

Aux/IAA Proteins Are Phosphorylated by Phytochrome in Vitro¹

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Auxin/indole-3-acetic acid (Aux/IAA) genes encode short-lived transcription factors that are induced as a primary response to the plant growth hormone IAA or auxin. Gain-of-function mutations in Arabidopsis genes, *SHY2/IAA3*, *AXR3/IAA17*, and *AXR2/IAA7* cause pleiotropic phenotypes consistent with enhanced auxin responses, possibly by increasing Aux/IAA protein stability. Semidominant mutations *shy2-1D*, *shy2-2*, *axr3-1*, and *axr2-1* induce ectopic light responses in dark-grown seedlings. Because genetic studies suggest that the *shy2-1D* and *shy2-2* mutations bypass phytochrome requirement for certain aspects of photomorphogenesis, we tested whether SHY2/IAA3 and related Aux/IAA proteins interact directly with phytochrome and whether they are substrates for its protein kinase activity. Here we show that recombinant Aux/IAA proteins from Arabidopsis and pea (*Pisum sativum*) interact in vitro with recombinant phytochrome A from oat (*Avena sativa*). We further show that recombinant SHY2/IAA3, AXR3/IAA17, IAA1, IAA9, and Ps-IAA4 are phosphorylated by recombinant oat phytochrome A in vitro. Deletion analysis of Ps-IAA4 indicates that phytochrome A phosphorylation occurs on the N-terminal half of the protein. Metabolic labeling and immunoprecipitation studies with affinity-purified antibodies to IAA3 demonstrate increased in vivo steady-state levels of mutant IAA3 in *shy2-2* plants and phosphorylation of the SHY2-2 protein in vivo. Phytochrome-dependent phosphorylation of Aux/IAA proteins is proposed to provide one molecular mechanism for integrating auxin and light signaling in plant development.

Indole-3-acetic acid (IAA or auxin) profoundly affects a plethora of cellular activities and is therefore indispensable for plant growth and development (Thimann, 1977). At the molecular level the hormone rapidly activates transcription of a select set of early genes that are thought to mediate, at least in part, the various effects of auxin (Guilfoyle, 1986; Theologis, 1986). Primary auxin response genes provide bidirectional molecular probes for elucidating auxin action and comprise three major classes known as the *SAUR*, *GH3*-like, and *Aux/IAA* gene families (Abel and Theologis, 1996). Functional promoter analyses of members of each class revealed conserved auxin response elements that led to the identification of their cognate DNA-binding proteins, termed auxin response factors (ARFs). On the other hand, little is known about the function and post-translational regulation of early auxin-induced gene products. However, recent molecular and genetic studies suggest Aux/IAA proteins play a central role in auxin signaling (Guilfoyle et al., 1998; Walker and Estelle, 1998).

Expression of *Aux/IAA* genes is stringently regulated at multiple levels, which is a hallmark of genes coding for regulatory proteins with transitory roles (Abel and Theologis, 1996). Large families of *Aux/IAA* genes (>25 members in Arabidopsis; P. Overvoorde and A. Theologis, personal communication) encode short-lived nuclear proteins that homo- and heterodimerize in vitro and in vivo (Abel et al., 1994; Kim et al., 1997). Aux/IAA proteins share four domains of conserved primary structure (Abel et al., 1995; see Fig. 1). Domains II and IV contain functional nuclear localization signals (Abel and Theologis, 1995) and domain III is predicted to adopt a $\beta\alpha\alpha$ -fold that is similar to the DNA-binding domain of certain oligomeric transcription factors in prokaryotes (Abel et al., 1994). Biophysical and biochemical studies of recombinant peptides demonstrated that domain III is folded and heterodimerizes in vitro (Morgan et al., 1999). It is interesting that the C-terminal regions of most ARF proteins contain Aux/IAA-like domains III and IV, which mediate intra- and interfamily protein-to-protein interactions of ARF and Aux/IAA family members (Kim et al., 1997; Ulmasov et al., 1997a). As shown in a transient expression system, several Aux/IAA proteins repress auxin-dependent activation of auxin response element-mediated reporter genes, likely by interacting with endogenous ARFs (Ulmasov et al., 1997b). Because ARF genes are apparently not induced by auxin (Ulmasov et al., 1999), it is thus conceivable that Aux/IAA proteins are intricately involved in (auto) regulatory circuits of auxin-dependent gene expression.

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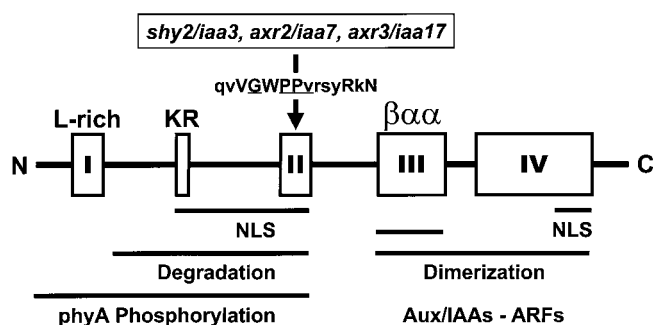


Figure 1. Structural and functional domains of Aux/IAA proteins. The primary structure conserved in the core of domain II is given above the designated box. Uppercase letters denote invariant amino acid residues. Positions affected by missense gain-of-function mutations of the indicated *Aux/IAA* genes are underlined. Bars below the structural domains delineate regions important to various protein activities or modifications. These include functionally identified signals for nuclear localization (NLS; Abel and Theologis, 1995) and protein degradation (Worley et al., 2000), as well as regions shown to mediate homo- and heterodimerization (Kim et al., 1997; Morgan et al., 1999) and to be modified by phytochrome A phosphorylation (this study).

The cloning of genes in *Arabidopsis* for semidominant mutations *shy2* (suppressor of *hy2* or short hypocotyl), *axr3* (auxin resistant), and *axr2* provided compelling genetic evidence for the importance of *Aux/IAA* genes in plant development. Missense mutations in *SHY2/IAA3* (Tian and Reed, 1999), *AXR3/IAA17* (Rouse et al., 1998), and *AXR2/IAA7* (Nagpal et al., 2000) alter invariant amino acids in domain II (see Fig. 1). The associated pleiotropic phenotypes are consistent with enhanced auxin responses (Timpte et al., 1994; Leyser et al., 1996; Kim et al., 1996, 1998; Tian and Reed, 1999). It is interesting that the gain-of-function mutation in domain II of *AXR3/IAA17* resides in a transferable protein degradation signal and stabilizes otherwise labile *Aux/IAA::luciferase* fusion proteins in heterologous expression systems (Worley et al., 2000). Thus missense mutations in domain II are likely to be hypermorphic because they may increase steady-state levels of *Aux/IAA* proteins in vivo.

Semidominant *shy2-1D* (Kim et al., 1996) and *shy2-2* (Reed et al., 1998) mutations were independently isolated as extragenic suppressors of the long hypocotyl phenotype of *hy2* and *hy3(phyB-1)* mutants, respectively. The *hy2* mutation blocks synthesis of the chromophore, phytochromobilin, and likely causes defects in all holophytochromes, whereas the *phyB-1* mutation is a null allele of *PHYB*, the gene coding for the red/far-red sensing photoreceptor, phytochrome B (Parks and Quail, 1991; Reed et al., 1993). The semidominant *SHY2/IAA3* alleles cause photomorphogenic characteristics in dark-grown seedlings such as true leaf development, cotyledon expansion, and reduced hypocotyl growth. A similar de-etiolated phenotype of dark-grown seedlings is observed as a consequence of the *axr3* and *axr2* mutations (Nagpal et al., 2000). Moreover, *shy2-1D* plants

express light-inducible genes, *CAB* and *PSBA*, in darkness (Kim et al., 1998). The collective data suggest that at least some *Aux/IAA* proteins may normally participate in light responses. When grown in light, young seedlings carrying loss-of-function alleles of *SHY2/IAA3* and *AXR2/IAA7* consistently have slightly longer hypocotyls than wild-type plants (Tian and Reed, 1999; Nagpal et al., 2000).

Because gain-of-function mutations in *SHY2/IAA3* induce ectopic photomorphogenesis in the dark and bypass phytochrome requirement for inhibiting hypocotyl elongation in the light, we tested whether *Aux/IAA* proteins directly interact with phytochrome and, if so, whether they are substrates for phytochrome protein kinase activity (Yeh and Lagarias, 1998). Here, we demonstrate that *SHY2/IAA3*, *AXR3/IAA17*, and other *Aux/IAA* proteins interact with and are phosphorylated by recombinant phytochrome A in vitro. We further show that the *SHY2-2* allele causes higher steady-state levels of the encoded protein and that *SHY2-2* is a phosphoprotein in vivo. We propose phytochrome-mediated phosphorylation of *Aux/IAA* proteins may be a regulatory event at which light and auxin signaling converge.

RESULTS

IAA Proteins Interact with Phytochrome A in Vitro

Genetic characterization of *shy2-1D* and *shy2-2* mutations derived from *hy2* and *phyB* suppressor screens, respectively, suggests interaction of *SHY2/IAA3* and *PHY* genes (Kim et al., 1998; Tian and Reed, 1999). Therefore, we tested whether *SHY2/IAA3* and related *Aux/IAA* proteins such as *AXR3/IAA17* from *Arabidopsis* and *Ps-IAA4* from pea (*Pisum sativum*) interact directly with phytochrome in vitro. To perform in vitro interaction assays we expressed *Aux/IAA* proteins as (His)₆-tagged fusion proteins in *Escherichia coli* and Strep-tagged *phyA* from oat (*Avena sativa*) in the yeast *Saccharomyces cerevisiae*. Recombinant *Aux/IAA* proteins were affinity-purified and functional *phyA* was prepared as described in "Materials and Methods." In our in vitro assay, interaction of both protein partners is revealed by cosedimentation of recombinant *Aux/IAA* proteins and Strep-tagged *phyA* with streptavidin agarose beads after co-incubation and repeated washes of the protein agarose complexes with detergent-containing buffer.

Protein analysis of control incubations by SDS-PAGE demonstrated quantitative binding of Strep-tagged *phyA* to streptavidin agarose. Recombinant oat *phyA* cosedimented with the agarose matrix, but was not detectable in the supernatant fraction (Fig. 2A). On the other hand, purified recombinant *Ps-IAA4* and *IAA17* protein did not bind to streptavidin agarose and remained in the supernatant (Fig. 2, B and C). Purified *IAA3* had considerable non-specific affinity to streptavidin agarose, which precluded fur-

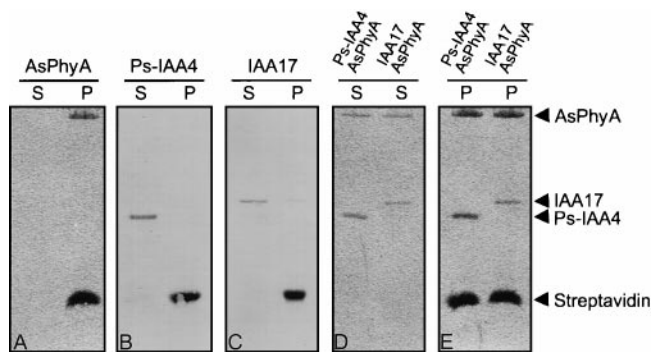


Figure 2. Aux/IAA proteins and phytochrome A interact in vitro. Recombinant oat phyA (AsPhyA) and IAA proteins were mixed with streptavidin agarose beads, incubated at room temperature, and treated as described in "Materials and Methods." Shown are SDS-PAGE resolved proteins of the binding reactions after Coomassie Blue staining for AsPhyA (A) and IAA protein (B and C) control incubations, as well as for the test incubations containing AsPhyA and IAA protein (D and E). S indicates the entire supernatant fraction after the first sedimentation step. P denotes the entire pellet fraction after the third washing step with detergent-containing buffer.

ther experimentation. It is important to note that when Ps-IAA4 or IAA17 and Strep-tagged phyA were combined in the same binding reactions, Ps-IAA4 and IAA17 cosedimented with immobilized phyA as evidenced by their presence in the agarose bead fraction after repeated washes (Fig. 2, D and E). The data suggest that at least some members of the Aux/IAA protein family have the ability to interact with recombinant oat phyA in vitro.

Phytochrome A Phosphorylates IAA Proteins in Vitro

Biochemical data demonstrate that phytochromes are light-regulated, autophosphorylating Ser/Thr protein kinases (Yeh and Lagarias, 1998; Fankhauser et al., 1999). Since we have shown that recombinant oat phyA interacts with Aux/IAA proteins in vitro,

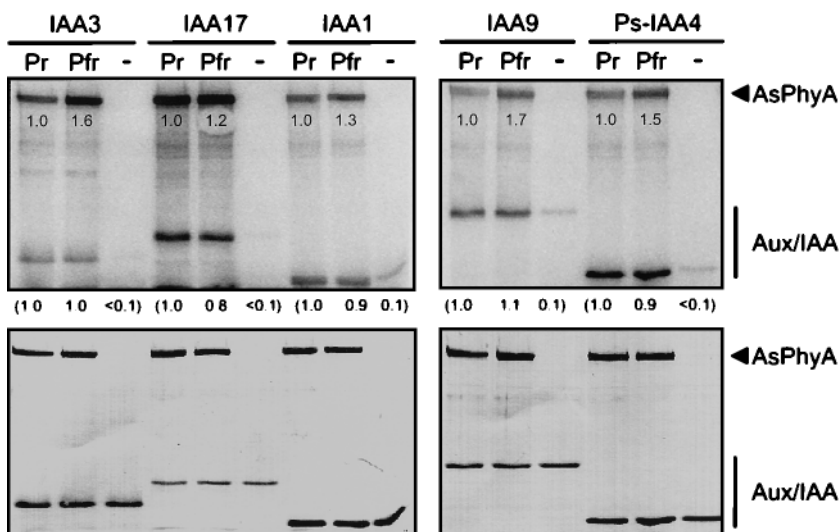
we further examined whether recombinant Aux/IAA proteins are substrates for the protein kinase activity of phyA. We performed kinase activity assays with recombinant oat phyA in the Pr and Pfr form and with recombinant Aux/IAA proteins, SHY2/IAA3, AXR3/IAA17, IAA1, IAA9, and Ps-IAA4, as potential protein kinase substrates.

In agreement with previous studies (Yeh and Lagarias, 1998; Fankhauser et al., 1999), recombinant oat phyA exhibited light-regulated autophosphorylation, which was about 2-fold higher for the Pfr form than for the Pr form (Fig. 3). On the other hand, the IAA proteins alone showed little or no autophosphorylation or non-specific binding of [³²P]ATP or [³²P]orthophosphate. However, phosphorylation of IAA proteins increased at least 10-fold upon addition of phyA, irrespective of its spectral form, indicating that all Aux/IAA proteins tested are substrates for the protein kinase activity of phyA in vitro. The amount of IAA17 and Ps-IAA4 phosphorylation was about three to five times higher than the amount of phyA-dependent phosphorylation of IAA1 or IAA3. Unlike the light-regulated autophosphorylation activity of phyA, phosphorylation of the IAA protein substrates by recombinant phyA was similar for the Pr form and Pfr form, suggesting that Aux/IAA phosphorylation by phyA is not light-dependent under the conditions tested (Fig. 3).

Phytochrome A Phosphorylates the N-Terminal Region of Ps-IAA4

We tested a small set of Ps-IAA4 deletions as substrates to locate, in a first approximation, phosphorylation sites of phyA on Ps-IAA4. We prepared various truncated, recombinant polypeptides encompassing different conserved domains in Ps-IAA4, including domains I-II, I-III, II-IV, and III-IV. Unlike the protein kinase assays described above, we used phyA pre-assembled in white light for the deletion analysis.

Figure 3. Phytochrome A phosphorylates Aux/IAA proteins in vitro. Recombinant oat phyA (AsPhyA) in the Pr form or Pfr form was used in kinase assays with recombinant IAA proteins as described in "Materials and Methods." To detect any autophosphorylation of IAA proteins, assays were also performed without AsPhyA. Shown are autoradiographs of the kinase reactions after SDS-PAGE and protein transfer to membranes (top), and Coomassie Blue staining of the transferred proteins (bottom). Amounts of phyA autophosphorylation and of Aux/IAA phosphorylation by phyA are expressed relative to the reactions containing the Pr form of phyA and are given below the phyA protein bands and below the top panels, respectively.



Again the data show absence of autophosphorylation for the full-length Ps-IAA4 protein and its truncated forms (Fig. 4). A C-terminal polypeptide of Ps-IAA4, which contains several Ser and Thr residues in conserved domains III and IV, as well as intervening and adjacent sequences, is not phosphorylated to detectable levels by recombinant oat phyA. The C-terminal region provides a negative control and thus support for the specificity of the phyA kinase activity toward Aux/IAA proteins *in vitro*. Likewise, a recombinant polypeptide corresponding to domain III of IAA3 (Morgan et al., 1999) is not a substrate of oat phyA (data not shown). On the other hand, the N-terminal region-encompassing domain I and II is a very potent substrate that is phosphorylated to high extent (Fig. 4). Consistent with this data, recombinant polypeptides spanning domains I through III and domains II through IV are also phosphorylated (data not shown). Experiments currently in progress will elucidate phosphorylation site(s) of phyA on Aux/IAA proteins.

Detection of SHY2/IAA3 *in Vivo*

To detect authentic IAA3 and to probe its phosphorylation status *in vivo* we raised polyclonal antibodies to recombinant IAA3, which were subsequently purified by affinity chromatography as described in "Materials and Methods." Because Aux/IAA proteins are encoded by a large gene family in Arabidopsis and share four domains of conserved primary structure (Abel et al., 1995), we used a series

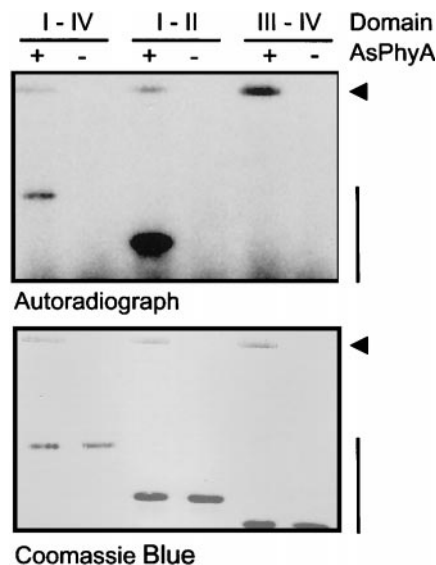


Figure 4. Deletion analysis of Ps-IAA4. Kinase assays were conducted in white light with (+) or without (-) recombinant oat phyA (AsPhyA) using full-length (I-IV) Ps-IAA4 (amino acids 1–189) and Ps-IAA4 deletions, domains I-II (amino acids 1–90), and domains III-IV (amino acids 86–189), as protein substrates. Shown are autoradiographs and Coomassie Blue protein staining of the resolved kinase reactions. The arrowhead and bar indicate the positions of oat phyA and of truncated PS-IAA4 polypeptides, respectively.

of *shy2* mutant alleles to expedite identification of the IAA3 protein *in vivo*. Based on the genetic characterization of *shy2-2* plants (Tian and Reed, 1999) and on the molecular characterization of mutations in conserved domain II of AXR3/IAA17 (Worley et al., 2000), the *shy2-2* gain-of-function mutation is hypothesized to stabilize IAA3, a protein with a predicted molecular mass of 21.5 kD. On the other hand, the loss-of-function alleles, *SHY2-22* and *SHY2-24*, are predicted to encode truncated IAA3 proteins of 19.5 and 7 kD, respectively (Tian and Reed, 1999).

Aux/IAA proteins from pea, Ps-IAA4 and Ps-IAA6, are of extremely low abundance and are difficult to detect by immunoblot analysis (Abel et al., 1994; Oeller and Theologis, 1995). To identify the Arabidopsis IAA3 protein and to analyze its steady-state levels *in vivo* we used metabolic labeling of wild-type and the various *shy2* mutant plants with [³⁵S]Met followed by immunoprecipitation of IAA3 from total labeled proteins. Affinity-purified IAA3 antibodies precipitate three major proteins within the range of molecular mass expected for IAA3 synthesized *in vivo* of 27, 23, and 19 kD (Fig. 5). The following observations suggest that the 23-kD protein represents endogenous IAA3: (a) Steady-state levels are auxin-inducible in wild-type seedlings (compare lane 3 and lane 4); (b) steady-state levels are appreciably higher in untreated *shy2-2* seedlings than in untreated or auxin-treated wild-type plants (compare lane 2 with lane 3 and lane 4); and (c) as expected, the 23-kD protein fails to be immunoprecipitated from *shy2-24* and *shy2-22* extracts (see lane 5 and lane 6). The specificity of the immunoprecipitation reactions was confirmed by competition with recombinant (His)₆-tagged IAA3 protein (Fig. 5, lane 7) and by the absence of the auxin-inducible 23-kD polypeptide in immunoprecipitations with preimmune serum (Fig. 5, lane 1).

Because the *shy2-2* mutation causes increased steady-state levels of the 23-kD protein (Fig. 5, lane 2), we tested whether its concentrations are sufficiently high to be detected by immunoblot analysis. Using 300 μg of total protein extract from 5-d-old dark-grown seedlings, affinity-purified IAA3 antibodies reproducibly recognize a protein of approximately 23 kD in extracts of *shy2-2* plants, but not in extracts prepared from *shy2-22* and *shy2-24* seedlings (Fig. 6). For wild-type extracts the amount of the 23-kD protein was near the detection limit and detection was not consistently achieved. It is interesting that the IAA3 protein accumulates to higher steady-state levels in *shy2-2* than in wild-type seedlings. In summary, the data indicate specific detection by metabolic labeling and immunoprecipitation of IAA3 *in vivo*.

SHY2-2 Is a Phosphoprotein *in Vivo*

In a first attempt to examine if the observed *in vitro* phosphorylation of IAA3 by phytochrome is func-

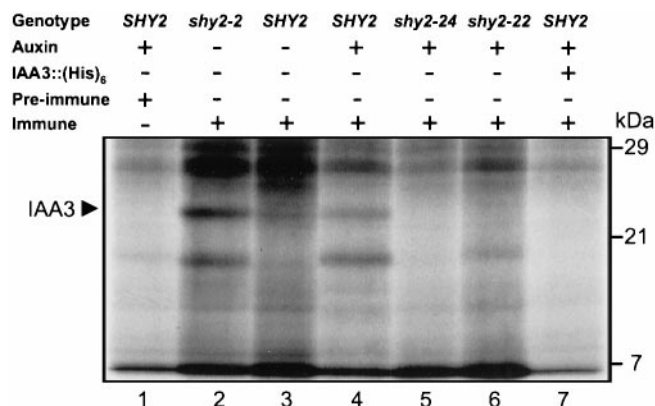


Figure 5. Immunoprecipitations with anti-IAA3 of [³⁵S]Met-labeled proteins synthesized in vivo. Etiolated seedlings of the indicated genotypes were metabolically labeled, treated with auxin, and extracted as described in "Materials and Methods." Shown is the autoradiograph (8 d of exposure) for resolved proteins after immunoprecipitations of the extracts with affinity-purified antibodies to IAA3 (lanes 2–7) or with preimmune serum (lane 1). For each immunoprecipitation reaction, 2.1×10^8 cpm of trichloroacetic acid-precipitable material were used. Incorporation of [³⁵S]Met into trichloroacetic acid-precipitable material was between 31% to 37% of the radiolabel measured in total tissue extracts. The arrowhead indicates a 23-kD protein identified as endogenous IAA3.

tionally relevant we tested whether the IAA3 protein is phosphorylated in vivo. To directly demonstrate in vivo phosphorylation, we used metabolic labeling with [³²P]orthophosphate followed by immunoprecipitation analysis (Fig. 7). Wild-type, *shy2-2*, and *shy2-24* seedlings were germinated for 5 d in the dark and labeled for 3 h in ambient white light in the presence (wild type, *shy2-24*) or absence (*shy2-2*) of auxin. After immunoprecipitation of total protein extracts and resolution of the precipitated protein complexes by SDS-PAGE, a protein of approximately 23 kD was detectable by autoradiography for the *shy2-2* extract (Fig. 7, lane 3). As expected, a [³²P]labeled protein of 23 kD could not be immunoprecipitated from *shy2-24* extracts, lending support for the specificity of the immunoprecipitation observed for *shy2-2* extracts (Fig. 7, lane 2). However, repeated attempts failed to demonstrate [³²P] labeling of IAA3 in wild-type seedlings treated with (Fig. 7, lane 1) or without (data not shown) auxin, possibly due to the lower steady-state level of IAA3 in wild-type seedlings than of the mutant IAA3 protein in *shy2-2* plants. Nonetheless, the data suggest that at least the mutant IAA3 protein, SHY2-2, is phosphorylated in vivo.

DISCUSSION

Aux/IAA Proteins Are Substrates of Phytochrome Kinase Activity in Vitro

Molecular and genetic studies suggest that Aux/IAA proteins play a central role in auxin signal transduction by regulating gene expression. However, the

structures, biochemical functions, and regulation of Aux/IAA polypeptides are largely unknown. In this communication we provide first experimental evidence for post-translational modification of Aux/IAA gene products. Using various recombinant Aux/IAA proteins from Arabidopsis and pea, which partially reflect the structural diversity of the Aux/IAA superfamily, we have demonstrated in vitro phosphorylation of Aux/IAA polypeptides by recombinant phyA from oat.

Recent biochemical studies have firmly established that eukaryotic phytochromes belong to a growing class of autophosphorylating eukaryotic Ser/Thr kinases with His kinase ancestry (Yeh et al., 1997; Lapko et al., 1999). The phytochrome protein kinase activity is stimulated by red light, which has been demonstrated for recombinant oat phyA (Yeh and Lagarias, 1998). To date, only a few plant proteins have been identified genetically or by yeast two-hybrid screens that are phosphorylated by phytochromes in a light-modulated manner (Reed, 1999). Among the substrates of recombinant oat phyA are the blue light photoreceptors, cry1 and cry2. Although the phosphorylation in vitro is not light regulated, red light stimulates cry1 phosphorylation in vivo (Ahmad et al., 1998). PhyA-mediated in vitro phosphorylation of phytochrome kinase substrate 1, a protein of unknown function, is modulated by light. The degree of phytochrome kinase substrate 1 phosphorylation in vivo is increased upon red light illumination and in plants overexpressing phyB, suggesting that phyB is also a protein kinase (Fankhauser et al., 1999).

Our in vitro data on Aux/IAA protein phosphorylation by recombinant oat phyA are reminiscent of in vitro studies with cry1 and cry2 (Ahmad et al., 1998). Although we have reproduced light-modulated autophosphorylation of phyA, phosphorylation of Aux/IAA proteins by recombinant oat phyA in vitro is apparently not modulated by light in our conditions. However, the prospect arises that biochemical activities of at least some Aux/IAA proteins are post-translationally regulated by phytochrome-mediated

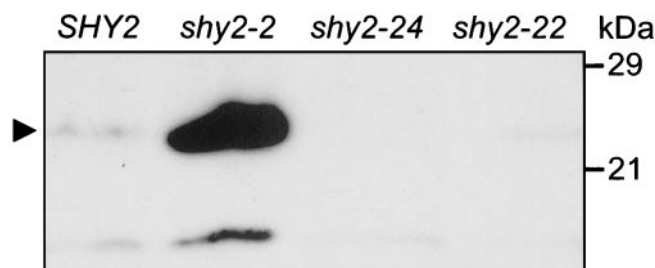


Figure 6. Detection of IAA3 proteins by immunoblot analysis. Seedlings of the indicated genotypes were grown for 5 d in darkness and 300 μ g of total proteins were resolved by SDS-PAGE, transferred to a membrane, and probed with affinity-purified IAA3 antibodies as described in "Materials and Methods." Detection by enhanced chemiluminescence was for 20 min of exposure time. The arrowhead indicates a 23-kD protein identified as endogenous IAA3.

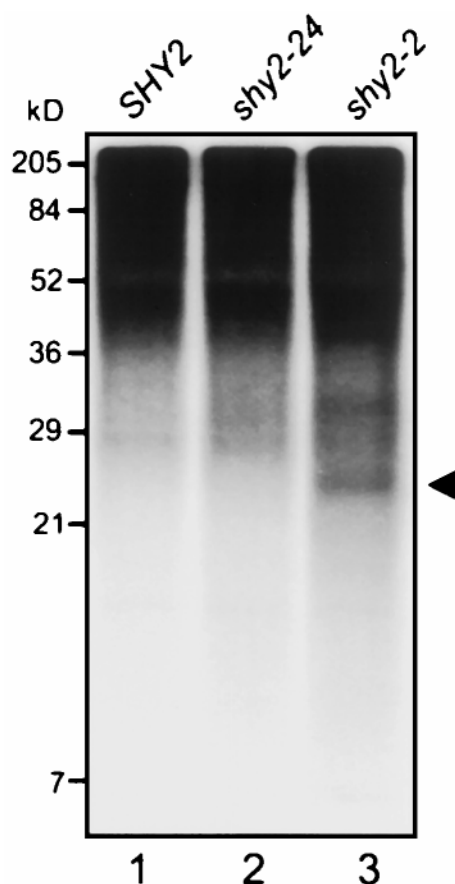


Figure 7. Immunoprecipitations of [32 P]orthophosphate-labeled IAA3 proteins synthesized *in vivo*. Etiolated seedlings of the indicated genotypes were radioactively labeled, treated with auxin (wild-type and *shy2-24*), and extracted as described in "Materials and Methods." Shown is the autoradiograph of resolved proteins after immunoprecipitations of the extracts with affinity-purified antibodies to IAA3 (10 d of exposure). The arrowhead indicates a 23-kD protein, likely representing IAA3. The labeled protein bands seen in the region above 36 kD are due to non-specific binding to protein A agarose beads (data not shown).

phosphorylation or that other factors in the nucleus are necessary for light specificity. Phosphorylation of additional Aux/IAA proteins by phytochromes *in vitro* is presently under investigation to examine regulation of the modification reaction by light and to assess specificity of Aux/IAA proteins as phytochrome kinase substrates.

IAA3 Is Overexpressed and Phosphorylated in *shy2-2* Plants

If *in vitro* phosphorylation of Aux/IAA proteins by phytochrome reflects biological relevance, it is to be expected that at least some Aux/IAA polypeptides are phosphoproteins *in vivo*. To investigate the phosphorylation status of select Aux/IAA proteins *in vivo* we initially focused on SHY2/IAA3 for the following reasons. First, the genetic data pointing to a

possibly direct interaction of Aux/IAA proteins and phytochromes is most indicative for *shy2/iaa3* mutations (Kim et al., 1996, 1998; Reed et al., 1998; Tian and Reed, 1999). Second, loss-of-function alleles of SHY2/IAA3 are available that may facilitate identification of endogenous IAA3 by immunochemistry.

Metabolic labeling of seedlings with [35 S]Met followed by immunoprecipitation with affinity-purified antibodies to IAA3 proved to be the only method for reliably detecting IAA3 in plant extracts. Immunoblot analysis did not consistently recognize the IAA3 polypeptide in up to 300 μ g of total wild-type protein. These observations are in agreement with previous reports describing biochemical studies of Aux/IAA proteins from pea, Ps-IAA4, and Ps-IAA6, which were found to be of extremely low abundance *in vivo* (Abel et al., 1994; Oeller and Theologis, 1995). As expected, the SHY2-2 gain-of-function allele, predicted to stabilize the encoded mutant IAA3 protein (Worley et al., 2000), and the SHY2-22 and SHY2-24 loss-of-function alleles, predicted to encode truncated IAA3 polypeptides (Tian and Reed, 1999), allowed for unambiguous identification of IAA3 *in vivo*. IAA3 synthesized *in planta* migrates in SDS-PAGE as a 23-kD protein, which closely matches the predicted molecular mass of 21.5 kD. Despite the complexity of the Aux/IAA family in Arabidopsis, only three major proteins with molecular weights in the range expected for IAA3 were immunoprecipitated with affinity-purified IAA3 antibodies. Again these data are reminiscent of immunoprecipitation studies in pea (Oeller and Theologis, 1995). Although Aux/IAA proteins share four conserved domains and are encoded by large gene families in higher plants (Abel et al., 1995), affinity-purified polyclonal antibodies to Ps-IAA4 and Ps-IAA6 recognize a limited set of proteins *in vivo* and are specific for the cognate Aux/IAA protein in immunoprecipitation assays (Oeller and Theologis, 1995). In summary, our data suggest specific detection *in vivo* of the Arabidopsis IAA3 polypeptide.

It is interesting that we found *in vivo* steady-state levels of [35 S]-labeled IAA3 to be significantly higher in *shy2-2* than in wild-type plants, exceeding even steady-state concentrations of IAA3 in auxin-treated wild-type seedlings. Moreover, mutant IAA3 protein accumulated in *shy2-2* plants to levels high enough to be detected by immunoblot analysis. Our biochemical data provide the first direct evidence for the hypothesis that gain-of-function mutations in domain II of AXR3/IAA17 (Rouse et al., 1998), SHY2/IAA3 (Tian and Reed, 1999), and AXR2/IAA7 (Nagpal et al., 2000) may stabilize the short-lived proteins. Furthermore, our data are in agreement with and support studies demonstrating that conserved domain II of Aux/IAA proteins constitutes a transferable protein degradation signal and that *shy2-2*-like mutations therein increase the stability of reporter fusion proteins (Worley et al., 2000; J. Callis, personal

communication). In collection, these studies provide additional compelling evidence for the importance of protein degradation in auxin responses (Gray and Estelle, 2000). Direct measurement of the metabolic half-lives of wild-type and mutant IAA3 proteins will be necessary to understand the consequences of these semidominant mutations on the stability and activity of IAA3 and of other Aux/IAA proteins.

Detection of endogenous IAA3 by metabolic labeling and immunoprecipitation assays enabled us to directly test whether IAA3 is phosphorylated *in vivo*. Labeling of *shy2-2* seedlings with [³²P]orthophosphate followed by immunoprecipitation with anti-IAA3 revealed a phosphoprotein of 23 kD. Failure to detect a [³²P]-labeled protein of similar size in immunoprecipitates from *shy2-24* extracts suggests specific precipitation of mutant IAA3 from *shy2-2* extracts. Although considered to be unlikely, we cannot entirely rule out the possibility that the 23-kD protein represents an Aux/IAA polypeptide that is highly similar to IAA3 and whose expression is enhanced in the *shy2-2* background. We were unable to detect [³²P] labeling of IAA3 in untreated or auxin-treated wild-type seedlings, which may be explained by lower steady-state levels of IAA3 in wild-type than of mutant IAA3 in *shy2-2* plants. In an alternate manner, it is possible that auxin treatment or the *shy2-2* mutation (P69S) *per se* affect turnover of phosphate ester groups in IAA3 and thus [³²P] labeling *in vivo*. Although we have shown that at least the *SHY2-2* allele of *IAA3* encodes a phosphoprotein *in vivo*, more powerful analytical tools such as two-dimensional gel electrophoresis will be required to probe the phosphorylation status of immunoprecipitated, [³⁵S]-labeled wild-type IAA3. Different degrees of phosphorylation may be revealed by differences in the electrophoretic mobilities of post-translationally modified IAA3 isoforms.

Functional Implications

Although the *in vivo* significance of the observed *in vitro* phosphorylation of Aux/IAA proteins by phyA remains to be further addressed, several observations are consistent with a functional role of Aux/IAA protein phosphorylation *in vivo*. First, genetic characterization of gain- and loss-of-function *shy2* mutations suggests functional interactions between *PHY* and *SHY2/IAA3* gene products (Kim et al., 1996, 1998; Reed et al., 1998; Tian and Reed, 1999). Higher steady-state levels of IAA3 in *shy2-2* plants indicate that the gain-of-function mutation is hypermorphic and that IAA3 may normally activate de-etiolation in response to light. Second, since most plant cells express *PHYA-PHYE* mRNAs at some level during the entire life cycle of Arabidopsis (Somers and Quail, 1995; Goosey et al., 1997), spatio-temporal co-expression of *PHY* and *Aux/IAA* genes is most likely determined by tissue-specific expression of *Aux/IAA* genes (Abel et al., 1995; Wong et al., 1996). Third,

phytochromes and Aux/IAA proteins are localized to the cell nucleus in light-grown plants (Abel et al., 1994; Sakamoto and Nagatani, 1996). Although nuclear transport of phytochromes is regulated by light (Kirchner et al., 1999; Yamaguchi et al., 1999; Hisada et al., 2000), nuclear localization of Aux/IAA proteins appears to be constitutive, although the dynamics of the translocation process remains to be studied (Abel et al., 1994; Abel and Theologis, 1995). Thus cellular co-expression and nuclear colocalization of phytochrome and Aux/IAA proteins provide opportunity for phytochrome-mediated phosphorylation of Aux/IAAs proteins to occur *in vivo*, which may be regulated by light-induced nuclear transport of the photoreceptors.

We are left to speculate about the biochemical consequences of potential phosphorylation of Aux/IAA proteins by phytochrome *in vivo*. As implicated by genetic and biochemical studies of semidominant *shy2-2* and *axr3-1* mutations (Kim et al., 1998; Tian and Reed, 1999; Worley et al., 2000), phytochrome-mediated phosphorylation of *SHY2/IAA3* would be most consistent with achieving a higher specific activity and/or nuclear concentration of IAA3. As expected, the *axr3-1* mutation does not change the ability of IAA17 to localize to the nucleus or to form homo- and heterodimers (Quellet et al., 2000). It is therefore conceivable that phosphorylation of IAA3 may regulate nuclear localization of IAA3, its metabolic stability, its ability to (auto) regulate transcription by interacting with other proteins, including Aux/IAAs and ARFs, its DNA-binding properties (A. Colón-Carmona and S. Abel, unpublished data), or combinations thereof. Various biochemical functions of transcription factors are affected by phosphorylation (Hunter and Karim, 1992), and multiple phosphorylation sites can play distinct and separable roles in regulating transcription factor activity (Komeili and O'Shea, 1999). It is interesting that phyA phosphorylation site(s) map to the N-terminal one-half of Ps-IAA4 (see Fig. 1). The corresponding region of all Aux/IAA proteins, encompassing conserved domains I and II, is Ser/Thr-rich and contains functionally identified, conserved signals for nuclear localization (Abel and Theologis, 1995) and protein degradation (Worley et al., 2000). We are currently mapping phytochrome phosphorylation sites on select Aux/IAA proteins as a prerequisite for studying the function of Aux/IAA phosphorylation *in vivo*.

Concluding Remarks

An increasing body of evidence suggests intimate interactions between phytochrome and hormone signaling (Neff et al., 2000). As for auxin, in addition to the cloning of *SHY2/IAA3* (Tian and Reed, 1999), a number of physiological and genetic studies indicate its involvement in photomorphogenesis. For example, auxin transport is affected in a light-dependent

manner (Jensen et al., 1998), which has been implicated in playing a role in the shade avoidance response (Morelli and Ruberti, 2000); loss of *NPH4/ARF7* results in a number of differential growth defects, including phytochrome-dependent hypocotyl curvature (Stowe-Evans et al., 1998; Harper et al., 2000) or characterization of *hy5*, which affects a bZIP transcription factor acting downstream of cryptochrome and phytochrome signaling, strongly suggests a link between light and auxin signal transduction (Oyama et al., 1997). The existence of such a link is further indicated by the phenotypic analyses of the *far-red insensitive219* mutation, suggesting that it defines a novel phyA signaling component. It is also interesting that the *FIN219* gene is auxin-inducible and highly similar to *GH3*, a primary auxin response gene (Hsieh et al., 2000). As discussed above, the collective data suggest that SHY2/IAA3 and additional Aux/IAA proteins are bona fide substrates of phytochrome. However, it remains to be seen whether phytochrome-mediated phosphorylation of Aux/IAA proteins provides a mechanism for integrating auxin and light signals in plant development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (ecotype Landsberg *erecta*) was obtained from the *Arabidopsis* Biological Resource Center at Ohio State University. Seeds of mutant lines *shy2-2*, *shy2-22*, and *shy2-24*, derived from Landsberg *erecta* (Tian and Reed, 1999), were kindly provided by Dr. J. Reed (University of North Carolina, Chapel Hill). Seeds were surface sterilized and plated on solid medium containing 8 g L⁻¹ phytagar (Gibco-BRL, Gaithersburg, MD), 15 g L⁻¹ Suc, and 2.15 g L⁻¹ (0.5×) Murashige-Skoog salts (Gibco-BRL), pH 5.6. For [³²P]orthophosphate-labeling experiments, seedlings were grown on low-phosphate agar medium as previously described (Chen et al., 2000). After stratification at 4°C for 2 to 3 d, seeds were germinated in a controlled environmental chamber at 22°C under illumination with fluorescent and incandescent light at a photon fluence rate of approximately 60 μmol m⁻² s⁻¹ for 16 h daily. Prior to growth in darkness, seeds were induced to germinate by treatment with 6 to 12 h of ambient white light.

Plasmid Construction and Purification of Recombinant Proteins

The full-length cDNAs of *IAA1*, *IAA3*, *IAA9*, and *IAA17* from *Arabidopsis*, and *Ps-IAA4* from pea (*Pisum sativum*) were generated by PCR and subcloned into pQE plasmids (Qiagen, Valencia, CA) to produce recombinant, C-terminally (His)₆-tagged proteins. Plasmid construction was essentially performed as described by Kim et al. (1997) and Morgan et al. (1999). Expression vectors coding for various C-terminally (His)₆-tagged, truncated *Ps-IAA4* derivatives, domain I-II (amino acids 1–90), domain I-III (amino acids 1–138), domain II-IV (amino acids 66–189), and

domain III-IV (amino acids 86–189), were constructed accordingly. All subcloned cDNA inserts were verified by DNA sequencing. Recombinant proteins were expressed in *Escherichia coli* M15[pREP] (Qiagen) and purified by affinity chromatography as described (Kim et al., 1997). Expression in the yeast *Saccharomyces cerevisiae* of Strep-tagged phyA from oat (*Avena sativa*), purification of the recombinant protein, and chromophore attachment were performed as described (Wu and Lagarias, 1996; Murphy and Lagarias, 1997).

Antibody Production and Purification

Antibodies to recombinant (His)₆-tagged IAA3 protein were produced in New Zealand White rabbits (Harlow and Lane, 1988). IAA3 rabbit antiserum and preimmune serum were purified by sequential affinity chromatography (Harlow and Lane, 1988) using total proteins from *E. coli* M15[pREP] and purified (His)₆-tagged IAA3 protein immobilized on CNBr-activated Sepharose-4B (Amersham-Pharmacia Biotech, Uppsala). To obtain antibodies preferentially recognizing variable spacer regions of the IAA3 protein, affinity-purified antibodies were passed over a Sepharose-4B column containing immobilized recombinant (His)₆-tagged IAA1 protein.

In Vitro Interaction Assays

Purified recombinant proteins, 2 μg of IAA protein, and 1 μg of Strep-tagged oat phyA were mixed in a total volume of 30 μL of kinase buffer (25 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, and 5 mM MgCl₂) with 5 μL (bed volume) of kinase buffer-washed streptavidin agarose beads. Binding reactions were incubated at room temperature for 45 min and mixed every 5 min. Agarose beads were sedimented by centrifugation and washed three times with 500 μL of detergent-containing buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, and 0.1% [v/v] NP-40). The attached proteins were solubilized by boiling in 1× SDS sample buffer. The proteins of the pellet and initial supernatant (entire fractions) were resolved on 12.5% (w/v) SDS-polyacrylamide gels (Laemmli, 1970). Proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes (Osmonics, Westborough, MA) before visualizing by Coomassie Blue staining.

Phytochrome Kinase Assays

Protein phosphorylation experiments were essentially performed as described (Yeh et al., 1997; Yeh and Lagarias, 1998). The reaction mixtures (total volume of 25 μL) contained kinase buffer (25 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, and 5 mM MgCl₂), 0.1 mM ATP, 1 μg of purified recombinant oat phyA in the Pr or Pfr form, and 2 μg of purified recombinant IAA protein (IAA1, IAA3, IAA9, IAA17, PS-IAA4, or truncated forms of PS-IAA4). Kinase reactions were started by adding 10 μCi of [^γ-³²P]ATP (ICN, 3,000 Ci mmol⁻¹) and were incubated at room temperature for 25 min. Reactions were stopped by the addi-

tion of 6 μL of $5\times$ SDS sample buffer. Proteins were resolved on 12.5% (w/v) SDS-polyacrylamide gels and transferred to PVDF membranes for autoradiographic analysis using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and for subsequent Coomassie Blue protein staining to verify protein loading.

Metabolic Labeling

For in vivo labeling experiments, 5-d-old dark-grown wild-type and mutant seedlings (1 g fresh weight) were submerged in 7 mL of incubation buffer consisting of 15 mM Suc, 1 mM KCl, 1 mM sodium citrate, and 1 mM 2-(*N*-morpholino) ethane sulfonic acid, pH 5.6 (Theologis et al., 1985). The seedlings were incubated at room temperature in ambient light under moderate shaking with or without 20 μM IAA in the presence of 1 mCi [^{35}S]Met (Trans label, ICN, $>1,000$ Ci mmol^{-1}) or 10 mCi [^{32}P]orthophosphate (ICN, 400–800 Ci mmol^{-1}) as follows: for the detection of [^{35}S]Met-labeled IAA3 proteins in vivo, seedlings were incubated with 1 mCi [^{35}S]Met for 4 h. Where indicated, treatment with 20 μM IAA was initiated after 2 h of metabolic labeling. For [^{32}P] labeling experiments, wild-type and mutant seedlings were incubated with 10 mCi [^{32}P]orthophosphate for 3 h.

Protein Extraction and Immunoprecipitation of IAA3

After the indicated incubation regimes, the radioactively labeled seedlings were frozen in liquid nitrogen, ground to a fine powder using a mortar and a pestle, and lyophilized overnight. Proteins were extracted with 1.0 mL of boiling extraction buffer (100 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.0, 140 mM β -mercaptoethanol, and 1% [w/v] SDS) to inactivate endogenous proteases (Vierstra and Quail, 1982). After re-extraction of the plant residues with 0.5 mL of buffer, the combined extract was cleared by centrifugation (20,000g for 20 min at 4°C). The supernatant was diluted 10-fold with buffer A, consisting of 20 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.0, 250 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, and a protease inhibitor cocktail (Complete, Boehringer Mannheim, Basel), or with buffer B (buffer A containing 5 mM EGTA, 50 mM β -glycerophosphate, 50 mM NaF, 10 mM Na_3VO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 100 $\mu\text{g mL}^{-1}$ RNase A, and 200 units mL^{-1} RNase T₁) to inactivate phosphatases and to degrade [^{32}P]-labeled RNA (Lanker et al., 1996). The diluted extracts were first precleared with 50 μL of protein A-agarose beads (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h. After removal of the agarose beads, affinity-purified IAA3 antibodies were added corresponding to 100 μL of crude antiserum and incubated with agitation for 5 to 6 h at 4°C. Fifty microliters of protein A agarose beads were subsequently added and incubated for 2 h at 4°C. The immunoprecipitate-agarose bead complexes were collected by centrifugation and washed essentially according to Oeller and Theologis (1995). In brief, the agarose beads were sedimented four times in 10 mL of wash buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, and 1% [v/v] Triton X-100), followed by treatments with 1 mL each

of wash buffer containing 0.4 M LiCl and 0.2 M LiCl, wash buffer, and wash buffer containing 1% (w/v) sodium deoxycholate and 0.1% (w/v) SDS. The beads were then washed twice with 20 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.4, 150 mM NaCl, and once with deionized water. Immunocomplexes were eluted into boiling $2\times$ SDS loading buffer and resolved on a 12.5% (w/v) SDS-polyacrylamide gel. Dried gels were analyzed by phosphorimaging (Storm 860, Molecular Dynamics) and by autoradiography (Oeller and Theologis, 1995) using BioMax film (Kodak, Rochester, NY).

Immunoblot Analysis

Proteins were extracted from lyophilized plant tissues as described above. Total proteins were separated by electrophoresis on 12.5% (w/v) SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 10% (w/v) skim milk in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.2% (v/v) Tween 20 for 45 min at room temperature. Blots were probed with affinity-purified IAA3 antibodies (2 h) followed by incubation with anti-rabbit IgG conjugated with horse radish peroxidase (Amersham, Buckinghamshire, UK) for 1 h at room temperature. Signals were detected using the enhanced chemiluminescence system (SuperSignal, Pierce, Rockford, IL).

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