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NPH4, a Conditional Modulator of Auxin-Dependent Differential Growth Responses in Arabidopsis[1]

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Although sessile in nature, plants are able to use a number of mechanisms to modify their morphology in response to changing environmental conditions. Differential growth is one such mechanism. Despite its importance in plant development, little is known about the molecular events regulating the establishment of differential growth. Here we report analyses of the *nph4* (nonphototropic hypocotyl) mutants of Arabidopsis that suggest that the NPH4 protein plays a central role in the modulation of auxin-dependent differential growth. Results from physiological studies demonstrate that NPH4 activity is conditionally required for a number of differential growth responses, including phototropism, gravitropism, phytochrome-dependent hypocotyl curvature, apical hook maintenance, and abaxial/adaxial leaf-blade expansion. The *nph4* mutants exhibited auxin resistance and severely impaired auxin-dependent gene expression, indicating that the defects associated with differential growth likely arise because of altered auxin responsiveness. Moreover, the auxin signaling events mediating phototropism are genetically correlated with the abundance of the NPH4 protein.

Most, if not all, plant growth can be considered differential in the sense that not all cells within a given organ are elongating equally at any given time. However, "differential growth responses" have been classically defined by the bending or movement of an organ resulting from unequal cellular growth in one position of the organ relative to an opposing position. As such, the generation of differential growth represents one adaptive mechanism by which plants are able to modify their morphology rapidly in response to changing environmental conditions. Examples of such responses include tropisms, modification of apical hook structures, and nastic movements of leaves (for reviews, see Darwin and Darwin, 1896; Firn and Digby, 1980; Palmer, 1985). Results from physiological studies conducted during the past 60 years have shown that auxins likely play an important role(s) in the establishment of differential growth (for reviews, see Trewavas, 1992; Hobbie and Estelle, 1994; Kaufman et al., 1995). Perhaps the most well-known interpretation of such data is found in the Cholodny-Went theory (Went and Thimann, 1937). This theory holds that tropic curvatures develop in response to an unequal distribution of auxin in the two sides of a curving organ, which arises as a result of lateral auxin transport. Despite considerable effort aimed at testing this theory (see Trewavas, 1992), very little is known about the coordinated regulation of differential growth at the molecular level by auxin or any other growth-promoting/-inhibiting substances.

In recent years the study of mutants has played an increasingly important role in the analysis of differential growth (for reviews, see Hobbie and Estelle, 1994; Estelle, 1996; Leyser, 1998). The *aux1*, *axr3*, and *hls1* mutants of Arabidopsis are especially notable. These mutants exhibit altered root gravitropic and thigmotropic responses (Maher and Martindale, 1980; Okada and Shimura, 1990; Pickett et al., 1990; Timpte et al., 1995), altered root and hypocotyl gravitropic responses (Leyser et al., 1996), and altered apical hook formation/maintenance (Guzman and Ecker, 1990; Hou et al., 1993; Lehman et al., 1996), respectively. The corresponding wild-type genes have been cloned for these mutants, and each of the encoded proteins has been hypothesized to regulate auxin-dependent processes. Specifically, the AUX1/amino acid permease-like protein may function in the basipetal transport of IAA (Bennett et al., 1996; Yamamoto and Yamamoto, 1998), the AXR3/IAA17 protein may act as an auxin-responsive transcriptional regulator (Rouse et al., 1998), and the putative HLS1/*N*-acetyltransferase may modify the transport or chemical structure of IAA in planta (Lehman et al., 1996).

Despite the intriguing nature of these gene products and their possible roles, phenotypic analyses of mutants indicate that the proteins encoded by these loci function in only one or a limited number of differential growth responses. Furthermore, the *hls1* and *axr3* mutants exhibit several additional defects not directly related to differential growth, including decreased hypocotyl and primary inflorescence length and changes in apical dominance (Lehman et al., 1996; Leyser et al., 1996). Hence, none of these proteins appears to have functions that are common to the suite of differential growth responses a plant possesses.

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Abbreviations: BL, blue light; EMS, ethyl methanesulfonate; RL, red light; WL, white light; YL, yellow light.

Another class of Arabidopsis mutants in which differential growth can be studied are the *nph* (nonphototropic hypocotyl) mutants (Liscum and Briggs, 1995). Of particular interest are the *nph4* mutants, which have been shown to exhibit not only disrupted hypocotyl and root phototropism, but also impaired hypocotyl gravitropism (Liscum and Briggs, 1996). It has been hypothesized that the NPH4 protein might act close to, or directly on, the differential growth response, giving rise to tropic curvatures (Liscum and Briggs, 1996). In this paper we present results from a number of physiological analyses of the *nph4* mutants that implicate NPH4 as a specific regulator of multiple auxin-dependent differential growth responses. Genetic and molecular studies further indicate that NPH4 represents a temporally early-acting, concentration-dependent modulator of an auxin-response pathway(s) leading to differential growth.

MATERIALS AND METHODS

All mutants used in these studies were of the Columbia ecotype of Arabidopsis and have been described elsewhere: *nph1-4* (Liscum and Briggs, 1995); *nph1-5* (Huala et al., 1997); *nph4-1*, *nph4-2*, and *nph4-3* (Liscum and Briggs, 1996); *tir5-1* (*nph4-4*) (Ruegger et al., 1997); *msg1-2* (*nph4-102*) (Watahiki and Yamamoto, 1997); *etr1-1* (Bleecker et al., 1988); *hy4-101* (Liscum and Hangarter, 1991); and *phyB-9* (Reed et al., 1993).

Growth Conditions

For seedling experiments, seeds were surface-sterilized and plated on nutrient medium solidified with 1.0% (w/v) agar, as described previously (Liscum and Briggs, 1995). One-half-strength Murashige and Skoog nutrient medium (Murashige and Skoog, 1962) without Suc was used for all but the auxin-sensitivity experiments. In these latter experiments, full-strength Murashige and Skoog medium supplemented with 2.0% (w/v) Suc was used. Cold treatment and RL exposure to induce uniform germination were as described previously by Liscum and Briggs (1995).

After induction of germination plates were handled in several different ways, depending on the experiment. For phototropic assays, plates were placed in darkness for 71.5 h and then transferred to unilateral BL (0.1 μmol m^{-2} s^{-1}) for 8 h. For assays of dark growth and apical hook structure, plates were placed in a vertical position in darkness for the indicated times. Vertical plate orientation caused seedlings to grow along the surface of the agar medium, thus allowing seedling images to be traced on the back side of the plates. When seedlings were to be exposed to ethylene, plates were placed in a desiccator to which 1 mL of pure ethylene was added each day after purging with ambient air (daily ethylene exposure was approximately 50 μL/L). For de-etiolation experiments, plates were placed in darkness for 23.5 h and then transferred to BL or RL for 96 h at the indicated fluence rates. For assays of RL-induced hypocotyl curvature, plates were placed in darkness for 60 h and then transferred to RL for 20 h at the indicated fluence rates. For auxin-sensitivity experiments, plates lacking auxin were incubated in darkness for 23.5 h and then transferred to YL (30 $\pm$ 5 μmol m^{-2} s^{-1}). After 48 h seedlings were transferred to vertically oriented plates containing auxin (IAA, 2,4-D, or NAA) at the indicated concentrations. After marking the positions of hypocotyl and root termini, plates were returned to YL for 72 h. For assays of gene expression, plates were placed in darkness for 23.5 h and then transferred to WL (45 $\pm$ 4 μmol m^{-2} s^{-1}) for 7 d before exposure to auxin.

For mature plant experiments, seeds were sown directly on Pro-Mix (Premier Horticulture, Red Hill, PA) saturated with 0.3% (w/v) Peter's nutrient solution (Scotts-Sierra Horticultural Products, Marysville, OH) and grown under constant WL (100–150 μmol m^{-2} s^{-1}). Plants were watered twice weekly with distilled water and once every other week with nutrient solution.

Light Sources

For the induction of germination and phytochrome-dependent hypocotyl growth inhibition experiments, RL was obtained by filtering light from gold fluorescent bulbs (F40/GO, Sylvania) through one layer of red acrylic (Rohm and Haas no. 2423, 3.18 mm thick; Cope Plastics, St. Louis, MO). For phototropism experiments, BL was obtained as described previously (Liscum and Briggs, 1995), and for cryptochrome-dependent hypocotyl-growth-inhibition experiments, BL was obtained by filtering light from blue fluorescent bulbs (F40B, Sylvania) through one layer of blue acrylic (Rohm and Haas no. 2424, 3.18 mm thick; Cope Plastics). For auxin-sensitivity experiments, YL was obtained by filtering light from cool-white fluorescent bulbs (F40CW.RS.WM, Sylvania) through one layer of yellow acrylic (Rohm and Haas no. 2208, 3.18 mm thick; Cope Plastics). For the growth of seedlings for RNA experiments, WL was obtained from unfiltered, cool-white fluorescent bulbs, and for the growth of adult plants, WL was obtained from Trimline T8 fluorescent bulbs (F32T8SP41, General Electric).

Genetic Analysis

Heterozygous (*nph4-1/NPH4-1*) F_1 plants were generated by pollinating wild-type plants with pollen from homozygous *nph4-1* plants. Complementation tests were performed using F_1 seedlings from crosses between homozygous mutants. The genetic mapping stock consisted of *nph4-1* individuals (aphototropic/agravitropic seedlings) selected from an F_2 population arising from self-pollination of F_1 plants derived from a cross of a *nph4-1/nph4-1* plant of the Columbia ecotype to a wild-type Landsberg *erecta* plant. The *nph4* genotype of F_2 mapping individuals was verified in the F_3 generation. PCR primers for simple-sequence-length polymorphism marker-based mapping (Bell and Ecker, 1994) were obtained from Research Genetics (Huntsville, AL). Linkage to the flanking markers nga106 and nga139 was determined by examination of 250 and 290 chromosomes, respectively. Map positions are relative to the latest recombinant-inbred map (http://genome-

www.stanford.edu/Arabidopsis/ww/Feb98Rimaps/html/chrom5.html).

Growth Measurements

Hypocotyl curvature responses (phototropism and RL-induced curvatures) were determined as described previously for phototropic and gravitropic responses of etiolated Arabidopsis seedlings (Liscum and Briggs, 1995). For the analysis of etiolated hypocotyl growth, seedling images traced on the back of growth plates (see above) were measured with a ruler to the nearest millimeter. Apical hook angles were measured as described by Liscum and Hangarter (1993a). Light-dependent hypocotyl growth inhibition was determined as described by Young et al. (1992). Growth of hypocotyls and roots after exposure to exogenous hormones was determined essentially as described by Lincoln et al. (1990).

Microscopy

Three-day-old etiolated seedlings were fixed and embedded in butyl-methyl-methacrylate, and ultramicrotome sections were made as described by Baskin and Wilson (1997). Sections were then stained using a modified periodic acid-Schiff's reagent method. Sections were first placed in acetone for 10 min to extract the embedded material, and then transferred to 1.0% (v/v) periodic acid for 15 min. After a 9-min wash in tap water, sections were placed in Schiff's reagent (Sigma) for 30 min, followed by a 9-min wash in tap water. Sections were finally washed for 1 min in distilled water.

Northern-Blot Analysis

Plates containing 7-d-old WL-grown seedlings were flooded with 10 mL of 100 μM IAA or solvent (0.04% [v/v] ethanol) and returned to WL for an additional 1 h. Seedlings were then immediately frozen in liquid nitrogen, and total RNA was extracted as described by Ausubel et al. (1995). RNA samples (20 μg) were separated on a 1.0% (w/v) agarose formaldehyde/Mops gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell), according to the method of Ausubel et al. (1995). Prehybridization, hybridization, and washing of blots were performed as described by Ausubel et al. (1995). Probes were labeled with ^{32}P by random priming (Prime-a-Gene Labeling System, Promega) and then purified from unincorporated label by chromatography (NucTrap columns, Stratagene). Hybridized membranes were exposed to Kodak X-Omat x-ray film.

RESULTS

Genetic Analysis of the *nph4* Locus

It was shown previously that *nph4* alleles do not segregate as simple recessive Mendelian traits in F_2 populations (Liscum and Briggs, 1995). Here we demonstrate that etiolated *NPH4/nph4* heterozygotes (F_1 plants) exhibit phototropic curvatures that are intermediate between, and significantly different from, those of either parental homozygote (Table I). These results indicate that *nph4* alleles are semidominant with respect to phototropism.

The *nph4* locus was mapped to the proximal arm of chromosome 5 between simple-sequence-length polymorphism markers (Bell and Ecker, 1994) nga106 and nga139 at approximately position 44 centimorgans (data not shown). A similar map position has been reported for two recently identified Arabidopsis mutants, *msg1* (Watahiki and Yamamoto, 1997) and *tir5* (Ruegger et al., 1997). The *msg1* mutants were identified in a screen for mutants that failed to exhibit hypocotyl curvature in response to unilaterally applied auxin, whereas the *tir5* mutants were identified by their resistance to auxin-transport inhibitors. Neither the *msg1-2* nor the *tir5-1* mutant was capable of complementing the *nph4* mutants in the F_1 generation, and no wild-type seedlings have segregated in the F_2 progeny from such F_1 plants (data not shown). Thus, the *msg1* and *tir5* mutants represent independently identified alleles of the *nph4* locus. The two *tir5* alleles (Ruegger et al., 1997) have been renamed *nph4-4* and *nph4-5* (M. Estelle, unpublished data), and the *msg1* mutants (Watahiki and Yamamoto, 1997) have been given *nph4* allele designations beginning with allele number *101* (K. Yamamoto, unpublished data).

Overall Morphogenesis of Dark- and Light-Grown *nph4* Plants Is Normal

It was proposed previously that the *NPH4* gene product might play an essential (Watahiki and Yamamoto, 1997), if not direct (Liscum and Briggs, 1996), role in the establishment of differential growth in Arabidopsis. This hypothesis was based mainly on data related to hypocotyl tropisms. Alternatively, it could be argued that the observed mutant tropic phenotypes arose because of changes in overall growth properties or cellular/tissue organization, rather than from specific defects related directly to differential growth. In an attempt to reconcile these opposing hypotheses, we examined a number of morphogenic properties in both dark- and light-grown *nph4* plants.

Table I. *Phototropic curvature in wild-type, heterozygous, and homozygous nph4 mutants*

Three-day-old seedlings were exposed to 7 h of continuous, unilateral BL at a fluence rate of 0.1 μmol m^{-2} s^{-1}, and then phototropic curvatures were determined (see "Materials and Methods"). Data represent the mean response ± SD. Numbers of seedlings are given in parentheses.

Genotype	Curvature	*t*	P[a]
	degrees		
NPH4/NPH4	52.3 ± 12.9 (65)	12.64[b]	<0.001
NPH4/nph4-1	32.2 ± 8.9 (124)	–	–
nph4-1/nph4-1	2.2 ± 5.3 (60)	24.19[c]	<0.001

[a] Genotypes being compared were considered significantly different if $P \leq 0.05$. [b] Student's *t* test comparison of mean responses between wild-type and heterozygous seedlings. [c] Student's *t* test comparison of mean responses between homozygous and heterozygous mutant seedlings.

Etiolated Growth

As shown in Figure 1, hypocotyls of etiolated wild-type and *nph4* seedlings exhibit similar straight-growth kinetics. Moreover, the overall cellular morphology and anatomical structure are similar between hypocotyls of wild-type and *nph4* seedlings (Fig. 2). Thus, it appears unlikely that disrupted tropic responses of etiolated *nph4* seedlings result from gross changes in hypocotyl morphogenesis.

Because apical hooks of etiolated seedlings are formed by the continual differential growth of cells on the inner and outer edges of the hook as they flow through the apical region (Silk and Erickson, 1978), we examined the apical hooks of *nph4* mutants. As illustrated in Figure 3A, *nph4* seedlings were partially hookless, indicating that this response is disrupted by *nph4* mutations. The dark-dependent hook-opening response of *nph4* seedlings was saturated after about 2.5 to 3 d, rather than 4 to 5 d as in the wild-type (Fig. 4; see also Liscum and Hangarter, 1993a). Apical hooks of *nph4* seedlings, however, were similar in appearance to the wild type upon germination (data not shown). Thus, it appears that the hookless phenotype of *nph4* seedlings resulted from an accelerated phase of opening. It is interesting that both wild-type and *nph4* seedlings exhibited an exaggerated apical hook when grown in the presence of ethylene (Fig. 3B). This result demonstrates that the cells within the apical hook of *nph4* seedlings are capable of exhibiting differential growth, and indicates that the hookless phenotype of air-grown *nph4* seedlings is not a result of a general defect in apical hook structure/maintenance. Taken together, the apical hook phenotypes of *nph4* seedlings suggest that NPH4 acts as a conditional modulator of differential growth.

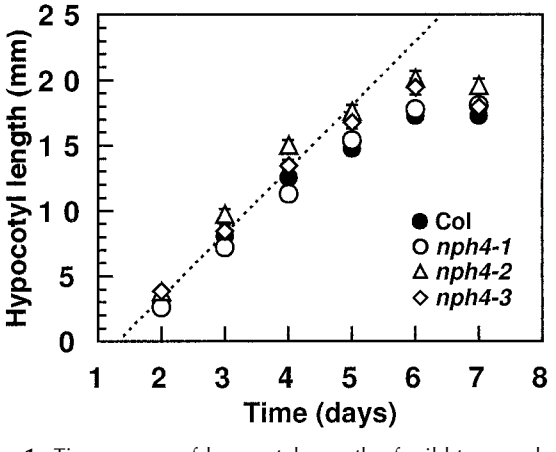

Figure 1. Time course of hypocotyl growth of wild-type and *nph4* seedlings in darkness. At the indicated times after induction of germination, seedlings were removed and hypocotyl lengths were measured to the nearest millimeter. Data represent the mean response of a minimum of 60 seedlings from two replicate experiments. The vertical error bars represent the SE values. Because the symbols often overlap, some individual symbols and error bars are not visible. The dotted line represents a regression (r^2 = 0.942) calculated for combined wild-type and *nph4* data during the period of linear growth (d 2, 3, and 4). Col, Wild-type Columbia ecotype.

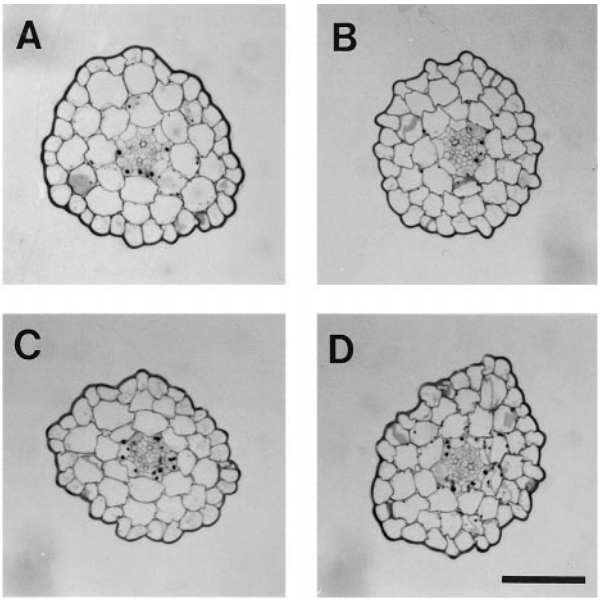

Figure 2. Cellular morphology of wild-type (A), *nph4-1* (B), *nph4-2* (C), and *nph4-3* (D) hypocotyls. All sections were taken from a region just below the apical hook of 3-d-old dark-grown seedlings, where phototropism is initiated (Orbovic and Poff, 1991), stained with periodic acid-Schiff's reagent, and viewed with bright-field optics. Morphologies are similar to those reported previously for etiolated Arabidopsis seedlings (Gendreau et al., 1997). Bar = 100 μm.

De-Etiolated Growth

As was observed with etiolated seedlings (Fig. 1), hypocotyl growth of deetiolated *nph4* seedlings was indistinguishable from that of the wild type (Fig. 5). In general, the growth and development of adult light-grown *nph4* plants also appeared normal. As shown in Table II, rosettes of mature *nph4* plants were similar to those of the wild type with respect to size and number of leaves. The growth and development of reproductive structures were also unaffected by *nph4* mutations, such that wild-type and *nph4* plants flowered with a similar timing (data not shown) and the resultant inflorescences were similar in size and number (Table II).

The only abnormal morphological feature invariably associated with light-grown *nph4* plants was the presence of epinastic or hyponastic rosette leaves (data not shown). The extent of leaf epinasty observed in this study was similar to that observed previously for the *nph4-102* allele (see Watahiki and Yamamoto, 1997). Although hyponasty has been reported for the *nph4-103* allele (Watahiki and Yamamoto, 1997), only epinasty was observed in other *nph4* alleles (data not shown; Watahiki and Yamamoto, 1997). The morphology of mature *nph4* plants, like that of seedlings, indicated that NPH4 is dispensable with respect to the overall morphological and developmental program of the plant. However, the epinastic/hyponastic character of *nph4* rosette leaves, which likely occurred as a result of abnormal differential growth of adaxial and abaxial leaf surfaces (Palmer, 1985; Klee et al., 1987), provides additional evidence that NPH4 acts as a modulator of differential growth.

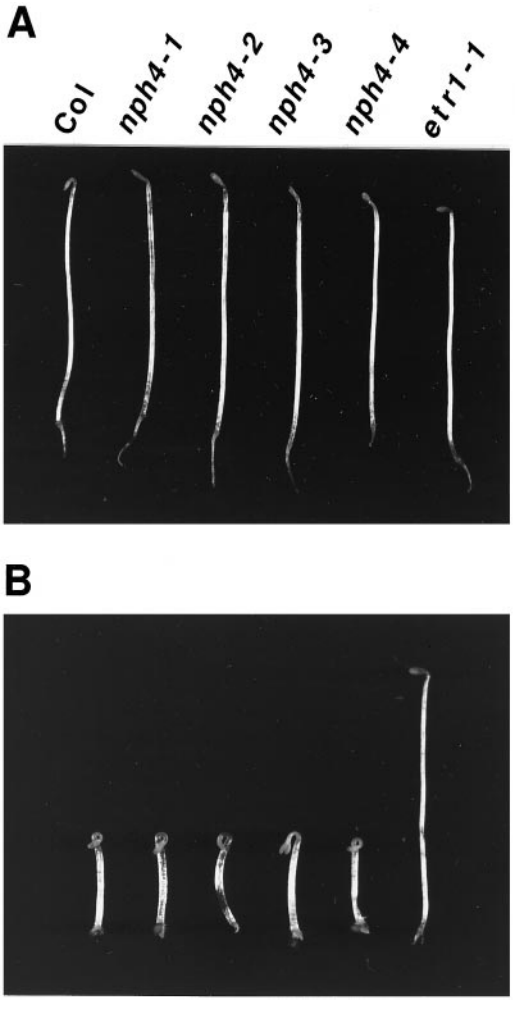

Figure 3. Morphogenesis of etiolated wild-type and *nph4* seedlings grown in air (A) and 50 μL/L ethylene (B). Photographs were taken after 4 d of growth in darkness. The ethylene receptor mutant *etr1-1* is shown as a negative control for ethylene responsiveness. Col, Wild-type Columbia ecotype.

Phototropic Impairment Is a Common Feature of *nph4* Mutants

Watahiki and Yamamoto (1997) reported that *nph4-101*, *nph4-102*, and *nph4-103* seedlings exhibited normal phototropic responses in unilateral WL. However, it has been shown previously that under conditions in which significant phytochrome photoactivation occurs in addition to phototropic photoreceptor activation (i.e. unilateral WL), considerable phototropic response is observed in *nph4* seedlings (Liscum and Briggs, 1996). Therefore, we examined the phototropic response of various *nph4* mutants in unilateral BL. As shown in Table III, *nph4-102* was only about 28% as responsive as the wild type after exposure to 8 h of unilateral BL, demonstrating that this allele is in fact phototropically impaired. The response of *nph4-102*, however, was more than twice as great as that observed in *nph4-1* (Table III). *nph4-4* seedlings exhibited a phototropic response that was intermediate between that of *nph4-1* and *nph4-102* (Table III). Thus, it appears that NPH4 is necessary for the generation of phototropic curvatures in unilateral BL, and that considerable quantitative variation occurs between the various *nph4* alleles.

Etiolated *nph4* Seedlings Lack RL-Induced Hypocotyl Curvature

Although the hypocotyls of etiolated Arabidopsis seedlings normally grow vertically upward (Mizra et al., 1984; Liscum and Hangarter, 1993b), they bend away from this orientation when exposed to RL (Fig. 6; also see Hangarter, 1997). This differential growth response apparently requires phytochrome B photoconversion, since the response was virtually eliminated in a *phyB* null mutant (Fig. 6). However, it does not require functional NPH1, because a *nph1* null mutant exhibited a wild-type response (Fig. 6). This latter result, together with the findings that the direction of RL-induced curvature was random (E. Liscum, unpublished data) and that phototropic curvatures in Arabidopsis were not induced by exposure to unilateral RL (Steinitz et al., 1985; Liscum and Briggs, 1996), indicates that this phytochrome-B-dependent curvature response is not a phototropic response. However, the *nph4* mutants lack this RL-induced curvature (Fig. 6), indicating that at least one component is shared between this response and phototropism. This result also illustrates an additional condition in which NPH4 function is required for the generation of differential growth.

Auxin Responsiveness Is Disrupted in *nph4* Seedlings

Auxin-Dependent Growth

The *nph4* mutants have been shown to represent a unique class of auxin-resistant mutants that are not cross-

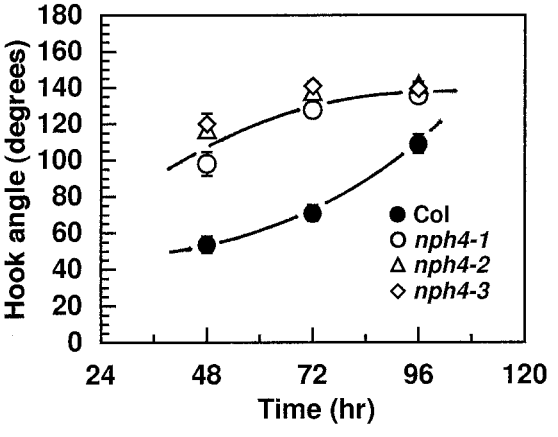

Figure 4. Time course of apical hook opening in dark-grown wild-type and *nph4* seedlings. Seedlings were grown in darkness on vertical plates. At the indicated times (after induction of germination), apical hook angles were determined (see "Materials and Methods"). Data represent the mean response of a minimum of 21 seedlings from two replicate experiments. The vertical error bars represent the SE values. Because the symbols often overlap, some individual symbols and error bars are not visible. Col, Wild-type Columbia ecotype.

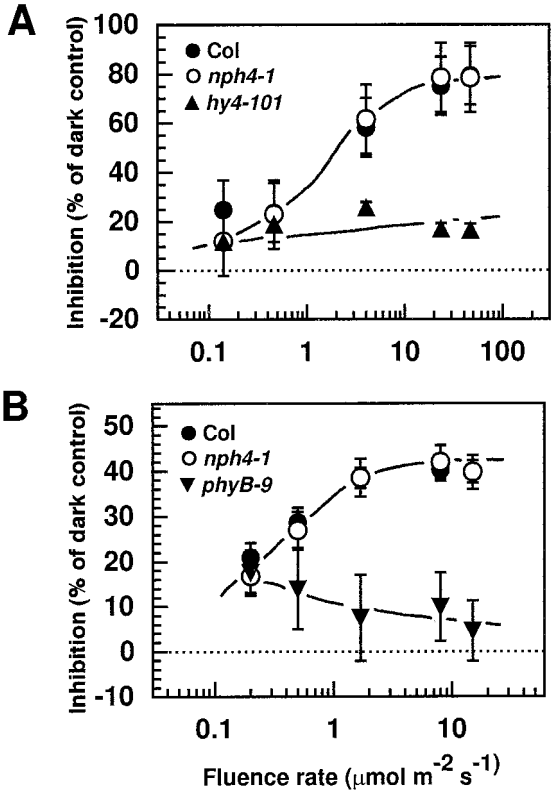

Figure 5. BL-dependent (A) and RL-dependent (B) hypocotyl growth inhibition in wild-type and *nph4* seedlings. After 23 h of growth in darkness, seedlings were transferred to continuous light at the indicated fluence rates shown for an additional 96 h. Control seedlings were kept in darkness for the entire growth period. Hypocotyl lengths were measured from digitized images of seedlings (see "Materials and Methods"). Data represent the mean response of at least 33 seedlings from two replicate experiments. Vertical error bars represent the combined SE values for dark- and light-grown seedlings. Because the symbols often overlap, some individual symbols and error bars are not visible. The response of cry1-deficient *hy4-101* seedlings is presented as a negative control. The response of phyB-deficient *phyB-9* seedlings is presented as a negative control. Col, Wild-type Columbia ecotype.

resistant to other growth regulators, and exhibit auxin resistance only in aerial organs (Watahiki and Yamamoto, 1997). Figure 7 shows the auxin resistance exhibited by hypocotyls of the *nph4-1*, *nph4-2*, and *nph4-3* mutants. Using a 50% inhibitory concentration as a measure of resistance we found that the *nph4-1*, *nph4-2*, and *nph4-3* mutants were 15- to 20-fold more resistant to IAA, 2,4-D, and 1-NAA than the wild type. In comparison, the *nph4-101*, *nph4-102*, and *nph4-103* mutants were only about 5-fold more resistant to 2,4-D than the wild type (Watahiki and Yamamoto, 1997). Together, these findings, along with those from analyses of apical hook structure and BL-dependent phototropism in etiolated seedlings (Figs. 3–5; Watahiki and Yamamoto, 1997), indicate that the *nph4-101*, *nph4-102*, and *nph4-103* mutants represent weak alleles, whereas the *nph4-1*, *nph4-2*, and *nph4-3* mutants represent strong alleles.

Table II. *Morphological features of adult wild-type and nph4 plants*

Seeds were sown directly on soil and allowed to germinate under constant WL (120 μmol m^{-2} s^{-1}) at 25°C. Measurements were made at 3 weeks (number of leaves) and 6 weeks (all other characteristics) after sowing. Data represent the mean response ± SD of a minimum of 16 plants. Col, Wild-type Columbia ecotype (genetic background of *nph4-2*).

Feature	Col	*nph4-2*[a]
No. of rosette leaves	11.8 ± 1.4	12.6 ± 1.6
Rosette diameter (mm)[b]	89.6 ± 13.6	80.4 ± 14.4
No. of inflorescences[c]	4.5 ± 0.8	4.9 ± 0.5
Length of inflorescence (cm)[d]	36.6 ± 5.6	40.3 ± 6.7
No. of lateral branches[e]	17.0 ± 7.2	20.7 ± 4.4

[a] Similar results were obtained with other *nph4* alleles. [b] Measured across the two largest opposing leaves. [c] Measured as the number of bolts emerging from the rosette. [d] Measured on the longest bolt. [e] Measured as secondary inflorescences arising from axils of primary inflorescences.

Auxin-Dependent Gene Expression

A number of genes have been identified in higher plants that are transcriptionally activated within 5 to 60 min of exposure to auxin (for review, see Abel and Theologis, 1996). In an attempt to determine how early in the auxin-response pathway(s) NPH4 functions, the steady-state mRNA levels of a number of these rapid primary-response genes were examined. As shown in Figure 8, expression of such genes was severely impaired in the *nph4* mutant background. In particular, mRNAs of *SAUR-AC1* and *IAA12* were undetectable, and *GH3*, *IAA4*, and *IAA6* mRNAs were detectable only after extended autoradiographic exposures (data not shown). *IAA2*, *IAA5*, and *IAA13* mRNAs, however, were clearly detectable in total RNAs from *nph4-1* and *nph4-2*, but were reduced in level relative to the wild type. The basal levels of expression (i.e. expression that was dependent on endogenous auxin) of all of the primary-response genes examined were also reduced. However, in some cases the effects were much smaller than those observed with respect to induction by exogenous auxin. For example, although a dramatic reduction in the abundance

Table III. *BL-dependent hypocotyl phototropism in dark-grown wild-type and various nph4 seedlings*

Three-day-old etiolated seedlings were exposed to 8 h of unilateral BL (0.1 μmol m^{-2} s^{-1}), and then phototropic curvatures were determined (see "Materials and Methods"). Data represent the mean response ± SE from a minimum of two replicate experiments. Numbers of seedlings are given in parentheses. Col, Wild-type Columbia ecotype.

Genotype	Curvature
	degrees
Col	49.0 ± 1.4 (75)
nph4-1	4.4 ± 0.9 (59)
nph4-4	8.0 ± 1.1 (49)
nph4-102	13.5 ± 0.9 (75)
nph1-5[a]	0.8 ± 0.9 (51)

[a] The response of this genotype is given as a negative control.

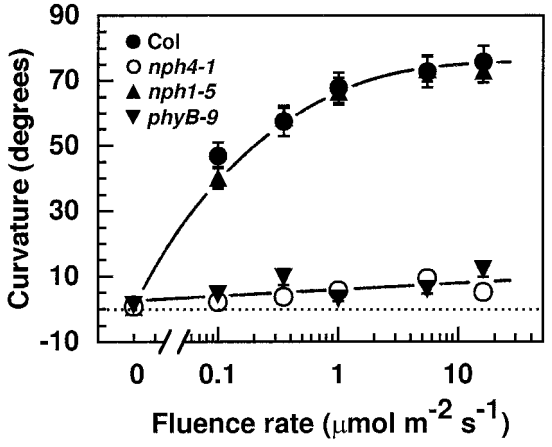

Figure 6. RL-dependent hypocotyl curvature in dark-grown wild-type and mutant seedlings. Sixty-hour-old seedlings were exposed to 20 h of continuous RL at the indicated fluence rates, and then curvatures were determined (see "Materials and Methods"). Data represent the mean response of a minimum of 33 seedlings from two replicate experiments. Vertical error bars represent the SE values. Because the symbols often overlap, some individual symbols and error bars are not visible. Col, Wild-type Columbia ecotype.

of *IAA2* and *IAA5* mRNAs was observed in auxin-treated *nph4* seedlings, the basal level of expression of these genes was only slightly reduced relative to the wild type (Fig. 8).

In contrast to the *nph4* mutants, no differences in mRNA abundances of the auxin primary-response genes were observed in the *nph1-4* mutant (Fig. 8). Although this finding was not unexpected, given the proposed role of NPH1 as an early step in the signaling pathway controlling phototropism (Liscum and Briggs, 1995; Huala et al., 1997), it does indicate that the molecular phenotypes of the *nph4* mutants were not simply a consequence of their aphototropic physiology (Liscum and Briggs, 1996). It remains to be determined if any of the early-response genes examined are actually necessary for the establishment of differential growth.

DISCUSSION

NPH4 Acts as a Conditional Modulator of Multiple Differential Growth Responses

Previous studies have shown that in addition to altered hypocotyl and root phototropism (Liscum and Briggs, 1996), *nph4* mutant seedlings exhibit impaired hypocotyl gravitropism (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997). In this study we demonstrate that apical hook and phytochrome-dependent hypocotyl curvatures of etiolated seedlings are also disrupted by *nph4* mutations. Furthermore, adaxial/abaxial leaf-surface expansion is altered in adult *nph4* plants, such that rosette leaves are either epinastic or hyponastic in appearance (Watahiki and Yamamoto, 1997; this paper). Under laboratory conditions all of these *nph4*-dependent alterations occur in the absence of any obvious changes in general growth or development. One interpretation of these results might be that differential growth responses are dispensable with respect to the overall morphological and developmental program of Arabidopsis. However, the conditional nature of the *nph4* phenotypes (i.e. aphototropism in BL versus considerable phototropism in WL, and a hookless phenotype in air versus a normal exaggerated hook in ethylene) indicate that redundant mechanisms exist to regulate differential growth. Hence, progression of a normal developmental program in the *nph4* background probably reflects the function of these redundant differential growth pathways, rather than a "noneffect" of the *nph4* mutations and dispensability of

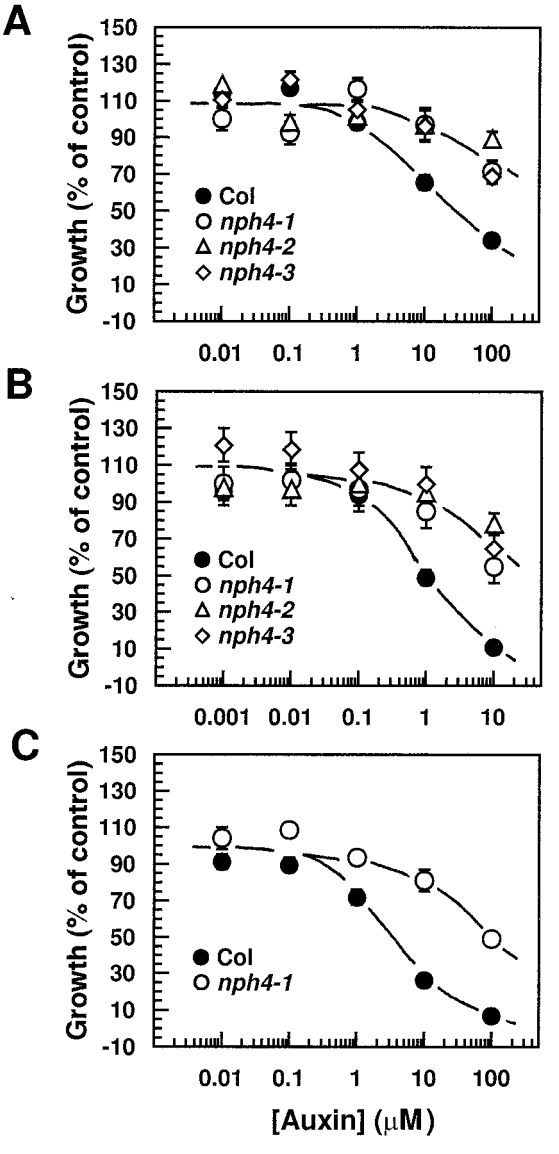

Figure 7. Dose responses of wild-type and *nph4* hypocotyls to exogenous IAA (A), 2,4-D (B), and 1-NAA (C). Three-day-old YL-grown seedlings were transferred to medium containing various concentrations of auxins (see "Materials and Methods"). Hypocotyl growth was measured 3 d later. Data represent the mean response (as a percent of controls) of a minimum of 90 seedlings from at least three replicate experiments. Vertical error bars represent the SE values. Controls were seedlings transferred to plates containing only solvent (0.04% ethanol). Because the symbols often overlap, some individual symbols and error bars are not visible. Col, Wild-type Columbia ecotype.

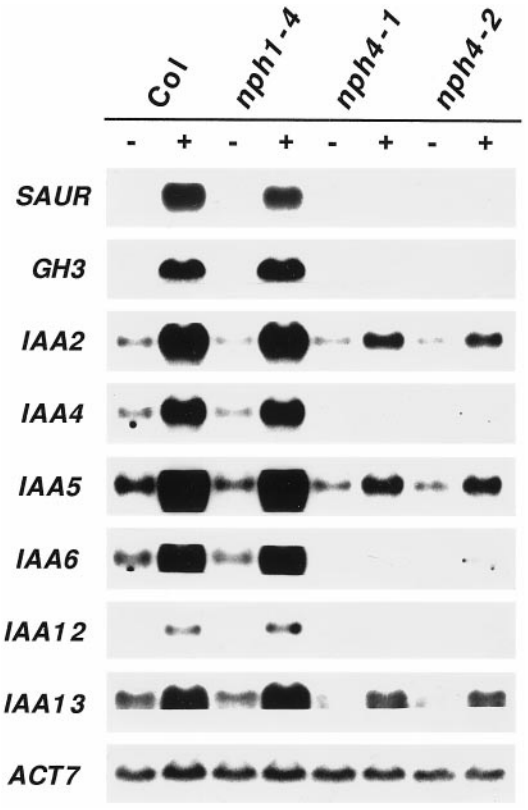

Figure 8. Expression of auxin-induced genes in wild-type and *nph4* seedlings. Total RNA was isolated from 7-d-old WL-grown seedlings that had been exposed to 100 μM IAA (+) or solvent (0.04% ethanol; −) for 1 h. Samples (20 μg each) were separated on a 1.0% agarose formaldehyde/Mops gel and then blotted to nylon. The blot was then hybridized with ^{32}P-labeled gene-specific probes against various auxin-induced genes: *SAUR-AC1* (Gil et al., 1994); *GH3* (Hagen et al., 1984); and *IAA2*, *IAA4*, *IAA5*, *IAA6*, *IAA12*, and *IAA13* (Abel et al., 1995). The blot was also hybridized with a labeled actin probe (*ACT7*; McDowell et al., 1996) as a loading control. RNA from *nph1-4* seedlings was used as an additional positive control. Similar overall results were observed in replicate experiments with both WL- and dark-grown seedlings (data not shown). The blot was rehybridized with multiple probes between strippings; thus, artificially flat upper and/or lower edges were generated on the *IAA5*, *IAA6*, and *IAA13* transcripts when individual panels for these genes were cropped from the entire blot for photographs. Col, Wild-type Columbia ecotype; *SAUR*, *SAUR-AC1*.

differential growth. Because NPH4 acts as a conditional modulator of differential growth, it represents an attractive molecule for future studies of differential growth regulation in the absence of potentially confounding pleiotropic effects, as occurs with many of the other apparent regulators of differential growth. Furthermore, the conditional nature of NPH4 action should allow us to genetically identify redundant modulators that are functioning under other conditions.

The *nph4* Mutants Comprise a Phenotypically Variable Allele Series

Although the *nph4* mutants were first identified by their ability to disrupt hypocotyl phototropism in etiolated seedlings (Liscum and Briggs, 1995, 1996), complementation studies presented here demonstrate that several additional *nph4* alleles have recently been identified in screens for seedlings exhibiting reduced auxin-induced hypocotyl curvature (Watahiki and Yamamoto, 1997) or sensitivity to auxin-transport inhibitors (Ruegger et al., 1997). Analyses of the different *nph4* mutants indicate that considerable phenotypic variation exists within this allele series. For instance, seedlings homozygous for the *nph4-102* allele (previously designated *msg1-2*) exhibit hypocotyl phototropism and apical hook closure that is considerably more like the wild type (Watahiki and Yamamoto, 1997) than the mutants homozygous for any of the originally identified *nph4* alleles, such as *nph4-1*. Furthermore, whereas "weak" *nph4* alleles (i.e. *nph4-102*) are more resistant to exogenously applied auxin than the wild type, they retain auxin sensitivity that is three to four times greater than that observed with "strong" *nph4* alleles (i.e. *nph4-1*). Such differences in auxin sensitivity are probably causal determinants of the aforementioned phenotypic differences between these alleles.

Allelic variation within the *nph4* allele series should not be surprising considering how the different mutant alleles were generated. The *nph4-1*, *nph4-2*, and *nph4-3* mutants were generated by fast-neutron bombardment (Liscum and Briggs, 1995, 1996), whereas the *nph4-4*, *nph4-5*, *nph4-101*, *nph4-102*, and *nph4-103* mutants were generated by EMS mutagenesis (Ruegger et al., 1997; Watahiki and Yamamoto, 1997). Fast neutrons usually induce deletions and/or large chromosomal rearrangements (Rédei and Koncz, 1992; Bruggemann et al., 1996), which result in the severe dysfunction or lack of the protein encoded by the mutated gene. As expected, all of the fast-neutron-generated *nph4* mutants are phenotypically strong mutants. In contrast, EMS usually causes G:C to A:T base substitutions that result in either missense or nonsense mutations (DuBridge and Calos, 1987). As would be predicted, both weak (i.e. *nph4-102*) and strong (i.e. *nph4-4*) alleles have been identified within the collection of EMS-generated *nph4* mutants.

Strong *nph4* Alleles Define a Threshold Step in the Phototropic Signal-Response Pathway

It was concluded from earlier studies that NPH4 likely functions as a signal transduction/response element acting downstream of the photoperception event(s) mediating phototropism (Liscum and Briggs, 1995, 1996). Because *nph4* mutants exhibit alterations in multiple differential growth responses, it is probable that NPH4 acts late in the phototropic signal-response pathway. The semidominant inheritance exhibited by the fast-neutron-generated *nph4* alleles indicates that the phototropic response is sensitive to the gene dosage of *NPH4*, and further implies that the magnitude of phototropic curvature is directly related to the abundance of the NPH4 protein. Therefore, we hypothesize that NPH4 is a concentration-dependent modulator of differential growth that functions late in the signal-response process(es) leading to phototropic curvatures. Previous photophysiological studies of phototropism in

Arabidopsis (Steinitz and Poff, 1986; Janoudi and Poff, 1991; Janoudi et al., 1992) and other species such as maize (Iino, 1987, 1990) have shown that the magnitude of phototropic curvature is kinetically limited by a postphotoperception component in the signal transduction chain. It is possible that NPH4 represents, or regulates the activity of, this previously predicted but unidentified gene product.

It is interesting to note that although the fast-neutron-generated *nph4* alleles exhibited semidominant inheritance, the EMS-generated alleles have been reported to segregate as simple recessive loci (Ruegger et al., 1997; Watahiki and Yamamoto, 1997). Of several plausible explanations for these apparently contradictory data, one in which allele strength determines the pattern of inheritance seems most likely. For example, heterozygotes carrying a weak *nph4* allele (i.e. *nph4-102*) could make enough active NPH4 protein to exceed a threshold level required for the establishment of a wild-type phototropic response, whereas heterozygotes having a strong *nph4* allele (i.e. *nph4-1*) would not and thus would appear partially mutant. Alternatively, the segregation of *nph4* alleles could be dependent on the physiological response being examined. As an example, all alleles might segregate as semidominant loci with respect to phototropism. A second alternative is that all *nph4* alleles are semidominant, independent of response, but that the "mutant" and "wild-type" classifications used in the initial genetic characterizations of the EMS-generated *nph4* mutations (Ruegger et al., 1997; Watahiki and Yamamoto, 1997) were too broad to clearly distinguish between recessive and semidominant inheritance. To test these latter possibilities we are currently generating a population that is heterozygous for the *nph4-102* allele (an apparent recessive allele), and will examine the dominance of this weak allele relative to phototropic response, for which semidominance has been observed.

Changes in Auxin Sensitivity of the *nph4* Mutants Likely Account for the Alterations in Differential Growth

Although most of the previously identified auxin-response mutants are disrupted with respect to at least one differential growth response (for reviews, see Hobbie and Estelle, 1994; Estelle, 1996; Leyser, 1998), nearly all are highly pleiotropic and exhibit multiple defects in addition to altered differential growth. Moreover, many apparently secondary effects of nonauxin growth regulators are observed in these mutants. In contrast, the *nph4* mutants represent a class of auxin-response mutants that can be used to assess the potential role(s) of auxin in the generation of differential growth in the absence of confounding phenotypic effects.

In addition to being resistant to exogenously applied auxin, the *nph4* mutants exhibit alterations in multiple differential growth responses. Moreover, changes in hormone responsiveness of the *nph4* mutants are limited to auxins, and among the phenotypes examined to date, most morphological defects appear to be confined to differential growth responses. The finding that auxin primary-response genes are expressed at dramatically reduced (or undetectable) steady-state levels in the *nph4* background indicates that NPH4 functions temporally early in an auxin-response pathway(s). Because many of the genes that were examined are normally transcriptionally activated within 5 min of exposure to auxin (for review, see Abel and Theologis, 1996), and the lag period between tropic stimulation and measurable curvature of hypocotyls and roots in Arabidopsis is approximately 5 to 20 min (Kiss et al., 1989; Orbovic and Poff, 1991), NPH4 activity is apparently required before, or concomitant with, cellular changes that actually drive differential growth. These observations, together with those gathered from analyses of other auxin-response mutants (for reviews, see Hobbie and Estelle, 1994; Estelle, 1996; Leyser, 1998), provide clear genetic evidence that auxin plays a critical role in the generation of differential growth patterns. Our results suggest further that the magnitude of differential growth is related directly to the auxin responsiveness of the plant, which can be modulated by NPH4. These conclusions are consistent with a broad interpretation of the Cholodny-Went theory (see Trewavas, 1992).

Whereas a definitive biochemical function for NPH4 awaits cloning of the *NPH4* locus, two obvious possible functions can be proposed based on the data accumulated to date: (a) NPH4 regulates lateral auxin transport (influx and/or efflux) or (b) NPH4 modulates auxin sensitivity at some step after the establishment of an auxin gradient. In terms of auxin influx, previous studies have shown that IAA and 2,4-D enter cells through an active influx carrier, whereas NAA enters via passive diffusion (Delbarre et al., 1996). Thus, if NPH4 regulates auxin influx, the *nph4* mutants would be expected to exhibit greater sensitivity to NAA than either IAA or 2,4-D. However, all of the *nph4* mutants examined were found to exhibit equivalent levels of resistance to IAA, 2,4-D, and NAA. With respect to auxin efflux, loss-of-function mutations affecting either the efflux carrier itself or some positively acting regulatory protein would be expected to cause increases in intracellular auxin concentration (see Lomax et al., 1995), thereby promoting dramatic changes in morphology. Increases in both hypocotyl elongation and apical dominance have been observed in light-grown Arabidopsis plants that overproduce IAA (Romano et al., 1995). However, no such morphological changes were observed in the strong *nph4* mutants. Moreover, to achieve the reduced basal levels of expression of the *IAA4* and *IAA6* genes observed in the *nph4* mutants, intracellular auxin concentrations might need to increase as much as 4.5 orders of magnitude (see Abel et al., 1995), which seems improbable. Therefore, it is unlikely that either auxin influx or efflux is dependent on NPH4 activity; therefore, we hypothesize that NPH4 plays a role in the modulation of auxin sensitivity. Such an activity could arise through direct binding of auxin or at some step removed from auxin binding (i.e. regulation of intracellular auxin signaling or auxin-dependent gene expression). These types of activities are consistent with the observed genetic and physiological phenotypes of the *nph4* mutants. We are currently in the midst of a chromosome walk to clone the *NPH4* locus, and in the near future would like to address these possibilities at the molecular level.

In conclusion, the results presented here demonstrate that the *NPH4* locus encodes an important conditional modulator of auxin-dependent differential growth. Further analysis of this locus and the encoded protein will certainly provide insight into the basic regulation of these adaptive growth responses, and lead to a more comprehensive understanding of the coordinated regulation of cellular growth by auxins.

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