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Disruptions in AUX1-Dependent Auxin Influx Alter Hypocotyl Phototropism in *Arabidopsis*

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ABSTRACT Phototropism represents a differential growth response by which plant organs can respond adaptively to changes in the direction of incident light to optimize leaf/stem positioning for photosynthetic light capture and root growth orientation for water/nutrient acquisition. Studies over the past few years have identified a number of components in the signaling pathway(s) leading to development of phototropic curvatures in hypocotyls. These include the phototropin photoreceptors (phot1 and phot2) that perceive directional blue-light (BL) cues and then stimulate signaling, leading to relocalization of the plant hormone auxin, as well as the auxin response factor NPH4/ARF7 that responds to changes in local auxin concentrations to directly mediate expression of genes likely encoding proteins necessary for development of phototropic curvatures. While null mutations in *NPH4/ARF7* condition an aphototropic response to unidirectional BL, seedlings carrying the same mutations recover BL-dependent phototropic responsiveness if co-irradiated with red light (RL) or pre-treated with either ethylene. In the present study, we identify second-site enhancer mutations in the *nph4* background that abrogate these recovery responses. One of these mutations—*map1* (*modifier of arf7 phenotypes 1*)—was found to represent a missense allele of *AUX1*—a gene encoding a high-affinity auxin influx carrier previously associated with a number of root responses. Pharmocological studies and analyses of additional *aux1* mutants confirmed that AUX1 functions as a modulator of hypocotyl phototropism. Moreover, we have found that the strength of dependence of hypocotyl phototropism on AUX1-mediated auxin influx is directly related to the auxin responsiveness of the seedling in question.

INTRODUCTION

As sessile organisms, plants maintain highly plastic developmental programs that afford a means to alter their growth and morphology in response to changes in prevailing environmental conditions. Not surprisingly, given their photoautotrophic growth, light represents one of the most important environmental signals impinging upon a plant. Plants utilize light not only as an energy source, but also as a vital source of temporal and spatial information (Chen et al., 2004; Franklin et al., 2005; Spalding and Folta, 2005; Sullivan and Deng, 2003). For example, discrimination of light quality, intensity and direction by a given organ of a plant informs that organ where it is in time and space relative to other organs on the same plant, as well as organs of neighboring plants. Such signals are interpreted and utilized to modulate development, morphology and position of organs for adaptive processes such as optimization of leaf/stem positioning for photosynthetic light capture, and root growth orientation for efficient water/nutrient acquisition (Celaya and Liscum, 2005; Galen et al., 2004, 2007a, 2007b; Iino, 2001, 2006; Inoue et al., 2007; Kimura and Kagawa, 2006; Spalding and Folta, 2005).

Phototropism, or the directional growth of a plant organ in response to directional blue light (BL), represents a predominant mechanism by which plants reposition body parts to achieve the aforementioned adaptation (Iino, 2001, 2006; Liscum, 2002). The BL-absorbing phototropins (phots) function as the primary photoreceptors controlling phototropism in aerial organs of higher plants (Jarillo et al., 2001; Ohgishi et al., 2004; Sakai et al., 2001; Whippo and Hangarter, 2003), while the BL-absorbing cryptochromes (crys) and principally red light (RL)-absorbing phytochromes (phys) can act as modulators of phot-dependent signal-response (Janoudi et al., 1997a, 1997b;

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Lariguet and Fankhauser, 2004; Ohgishi et al., 2004; Parks et al., 1996; Stowe-Evans et al., 2001; Whippo and Hangarter, 2003, 2004). While considerable advances have been made in the identification and characterization of molecular components of the phot1 signal–response system mediating phototropism under low-light conditions (Esmon et al., 2006; Harper et al., 2000; Inada et al., 2004; Lariquet et al., 2006; Motchoulski and Liscum, 1999; Park et al., 2002; Pedmale and Liscum, 2007; Sakai et al., 2000; Stowe-Evans et al., 1998; Tatematsu et al., 2004; Yang et al., 2004), much less is known about how crys and phys influence phot1-dependent phototropism (Iino, 2006; Liscum, 2002; Whippo and Hangarter, 2006).

Most current models of tropic responsiveness in both aerial organs (Esmon et al., 2005; Kimura and Kagawa, 2006; Tatematsu et al., 2004; Whippo and Hangarter, 2006) and roots (Swarup et al., 2005; Wisniewska et al., 2006) represent modifications of the classic Cholodny–Went hypothesis in which differential accumulation of, and response to, the growth regulator auxin is key to the development of tropic curvatures (Went and Thimann, 1937). Molecular support for a central role for auxin in tropic responses has been accumulated in recent years through studies of various *Arabidopsis* mutants affecting both auxin transport (Abas et al., 2006; Blakeslee et al., 2004; Friml et al., 2002; Noh et al., 2003) and auxin responsiveness (Harper et al., 2000; Park et al., 2002; Swarup et al., 2005; Tatematsu et al., 2004; Yang et al., 2004).

The identification and subsequent study of the auxin-responsive transcriptional activator, NPH4/ARF7, as a key regulator of hypocotyl phototropism and gravitropism in *Arabidopsis* (Harper et al., 2000; Liscum and Briggs, 1996; Stowe-Evans et al., 1998) has lead to a proposed mechanistic model for how auxin responsiveness acts as a driving factor in the development of phototropic curvatures (Esmon et al., 2005; Liscum and Reed, 2002; Tatematsu et al., 2004). First, phot1-dependent signaling in response to irradiation with unidirectional BL leads to an alteration in basal longitudinal polar auxin transport (PAT) (Blakeslee et al., 2005; Geisler and Murphy, 2006; Kramer and Bennett, 2006; Wisniewska et al., 2006) such that a lateral gradient of the hormone is established across the hypocotyl, with increased auxin levels in the 'shaded flank' (side farthest from incident light) as compared to the 'lit flank' (side closest to incident light) (Esmon et al., 2006; Fuchs et al., 2003; Iino, 2001). Next, perception of threshold levels of auxin in cells within the 'shaded flank' by the TIR1/AFB F-box proteins facilitates binding of substrate Aux/IAA proteins to the $SCF^{TIR1/AFB}$ ubiquitin ligase complex to stimulate proteosome-dependent degradation of those substrates (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005; Tan et al., 2007). Two known SCF^{TIR1} substrates are MSG2/IAA19 and AXR5/IAA1, both of which appear to heterodimerize with, and repress the action of, NPH4/ARF7 (Park et al., 2002; Tatematsu et al., 2004; Yang et al., 2004). Thus, in the absence of phototropic stimulation, NPH4/ARF7 in the hypocotyl is found primarily as a heterodimer with Aux/IAA proteins, like MSG2/IAA19 and AXR5/IAA1, and is transcriptionally inactive. However, after phototropic stimulation, when auxin accumulates in the 'shaded flank' and Aux/IAA proteins are degraded, NPH4/ARF7 is able to homodimerize and function as a potent transcriptional activator to stimulate expression of genes, presumably encoding proteins necessary for the development of phototropic curvatures (Esmon et al., 2005; Liscum and Reed, 2002; Tatematsu et al., 2004). A recent study has identified a number of apparent NPH4/ARF7 target genes whose transcripts accumulate in an auxin-dependent manner specifically in the 'shaded flank' of phototropically stimulated *Brassica oleracea* seedlings, prior to, or concomitant with, curvature (Esmon et al., 2006). Consistent with the latter portion of the aforementioned model, two of these genes encode α-expansins (Esmon et al., 2006)—enzymes that catalyze cell wall extension (Cosgrove, 2000; Kende et al., 2004; Li et al., 2003) that is prerequisite for cell elongation processes like those occurring in the shaded flank during development of tropic curvatures (Iino, 2001).

While it is clear that NPH4/ARF7 is a critical regulator of phot1-dependent phototropism (Esmon et al., 2006; Harper et al., 2000; Liscum and Briggs, 1995, 1996; Stowe-Evans et al., 1998; Tatematsu et al., 2004), it is apparently not the only ARF capable of regulating phototropic responsiveness, since the aphototropic hypocotyl phenotype of *nph4/arf7*-null mutants is conditional (Harper et al., 2000; Liscum and Briggs, 1996; Stowe-Evans et al., 2001). For example, *nph4/arf7*-null mutant seedlings pretreated with ethylene prior to exposure to unidirectional BL recover ~60% of the response observed in wild-type seedlings treated with BL alone (Harper et al., 2000). Importantly, this ethylene-dependent response requires auxin responsiveness and appears to result from at least partial recovery of auxin-dependent gene expression (Harper et al., 2000). Ethylene exposure is not the only treatment that can condition recovery of phot1-dependent phototropism in the *nph4/arf7* background. Exposure of *nph4/arf7*-null mutant seedlings to RL prior to, or concurrent with, stimulation with unidirectional BL also results in a suppression of the aphototropic phenotype observed in *nph4/arf7* seedlings exposed to BL alone (Liscum and Briggs, 1996; Stowe-Evans et al., 2001). RL alone is, however, incapable of stimulating hypocotyl phototropism in *Arabidopsis* (Liscum and Briggs, 1996; Steinitz et al., 1985). It is presumed that the RL-dependent recovery of BL-induced phototropism in *nph4/arf7* mutants also requires conditional activation of auxin-dependent gene expression (Stowe-Evans et al., 2001).

In this study, we exploited the conditional nature of the *nph4/arf7* aphototropic phenotype in a genetic screen to identify loci that are required for recovery of phototropism in the absence of NPH4/ARF function. It was surprising to find that *AUX1*, which encodes a high-affinity auxin influx carrier (Yang et al., 2006), represents one such locus, since most functions ascribed to AUX1 are root-specific and no hypocotyl-specific function was previously known. Our studies clearly show that AUX1-facilitated auxin influx is an important player in the establishment of phototropic responses in *Arabidopsis*

hypocotyls, especially when seedlings lack NPH4/ARF7 function and basal auxin responsiveness is compromised.

RESULTS

Isolation and Characterization of Second-Site Mutations that Disrupt Condition-Dependent Recovery of Hypocotyl Phototropism in the *nph4/arf7*-Null Mutant Background

As introduced earlier, *nph4* seedlings recover a phototropic response to unidirectional BL if co-irradiated or pre-treated with RL (Liscum and Briggs, 1996; Stowe-Evans et al., 2001). This RL response has been shown to be specifically mediated by phyA (Stowe-Evans et al., 2001). In this context, it is interesting to note that unidirectional UV-A light (UV-A) alone, which is absorbed efficiently by both phot1 (Christie et al., 1998) and phyA (Butler, 1964; Pratt and Briggs, 1966; Vierstra and Quail, 1983), can stimulate phototropic responses in *nph4* and wild-type that are similar to those observed when seedlings are irradiated with BL and RL together (Figure 1A; Liscum and Briggs, 1996). This UV-A responsiveness was the basis of a simple screen for second-site mutations that affect recovery of phototropism in an *nph4* background. In brief, ~40 000 3 d old etiolated ethyl methanesulfonate (EMS)-mutagenized *nph4-1* M2 seedlings (representing ~10 000 M1 parental lines) were exposed to 8 h of unidirectional UV-A (0.5 μmol m^{-2} s^{-1}) and putative enhancer mutants were selected as seedlings, exhibiting little to no hypocotyl phototropism. Because the *nph4-1* allele is a deletion/rearrangement null allele (Harper et al., 2000), any recovered mutations in this background must, by default, represent second-site lesions.

Of 13 M2 seedlings selected for an aphototropic phenotype in the *nph4-1* background, three exhibited a clear heritable phenotype in subsequent generations under the same conditions (data not shown). Each of these second-site mutations segregated as a single recessive trait in F2 progeny from backcrosses to the *nph4-1* progenitor (data not shown), and each represents a lesion at a distinct locus, since F1 progeny from pair-wise crosses between mutants (carried in the *nph4-1* background) exhibited UV-A-induced recovery of phototropism (data not shown). Based on the aforementioned results, the three mutants were named *map1*, *map2*, and *map3* (*map* for *m*odifier of *a*rf7/*n*ph4 *p*henotypes). As shown in Figure 1B, each of the *map* mutations suppressed recovery of phototropism normally observed in *nph4* seedlings co-irradiated with BL and RL, as was expected based on the UV-A phenotypes for which they were selected. However, only *nph4map3* retained the ethylene-stimulated recovery response of *nph4* (Figure 1B), indicating that *map1* and *map2* do not specifically alter the phyA-stimulated response, whereas *map3* may.

It is interesting to note that *nph4* seedlings carrying a homozygous loss-of-function mutation in the most closely related *ARF*, *ARF19* (Remington et al., 2004), exhibited a normal recovery of phototropic responsiveness in RL plus BL conditions, but were completely unresponsive in ethylene plus BL (Figure 1B). These findings imply: (1) that ARF19 may not be redundant to NPH4/ARF7 under RL plus BL conditions, or, if it is, that additional ARFs must also participate, and (2) that ARF19 alone is sufficient to compensate for the absence of NPH4/ARF7 under ethylene pretreatments. This latter conclusion is consistent with previous studies showing that ARF19 and NPH4/ARF7 functions overlap at least partially (Li et al., 2006; Okushima

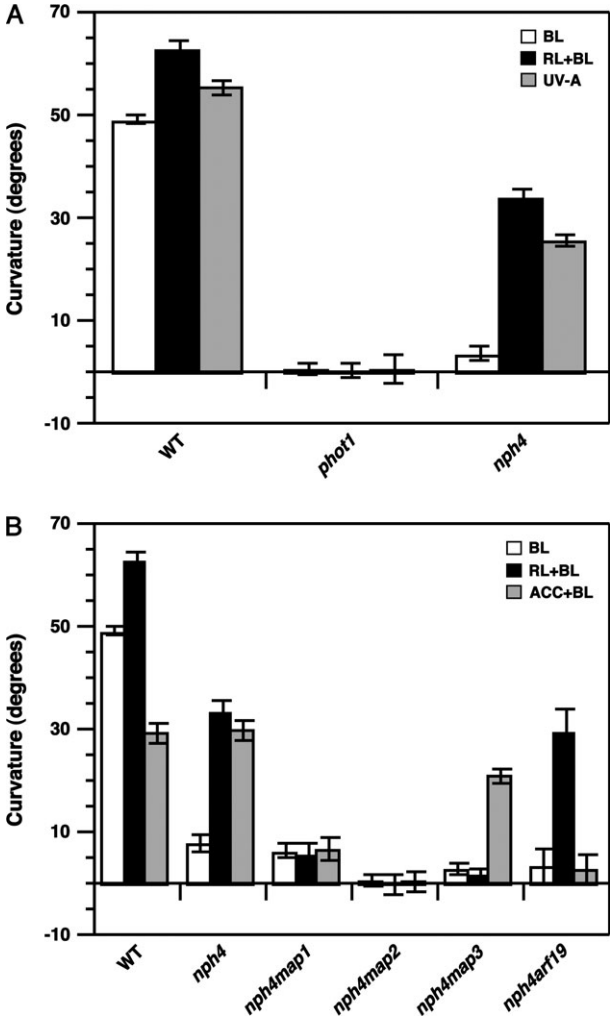

Figure 1. Hypocotyl Phototropic Responses of Wild-Type and Mutant Seedlings Exposed to Unidirectional UV-A Light and Unidirectional Blue Light, With and Without Red Light Co-Irradiation or Ethylene Pretreatment.

(A) Phototropic curvatures of etiolated wild-type Col-O (WT), *phot1*, and *nph4* seedlings exposed to 4 h of unidirectional BL (0.1 mmol m^{-2} s^{-1}), unidirectional UV-A light (0.2 mmol m^{-2} s^{-1}), or unidirectional BL supplemented with RL (1.6 mmol m^{-2} s^{-1}) from above. Data represent mean response from a minimum of 60 seedlings from at least two independent replicate experiments. Error bars represent SE.

(B) Phototropic curvatures of etiolated wild-type Col-O (WT), *nph4*, *nph4map*, and *nph4arf19* seedlings exposed to 4 h of unidirectional BL, unidirectional BL supplemented with RL, or unidirectional BL after growth on ACC (1 μM). Data represent mean response from a minimum of 80 seedlings from at least two independent replicate experiments. Error bars represent SE.

et al., 2005, 2007; Wilmoth et al., 2005), and that *ARF19* transcription is up-regulated by ethylene treatment (Li et al., 2006). Given the phenotypes of *nph4map3* seedlings as compared to those of *nph4arf19* (Figure 1B), it seems possible that *MAP3* encodes a redundantly acting ARF (other than ARF19), or, at a minimum, a component of the response system shared by this ARF and NPH4/ARF7. It is worth noting that a loss-of-function mutant in the *ARF8* locus—another member of the *NPH4/ARF7 and ARF19* subclade (Remington et al., 2004)—exhibits a BL-induced phototropic response that is only about 80% of the wild-type response (Tian et al., 2004). As such, *ARF8* represents a potential target locus of the *map3* mutation. Future genetic mapping of *MAP3* will determine if this is a viable hypothesis.

The conditional phenotypes of the *nph4* mutants require that the phot1 signal–response pathway functioning upstream of NPH4/ARF7 remains intact (Harper et al., 2000; Liscum and Briggs, 1996) and, as such, second-site mutations affecting elements of the phot1 pathway would be expected to give rise to a non-conditional phenotype similar to that observed in the *nph4map1* and *nph4map2* mutants. Thus, the *nph4map1* and *nph4map2* mutants were out-crossed to *nph4phot1* and *nph4nph3* double mutants to determine if either of these second-site lesions represented new *phot1* or *nph3* alleles. While all F1 progeny from crosses of *nph4map1* to *nph4phot1* and *nph4nph3* exhibited UV-A-induced phototropism, F1 and F2 progeny from crosses of *nph4map2* to *nph4nph3* failed to segregate any seedlings exhibiting a UV-A-induced phototropic response (data not shown). These results indicate that *map1* represents a lesion in either a previously uncharacterized primary signal–response element or a common modulatory element, whereas *map2* represents a new *nph3* allele (*nph3-9*). Sequencing of *NPH3* confirmed this latter conclusion and showed that the *map2/nph3-9* allele contains a G-to-A transition that changes E_{647} to K in the mutant nph3 protein.

map1 Represents a New Allele of *AUX1*—A Gene Encoding a High-Affinity Auxin Influx Carrier

Ethylene-stimulated recovery of phototropism in the *nph4* background, in addition to requiring a functional phot1-signaling pathway upstream of NPH4/ARF7 itself, has been shown to require auxin responsiveness (Harper et al., 2000). Based on this knowledge, the observed failure of *nph4map1* mutant seedlings to respond to either RL or ethylene pretreatment (Figure 1B) suggested that the *map1* lesion may affect phototropism via influences on auxin homeostasis or signal–response.

Bulked-segregant analysis (see Methods; Lukowitz et al., 2000) showed that the *MAP1* locus is genetically linked to the SSLP marker nga168 on the distal arm of chromosome 2. Five known or predicted auxin-response genes are located within 2 Mb of nga168 (Arabidopsis Genome Initiative, 2000; Wortman et al., 2003): *ETTIN/ARF3* (Liscum and Reed, 2002; Nemhauser et al., 2000), *SAUR-AC1/AtSAUR15* (Gil et al., 1994; Hagen and Guilfoyle, 2002), *AtSAUR46* (Hagen and Guilfoyle, 2002), *AUX1* (Bennett et al., 1996), and *TCH3* (Antosiewicz et al., 1995; Benjamins et al., 2003). Although none of these genes had been previously functionally associated with hypocotyl tropic responses, *AUX1* was particularly interesting as a potential candidate for *MAP1* since *nph4map1* seedlings exhibited alterations in root gravitropism reminiscent of those observed in *aux1* mutants (Figure 2A; Bennett et al., 1996; Mizra et al., 1984; Swarup et al., 2004). The reduced root gravitropism common to both *nph4map1* and *aux1* mutants was retained in F1 progeny from a cross of *nph4map1* to *aux1-7* (data not shown), suggesting that *map1* does in fact represent a new *aux1* allele. Sequencing of *AUX1* in the *nph4map1* background showed that it contains a C-to-T transition that converts Ala_{94} to a Val. Within the AUX/LAX (Like-AUX1) subfamily of amino acid/auxin:proton symport permeases (AAAPs) (Fischer et al., 1998; Swarup et al., 2004), Ala_{94} is an invariant residue within a highly conserved motif (Figure 2B) that resides on the hydrophilic face of the second of 11 transmembrane domains (Figure 2C and 2D; Kerr and Bennett, 2007; Swarup et al., 2004). The *map1* allele has been re-named *aux1-201* to reflect its molecular identity.

A Combination of Allele- and Condition-Specific Phototropic Responses Are Observed in Various *aux1* Missense Mutants

The isolation of the *aux1-201/map1* allele as a modifier of *nph4* hypocotyl phototropism was initially surprising, since nearly all previously reported *aux1* phenotypes were restricted to the root (Bennett et al., 1996; Marchant and Bennett, 1998; Marchant et al., 1999; Swarup et al., 2004). It therefore became important to address whether the *aux1-201* mutation could condition a defective phototropic response on its own, and whether other *aux1* alleles (Figure 2C) could influence phototropism, either solely or in combination with *nph4*. Figure 3A demonstrates that although none of the *aux1* single mutants tested, including *aux1-201*, exhibited alterations in BL-induced phototropism on the order of that observed for *hypo*-phototropic mutants like *nph4* (Figure 1), several alleles (*aux1-7*, *aux1-105*, and *aux1-201*) did exhibit a statistically significant reduction in response. These results suggest that while AUX1 is not a major regulator of hypocotyl phototropism, it may function as a modulator of the response. Pharmacological data provide additional support for this conclusion. As shown in Figure 4, the potent auxin influx inhibitor 1-naphthoxyacetic acid (1-NOA) (Imhoff et al., 2000), which can phenocopy the auxin-insensitive and agravitropic root phenotypes observed in *aux1* mutants (Ottenschlager et al., 2003; Parry et al., 2001; Yang et al., 2006), had only a minor effect on hypocotyl phototropism in wild-type seedlings.

Although only a minor function for AUX1 in hypocotyl phototropism is predicted from results with either *aux1* single mutants or wild-type seedlings treated with 1-NOA, the apparent modulatory role of AUX1 becomes much more obvious in the absence of NPH4/ARF7 function (Figure 3B). For example, the previously described strong loss-of-function *aux1* allele, *aux1-7* (Swarup et al., 2004; Yang et al., 2006), impairs

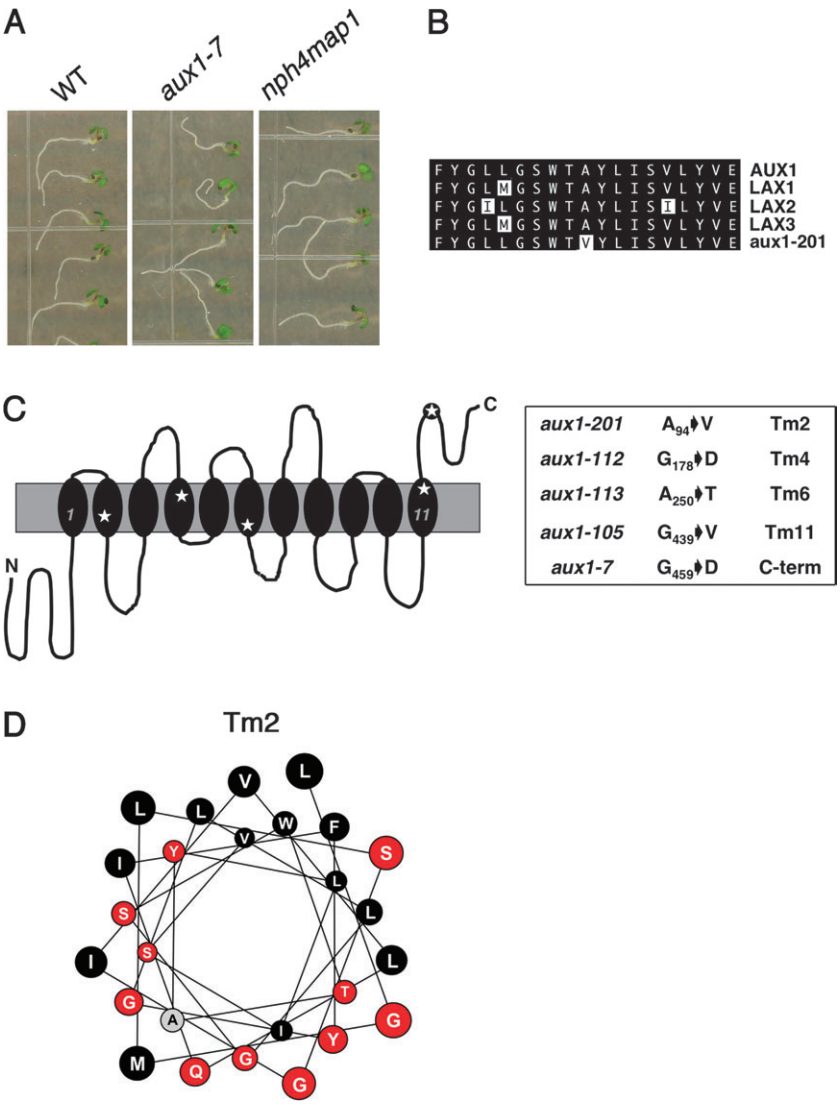

Figure 2. Identification of *AUX1* as a Genetic Modifier of *arf7* Phenotypes.

(A) Root gravitropic phenotypes of wild-type Col-O (WT), *aux1-7*, and *nph4map1/aux1-201* seedlings. Seedlings were grown in plates along the surface of the agar medium for 5 d in white light, then the plates were rotated 90° to induce a gravitropic response (downward curvature of the root; upward curvature of the hypocotyl).

(B) Position of the *aux1-201* Ala to Val missense mutation within a conserved motif of AUX/LAX family of auxin influx carriers.

(C) Positions and identities of various *aux1* missense alleles used in this study. Topology of AUX1 is adapted from Swarup et al. (2004) and shows the 11 predicted transmembrane (Tm) spans in linear sequence with Tm1 shown on the far left and Tm11 on the far right. Stars indicate the position of various missense alleles described in the box to the right.

(D) Helical wheel projection of the second transmembrane span (Tm2) of AUX1. Hydrophobic residues are in black, hydrophilic residues are in red, and the Ala that is changed to Val in the *aux1-201* allele is shown in grey. Projection adapted from Kerr and Bennett, 2007.

RL-dependent recovery of BL-induced phototropism in the *nph4*-null mutant background to a similar extent as the *aux1-201* allele (Figure 3B). A comparable lack of responsiveness was also observed in the *nph4aux1-105* double mutant (Figure 3B). As shown in Figure 4, treatment of *nph4* seedlings with 1-NOA results in a concentration-dependent inhibition the phototropic response normally observed in RL plus BL conditions. In fact, *nph4* seedlings treated with 30 μM 1-NOA exhibited an aphototropic response that was indistinguishable from that of *nph4aux1-7*, *nph4aux1-105*, and *nph4aux1-201* double mutants in RL plus BL (Figures 3B and 4). It is, however, interesting to note that the *aux1-112* allele, which has been reported to represent a strong allele relative to root gravitropism and to have undetectable levels of AUX1 protein in root microsomal membrane fractions (Swarup et al., 2004), only influences hypocotyl phototropism when NPH4/ARF7 is absent, and, even then, not nearly to the extent observed in the *aux1-7*, *aux1-105*, or *aux1-201* backgrounds (Figure 3). The response of the *aux1-112* allele was most similar to that of *aux1-113*—a known partial loss-of-function allele (Swarup et al., 2004)—which was indistinguishable from wild-type in either BL alone or RL plus BL conditions (Figure 3B).

Penetrance of the *aux1-201* Allele Is Phenotype-Dependent

The similar phenotypic effects of the *aux1-7*, *aux1-105*, and *aux1-201* alleles on hypocotyl phototropism would suggest that *aux1-201*, like *aux1-7* and *aux1-105* (Swarup et al., 2004; Yang et al., 2006), represents a strong loss-of-function allele. However, as shown in Figure 5, this prediction is not supported by experimental evidence. Rather, *aux1-201* appears to exhibit varying penetrance, depending upon the physiological response being examined. For example, while the *aux1-201* mutation strongly influenced hypocotyl phototropism (Figures 1B and 3), it had a more moderate affect on root gravitropism, exhibiting a response intermediate between that of the weak *aux1-113* and strong *aux1-7* and *aux1-105* alleles (Figure 5A). An even weaker influence of

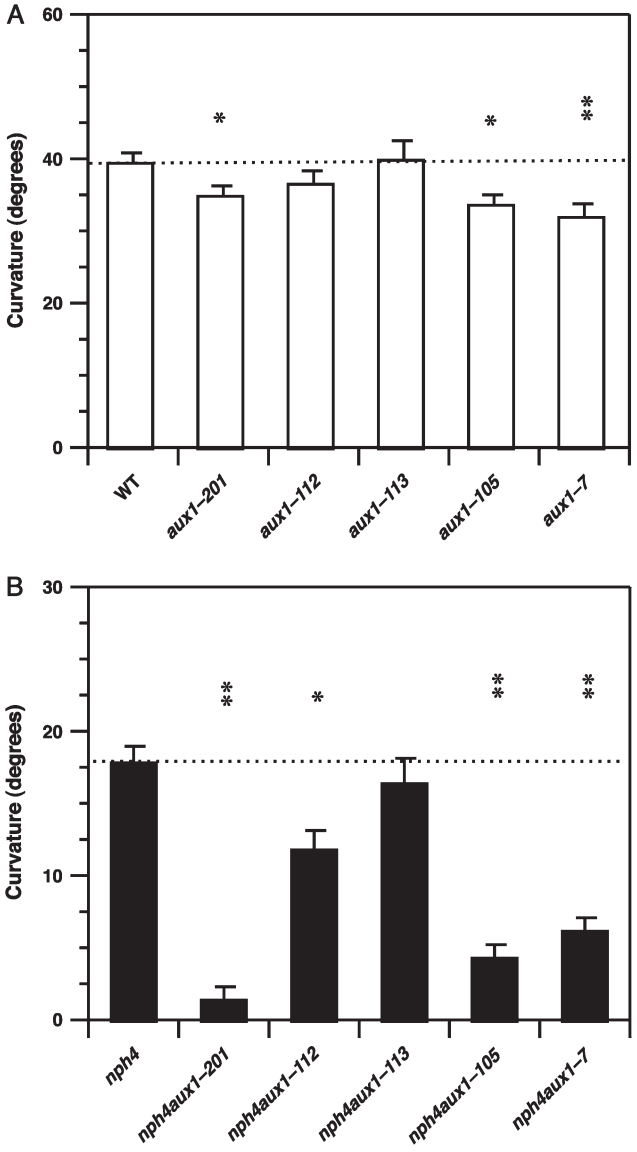

Figure 3. Hypocotyl Phototropism in Wild-Type and *aux1* Mutant Seedlings.

(A) Phototropic curvatures of wild-type Col-O (WT) and various *aux1* missense mutants exposed to 4 h of unidirectional BL (0.1 mmol m^{-2} s^{-1}). Although not all *aux1* alleles are carried in the Col-O background (see Methods), only the Col-O response is shown, as other wild types responded similarly (data not shown). Data represent mean response from a minimum of 107 seedlings from at least two independent replicate experiments. Error bars represent SE. Asterisks denote responses significantly different from that observed in wild-type by Student's *t*-test (*, $0.01 > P > 0.001$; **, $P < 0.001$).

(B) Phototropic curvatures of *nph4*-null single and *nph4aux1* double mutants exposed to 4 h of unidirectional BL supplemented with RL (1.6 mmol m^{-2} s^{-1}) from above. Data represent mean response from a minimum of 75 seedlings from at least two independent replicate experiments. Error bars represent SE. Asterisks denote responses significantly different from that observed in the *nph4*-null single mutant by Student's *t*-test (*, $0.01 > P > 0.001$; **, $P < 0.001$).

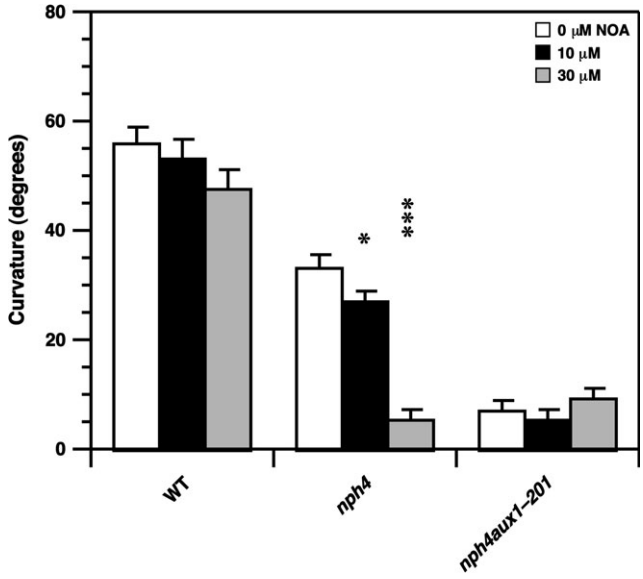

Figure 4. Influence of the Auxin Influx Inhibitor, 1-NOA, on Hypocotyl Phototropism in Wild-Type, *nph4 and nph4aux1-201* Seedlings.

Seedlings were grown for 3 d in darkness on agar-solidified medium containing the indicated concentration of 1-NOA, then exposed to 4 h of unidirectional BL (0.1 mmol m^{-2} s^{-1}). WT, wild-type Col-O. Data represent mean response from a minimum of 72 seedlings from at least two independent replicate experiments. Error bars represent SE. Asterisks denote responses significantly different from that observed in *nph4* controls (no NOA exposure) by Student's *t*-test (*, $0.05 > P > 0.01$; **, $P < 0.001$).

the *aux1-201* allele was observed when root elongation in the presence of exogenous IAA was examined (Figure 5B). In this latter case, *aux1-201* was the weakest of the *aux1* alleles examined.

In contrast to *aux1-201*, the previously described strong loss-of-function alleles, *aux1-7* and *aux1-105* (Bennett et al., 1996; Pickett et al., 1990; Swarup et al., 2004), exhibited strong loss-of-function phenotypes for all three responses examined in this study, independently of whether the response was occurring in the root (Figure 5) or hypocotyl (Figure 3). The *aux1-112* and *aux1-113* mutants also exhibited consistent and essentially condition-independent penetrance, although considerably weaker in severity than those of the *aux1-7* and *aux1-105* alleles (Figures 3 and 5). While the partial loss-of-function phenotypes of *aux1-113* were as expected, based on previous studies, those of *aux1-112* were somewhat surprising, given that this allele had been reported to lack immunodetectable aux1 protein in root microsomal membranes (Swarup et al., 2004). The results presented here imply that there is in fact functional aux1 protein in the *aux1-112* mutant.

BL-Induced Phototropism in *nph4 is* Recovered in the Presence of Exogenous IAA

It has been proposed that phyA- and ethylene-dependent recovery of BL-induced phototropism in the *nph4* background

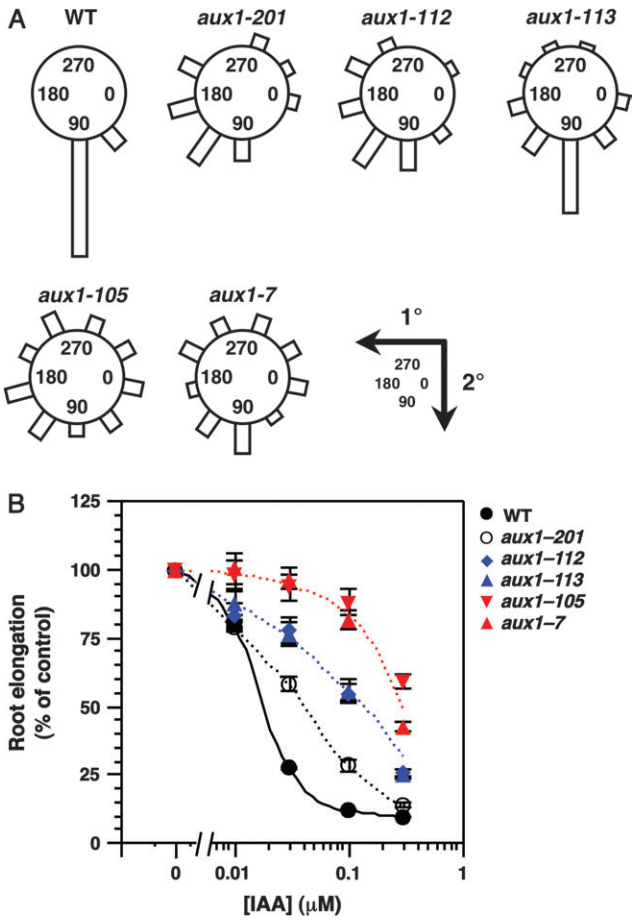

Figure 5. Root Gravitropism and Auxin Sensitivity in *aux1* Missense Alleles.

(A) Root gravitropic re-orientation response of wild-type Col-O (WT) and *aux1* mutant seedlings. Seedlings were grown on vertically oriented plates for 3 d in darkness, followed by 90° rotation of the plate to induce change in root growth orientation (gravirresponse). After 24 h, growth in darkness, root orientations were determined relative to the seedling axis upon re-orientation (see insert, lower right). Although not all *aux1* alleles are carried in the Col-O background (see Methods), only the Col-O response is shown, as other wild-types responded similarly (data not shown). Data are projected on a wheel diagram that reflects root orientation at the end of the experiment, where 0° is no change in growth direction after re-orientation, 90° is complete downward re-orientation (note nearly perfect downward re-orientation of WT roots), 180° is growth in the polar opposite horizontal direction, and 270° is upward growth. Data represent percent of total seedlings in each response range from a minimum of 63 seedlings from at least two replicate experiments.

(B) Root elongation response of wild-type Col-O (WT) and *aux1* missense alleles after exposure to IAA. Seedlings were grown on vertically oriented plates for 3 d in white light, then transferred to new vertically oriented plates containing the indicated concentration of IAA and the position of the root tip for each seedling was noted on the plates. Seedlings were allowed to grow for an additional 3 d, after which total root elongation was determined. Data represent the mean response (presented as percent of controls; no auxin treatment) from a minimum of 70 seedlings from at least two replicate experiments. Error bars represent SE.

occurs through the activation of a partially redundant ARF system (Liscum, 2002; Stowe-Evans et al., 2001). It seems likely that activation of a secondary ARF system could occur through one of three mechanisms: (1) increased expression of the secondary ARF, (2) post-translational modification of the secondary ARF to increase its sensitivity to auxin, or (3) alterations in the endogenous auxin level to that within the effective activity range of the secondary ARF.

The observed aphototropic phenotype of the *nph4arf19* double mutant grown on 1-aminocyclopropoane-1-carboxylic acid (ACC) (Figure 1B), together with the knowledge that *ARF19* is transcriptionally upregulated by ethylene treatment (Li et al., 2006), suggests that the ethylene-dependent recovery of phototropism in the *nph4* background (Figure 1) is dependent upon the ethylene-induced expression of *ARF19*. That ARF19 represents a secondary ARF involved in the regulation of phototropism may not be too surprising, since *ARF7* and *ARF19* represent phylogenetic sister loci (Liscum and Reed, 2002; Remington et al., 2004) previously shown to have overlapping function in root, shoot and leaf growth control (Okushima et al., 2005; Wilmoth et al., 2005). Retention of phyA-dependent recovery of BL-induced phototropism in the *nph4/arf7arf19* double mutant (Figure 1B), however, indicates that ARF19 is not sufficient for this recovery response, and implicates the role of another ARF.

Although it is currently unknown which ARF(s) might provide redundant function for NPH4/ARF7 under RL plus BL conditions, it is interesting to note that *nph4* seedlings recovered BL-induced phototropism in the absence of a RL co-irradiation when grown on the native auxin indole-3-acetic acid (IAA) (Figure 6). These results suggest that if activation of a second ARF system is essential for recovery of BL-induced phototropism in the *nph4* background (Harper et al., 2000; Stowe-Evans et al., 2001), then simply increasing the intracellular free auxin level beyond some critical threshold may be all that is necessary to stimulate the second system. In a finding consistent with this proposal, *nph4aux1-201* double mutants, which are presumed to be defective in auxin influx, failed to recover a phototropic response when grown on the same (Figure 6) or higher (data not shown) concentrations of IAA.

Previous studies have shown that multiple root defects normally observed in *aux1* mutants can be suppressed by growth on the synthetic auxin, 1-napthalene acetic acid (NAA) (Marchant et al., 1999, 2002; Rahman et al., 2002; Yamamoto and Yamamoto, 1998), which enter cells exclusively by facilitator-independent diffusion and thus bypasses the necessity for AUX1 function (Delbarre et al., 1996). Thus, it was surprising to find that while normal root gravitropism was recovered in *nph4aux1-201* double mutants grown on NAA (data not shown), BL-induced phototropism was not similarly recovered (Figure 6). However, it is interesting to note that NAA was much less effective than IAA in conditioning phototropic recovery in *nph4* single mutants (Figure 6). One possible explanation for this latter observation is that hypocotyl growth is simply more inhibited in the presence of NAA than

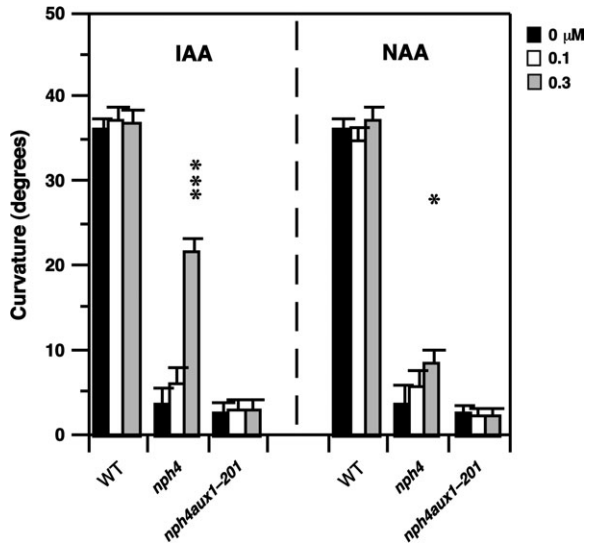

Figure 6. Hypocotyl Phototropism in Wild-Type, *nph4 and nph4aux1-201* Seedlings Grown on Auxin.

Seedlings were grown on medium containing the indicated concentration of auxin (IAA or NAA) for 3 d in darkness and then exposed to 4-h unidirectional BL (0.1 mmol m^{-2} s^{-1}) to induce phototropic curvature. Data represent the mean responses from a minimum of 40 seedlings from at least two replicate experiments. Error bars represent SE. Asterisks denote responses significantly different from that observed in *nph4* controls (no auxin exposure) by Student's *t*-test (*, $0.05 > P > 0.01$; **, $P < 0.001$).

IAA, thus abrogating much of the potential for a differential growth response to develop. Previous studies have shown that while little to no hypocotyl growth inhibition is observed in *nph4* seedlings at the concentrations of IAA tested here or higher, ~20% growth inhibition is observed on the same concentrations of NAA (Stowe-Evans et al., 1998). Such an impact on growth might explain not only the reduced recovery of *nph4* single mutants, but also the lack of recovery in the *nph4aux1-201* double mutant.

RL Does Not Affect Total Free Auxin Levels in Hypocotyls, But May Increase Symplastic/Apoplastic AUX1-Dependent Partitioning of Auxin

Results from two sets of experiments discussed here suggest that the phyA-dependent recovery of BL-induced hypocotyl phototropism in the *nph4* mutants requires an AUX1-mediated increase in intracellular auxin. First, this recovery response is abrogated when auxin influx is reduced, either through mutation of the AUX1 influx carrier (Figure 3B) or via pharmacological treatment with 1-NOA (Figure 4), which inhibits carrier-dependent influx (Imhoff et al., 2000; Yang et al., 2006). Second, *nph4* seedlings grown on IAA recover a phototropic response when exposed to unidirectional BL alone (Figure 6), thus bypassing the necessity for phyA activation. In contrast, *nph4aux1* double mutants fail to recover a response, even in the presence of IAA (Figure 6). While these results do not suggest a particular mechanism by which this phyA-dependent response could incorporate AUX1 function, one can imagine variations on two simple models: one involving direct influences of phyA signaling on AUX1 abundance and/or activity, and a second involving phyA-modulated changes in IAA metabolism such that free auxin content increases. In the latter model, AUX1 would not be directly influenced by phyA, but rather would be homoestatically necessary to cope with the increased levels of auxin. Experiments described below were aimed at testing components of each of these models.

In order to test whether phyA signaling influences auxin metabolism, total free IAA levels (apoplastic and symplastic together) were determined in hypocotyls of 3 d old etiolated seedlings by liquid chromatography-selected reaction monitoring-mass spectroscopy (GC-SRM-MS) (Edlund et al., 1995). As shown Figure 7A, hypocotyls from seedlings co-irradiated with BL and RL contain levels of IAA that were not significantly different from those exposed to unidirectional BL alone, suggesting that phyA signaling is not influencing gross auxin metabolism. The observation that wild-type and *phyA* hypocotyls contained similar levels of IAA (Figure 7A) provides conclusive evidence that steady-state levels of auxin are not affected by phyA signaling, at least not under the conditions tested here. Given these results, it was not surprising to find that the *aux1-201* mutation had no significant affect on total free auxin levels (Figure 7A).

While the experiments discussed above demonstrate that the total auxin pool is not influenced by phyA signals, they do not address whether *AUX1* expression and/or protein function might be stimulated under phyA activating conditions to increase symplastic levels of IAA. To address this latter question at the level of transcriptional control, we grew siblings from an $AUX1_{promoter}$::*uidA* (GUS) reporter transgenic line (Marchant et al., 1999) under various light conditions and then assayed their GUS activity both qualitatively (Figure 7B) and quantitatively (Figure 7C). Consistent with previous reports (Marchant et al., 1999; Swarup et al., 2001, 2004), we observed very strong GUS staining in the primary root tip (Figure 7B, left panel insets). Though hypocotyl-localized *AUX1* expression had not previously been reported, we also observed clear GUS activity in hypocotyls of etiolated seedlings (Figure 7B and 7C). Interestingly, seedlings grown under BL had reduced levels of GUS activity compared to dark-grown siblings, while seedlings grown in RL plus BL conditions identical to those used for phototropic assays exhibited higher levels of GUS staining than either mock-irradiated or BL treated siblings (Figure 7B and 7C). The observed reduction in GUS activity in hypocotyls of BL-treated seedlings was not surprising, since previous studies in *Arabidopsis* and *Brassica oleracea* had shown a repressive influence of BL on *AUX1* expression (Barrett et al., 2007; Esmon et al., 2006). In contrast, the finding that *AUX1* promoter activity was enhanced in RL plus BL conditions represents a novel finding, as *AUX1* expression under such conditions had not been previously reported. Relative to the development of phototropic curvatures, increased *AUX1* expression in RL plus BL

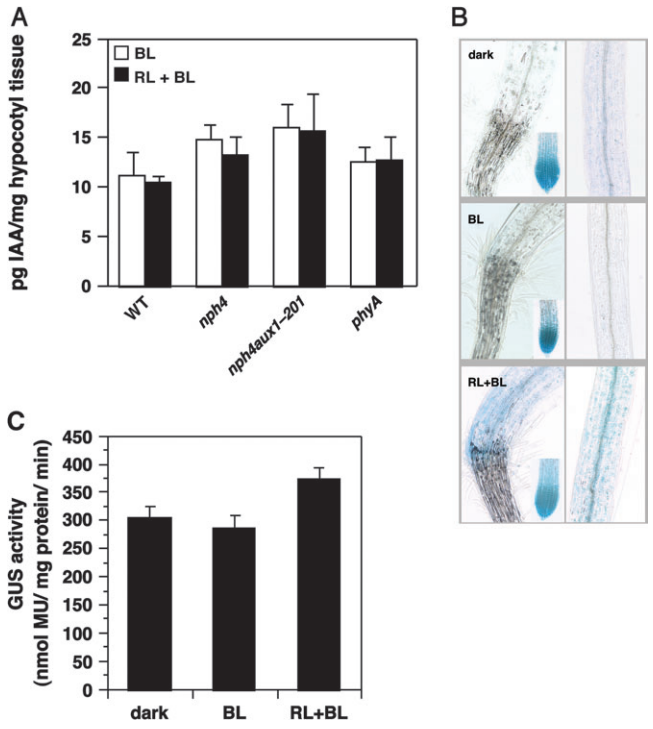

Figure 7. Total Auxin Content and *AUX1* Promoter Activity in Hypocotyls of Phototropically Stimulated Seedlings.

(A) Total free IAA contents in hypocotyls of wild-type Col-O (WT), *nph4*, *nph4aux1-201*, and *phyA* seedlings exposed to phototropic stimuli. IAA contents were determined by gas chromatography–selected reaction monitoring–mass spectroscopy (GC–SRM–MS) as described in the Methods. Data represent the mean IAA content from three independent biological samples. Error bars represent standard deviation.

(B) β-glucuronidase (GUS) activity staining in etiolated $AUX1_{Promoter}$*::uidA* seedlings exposed to different light conditions. Seedlings were grown in darkness for 3 d, then placed in unidirectional BL (0.1 mmol m^{-2} s^{-1}) (middle panel; BL), unidirectional BL supplemented with RL (1.6 mmol m^{-2} s^{-1}) from above (bottom panel; BL+RL), or kept in darkness (top panels, dark) for an additional 4 h, followed by staining for GUS activity (see Methods). Left portion of figure shows GUS staining in the hypocotyl:root junction, while insert shows staining in the root tip (positive control); right portion of figure shows staining in the elongation zone of the hypocotyl.

(C) β-glucuronidase (GUS) activity in hypocotyl extracts from etiolated $AUX1_{Promoter}$*::uidA* seedlings exposed to different light conditions. Seedlings were grown in darkness for 3 d, then placed in unidirectional BL (0.1 mmol m^{-2} s^{-1}; BL), unidirectional BL supplemented with RL (1.6 mmol m^{-2} s^{-1}; BL+RL), or kept in darkness (dark) for an additional 4 h, followed by tissue collection and fluorimetric GUS activity assay (see Methods). Data represent the mean response of two biological replicates assayed over four incubation times. Error bars represent SE.

conditions would be expected to result in a larger pool of active AUX1 protein, which would, in turn, facilitate greater auxin influx and elevated intracellular auxin levels. It remains to be determined whether higher levels of AUX1 protein are in fact produced in RL plus BL conditions, and if AUX1 transport activity is also altered in response to RL.

DISCUSSION

A number of previous studies have demonstrated that BL-induced hypocotyl phototropism in *Arabidopsis* is dependent upon the presence of the auxin-regulated transcriptional activator NPH4/ARF7 (Harper et al., 2000; Liscum and Briggs, 1996; Stowe-Evans et al., 1998; Watahiki and Yamamoto, 1997; Watahiki et al., 1999). The logical implication of this finding is that new auxin-induced gene expression is a prerequisite for development of phototropic curvatures (Harper et al., 2000). Identification of a number of genes in *B. oleracea* that are expressed in both an auxin-dependent and tropic stimulus-induced fashion, and exhibit NPH4/ARF7-dependence for auxin inducibility, provide tantalizing support for this idea (Esmon et al., 2006). It therefore seems appropriate to hypothesize that the observed recovery of BL-induced phototropism in *nph4*-null mutants co-irradiated with RL (Liscum and Briggs, 1996; Stowe-Evans et al., 2001) or pretreated with ethylene (Harper et al., 2000) is dependent upon the action of a second conditionally activated ARF system (Liscum, 2002). The initial objective of the present study was to utilize the conditional aphototropic nature of the *nph4* mutants in a genetic enhancer screen to identify loci encoding proteins potentially associated with the activation of this hypothesized redundant ARF system.

Of the three mutant loci identified in our second-site enhancer screen, two—*map1* and *map2*—were characterized at the molecular level. While *map2* was found to contain a missense mutation in *NPH3*—a gene encoding a phot1-interacting BTB domain-containing protein previously shown to be necessary for phototropic signaling (Liscum and Briggs, 1995, 1996; Motchoulski and Liscum, 1999; Pedmale and Liscum, 2007)—*map1* was found to represent a new allele of *AUX1*—a gene encoding a high-affinity auxin influx carrier (Bennett et al., 1996; Marchant et al., 1999; Yang et al., 2006) involved in root gravitropism (Maher and Martindale, 1980; Mizra et al., 1984) but not previously associated with hypocotyl phototropism. Although *map3* has not been characterized in detail here, it is interesting to note that in contrast to the *map1nph4* and *map2nph4* seedlings, *map3nph4* seedlings recovered a phototropic response in ethylene plus BL conditions (Figure 1B). Thus, it appears that *map3* represents a lesion in a component specific to the phyA-dependent RL-induced recovery pathway. A molecular test of this conclusion awaits cloning of the *MAP3* locus.

Hypocotyl Phototropism Is Modulated by AUX1

The identification of an *aux1* allele in our enhancer screen was initially surprising, since the morphological phenotypes previously reported for *aux1* mutants were generally root-specific (Bennett et al., 1996; Estelle and Somerville, 1987; Marchant et al., 1999; Maher and Martindale, 1980; Mizra et al., 1984; Pickett et al., 1990). Yet, results from three separate experiments indicate that loss of phototropic recovery in the *nph4map1/aux1-201* mutant did in fact occur because of a

defect in AUX1 function, rather than from a dysfunctional protein product of a second linked gene. First, alterations in the phototropic response of *nph4* could also be conditioned by several independent *aux1* alleles (Figure 3B). Second, *nph4* seedlings treated with 1-NOA—an apparent inhibitor of AUX1-facilitated auxin influx (Imhoff et al., 2000; Yang et al., 2006)—were phenotypically indistinguishable from *nph4map1/aux1-201* seedlings (Figure 4). Third, subtle but statistically significant reductions in BL-induced phototropism were observed in several *aux1* alleles in the presence (Figure 3A) of NPH4/ARF7 activity.

Although these findings clearly demonstrate that AUX1 is an important modulator of hypocotyl phototropism, they raise an important question given results from previous studies of *aux1* mutants: Do the alterations in hypocotyl phototropism observed in *aux1* and *nph4aux1* seedlings result from hypocotyl-autonomous changes in AUX1 activity or from root-localized AUX1 dysfunction that subsequently influences the hypocotyl? Organ autonomy could be discounted off hand if *AUX1* were simply not expressed in hypocotyl or other aerial tissues. However, it was recently reported that *AUX1* is in fact expressed in young leaf and shoot meristematic tissues of light-grown *Arabidopsis* and likely facilitates IAA loading into leaf vasculature, where it is transported to the root to promote lateral root formation (Marchant et al., 2002). In addition, we have observed GUS activity in etiolated hypocotyls of $AUX1_{promoter}$::*uidA* transgenic lines, and that this activity is enhanced in RL plus BL conditions (Figure 7B and 7C), under which the *aux1*-dependent phototropic phenotypes are most pronounced (Figure 3B). Together, these results suggest that the phototropic response examined here is influenced by hypocotyl-specific function of AUX1—a function that, until this study, had been unknown.

Allele-Specific Hypocotyl and Root Phenotypes of *aux1* Mutants Suggest Genotype–Environment–Organ/Tissue Regulation of AUX1 Function

It is now clear that AUX1 function is important in a variety of plant organs—not only the root where *aux1* mutant phenotypes were first described (Maher and Martindale, 1980), but also at sites of phloem loading in the shoot apex and young leaves (Marchant et al., 2002), and, as shown here, within the hypocotyl of an etiolated seedling (Figure 3). However, results from the present study further suggest that AUX1 function is not identical in each of these disparate sites. This is not to suggest that AUX1 has a function aside from that of an auxin influx carrier (Yang et al., 2006), but rather that the strength of carrier activity can be enhanced or repressed by tissue/organ-, growth condition- and genotype-dependent factors.

Several pieces of experimental evidence provide support for the aforementioned conclusion. First, as should be clear from the phenotypic summary shown in Table 1, no single *aux1* mutant phenotype can be easily used as a diagnostic tool to predict the strength of various missense alleles. For example, while the *aux1-7* and *aux1-105* alleles consistently exhibited strong loss-of-function phenotypes, other alleles varied in phenotypic strength from response to response. The *aux1-112* allele, despite conditioning relatively strong loss-of-function root phenotypes consistent with the reported absence of detectable aux1-112 protein in root microsomes (Swarup et al., 2004), resulted in a hypocotyl phototropic response that was little different from wild-type (Figure 3), suggesting that functional aux1-112 protein is present in hypocotyl tissues. In contrast, *aux1-201/map1* was the weakest allele of those examined with respect to alterations in auxin sensitivity of the root (Figure 5B), but one of the strongest alleles with respect to altered hypocotyl phototropism (Figure 3).

Table 1. Phenotypic Strengths of the Various *aux1* Alleles for Different Responses[a]

Allele	Hypocotyl phototropism	Root gravitropism	Auxin sensitivity[b]
aux1-201	+++	++	+
aux1-112	+	++	++
aux1-113	Wt[c]	+	++
aux1-105	+++	+++	+++
aux1-7	+++	+++	+++

a Plus signs refer to strength of loss-of-function phenotype, with + being a weak phenotype, ++ being moderate, and +++ being strong.
b Refers to sensitivity of the root to exogenous IAA or 2,4-D.
c Not different from wild-type as a single mutant or *nph4-1* in the case of *nph4aux1-113* double mutants.

AUX1 Likely Influences ARF-Dependent Hypocotyl Phototropism by Increasing the Symplastic-to-Apoplastic Partitioning of Free Auxin

The classic Cholodny–Went hypothesis represents the most basic description of how auxin is likely linked to the regulation of shoot and root tropisms. In brief, this hypothesis holds that tropic stimuli induce the lateral redistribution of auxin, resulting in unequal accumulation of the hormone between opposing flanks of a responding organ such that differential growth is promoted (Went and Thimann, 1937). The applicability of this model is dependent upon the regulated movement of auxin between cells. Although the size of IAA (MW = 175.19)—the most abundant native auxin—is not prohibitive to its passive diffusion between cells, free IAA is a weak acid (pK = 4.8) whose pH-dependent ionic state determines diffusibility; the undissociated protonated form of IAA (IAAH) is lipophilic and fully membrane permeable, whereas the anionic form (IAA^-) is membrane impermeable (Kerr and Bennett, 2007; Kramer and Bennett, 2006). These properties of IAA led to the development of a simple chemiosmotic hypothesis to explain auxin mobility within a plant (Raven, 1975; Rubery and Sheldrake, 1974). In particular, under acidic conditions, such as occur within the apoplast (pH $\sim$ 5.5), most free IAA will exist as IAAH and will thus be able to diffuse into

adjacent cells along favorable concentration gradients, while, under neutral or slightly basic conditions (e.g. within the cytoplasm), a majority of the free IAA will exist as IAA^- and will thus require carrier-facilitated transport to be effluxed from cells, even along a favorable concentration gradient. Recent studies have demonstrated that members of the PIN and MDR/PGP protein families function as auxin efflux carriers central to this hypothesis (Geisler et al., 2005; Petrasek et al., 2006; Terasaka et al., 2005).

It has been argued that PIN and MDR/PGP-mediated efflux of IAA, together with passive diffusional influx, is sufficient to account for the intercellular directional flow of auxin required for normal growth and development of a plant (Blakeslee et al., 2005). However, knowledge that AUX1 functions as a high-affinity auxin influx carrier (Yang et al., 2006), together with the agravitropic root phenotypes of *aux1* loss-of-function mutants (Bennett et al., 1996; Marchant et al., 1999; Swarup et al., 2004, 2005), indicate that this contention is not entirely valid. In fact, Bennett and colleagues have proposed that carrier-based auxin influx, rather than passive diffusion, represents the principal means by which auxin moves into cells and that it is the combined action of AUX1/LAX, PIN, and MDR/PGP proteins that mediates auxin-dependent responses such as root gravitropism and lateral root formation (Kerr and Bennett, 2007; Kramer and Bennett, 2006).

Results presented here show that, in addition to its previously described root- and meristem/leaf-specific functions (Bennett et al., 1996; Marchant et al., 1999, 2002; Pickett et al., 1990; Rahman et al., 2002; Swarup et al., 2001, 2005), AUX1 is an important modulator of hypocotyl phototropism (Figure 3). However, whether AUX1 provides the principle or a supplemental means of auxin influx necessary for phototropism appears to depend upon the basal auxin responsiveness of the hypocotyl (Figure 3), which is determined in large part by NPH4/ARF7 (Harper et al., 2000; Okushima et al., 2005; Stowe-Evans et al., 2001; Tatematsu et al., 2004). For example, AUX1 function appears mostly dispensable with respect to hypocotyl phototropism when NPH4/ARF7 function is normal, since seedlings homozygous for apparent physiological null *aux1* mutations, *aux1-7* or *aux1-105* (Swarup et al., 2004), retain ~75% of their phototropic responsiveness (Figure 3A). In contrast, AUX1 function is critical for hypocotyl phototropism when auxin responsiveness is compromised, as seedlings homozygous for either of the aforementioned *aux1* alleles, as well as the *nph4-1*-null allele, are only 25–35% as responsive as *nph4-1* single mutants (Figure 3B).

Because AUX1 functions as an auxin influx carrier (Yang et al., 2006) and total free auxin levels are not altered in phototropically stimulated (with or without RL pretreatment) seedlings (Figure 7A), we hypothesize that AUX1 acts as a modulator of hypocotyl phototropism by increasing the symplastic-to-apoplastic partitioning of free auxin. As just discussed, it appears that the impact of AUX1-dependent auxin influx can be supplemental or principle with respect to hypocotyl phototropism, depending upon whether NPH4/ARF7 is functional or not, respectively. This suggests that in wild-type seedlings exposed to unidirectional BL alone, cells in the shaded flank of the hypocotyl accumulate intracellular auxin at levels that are supra-optimal with respect to the development of ARF-dependent phototropism. Such intracellular concentrations of auxin would, of course, be determined by the combined influx (both passive and AUX1-dependent) and efflux (PIN and MDR/PGP-dependent) of IAA (Blakeslee et al., 2005; Kerr and Bennett, 2007; Kramer and Bennett, 2006). The aphototropic phenotype of *nph4-1* single mutant seedlings exposed to unidirectional BL alone (Figure 1A; Liscum and Briggs, 1996; Stowe-Evans et al., 1998), however, implies that the aforementioned intracellular auxin concentrations are in fact only supra-optimal for the activation of NPH4/ARF7-dependent processes, but are below the threshold necessary to activate any partially redundant secondary ARF system. Recovery of BL-induced phototropism in *nph4* seedlings pretreated with ethylene or co-irradiated with RL suggest that a second ARF is, however, activated under these latter conditions (Harper et al., 2000; Liscum, 2002; Liscum and Stowe-Evans, 2000; Stowe-Evans et al., 2001).

The finding that *nph4arf19* double mutants fail to recover BL-induced phototropism under ethylene pretreatment conditions (Figure 1B) supports the conclusion that activation of a second ARF is required for the recovery response. While ethylene-induced expression of *ARF19* (Li et al., 2006) would seem to represent a straightforward explanation for why ethylene pretreatment is able to suppress the aphototropic phenotype of *nph4*-null mutant seedlings, the failure of *nph4-1aux1-201/map1* seedlings to respond phototropically like *nph4-1* single mutants (Figure 1B) indicates that such a conclusion is incomplete. Rather, it would appear that while the abundance of ARF19 should increase in response to ethylene pretreatment, its mere presence is not enough to ensure recovery of auxin-dependent responses normally regulated by NPH4/ARF7 that are necessary for development of phototropic curvature unless intracellular auxin levels are sufficiently high. AUX1 appears to be the key determinant in this latter regard, such that in the absence of normal AUX1 function, diffusional influx of auxin is apparently not strong enough to counter efflux carrier action to achieve intracellular auxin concentrations sufficient to activate ARF19. In contrast, when AUX1 is functional, the ratio of total auxin influx (passive and AUX1-mediated) to efflux is apparently high enough to achieve such threshold levels of intracellular auxin. Although the secondary ARF mediating recovery of phototropism in *nph4* mutants under RL pretreatment conditions has yet to be identified, we predict a similar role for AUX1 in regulating intracellular auxin levels and thus responsiveness of the secondary ARF, since this response is also impaired in *nph4* seedlings when AUX1 function is disrupted either genetically (Figure 3B) or pharmacologically (Figure 4). We thus conclude that, at least in the case of hypocotyl phototropism, the level of dependence on AUX1-mediated auxin influx for response is directly related to the auxin sensitivity of the dominant ARF system functioning at

the time of assay; when NPH4/ARF7 is functional, AUX1 function is largely dispensable, while when another ARF(s) is operating in lieu of NPH4/ARF7, AUX1 function becomes extremely important.

METHODS

Plant Material and Growth Conditions

With the exception of the *map* mutants, the *Arabidopsis* mutant and transgenic lines used here have been described previously: *nph4-1* (Liscum and Briggs, 1996); *nph4-1arf19-1* (Okushima et al., 2005); *aux1-7* (Maher and Martindale, 1980; Pickett et al., 1990); *aux1-105*, *aux1-112*, and *aux1-113* (Swarup et al., 2004); and $AUX1_{Promoter}$::*uidA* promoter fusion transgenic (Marchant et al., 1999); *phyA–211* (Nagatani et al., 1993). The *nph4-1*, *nph4arf19*, *aux1-7*, *map1*, *map2*, *map3*, and *phyA* mutations are carried in the Columbia (Col-O) accession, and the *aux1-105*, *aux1-112*, and *aux1-113* mutations are in Wassilewskija (Ws) accession. For all experiments, seeds were surface sterilized with 30% bleach, rinsed five times with sterile water, and planted on half-strength Murashige and Skoog (MS) medium with 1% agar without any supplements, unless otherwise noted. To assure uniform germination, seeds were cold and red-light treated as described previously (Liscum and Hangarter, 1993). All plant growth was done at 22°C.

The *nph4aux1* double mutants were generated by crossing *nph4* and various *aux1* alleles, allowing the F_1 population to self-pollinate, and then selecting seedlings exhibiting auxin-resistant root elongation. In particular, F_2 seedlings were grown on full-strength Murashige–Skoog (MS; Murashige and Skoog, 1962) media supplemented with 2% sucrose, 1% agar, and 0.1 mM 2,4-Dichloro-phenoxyacetic acid (2,4-D). This concentration of 2,4-D dramatically reduced primary root elongation in wild-type seedlings, but allowed easily observable root elongation in various *aux1* mutants (data not shown). F_2 seedlings with elongated roots were transferred to soil, grown under greenhouse conditions for tissue collection (on liquid N_2) and F_3 seed production. Genomic DNA extracted (Edwards et al., 1991) from frozen rosette tissue was used to determine the *NPH4* genotype using multiplex PCR methods described previously (Wilmoth et al., 2005). Homozygous *nph4-1* lines were further analyzed using either cleaved amplified polymorphic sequence (CAPS; Konieczny and Ausubel, 1993) or derived CAPS (dCAPS; Michaels and Amasino, 1998; Neff et al., 1998) to determine if the given *aux1* mutation was also homozygous, as would be expected from the auxin-hyposensitive root phenotype of the F_2 plant. Primer and restriction enzyme information is available at http://www.biosci.missouri.edu/liscum/newmarkers.html.

To isolate the *aux1-201* allele away from the *nph4-1* background in which it was identified, *nph4aux1-201/map1* was crossed to Col-O wild-type, the F_1 progeny allowed to self-pollinate, and then F_2 seedlings were grown on MS medium supplemented with 2,4-D as described above. Seedlings exhibiting auxin-hyposensitive root elongation were then grown in soil, genomic DNA isolated, and analyzed for the presence of the *nph4-1* lesion using the multiplex PCR described above. While this PCR can identify homozygous *nph4-1* plants, it cannot distinguish between heterozygous and wild-type plants (Wilmoth et al., 2005; R. Harper and E. Liscum, unpublished). Therefore, 3 d old etiolated F_3 progeny from plants exhibiting auxin-hyposensitive root elongation and scoring positive for a wild-type allele of NPH4/ARF7 were subsequently grown on half-strength MS medium (1% agar, lacking sucrose and hormone) and scored for hypocotyl gravitropism. As both *NPH4/nph4-1* heterozygotes and *nph4-1* homozygotes exhibit defects in NPH4/ARF7-dependent hypocotyl growth responses, including gravitropism (Li et al., 2006; Stowe-Evans et al., 1998), normal hypocotyl gravitropism can only occur in seedlings wild-type for *NPH4/ARF7*. Finally, the homozygosity of the *aux1-201* allele in such seedlings was confirmed by CAPS marker analysis (see http://www.biosci.missouri.edu/liscum/newmarkers.html).

Phototropic and Gravitropic Assays

For phototropic assays, 64 h old (post-germination induction) etiolated seedlings were unidirectionally irradiated for 4 h with either UV-A light (0.2 mmol m^{-2} s^{-1}) for the isolation of *map* mutants, or BL (0.1 mmol m^{-2} s^{-1}) for the characterization of mutants. For RL recovery/enhancement studies, seedlings irradiated with unidirectional BL were concurrently exposed to RL (1.6 mmol m^{-2} s^{-1}) from above. The light sources and determination of phototropic curvatures of hypocotyls were determined as described previously (Liscum and Briggs, 1995; Stowe–Evans et al., 1998). When the effects of various compounds on phototropic curvatures were to be determined, the compound was added to the medium prior to plating of seed such that seedlings were exposed to the compound during their entire growth period.

For gravitropic assays, plates were oriented vertically after induction of germination and seedlings allowed to grow along the surface of the agar for 3 d in darkness. Plates were then rotated 90° and returned to darkness for an additional 24 h, after which gravitropic curvatures of the roots were measured (Liscum and Briggs, 1995).

Assay for Auxin Sensitivity of Root Elongation

Seedlings were grown on vertical plates containing full-strength MS medium with 1% sucrose and 1% agar, in continuous white light (Stowe-Evans et al., 1998) for 3 d, then transferred to new MS plates supplemented with the indicated concentrations of IAA. After transfer, the position of the primary root tips were marked the plates incubated in constant WL in a vertical position for an additional 3 d, after which the final position of the root tip was marked. The total root elongation was then measured as the distance between the two marks and expressed as a percent elongation of an untreated control.

GC–SRM–MS Measurements of Free IAA

For each sample, 20–30 hypocotyls (10–20 mg fresh weight) were pooled. The samples were extracted and purified, and free IAA was then analyzed by gas chromatography–selected reaction monitoring–mass spectroscopy (GC–SRM–MS) as previously described (Edlund et al., 1995). Calculation of isotopic dilution was based on the addition of 500 pg [$^{13}C_6$]IAA/sample. Four biological replicates were analyzed for each genotype and treatment.

β-Glucuronidase Activity Assays

For whole seedling GUS staining, 3 d old etiolated *AUX1$_{Promoter}$::uidA* seedlings exposed to BL, BL plus RL, or mock-irradiated were stained by incubation overnight in buffer (pH 7.0) containing 10 mM Na_2EDTA, 100 mM $NaH_2PO_4{\bullet}H_2O$, 0.5 mM $K_4FE(CN)_6{\bullet}3H_2O$, 0.1% Triton X-100, and 0.5 mg/ml X-Gluc at 37°C (Marchant et al., 1999). Following staining, GUS reaction products were observed by light microscopy.

For quantitative measures of GUS activity, 3 d old etiolated seedlings were grown on half-strength MS media and subjected to the same light treatments used in Figure 7B. Following light treatment, seedlings were removed from the plate and hypocotyls were collected and stored at –80°C in GUS extraction buffer (50 mM $NaHPO_4$, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% Sarkosyl and 0.1% Triton X-100) until assayed by flourimetry (Jefferson et al., 1987; Ulmasov et al., 1997). Samples from each condition were incubated with 1 mM 4-methylumbelliferyl-β-D-glucuronide (Research Organics, Cleveland, OH) at 37°C before the reaction was stopped with 1 M Na_2CO_3. Measurements were made using a plate reader (Synergy™ HT Multi-Detection Microplate Reader, BioTek, Winooski, VT) with enzyme activities calculated based on florescence of known concentrations of 4-methylumbelliferone (Sigma, St Louis, MO).

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