SHY2* gene.

IAA3/SHY2 is a member of the Aux/IAA family that is comprised of over 25 members in *Arabidopsis* and is also found in other plant species (Guilfoyle, 1998). Aux/IAAs are short-lived nuclear proteins and have four conserved domains (Fig. 3). They form homo- and hetero-dimers through the domains III and IV (Kim et al., 1997; Ulmasov et al., 1997a). The domain III of Aux/IAA proteins contains a sequence related to baa dimerization and DNA binding domains. Aux/IAA proteins can modify gene expression driven by auxin-responsive promoter elements (Ulmasov et al., 1997b). Furthermore, Aux/IAA proteins can interact with auxin response transcription factors (ARFs) through the domains III and IV (Ulmasov et al., 1997a). Thus, Aux/IAA proteins were suggested to control down stream auxin-responsive genes in association with ARFs or by directly binding to an auxin-responsive DNA element as a homo- or hetero-dimer. However, in vivo functions of the Aux/IAA proteins have not been established except for in IAA17/AXR3, which functions as a regulator in auxin responses (Rouse et al., 1998). Here, we identified IAA3/SHY2 as a potential regulator of both auxin and light responses in plants.

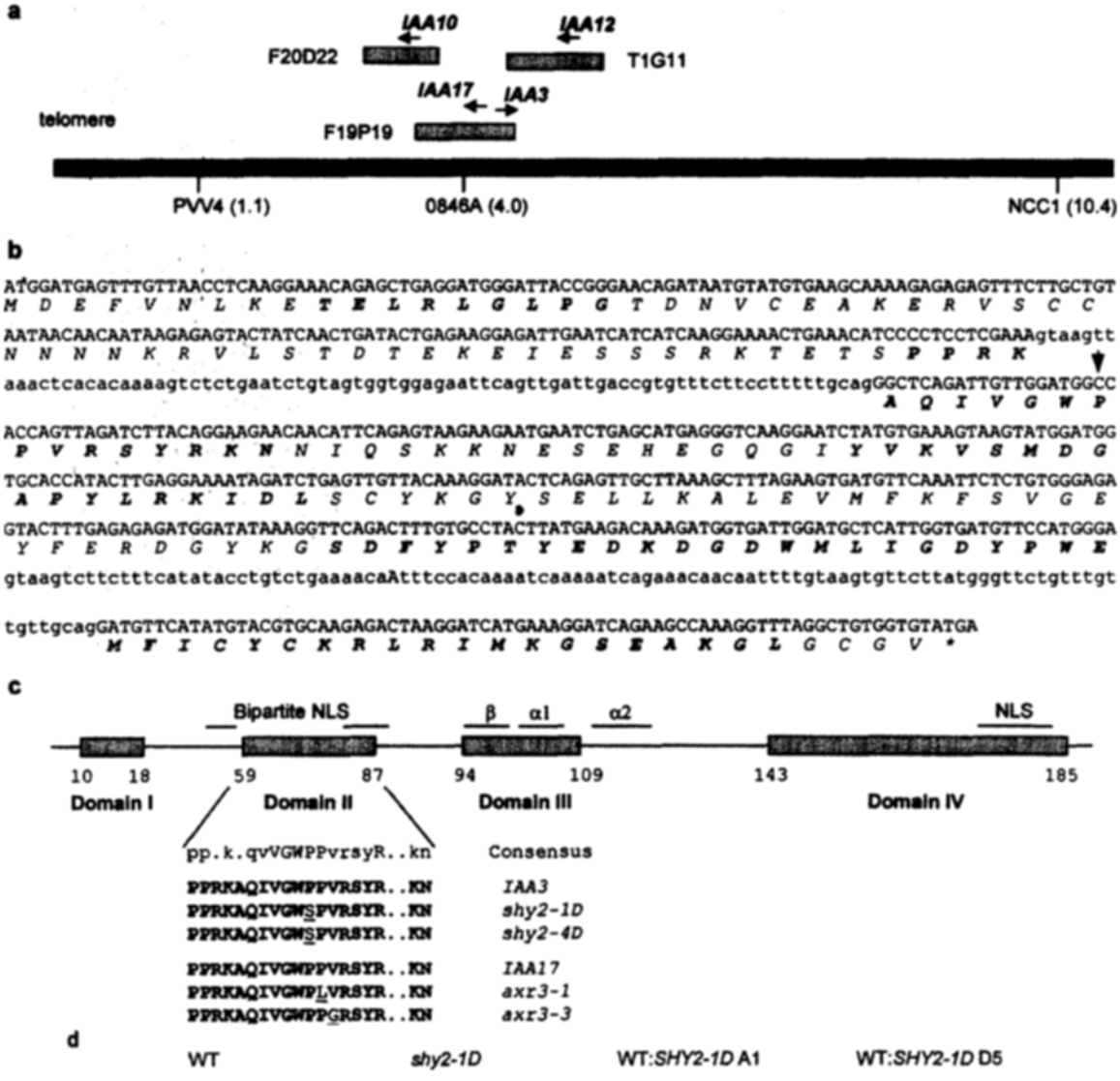

Figure 3. Cloning of the *IAA3/SHY2* gene. **a.** Physical map between the *NCC1* and *PVV4* loci on the chromosome 1. Only the BAC clones containing the *Aux/IAA* genes are shown. **b.** Nucleotide and deduced amino acid sequences of the *IAA3/SHY2* gene. The mutation sites in *shy2-1D* and *shy2-4D* are noted by arrows. Both mutations changed C to T. The four conserved domains are shown in bold case. **c.** Domain structure of IAA3/SHY2. The domain II sequences of the wild type and mutant IAA3/SHY2 and the wild type and mutant IAA17/AXR3 are shown. The mutated amino acid residues are underlined. **d.** Transformation of wild type *Arabidopsis* with the mutant *shy2-1D* gene. Shown from left to right are the wild type (WT), *shy2-1D*, and two independent transgenic lines (A1, D5). Seedlings were grown for 6 days in darkness. Scale bars, 1 mm.

shy2-1D Is a Gain-of-Function Mutation

When homozygotic *shy2-1D/shy2-1D*, heterozygotic *SHY2/shy2-1D*, and triploidic *SHY2/SHY2/shy2-1D* seedlings were grown in darkness, the average hypocotyl lengths were 5.5 mm, 6.7 mm, and 6.5 mm, respectively. The phenotypic severity followed the dosage of the mutant allele. *shy2-1D* is, thus, likely a gain of function mutation. The gain of function phenotypes of *shy2-1D* could be due to hypermorphism of the IAA3/SHY2 function or due to neomorphism unrelated to the normal cellular function of IAA3/SHY2. We suggest that wild type IAA3/SHY2 is involved in the light and auxin-mediated developmental control and that *shy2-1D* is a highly hypermorphic mutation that causes constitutive light and auxin responses. This suggestion is supported by the mutant phenotypes, and the previous reports on the role of Aux/IAA proteins in auxin responses.

Interaction of the Mutant and Wild Type IAA3/SHY2 with Other Aux/IAAs

Interaction of IAA3/SHY2 with other Aux/IAAs has previously been shown only for IAA1 (Kim et al., 1997). We found that IAA3/SHY2 interacts at least with two other Aux/IAA proteins, PAP1 and PAP2 (GenBank accession numbers, AF088281 and AF087936, respectively), by the yeast two hybrid system. The mutant and wild type IAA3/SHY2 produced a similar level of the β-galactosidase reporter activity that resulted from their interaction with PAP1 and PAP2 in the yeast two hybrid assay (Fig. 4). The mutation, thus, does not alter the interaction of IAA3/SHY2 with the PAP1 and PAP2 in the yeast two hybrid system. The dominant gain of function alleles of *IAA3/SHY2* and *IAA17/AXR3* arise by mutations only in the restricted amino acid residues of the conserved domain II to modify auxin responses (Fig. 3c). These amino acid residues must be critical for a regulated function of Aux/IAA proteins but are not likely to be involved in the protein-protein interaction among the Aux/IAA family members.

Possible Functions of IAA3/SHY2 in Arabidopsis Development

Light is probably the most important environmental factor that regulates plant development throughout the life cycle (Furuya and Schäfer, 1996; von Arnim and Deng, 1996; Barnes et al., 1997). However, light signals do not act autonomously, but should

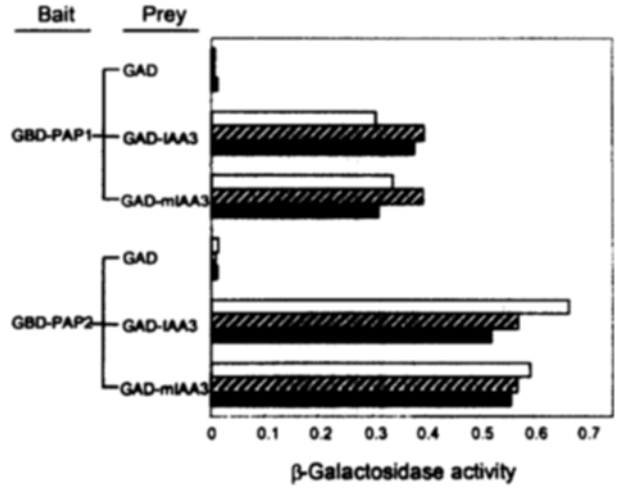

Figure 4. Yeast two hybrid analysis of the interaction of the mutant and wild type IAA/SHY2 with other Aux/IAA proteins. The two *Aux/IAA* genes, *PAP1* and *PAP2*, were fused to the GAL4 DNA binding domain (GBD) sequence to produce GBD-PAP1 and GBD-PAP2, respectively. The mutant and wild type *IAA3/SHY2* sequences were fused to the GAL4 activation domain (GAD) sequence of the pGAD424 vector to produce GAD-IAA3 and GAD-mIAA3, respectively. The GAD424 vector without the *IAA3/SHY2* sequence was used as a control. Three independent yeast colonies were used for the measurement of β-galactosidase activity resulting from the interaction between the proteins. The b-galactosidase activity is noted as an arbitrary unit.

be integrated with endogenous developmental signals such as auxin (Nick et al., 1992; Davis, 1995; Robson and Smith, 1996; Behringer and Davies, 1997; Kraepiel and Miginiac, 1997). An example is seen in Figure 2a. Wild type seedlings showed reduced responsiveness to auxin in inhibition of hypocotyl elongation in light, when compared to that in darkness. On the other hand, seedling responses to light can be altered by changing auxin physiology such as auxin transport (Jensen et al., 1998) or auxin content (Kraepiel et al., 1995). However, a molecular mechanism of the interaction between light and auxin signaling has not been revealed. We propose that IAA3/SHY2 is a molecule that networks these two signals. Aux/IAA proteins and auxin response transcription factors (ARFs) are known to form hetero-dimers among one another. Light-mediated modulation of expression, stability or activity of IAA3/SHY2 would modify auxin responses (expression of downstream auxin responsive genes) through changes of the content or activity of the heteromeric complex resulting from the interaction between IAA3/SHY2 and other Aux/IAAs or ARFs. The differential combinatorial arrays among these proteins in different organs and in

different light environments will lead to a proper developmental decision for plants to adapt to ever changing light environments.

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NOTES ADDED

While this manuscript was in preparation, we learned that cloning of the *IAA3/SHY2* gene and some of the auxin-related phenotypes of *shy2* mutants were independently reported by Tian and Reed (1999).

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