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The *NPH4* Locus Encodes the Auxin Response Factor ARF7, a Conditional Regulator of Differential Growth in Aerial Arabidopsis Tissue

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Organ bending through differential growth represents a major mechanism by which plants are able to adaptively alter their morphology in response to local changes in the environment. Two plant hormones, auxin and ethylene, have been implicated as regulators of differential growth responses; however, the mechanisms by which they elicit their effects remain largely unknown. Here, we describe isolation of the *NPH4* gene of Arabidopsis, which is conditionally required for differential growth responses of aerial tissues, and we report that *NPH4* encodes the auxin-regulated transcriptional activator ARF7. The phenotypes of *nph4* mutants, which include multiple differential growth defects associated with reduced auxin responsiveness, including impaired auxin-induced gene expression, are consistent with the predicted loss of function of a transcriptional activator, and these phenotypes indicate that auxin-dependent changes in gene transcription are prerequisite for proper organ bending responses. Although NPH4/ARF7 appears to be a major regulator of differential growth, it is not the sole regulator because phenotypes of *nph4* null mutants were suppressed by application of ethylene. This latter finding illustrates the intimate connection between auxin and ethylene in the control of growth in higher plants.

INTRODUCTION

Plants have evolved movement strategies that involve organ bending to respond adaptively to environmental signals. Dramatic and rapid changes in plant morphology can result from differential growth, that is, unequal cellular growth in one position of an organ relative to an opposing position. Examples of differential growth responses include stem and root tropisms, modification of apical hook structures, and nastic movements of leaves (reviewed in Darwin and Darwin, 1896; Palmer, 1985). Each of these examples of stimulus-driven organ bending represents a process by which plants maximize the positive attributes of their environment while minimizing the negatives.

Two plant hormones, auxin and ethylene, have been implicated as regulators of differential growth responses (Went and Thimann, 1937; Davies, 1987; Kaufman et al., 1995). Although each of these hormones is capable of modulating growth when applied externally, the relative contribution of each in response to changes in their endogenous concentrations, and the sensitivities to either, has been difficult to reconcile (Davies, 1987). Much of this ambiguity stems from functional overlap between the auxin and ethylene signal and response, as well as their biosynthetic, pathways. For example, auxin stimulates ethylene production (Yang and Hoffman, 1984), which in turn stimulates the expression of genes, such as *HOOKLESS 1* (*HLS1*; Lehman et al., 1996), that are involved in auxin homeostatic processes. Results from recent genetic and molecular studies suggest that auxin may be the major regulator of differential growth responses, with ethylene modifying the auxin responses (Romano et al., 1993; Lehman et al., 1996; Chen et al., 1998; Luschnig et al., 1998; Madlung et al., 1999).

The mechanism by which auxin modulates plant growth is still not understood; however, the gene activation hypothesis proposes that auxin regulates the transcription of specific mRNAs that encode proteins necessary for growth control (Key, 1969). Numerous target genes for auxin action,

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as well as regulatory loci controlling auxin-dependent gene expression, have now been identified (reviewed in Abel and Theologis, 1996; Sitbon and Perrot-Rechenmann, 1997; Guilfoyle et al., 1998a, 1998b). Although auxin-regulated transcription of proteins directly involved in growth control, such as α-expansins (Cosgrove, 1999), only recently has begun to be explored (Hutchison et al., 1999), considerable effort has been dedicated to the study of regulatory proteins. Two families of proteins that regulate auxin-dependent gene expression, the Aux/IAA proteins (Abel and Theologis, 1996; Guilfoyle, 1998) and the auxin response factors (ARFs; Guilfoyle et al., 1998a), have received the most attention.

The *Aux/IAA* genes are expressed in response to auxin and encode short-lived, small hydrophilic nuclear proteins that function as transcriptional repressors in transient assay systems (reviewed in Abel and Theologis, 1996; Guilfoyle, 1998). Like the Aux/IAA proteins, ARF proteins also function as transcriptional regulators (reviewed in Guilfoyle et al., 1998a); however, at least some appear to be transcriptional activators (Ulmasov et al., 1999a). Unlike the *Aux/IAA* genes, *ARF* gene expression does not appear to be sensitive to auxin (Ulmasov et al., 1999b). The Aux/IAA and ARF proteins are related to each other through two domains of conserved sequences that appear to be necessary for dimerization within and between these two classes of proteins (Kim et al., 1997; Ulmasov et al., 1999b). Because only the ARF proteins have been shown to exhibit clear DNA binding activity, auxin has been proposed to modulate gene expression through modification of ARF activity by way of cell- and tissue-specific auxin-dependent ARF–ARF and ARF–Aux/IAA dimerization (Ulmasov et al., 1999a, 1999b).

The functional roles of Aux/IAA and ARF proteins are beginning to be elucidated through the molecular analysis of Arabidopsis mutants found to contain lesions in *Aux/IAA* or *ARF* genes. For example, mutations in the *SHORT HYPOCOTYL 2 (SHY2)/IAA3* and *AUXIN RESISTANT 3 (AXR3)/IAA17* loci have been shown to have effects on similar auxin-dependent responses, including root elongation and proliferation, root gravitropism, hypocotyl elongation in light-grown seedlings, and inflorescence development (Leyser et al., 1996; Rouse et al., 1998; Tian and Reed, 1999). Mutations in two *ARF* genes, *ETTIN (ETT)/ARF3* and *MONOPTEROS (MP)/ARF5*, suggest that auxin-regulated transcription is essential for proper pattern formation. Specifically, *ett* mutations alter regional identity within floral meristems to disrupt proper patterning of reproductive organs (Sessions et al., 1997), whereas *mp* mutations disrupt body axis formation and vascular patterning within the embryo (Hardtke and Berleth, 1998). Together, analyses of these mutants provide genetic evidence that auxin-dependent changes in gene expression are important factors regulating plant development. The *shy2* and *axr3* mutants also provide compelling support for the gene activation hypothesis for regulation of differential growth, because both disrupt at least one bending response: root gravitropism (Leyser et al., 1996; Tian and Reed, 1999). However, because the phenotypes of these mutants are highly pleiotropic, the altered gravitropic responses may be the result of earlier effects on development rather than direct effects of changes in gene expression on growth. Conclusive evidence in support of the gene activation hypothesis would require the identification of an *aux/iaa* or *arf* mutant that specifically alters differential growth while leaving the overall developmental program intact.

Analyses of the *nph4* mutants of Arabidopsis suggest that the NPH4 protein may represent a specific regulator of differential growth (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Watahiki et al., 1999). Originally identified by their dramatically reduced phototropic responses (Figures 1A and 1B; Liscum and Briggs, 1995), the *nph4* mutants have been found to exhibit several additional differential growth defects, including altered stem gravitropism, phytochrome-dependent stem curvature, apical hook maintenance, and abaxial/adaxial leaf blade expansion (Liscum and Briggs, 1996; Stowe-Evans et al., 1998; Watahiki et al., 1999). The severely impaired auxin-induced hypocotyl bending, hypocotyl growth inhibition,

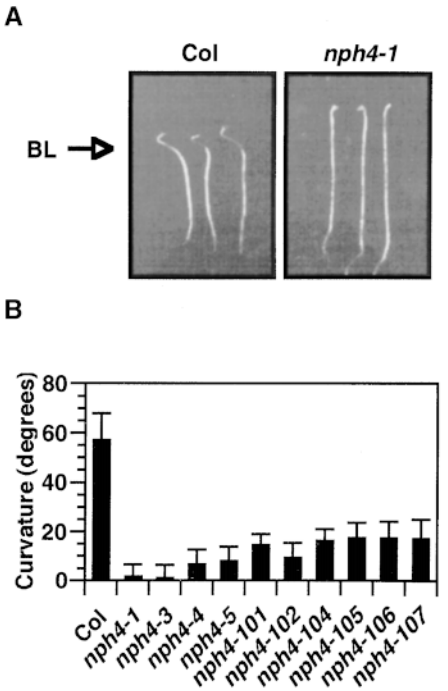

Figure 1. Phototropic Response of 3-Day-Old Dark-Grown Wild-Type (Col) and *nph4* Seedlings.

(A) Seedlings exposed to 8 hr of unilateral blue light (BL; 0.1 μmol m^{-2} sec^{-1}) from the left. Col, Columbia ecotype.

(B) Quantitative analysis of hypocotyl phototropism in 3-day-old dark-grown seedlings exposed to 8 hr of unilateral blue light. Data represent the mean response of a minimum of 20 seedlings for each genotype. Error bars indicate SD.

and gene expression responses in the *nph4* mutant background have led to the hypothesis that the NPH4 protein functions as a modulator of auxin-dependent differential growth (Stowe-Evans et al., 1998). In this study, we show that *NPH4* encodes the auxin response factor ARF7 and that this transcriptional activator is conditionally required for the modulation of differential growth of aerial tissues in Arabidopsis. These findings provide support for the gene activation hypothesis. Furthermore, the findings that ethylene can suppress the phenotypic abnormalities of *nph4* mutants illustrate the functional overlap occurring between auxin and ethylene response pathways, findings that should provide an ideal system for future studies of this interaction.

RESULTS

Physical Mapping and Cloning of *NPH4*

Previous work has shown that the *NPH4* locus mapped to the proximal arm of chromosome 5, between simple sequence length polymorphism (SSLP) markers nga106 and nga139 (Ruegger et al., 1997; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Figure 2A). We used a combination of polymerase chain reaction (PCR)–based marker systems to define the rough physical location of the *NPH4* locus within this genetic interval to a region of ~250 kb represented by the proximal half of yeast artificial chromosome clone CIC3F12 (Figure 2A). DNA hybridization analysis using the transformation-competent artificial chromosome clone K13O21 (Figure 2A) as a probe identified a 6.9-kb fragment in EcoRI-XbaI double-digested DNA from a wild-type plant that was absent in DNA from the fast neutron–generated *nph4-1* mutant (Figure 2B). When cloned and used as a probe of RNA gel blots, this 6.9-kb fragment hybridized with a transcript of ~5 kb from 2.5-day-old etiolated wild-type seedlings that was undetectable in *nph4-1* seedlings (Figure 2C), suggesting that this DNA fragment might contain the *NPH4* locus.

Searches of the GenBank library with sequence from one end of the 6.9-kb DNA fragment identified several related cDNAs that represent various members of the ARF family of transcriptional regulators (Ulmasov et al., 1999a, 1999b). The closest sequence identity was observed with a pair of cDNAs—*ARF7* and *BIPOSTO* (GenBank accession numbers AF022368 and AF042195, respectively). The sequences of the *ARF7* and *BIOPOSTO* cDNAs differ by just 12 bp and are predicted to encode proteins differing in sequence by only eight amino acids. Because these cDNAs were isolated from the same genetic background, they appear to represent independent isolates of the same gene, with the differences arising from errors in sequencing. The sequence of genomic DNA representing ~2.3 kb of upstream untranslated sequence, the *ARF7*/*BIPOSTO* open reading frame, and ~200 bp of downstream untranslated sequence has been determined for the wild-type gene (GenBank accession number AF186466) and several *nph4* mutants, and we have found mutations within the open reading frame in each mutant allele (Figure 2D). Comparisons of *NPH4* genomic sequence with *ARF7* and *BIPOSTO* cDNA sequences also have allowed us to identify sequencing errors in both cDNAs. Of nine total single-base discrepancies identified, only two result in amino acid sequence differences relative to the published ARF7 sequence (Ulmasov et al., 1999a), namely, A-627 to S and H-638 to Q. A third amino acid difference, the addition of a W at position 271, results from the addition of one codon (GTG) at the exon 9/intron 8 junction.

nph4 Mutations and Their Predicted Effects on the NPH4/ARF7 Protein

Although we have not determined the precise nature of the *nph4-1* lesion, PCR analysis indicates that a rearrangement, most likely an inversion with an internal deletion, breaks the coding sequence between exons 11 and 12 (Figures 2B and 2D; R.M. Harper and E. Liscum, unpublished results). We believe that *nph4-1* represents a null allele because no *NPH4* mRNA has been observed by either RNA gel blot (Figure 2C) or reverse transcription (RT)–PCR (Figure 3A) analysis. A second fast neutron–generated allele, *nph4-3*, also carries a complex mutation: a 59-bp deletion/4-bp insertion occurring across the junction between exon 7 and intron 7 (Figure 2D). This mutant produces a *nph4* mRNA at a reduced level relative to *NPH4* (Figure 3A) that is predicted to encode a protein corresponding to the first third of the DNA binding domain of NPH4/ARF7. The nph4-3 protein, if present, would be 192 amino acids long, the first 182 amino acids being identical to the wild-type protein and the last 10 amino acids being novel (Figure 3B). A third fast neutron–generated allele, *nph4-109*, contains a single-base deletion (Figure 2D) that results in a frameshift and premature stop codon at position 709. Each of the remaining seven alleles that have been sequenced contain a single-base substitution: in *nph4-101*, *nph4-103*, *nph4-104*, *nph4-105*, and *nph4-106*, this results in premature stop codons within the Q-rich middle region of the NPH4/ARF7 protein, and those in *nph4-102* and *nph4-107* disrupt splice site junctions within the DNA binding domain coding region (Figures 2D and 3B). The aphototropic phenotype of the latter two mutant alleles is weaker than that of the homozygous *nph4-1* null alleles (Figure 1B; Stowe-Evans et al., 1998), suggesting that the former plants are likely to express *nph4* mRNA that is alternatively spliced to give rise to partially functional protein. Although the *nph4-107* allele has not been examined, RT-PCR results indicate that the *nph4-102* splice site mutant does in fact contain transcript. However, the expression or stability of the *nph4-102* mRNA is dramatically reduced relative to the wild type (Figure 3A). We have not determined how the *nph4-102* mRNA is spliced.

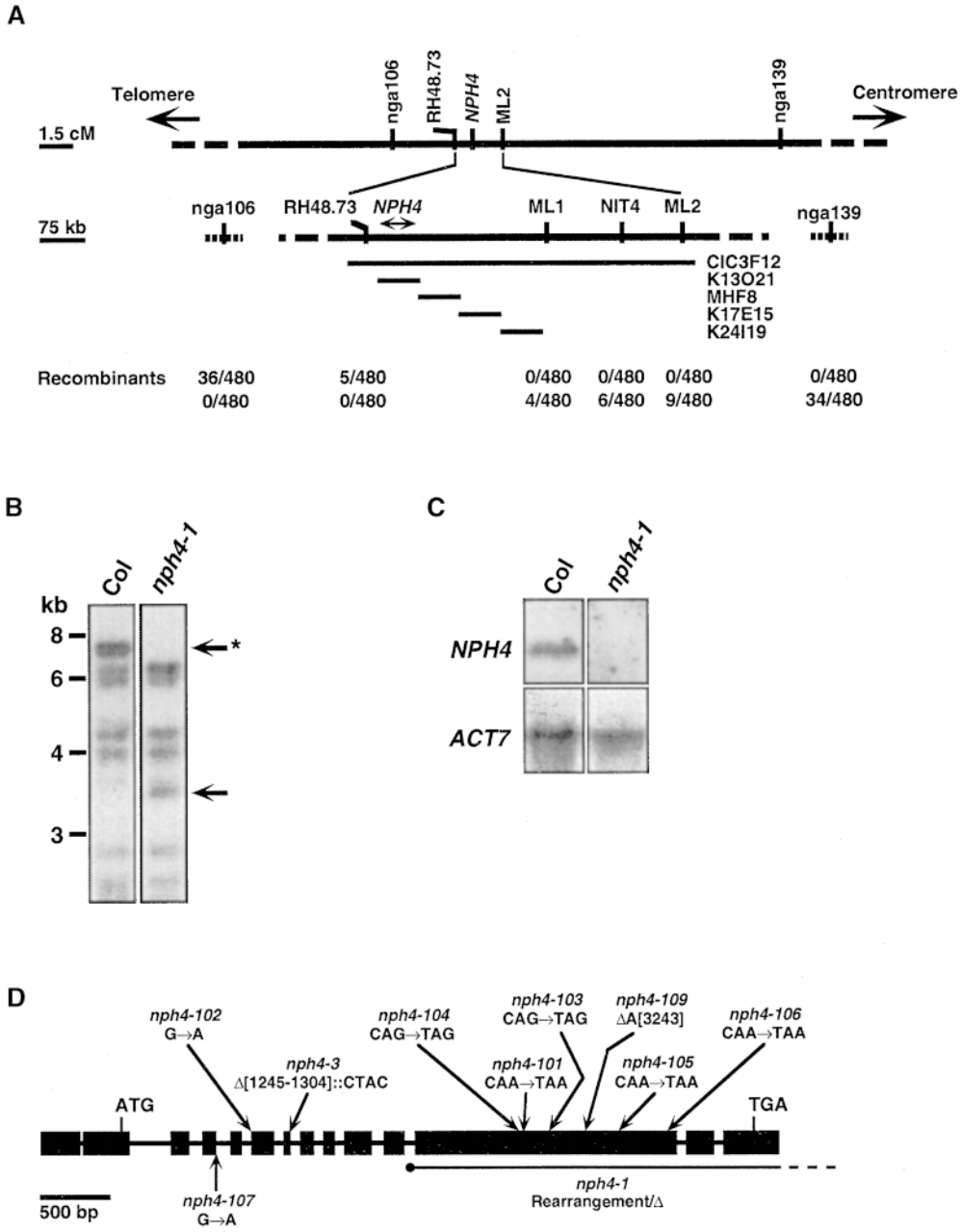

Figure 2. Map-Based Cloning and Structure of the *NPH4* Gene.

(A) Mapping of the *NPH4* locus on the proximal arm of chromosome 5. Genetic linkage map is shown at top; physical linkage map is shown in the middle; and numbers of recombinant chromosomes out of the total examined are shown at bottom. nga106, nga139, and ML2 are SSLP markers (Bell and Ecker, 1994); NIT4 is a cleaved amplified polymorphic sequence marker (Bartel and Fink, 1994; modified as described in Methods); ML1 is a simple nucleotide polymorphism marker (Cho et al., 1999); and RH48.73 is an amplified fragment length polymorphism marker (Liscum, 1999). CIC3F12 is a yeast artificial chromosome clone (Creusot et al., 1995; see Methods); MHF8 is a P1 phagemid clone (Liu et al., 1995); and K13O21, K17E15, and K24I19 are transformation-competent artificial chromosome clones (Liu et al., 1999). Arrows indicate that the chromosome continues beyond the region shown. cM, centimorgans.

(B) DNA gel blot made from Columbia (Col) and *nph4-1* genomic DNA double digested with EcoRI and XbaI and hybridized with ^{32}P-labeled K13O21 (see **[A]**). Arrows indicate polymorphic bands. The asterisk indicates the band cloned from wild-type DNA that was used as a probe in **(C)**. Numbers at left denote size markers in kilobases.

(C) RNA gel blot made with total RNA from 7-day-old Col and *nph4-1* seedlings and hybridized with the 6.9-kb DNA fragment described in **(B)** or with *ACT7* (McDowell et al., 1996) as a control.

Aphototropism of the *nph4-1* Null Mutant Is Suppressed by Ethylene Action

As described earlier, the *nph4* mutants of Arabidopsis represent a class of mutants that is disrupted with respect to several auxin-dependent differential growth responses. However, many of these phenotypes are conditional, depending on particular growth conditions. For example, air-grown *nph4* seedlings are partially hookless in appearance, whereas siblings exposed to ethylene exhibit an exaggerated apical hook typical of ethylene-treated wild-type seedlings (Stowe-Evans et al., 1998). This ethylene-dependent suppression of the phenotype of *nph4* mutants prompted us to examine whether ethylene action could influence other mutant phenotypes of *nph4* plants.

As shown in Figure 4A, a considerable phototropic response was recovered when seedlings homozygous for the *nph4-1* null allele were grown in ethylene (50 μL/L) instead of unsupplemented air. In contrast, the aphototropic phenotype of the phototropism photoreceptor mutant *nph1-5* (Christie et al., 1998) was not affected by ethylene treatment (Figure 4A). Interestingly, ethylene caused a depressed phototropic response in wild-type seedlings (Figure 4A). Because high concentrations of ethylene induce a typical "triple response" in etiolated Arabidopsis (Guzman and Ecker, 1990), the reduced phototropic curvature observed in ethylene-grown wild-type seedlings probably resulted from decreased overall growth capacity. This conclusion is supported by the finding that an ethylene receptor mutant, *etr1-1* (Bleecker et al., 1988; Schaller and Bleecker, 1995), and signal transduction mutant *ein2-1* (Alonso et al., 1999) exhibited no change in phototropic response when grown in ethylene (Figure 4A).

Etiolated *nph4* seedlings exhibit wild-type ethylene sensitivity with respect to hypocotyl elongation (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998); thus, growth inhibition probably explains why the recovered phototropic response of *nph4-1* seedlings did not exceed the response of ethylene-treated wild-type seedlings but was merely the same as the wild-type response. Ethylene-dependent growth inhibition does not, however, explain why the aphototropic phenotype of the *nph4* mutants was suppressed. Because *nph4-1* seedlings have wild-type concentrations of ethylene (Woeste et al., 1999), both in the presence and absence of auxin (Figure 4C), the trivial explanation that *nph4* seedlings are aphototropic in the absence of exogenous ethylene because they fail to properly synthesize ethylene is untenable.

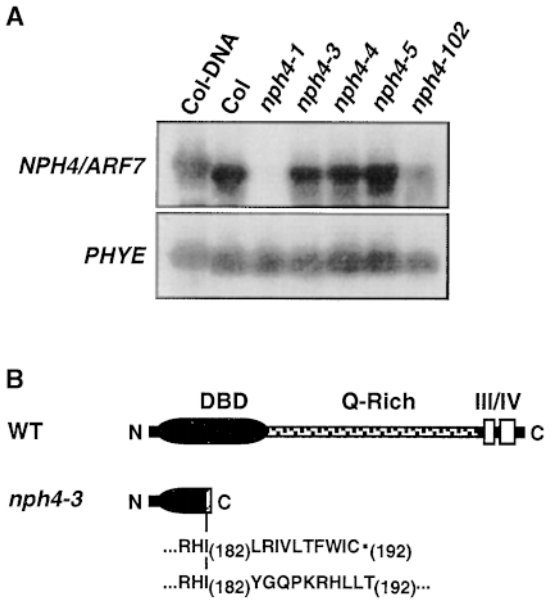

Figure 3. Expression of *NPH4/ARF7* in *nph4* Mutant Backgrounds.

(A) RT-PCR analysis of steady state amounts of *NPH4/ARF7* transcript in 2.5-day-old dark-grown wild-type (Col) and *nph4* mutants. RT-PCR products were detected by DNA gel blot analysis using ^{32}P-labeled *NPH4/ARF7* (top) or *PHYTOCHROME E* (*PHYE*; bottom). Genomic DNA from Col (Col-DNA) was used as a control template for both genes. Because the amplimers from genomic DNAs contain intron sequences, they are larger than the amplimers from RNA templates.

(B) Structure of wild-type (WT) NPH4/ARF7 (Ulmasov et al., 1999b) and nph4-3/arf7 proteins predicted from *nph4-3* sequence. Amino acid changes in nph4-3/arf7 (top line) are shown relative to the wild-type sequence (bottom line). C, C terminus; DBD, DNA binding domain; N, N terminus; Q-Rich, glutamine rich; III/IV, motifs homologous with domains III and IV of the C-terminal protein–protein interaction domain of Aux/IAA proteins (Guilfoyle, 1998).

We also have examined the phototropic response of wild-type and mutant seedlings grown on various concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene (Yang and Hoffman, 1984), to determine whether the positive and negative effects of ethylene on phototropism could be separated on the basis of ethylene dose responses. Consistent with their behavior

Figure 2. (continued).

(D) Structure of the *NPH4* gene and position of *nph4* mutations. The locations of start (ATG) and stop (TGA) codons are indicated. Exon (boxes) and intron (lines) positions were determined by comparing the genomic DNA sequence with the sequences of *ARF7* and *BIPOSTO* cDNAs. The position and identity of various *nph4* mutations are shown. For purposes of nucleotide numbering, the adenine of the start codon is considered position 1. The dashed line indicates that the end of the rearrangement is unknown.

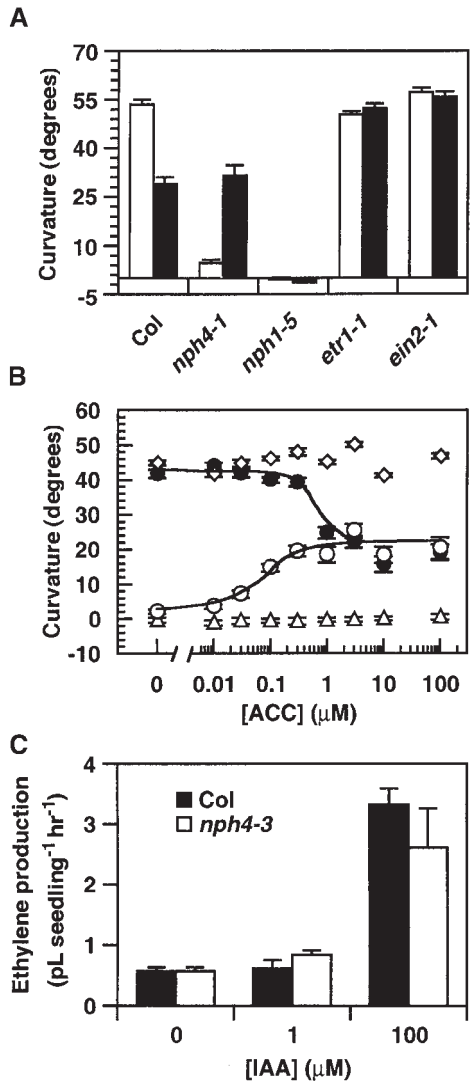

Figure 4. Effects of Ethylene on Phototropism of Wild-Type (Col) and *nph4* Seedlings.

(A) Phototropic curvature of 3-day-old seedlings grown in darkness under ambient air conditions (filled bars) or exposed to 50 μL/L ethylene (open bars) and then exposed to 8 hr of unilateral blue light (0.1 μmol m^{-2} sec^{-1}). Data represent the mean response of at least 40 seedlings from at least two replicate experiments. Error bars indicate SE.

(B) Dose–response curve for phototropism of wild-type (filled circles), *nph4-1* (open circles), *nph1-5* (open triangles), and *etr1-1* (open diamonds) seedlings grown on ACC. Seedlings were grown in darkness for 3 days on medium containing the indicated concentration of ACC and then exposed to 8 hr of unilateral blue light (0.1 μmol m^{-2} sec^{-1}). Data represent the mean response of at least 40 seedlings from at least two replicate experiments. Error bars indicate SE. Because errors are small, many error bars are not visible.

(C) Ethylene production in wild-type and *nph4* mutant seedlings. Seedlings were grown in sealed glass vials on a nutrient medium–agar plug for 72 hr in darkness. For auxin treatments, the nutrient medium was supplemented with the indicated concentration of IAA. Ethylene accumulation was measured as described in Methods. Values represent the mean response from three replicate experiments. Error bars indicate SD.

in high ethylene conditions (Figure 4A), *etr1-1* and *nph1-5* mutants exhibited no change in phototropic responsiveness when grown on ACC (Figure 4B). Wild-type seedlings grown on concentrations of ACC ⩾1 μM exhibited an inhibition of phototropic curvature (Figure 4B) equivalent to that observed in 50 μL/L ethylene (Figure 4A). This inhibition response correlates well with an effective dose (1 μM) required for half-maximal ACC-dependent inhibition of hypocotyl growth in both wild-type and *nph4* seedlings (E.L. Stowe-Evans, unpublished results). Maximal recovery of phototropism in *nph4* seedlings was observed over a range of ACC concentrations similar to that required for inhibition of phototropism in the wild type. However, the threshold for the recovery response was 10 to 30 times lower than the threshold for the inhibition response in the wild type, indicating that the recovery response in *nph4* seedlings is not associated with growth inhibition.

Altered Hypocotyl Gravitropism of *nph4* Is Suppressed by Ethylene Action

In addition to exhibiting a reduced gravitropic curvature in response to a reorientation stimulus (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997), hypocotyls of *nph4* seedlings exhibit randomized growth orientation that tends toward horizontal when grown unsupported in the absence of a reorientation stimulus (Figure 5A; Watahiki et al., 1999). Although the growth orientation of wild-type seedlings was unaffected, normal growth orientation of *nph4* seedlings was recovered in the presence of ACC (Figure 5B). The randomized growth orientation phenotype in both *nph4* mutants examined was suppressed with an ACC threshold of <0.1 μM, again indicating that simple growth inhibition does not represent the causal mechanism for suppression of the mutant phenotypes by ethylene.

Auxin Transport and Response Systems Are Required for the Ethylene-Dependent Suppression of the Mutant Phenotypes of *nph4* Mutants

Results from our studies on phototropism and gravity-dependent growth orientation presented here (Figures 4 and 5) suggest that ethylene, at concentrations suboptimal for hypocotyl growth inhibition, enhances the activity or abun-

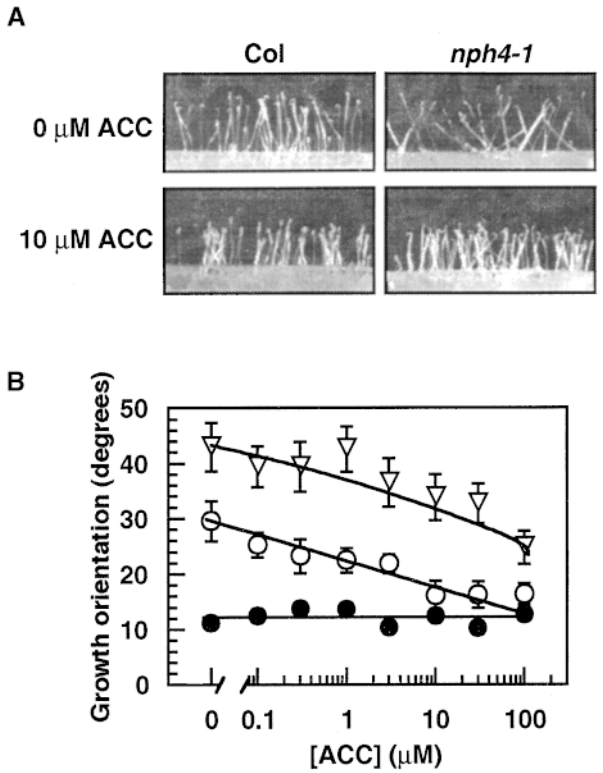

Figure 5. Effects of Ethylene on Gravity-Dependent Growth Orientation of Wild-Type (Col) and *nph4* Seedlings.

(A) Seedlings grown on horizontally oriented plates in the presence or absence of ACC.

(B) Dose–response curve for hypocotyl gravitropic growth orientation of wild-type (filled circles), *nph4-1* (open circles), and *nph4-3* (open inverted triangles) seedlings grown on ACC. Seedlings were grown in darkness for 3 days on vertically oriented plates containing the indicated concentration of ACC. Data represent the mean response of at least 45 seedlings from at least two replicate experiments. Error bars indicate SE. Because errors are small, many error bars are not visible.

dance (or both) of some component required for differential growth, a component that is limiting in the absence of NPH4/ARF7. Because ethylene can have several effects on auxin physiology (Burg and Burg, 1966, 1967; Lieberman and Knegt, 1977; Schwark and Schierle, 1992; Visser et al., 1996), perhaps the action of ethylene somehow enhances auxin responsiveness in the *nph4* mutants, thereby suppressing the mutant phenotypes. If this hypothesis is correct, any conditions that disrupt auxin responses independently of NPH4/AFR7 action would be expected to negate the effects of ethylene. Alternatively, if ethylene action is completely separable from auxin action, additional defects in auxin responsiveness would be without effect on suppression of the phenotypes of the *nph4* mutants.

To test these hypotheses, we examined the effects of *N*-1-naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport (Lomax et al., 1995), on the ability of ethylene to suppress the hookless and aphototropic phenotypes of *nph4-1* seedlings. As shown in Figure 6A, although air-grown *nph4-1* exhibited a partially hookless phenotype, siblings grown on ACC exhibited an exaggerated apical hook similar to that of the ACC-grown wild-type seedlings. However, this ACC-dependent exaggerated apical hook was negated by NPA for both wild-type and *nph4-1* seedlings, with similar dose dependencies in each case. As shown in Figure 6B, NPA also negated the phototropic response of *nph4-1* conditioned by growth on ACC (Figure 4B). Despite differences in their basal phototropic responses in the absence of NPA, wild-type and *nph4* seedlings appeared to exhibit similar

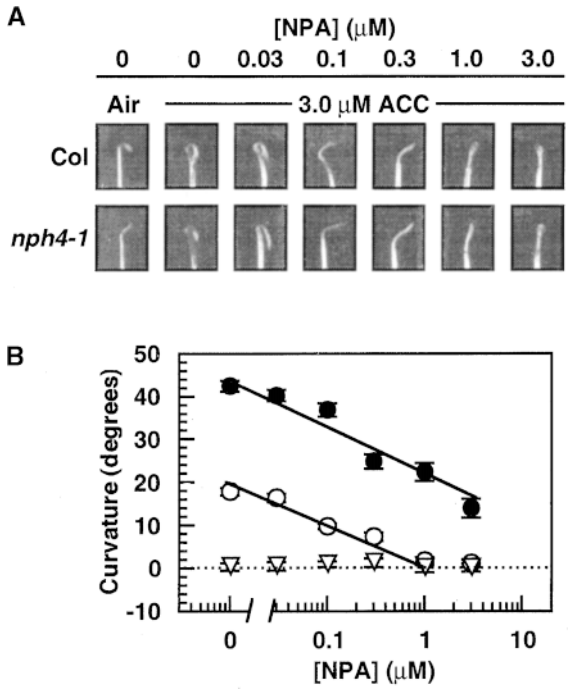

Figure 6. Effects of the Polar Auxin Transport Inhibitor NPA on Ethylene-Dependent Changes in Apical Hook Structure and Phototropism in Wild-Type (Col) and *nph4-2* Seedlings.

(A) Hook regions of 3-day-old seedlings grown in darkness on 3.0 μM ACC or in the absence of ACC (Air), with various concentrations of NPA.

(B) Dose–response curve for phototropism of wild-type (filled circles), *nph4-1* (open circles), and *nph1-5* (open inverted triangles) seedlings grown on NPA. Seedlings were grown as described in Figure 1B, except that they were grown in the presence of 0.3 μM ACC and the indicated concentration of NPA. Data represent the mean response of at least 33 seedlings from at least two replicate experiments. Error bars indicate SE. Because errors are small, many error bars are not visible.

sensitivities with respect to the effects of NPA on phototropism, as indicated by their parallel dose–response curves (Figure 6B). These similarities in sensitivities of wild-type and *nph4-1* seedlings to NPA are consistent with the hypothesis that ethylene exerts its effects on *nph4* seedlings by influencing auxin responsiveness.

Although NPA generally is accepted as being a specific inhibitor of polar auxin transport, the molecular target or targets and modes of action of NPA remain largely unknown (reviewed in Lomax et al., 1995). As such, with regard to the role of ethylene in auxin-mediated processes, conclusions based solely on pharmacological studies should be viewed skeptically. Hence, we also used a genetic approach to address the role of auxin in ethylene-dependent recovery of phototropism in *nph4-1* seedlings; that is, we disrupted additional genes involved in auxin responsiveness. As shown in Figures 7A and 7B, seedlings homozygous for either the weak *axr1-3* allele (Lincoln et al., 1990) or the strong *hls1-26* allele (Hou et al., 1993) were phototropically indistinguishable from wild-type seedlings when grown in air or ethylene. However, when either mutation was combined with the *nph4-1* null allele as a double homozygote, the effects of ethylene on the aphototropic response of *nph4* seedlings were negated (Figures 7A and 7B). Thus, although the auxin responsiveness appears not to be limiting with respect to phototropism in *arx1-3* and *hls1-26* single-mutant backgrounds, it becomes limiting when *NPH4* is also mutated, providing additional support for the hypothesis that the effects of ethylene on the phenotypes of the *nph4* mutants occur because of changes in auxin responsiveness.

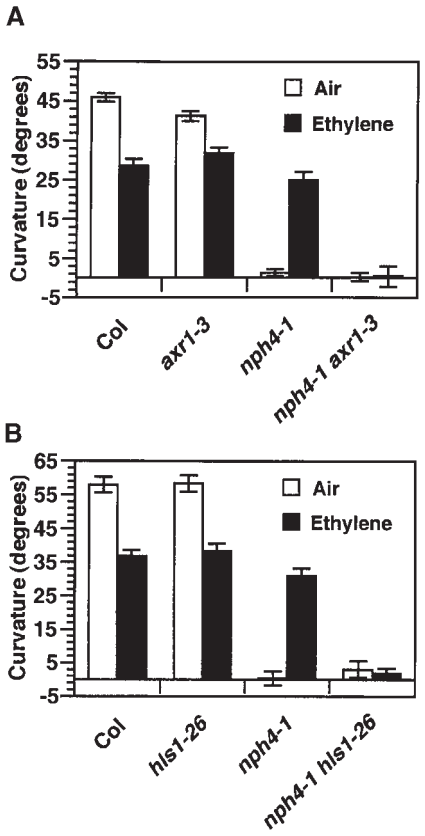

Figure 7. Effects of Auxin Response Mutations on Phototropism.

(A) Phototropism in 3-day-old wild-type (Col), *axr1-3*, *nph4-1*, and *nph4-1 axr1-3* double mutant seedlings grown in ambient air (open bars) or 50 μL/L ethylene (filled bars), then exposed to 8 hr of unilateral blue light (0.1 μmol m^{-2} sec^{-1}). Data represent the mean response of at least 41 seedlings from at least two replicate experiments. Error bars indicate SE.

(B) Phototropism in 3-day-old Col, *hls1-26*, *nph4-1*, and *nph4-1 hls1-26* double mutant seedlings treated the same as the seedlings in **(A)**, except that 10 hr of unilateral blue light was given. Data represent the mean response of at least 26 seedlings from two replicate experiments. Error bars indicate SE.

Ethylene Does Not Appear to Mediate Changes in Gross Auxin Sensitivity in the *nph4* Mutant Background

The studies discussed earlier in which NPA (Figure 6) and auxin response mutations (Figure 7) were used to disrupt auxin responsiveness suggest that ethylene-dependent changes in auxin responsiveness account for the ethylene-mediated suppression of the phenotypes of the *nph4* mutants. However, these studies did not address how ethylene might alter auxin responsiveness. In an initial attempt to answer this question, we have examined whether ethylene treatment resulted in dramatic changes in auxin responsiveness of wild-type and *nph4* seedlings by measuring auxin-dependent changes in hypocotyl growth and gene expression (Figures 8A and 8B). Although some increase in auxin responsiveness was observed in wild-type seedlings exposed to ACC, for both hypocotyl growth at low concentrations of auxin (Figure 8A) and the expression of auxin-induced genes (Figure 8B; data not shown), no dramatic changes in responsiveness were observed with similarly treated *nph4-1* seedlings (Figure 8). These results suggest that the effects of ethylene on the phenotypes of the *nph4* mutants result from subtle rather than dramatic changes in auxin responsiveness.

DISCUSSION

Most of the phenotypic alterations in the *nph4* mutants appear to be associated with auxin-dependent differential growth responses in aerial tissues (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998). With the cloning of the *NPH4* gene presented here, a hypothesis for the molecular basis for the phenotypes of *nph4* mutants now can be developed. This hypothesis must take into account the findings that multiple phenotypes of the *nph4* null mutant can be suppressed by exposure to ethylene.

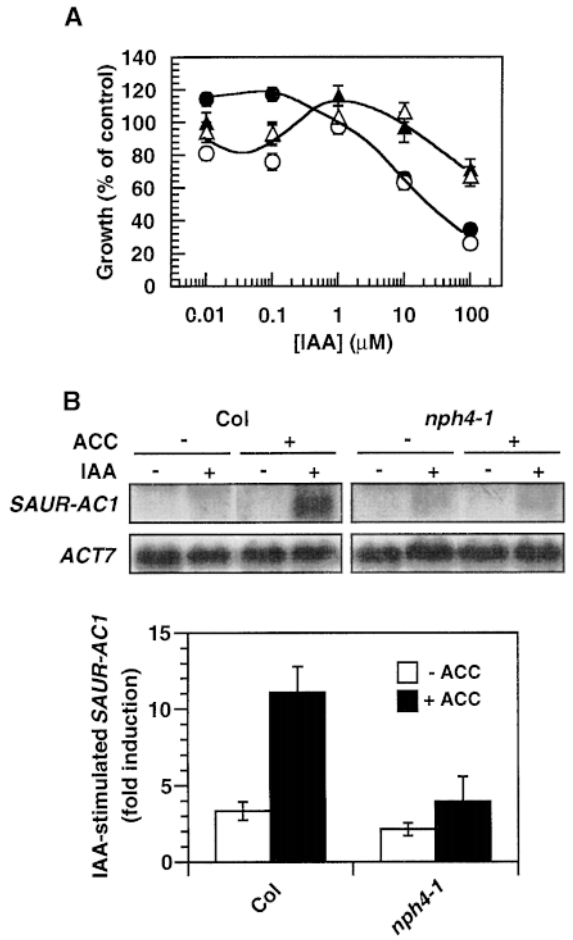

Figure 8. Effects of Ethylene on Auxin-Sensitive Growth and Gene Expression.

(A) Dose–response curve for auxin-dependent hypocotyl growth inhibition of seedlings grown in ambient air (filled circles, wild type; filled triangles, *nph4-1*) or in the presence of 0.3 μM ACC (open circles, wild type; open triangles, *nph4-1*). Data represent the mean response of at least 29 seedlings from at least three replicate experiments. Error bars indicate SE.

(B) Effects of ethylene on auxin-induced *SAUR-AC1* expression in wild-type (Col) and *nph4-1* seedlings. Seedlings were grown and treated with IAA, and RNA was analyzed as described previously (Stowe-Evans et al., 1998), except that one set of seedlings was grown in the presence of 0.3 μM ACC. Representative RNA gel blots probed with *SAUR-AC1* (Gil et al., 1994) and *ACT7*are shown at top. At bottom are quantitative data representing the mean response (*n*-fold induction by IAA relative to no IAA control) of three replicate RNA gel blots analyzed by densitometry. All data are normalized relative to an *ACT7* control. Error bars indicate SD. Similar results were obtained with *IAA6* and *IAA13* (E.L. Stowe-Evans and E. Liscum, unpublished results). (–), no added hormone; (+), hormone added.

NPH4/ARF7–Induced Gene Expression Is an Important Component of Auxin-Dependent Differential Growth in Aerial Tissues

It has long been known that auxins can modulate plant growth (reviewed in Went and Thimann, 1937); however, the molecular mechanisms by which auxins elicit these effects have remained more elusive. As discussed earlier, a gene activation hypotheses has been proposed to explain the effects of auxin on plant growth (Key, 1969). Despite evidence in support of this hypotheses (reviewed in Abel and Theologis, 1996), a direct causal relationship between auxin-mediated changes in gene expression and changes in specific growth processes had not been demonstrated. Cloning the *NPH4* locus represents a considerable step toward demonstrating a causal link between auxin-induced gene expression and control of growth.

NPH4 encodes the auxin response factor ARF7, which is a member of a large family of auxin-responsive transcriptional regulators in Arabidopsis (reviewed in Guilfoyle et al., 1998a). In general, ARF proteins are composed of three domains: an N-terminal DNA binding domain, a C-terminal protein–protein interaction domain, and a middle region of variable length and amino acid composition that separates the DNA binding domain and the C-terminal domain (see Figure 3B; Ulmasov et al., 1999a, 1999b). The middle region of an ARF determines whether the protein functions as a transcriptional activator or a repressor (Ulmasov et al., 1999a). Not surprisingly, given the observed reductions in auxin-induced gene expression in the *nph4* background (Stowe-Evans et al., 1998), the middle region of NPH4/ARF7 has been shown to function as an activator domain in transient expression assays (Ulmasov et al., 1999a). This latter finding, together with the differential growth-specific loss-of-function phenotypes of *nph4* null mutants, indicates that activation of gene expression by auxin-induced NPH4/ARF7 function is an important component of auxin-dependent localized growth responses.

nph4 Mutants Reveal Functional Properties of the N-Terminal DNA Binding and C-Terminal Protein–Protein Interaction Domains of NPH4/ARF7

Analyses of the *nph4* mutants also provide useful information for deciphering the biological functions of the domains of NPH4/ARF7 identified by biochemical studies (Ulmasov et al., 1999a, 1999b). When assayed in a carrot transient expression system, ARF proteins modulate auxin-dependent transcription either through direct DNA binding by way of the DNA binding domain or through assumed associations with ARFs already bound to DNA by way of the C-terminal protein–protein interaction domain (Ulmasov et al., 1999a). NPH4/ARF7, in particular, was shown to function as a transcriptional activator, whether as a full-length protein or as a

truncated protein in which the DNA binding domain had been deleted (Ulmasov et al., 1999a), suggesting that the C-terminal protein–protein interaction domain plays a critical role in NPH4/ARF7 function in the plant.

The loss-of-function phenotypes of *nph4* mutants carrying alleles containing premature stop codons that truncate the NPH4/ARF7 protein upstream of the C-terminal domain (i.e., *nph4-101*, *nph4-103*, *nph4-104*, *nph4-105*, and *nph4-106*) provide support for the aforementioned hypothesis. However, findings that the phenotypes of such mutants are less severe than those of the apparent null mutant *nph4-1* (Figure 1B; Stowe-Evans et al., 1998) indicate that NPH4/ARF7 retains some activity in the absence of the C-terminal protein–protein interaction domain. A similar conclusion can be drawn from analyses of mutant alleles of the *MP* locus. Although true null alleles of *MP* are presumed lethal (Hardtke and Berleth, 1998), which precludes any direct comparison between partial and complete loss-of-function alleles, a variation in phenotypic severity has been observed among existing *mp* alleles that contain premature stop codons preceding the ARF5 C-terminal domain (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998).

Although removal of the C-terminal domain from several ARFs disrupts stable binding of those ARFs to artificial palindromic auxin response elements in vitro, presumably by abolishing dimerization of the ARF proteins (Ulmasov et al., 1999b), *nph4* alleles lacking the C-terminal domain demonstrate that the DNA binding and the middle region activation domains are sufficient for partial biological response—suggesting either that NPH4/ARF7 dimerization is unnecessary for partial auxin-dependent transcriptional activation or that dimerization can occur at some site outside the C-terminal protein–protein interaction domain in vivo. Many native auxin response elements are actually composite elements, composed of the TGTCTC auxin response element and a constitutive or coupling element rather than simple palindromes (Guilfoyle et al., 1998b). Hence, truncated nph4/arf7 proteins present in the *nph4* alleles that lack the C-terminal domain may be able to form partially functional heterodimers with factors bound to coupling elements.

The *nph4-3* allele, which contains a premature stop codon within the DNA binding domain, is a particularly interesting allele. As might be expected, the phenotypes of this mutant were similar to those of the *nph4-1* null mutant, except for showing a stronger randomized gravitropic growth orientation under all conditions (Figure 5). If we assume that the mRNA shown to be present in the *nph4-3* allele produces a truncated nph4 protein, this observation suggests that the truncated nph4-3/arf7 DNA binding domain can function as a dominant negative inhibitor of auxin-dependent growth. That a nph4-3/arf7 protein would be able to act as a competitive repressor of auxin-induced transcription by direct binding to auxin response elements seems unlikely, however, because the *nph4-3* truncation occurs in the middle of the subdomain shown to be essential for DNA binding of ARF proteins (Ulmasov et al., 1999a). The most plausible explanation for the apparent dominant negative effects of the *nph4-3* mutation is that the truncated nph4-3/arf7 protein interacts with (and thus removes from action) an additional transcriptional activator, which might be either another ARF or a coupling factor, thereby preventing auxin-induced transcription. Although no studies have specifically addressed whether the DNA binding domain also can mediate protein–protein interactions, analysis of the DNA binding properties of ARF1 suggested that such a property might exist. Specifically, the ARF1 protein that lacks the C-terminal protein–protein interaction has been shown to bind more stably to palindromic auxin response elements than to auxin response element half-sites, suggesting that this truncated ARF is binding as a dimer, with the dimerization occurring through the DNA binding domain (Ulmasov et al., 1999b). However, exactly how a partial NPH4/ARF7 DNA binding domain can function in an apparent dominant negative fashion remains to be determined.

Ethylene Acts as a Modulator of Auxin-Dependent Differential Growth

In this study, we have shown that ethylene is able to suppress multiple differential growth defects of *nph4* mutants. These findings suggest that ethylene enhances the activity or sensitivity of a redundant system that is capable of mediating differential growth, presumably a system that involves changes in gene expression. In fact, this redundant system appears to be an auxin response system because further disruptions of auxin responsiveness, whether through mutation or pharmacological manipulation, negated the suppressive influence of ethylene on phenotypes of the *nph4* mutants. For instance, the effects of ethylene on the aphototropic response of *nph4* seedlings were counteracted by mutations in the *AXR1* locus (Figure 7; E.L. Stowe-Evans and E. Liscum, unpublished results). *AXR1* encodes a component of a ubiquitin-activating enzyme (E1) complex that activates ubiquitin-like proteins believed to be involved in the degradation of repressors of auxin responses (reviewed in del Pozo and Estelle, 1999). Previous studies suggested that NPH4/ARF7 and AXR1 function in independent pathways to regulate tropic responses (Watahiki et al., 1999); the results presented here are consistent with those findings and further suggest that AXR1-dependent regulation of differential growth is partially redundant to the NPH4/ARF7–dependent process.

We also failed to observe ethylene-dependent normalization of the phenotypes of the *nph4* mutants when either polar auxin transport or HLS1 activity was disrupted. *HLS1* encodes a putative *N*-acetyltransferase proposed to function as a regulator of local auxin concentrations (Lehman et al., 1996); thus, ethylene-dependent increases in active auxin pools within target cells/tissues might stimulate the activity of a redundant differential growth modulating system. However, activation of such a redundant system did

not appear to grossly alter auxin responsiveness in the *nph4* background, because neither auxin-dependent growth inhibition nor gene expression was much enhanced in response to ethylene treatment.

Previous studies have shown that ethylene can influence both polar (Burg and Burg, 1967; Suttle, 1988; Schwark and Schierle, 1992) and lateral (Burg and Burg, 1966; Lee et al., 1990; Schwark and Schierle, 1992) auxin transport. Moreover, a direct interaction between ethylene and auxin transport recently has been established with the cloning of the *AGRAVITROPIC 1 (AGR1)/ETHYLENE-INSENSITIVE ROOT 1 (EIR1)/PIN FORMED 2 (PIN2)* gene of Arabidopsis (hereafter referred to as *AGR1*), which encodes a transmembrane protein that probably represents a component of a polar auxin efflux system in primary roots (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Mutations in the *AGR1* locus, aside from disrupting polar auxin transport (Chen et al., 1998; Luschnig et al., 1998), result in decreased sensitivity of the root to both endogenous and exogenous ethylene (Bell and Maher, 1990; Roman et al., 1995; Chen et al., 1998). Hence, one target for ethylene action in root growth inhibition appears to be AGR1 function (Palme and Gälweiler, 1999; Rosen et al., 1999).

Presumably, an interaction between ethylene and auxin transport also might be occurring in aerial tissues. If so, ethylene-dependent changes in auxin transport could result in the subtle changes in auxin responsiveness that appear to be necessary for the suppression of differential growth defects in the *nph4* mutants. For example, an increase in local auxin concentration as a result of ethylene-dependent changes in auxin transport might allow for the partial activation of an ARF having a lower sensitivity to auxin. However, because no data are currently available to definitively address this hypothesis of auxin transport, it is equally plausible that the ethylene effects on *nph4* might reflect an enhanced auxin sensitivity of a redundant ARF system through changes in expression or activity of that ARF protein. Both hypotheses ultimately predict that in the absence of ethylene, NPH4/ARF7 is the predominant ARF regulating gene expression in response to auxin in cells of aerial tissues exhibiting differential growth, whereas in the presence of ethylene, one or more additional ARFs also may be functioning.

In summary, we have cloned the *NPH4* gene of Arabidopsis, which when mutated disrupts multiple differential growth responses within aerial tissues, and we found that the gene encodes the auxin-responsive transcriptional activator ARF7. The cloning of this locus and various analyses of mutant alleles provide evidence directly linking auxin-induced gene transcription to auxin-dependent changes in growth. Despite the clear requirement for NPH4/ARF7 in the regulation of differential growth, the conditional nature of the *nph4-1* null allele indicates that one or more partially redundant systems must exist. We anticipate that the *nph4* mutants may be very helpful in elucidating the mechanism by which ethylene and other environmental conditions, such as phytochrome activation (Liscum and Briggs, 1996; E.L. Stowe-Evans and E. Liscum, unpublished results), stimulate the activity of a redundant ARF system(s). In particular, we should be able to identify factors involved in the suppression of the phenotypes of the *nph4* mutants through isolation and characterization of second-site mutations that convert conditional phenotypes into constitutive phenotypes; in turn, we can directly ask whether any such factors are involved in signaling, auxin-transport processes, transcriptional regulation, or other aspects of auxin-mediated differential growth.

METHODS

Plant Materials, Growth Conditions, and Analyses of Seedling Responses

For all experiments, seeds of *Arabidopsis thaliana* were surface-sterilized and plated on nutrient medium solidified with 1.0% agar (w/v), as described previously (Liscum and Briggs, 1995). Murashige and Skoog nutrient medium (Murashige and Skoog, 1962) at one-half strength and without sucrose was used for all experiments except the auxin-dependent hypocotyl growth inhibition and gene expression studies, for which full-strength Murashige and Skoog medium supplemented with 2.0% (w/v) sucrose was used. Cold treatment and exposure to red light to induce uniform germination were as described previously by Liscum and Briggs (1995). Unless otherwise noted, ethylene and auxin treatments were performed as described previously (Stowe-Evans et al., 1998).

Phototropic responses were measured as described previously for hypocotyls of dark-grown Arabidopsis seedlings (Liscum and Briggs, 1995). Hypocotyl growth orientation was measured in dark-grown seedlings as described by Liscum and Hangarter (1993). Auxin-dependent hypocotyl growth inhibition was determined as described previously (Stowe-Evans et al., 1998), except that where appropriate, 1-aminocyclopropane-1-carboxylic acid (ACC) was included during the first 3 days of growth, after which time all seedlings were transferred to ACC-free plates containing indole-acetic acid (IAA).

Ethylene Measurement

For each treatment, seedlings were grown on a 10-mL Murashige and Skoog nutrient medium–agar plug inside a 44-mL glass vial sealed with a screw cap containing a Teflon septum. After cold treatment and red light exposure to induce uniform germination of seeds (Liscum and Briggs, 1995), the vials were wrapped in foil and transferred to a light-tight box. Seedlings were allowed to grow for 3 days, after which 30 mL of headspace air was removed from each vial and simultaneously replaced with ethylene-free air. The sample removed was concentrated in a cold trap, released by heating with boiling H_2O, and passed onto a gas chromatograph for ethylene determination (Spollen et al., 2000). The number of seedlings present in each vial was determined and used to calculate the data presented in Figure 4C.

Fine-Structure Mapping and Cloning of the *NPH4* Locus

A recombinant population was generated by crossing the *nph4-1* mutant in the Columbia (Col) ecotype to wild-type Landsberg *erecta* (L*er*), allowing the F_1 plants to self-pollinate, and selecting aphototropic plants (*nph4-1/*−) from the resulting F_2 progeny. Because *nph4* segregates as a semidominant trait (Stowe-Evans et al., 1998), the phototropic response was rechecked in the F_3 generation to eliminate heterozygotes from the mapping population. Genomic DNA was prepared (Edwards et al., 1991) from 240 recombinant *nph4-1/nph4-1* homozygotes and then used for polymerase chain reaction (PCR)–based mapping as described.

Amplified fragment length polymorphism (AFLP)–based mapping was performed as described elsewhere (Liscum, 1999). The tightly linked AFLP fragment RH48.73 (see Figure 2A) was isolated from acrylamide gels (Sambrook et al., 1989) and reamplified with oligonucleotides homologous with the AFLP adapters but having 5′ extensions containing a restriction enzyme recognition site that had been engineered into them. EcoRI-adapter sequences were primed with an oligonucleotide containing an EcoRI site (5′-GCGGAATTCCTC-GTAGACTGCGTACCAATTC-3′), and MseI-adapter sequences were primed with an oligonucleotide containing an XbaI site (5′-CGTCTA-GAGACGATGAGTCCTGAGTAA-3′). The resulting PCR products were double-digested with EcoRI and XbaI, subcloned into compatible sites within pBluescript SK+ (Stratagene, La Jolla, CA), and sequenced. RH48.73-specific oligonucleotides (5′-GTAAAGCTGTGT-TGATGATA-3′ and 5′-GAATACAAATATCTATCTGAGC-3′) then were generated and used to screen a pooled yeast artificial chromosome DNA library (Creusot et al., 1995) by PCR.

All additional mapping utilized the newly identified ML1–simple nucleotide polymorphism and ML2–simple sequence length polymorphism (SSLP) markers and a modified NIT4-cleaved amplified polymorphic sequence marker. ML1 was identified by comparing the sequence of L*er* genomic DNA with the corresponding Col sequence represented by the proximal end of the P1 clone, MTM4 (see http://www.kazusa.or.jp/arabi/chr5/pmap/P1_map_7.html and http://www.kazusa.or.jp/arabi/endseq/). Ecotype-specific ML1 products were amplified by PCR (26 cycles with 30 sec of denaturation, annealing, and extension and an annealing temperature of 56°C) by using ecotype-specific forward primers differing by just the simple nucleotide polymorphism at the 3′ terminal position (Col-specific oligonucleotide: 5′-CACATAATCGAGCTGCCTCC-3′; L*er*-specific oligonucleotide: 5′-CACATAATCGAGCTGCCTCG-3′) and a common reverse primer (CCATAGGCCATCGAGAGTTTC). ML2 represents a CA dinucleotide repeat identified on the distal end of P1 clone, MQJ16 (see http://www.kazusa.or.jp/arabi/chr5/pmap/P1_map_7.html and http://www.kazusa.or.jp/arabi/endseq/), which is polymorphic between the Col and L*er* ecotypes. PCR amplification with ML2-specific primers (5′-GAGGTTTATGGATTCGTAGACA-3′ and 5′-TTAGGAACAAAAGCA-GGATTAG-3′) and subsequent product resolution on 4.0% (w/v) agarose gels were performed as described previously for other SSLP markers (Bell and Ecker, 1994). New *NIT4*-specific primers (5′-GATTTCAACTGCTCCACAAGAC-3′ and 5′-TTGATGATGAACGGA-AACTATAAA-3′) were designed to allow for the amplification of a 405-bp PCR product (containing a single MboII site) in L*er* products that is missing in Col products.

RNA preparation and gel blot analyses were performed as described by Ausubel et al. (1995). DNA gel blot analysis was performed as described by Evola et al. (1986). Sequence comparisons of NPH4 genomic sequence (GenBank accession number AF186466) with database entries were performed by using GappedBLAST (Altschul et al., 1997). Sequence analyses, cDNA and genomic sequence alignments, and amino acid sequence predictions were performed using the DNASTAR software package (DNASTAR, Madison, WI).

Reverse Transcription–PCR Analysis

For expression analysis, total RNA was extracted from 2.5-day-old dark-grown seedlings by using an RNeasy plant minikit (Qiagen, Chatsworth, CA). *NPH4* and *PHYTOCHROME E* (*PHYE*; Clack et al., 1994) transcripts were assayed simultaneously in separate tubes with their respective gene-specific primers by using identical RNA aliquots. Reverse transcription–PCR (RT-PCR) reactions by using 100 ng of total RNA per sample (or 10 ng of genomic DNA as a control) were performed with the Access RT-PCR system (Promega), according to the manufacturer's directions. Amplifications were done for 30 cycles with an annealing temperature of 54°C and an extension time of 4 min. Products then were separated by electrophoresis on an 0.8% (w/v) agarose gel, blotted, and hybridized with ^{32}P-labeled gene-specific DNA probes.

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