Arabidopsis NPH3: A NPH1 Photoreceptor–Interacting Protein Essential for Phototropism

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Phototropism of *Arabidopsis thaliana* seedlings in response to a blue light source is initiated by nonphototropic hypocotyl 1 (NPH1), a light-activated serine-threonine protein kinase. Mutations in three loci [NPH2, root phototropism 2 (RPT2), and NPH3] disrupt early signaling occurring downstream of the NPH1 photoreceptor. The NPH3 gene, now cloned, encodes a NPH1-interacting protein. NPH3 is a member of a large protein family, apparently specific to higher plants, and may function as an adapter or scaffold protein to bring together the enzymatic components of a NPH1-activated phosphorylase.

Plants are able to sense and respond to changes in light quality, quantity, and direction through the action of a number of photoreceptors and associated signal-response systems. A variety of photoreceptor molecules, such as the red or far-red light–absorbing phytochromes (phy) and blue light–absorbing cryptochromes (cry) (1, 2) have been characterized. Components functioning downstream of such photoreceptors include two photoreceptor-interacting proteins PIF3 and PKS1, which are both phytochrome-interacting proteins (3). Potential postperception signaling components identified through mutational analyses (1, 2) include a set of genes in *Arabidopsis* (NPH2, RPT2, and NPH3) that is required for phototropism, or the bending response of plant organs toward or away from directional light stimuli (4, 5).

Null mutations in the NPH3 locus abolish phototropic responses of etiolated seedlings to blue light at a low fluence rate (4) (Fig. 1A). A similar aphototropic phenotype is observed with seedlings carrying null mutations at the NPH1 locus (4) (Fig. 1A), which encodes NPH1, a primary photoreceptor for phototropism (6–8). However, mutations in neither NPH1 nor NPH3 affect other light-dependent responses, such as blue light– and red light–dependent hypocotyl growth inhibition (Fig. 1, B and C). Moreover, despite efforts to identify additional alterations in development, the only clear phenotypic changes observed in *nph1* and *nph3* mutants are those associated with phototropism (9). The phototropism-specific phenotypes of the *nph1* and *nph3* mutants imply that NPH1 and NPH3 act in the same genetic pathway and suggest that NPH3 may function biochemically close to the photoperception event mediated by NPH1.

We have cloned the *NPH3* gene by positional cloning (Fig. 2A). Several predicted genes mapping to the region containing the *NPH3* locus (Fig. 2A) were sequenced in mutant backgrounds to identify the *NPH3* gene (Fig. 2B). A full-length *NPH3* cDNA was isolated (10) and found to encode a protein of 745 amino acids (11) (Fig. 2C). *NPH3* is, however, part of a family of proteins in *Arabidopsis* (12). Although *NPH3*-related sequences have also been found in other plant species, no paralogous sequences have been found outside the plant kingdom (9).

Four regions of sequence conservation have been identified within the *NPH3* family (12) (Fig. 2C). Region IV exhibits the highest level of sequence identity within the family (≥52.5%, relative to *NPH3*), with two motifs being most prominent: LYRAID and HAAQNERLPL (Fig. 2C). The functional importance of these conserved sequence motifs is currently unknown. However, Tyr<sup>434</sup> within the LYRAID motif is part of a conserved phosphorylation site ([RK]-x(2,3)-[DE]-x(2,3)-Y; where x(2,3) can be any two or three amino acids) (14), in which the Arg, Asp, and Tyr are invariant across the entire *NPH3* family (12), and deletion of this residue in the *nph3-2* mu-

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**Fig. 1.** Physiological characteristics of *nph1* and *nph3* mutants. (A) Hypocotyl phototropism in 3-day-old etiolated wild-type (WT) and mutant seedlings exposed to 8 hours of unilateral blue light (25). (B) Blue light– and (C) red light–dependent hypocotyl growth inhibition (25) in wild-type and mutant seedlings. The *cry1-101* (26) and *phyb-9* (27) mutants are shown as negative controls for (B) and (C), respectively. Because symbols often overlap, some symbols and error bars (standard error) are not visible.

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REPORTS

Fig. 2. NPH3 cloning, gene structure, and amino acid sequence. (A) Mapping of NPH3 on chromosome 5. Initial mapping placed NPH3 between simple sequence length polymorphism (SSLP) marker nga129 (28) and restriction fragment length polymorphism marker g2368 (29). Sequence of phagemid P1 clone, MSJ1 (AB008268), was used to generate two SSLP markers, AM40 and AM80 (30), which were used to narrow the potential NPH3 coding region to three predicted genes: msj1.9, msj1.10, and msj1.11 (31). msj1.10 was identified as NPH3 by sequencing multiple nph3 mutant alleles (24). T3J14 is a BAC clone (22). Numbers of recombinants for each marker are shown on the bottom. cM, centimorgan. (B) Structure of the NPH3 gene and positions of nph3 mutations. The locations of start (ATG) and stop (TGA) codons are indicated. Exon (boxes) and intron (lines) positions were determined by a comparison of the genomic and cDNA sequences. Position and identity of various nph3 mutations are indicated. (C) Deduced amino acid sequence (73) of NPH3. Amino acid residues are numbered at the right. Bold-faced residues indicate regions of sequence conserved across the NPH3 family (12). Motifs of highest sequence conservation are boxed. The BTB/POZ and coiled-coil domains are underlined with single and double lines, respectively. Locations of nph3 mutations are indicated by asterisks. In the nph3-1 allele, amino acids 272 to 295 are replaced with residues (LHIGYFSLKHFHKHLVYISQSSIH) before insertion of a stop codon at position 296.

Fig. 3. Plasma membrane localization and in vivo modification of NPH3. (A) Immunoblot analysis of NPH3 localization after cell fractionation (32) of 3-day-old seedlings that were grown in the dark. Samples containing 50 μg of soluble (Sol), 15 μg of microsomal membrane (MM), or 1.5 μg of plasma membrane (PM) protein were resolved by SDS-PAGE and blotted to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for subsequent immunodetection of NPH3 (left two panels) and NPH1 (right panel) proteins (21, 33). nph3-1 microsomal membranes represent a negative control for NPH3 antibody specificity, because any NPH3 protein in this mutant lacks the COOH-terminus (see Fig. 2B), which was used as antigen to generate the antisera (33). Molecular masses (in kilodaltons) are indicated at the left. Arrow and asterisk indicate the position of the NPH3 and NPH1 proteins, respectively. (B) In vivo modification of NPH3. Three-day-old healthy wild-type seedlings were grown in the dark were mock irradiated (D), or exposed to 10^3 μmol m^-2 of red (R) or blue (B) light (25), before the preparation of cell fractions (22). Fifty micromolars of soluble protein (B-Sol lane) or 15 μg of microsomal membrane protein (all other lanes) were resolved with SDS-PAGE and blotted to PVDF membrane; and NPH3 protein was visualized by immunodetection (23). Molecular masses (in kilodaltons) are indicated at the left. Arrow and asterisks (double asterisks indicate a change specific to the nph1-5 genotype) indicate the positions of the "unmodified" and "modified" NPH3, respectively, relative to NPH3 from seedlings that were grown in the dark. Although increased mobility of NPH3 in membranes of blue-light-treated wild-type seedlings appears to result from dephosphorylation, limited proteolysis may also contribute to mobility changes observed in nph1-5 membranes (9).
A similar enhanced mobility of NPH3 was observed in membranes from nph1-5 mutant seedlings (Fig. 3B) that were grown in the dark (6, 7). One interpretation of these observations is that NPH3 normally interacts with NPH1 in seedlings that were grown in the dark, either directly or indirectly through a protein complex, preventing its modification by other factors, whereas blue light–induced changes in NPH1, such as autophosphorylation (7), or simple removal of NPH1, expose sites on NPH3 for modification.

Given the apparent genetic coupling of their protein activities (4) (Fig. 1), colocalization to the plasmalemma (6, 7) (Fig. 3A), and potential interaction suggested by in vivo irradiation studies (Fig. 3B), we used a yeast two-hybrid assay (19) to directly test whether NPH1 and NPH3 physically interact. Although all combinations of NPH1 and NPH3 fragments shown in Fig. 4A were tested, significant interactions were only observed between coiled-coil–containing NPH3 polypeptide fragments and the N-terminal two-thirds of NPH1 (Fig. 4B), which contains the chromophore-binding LOV (light, oxygen, or voltage) domains (6, 7). Two hybrid results were confirmed by in vitro interaction studies, in which we assayed the ability of a radially located N-terminal NPH1 fragment to be immunoprecipitated by antibodies against a cellulose binding domain (CBD), in the absence or presence of nonlabeled CBD-NPH3 fusion proteins (Fig. 4C). Although CBD antibodies were incapable of precipitating NPH1 itself, a NPH1-containing immunoprecipitate was obtained when coiled-coil–containing NPH3-CBD fusion protein (NPH3C2-CBD) was included in the precipitation reaction (Fig. 4C). The NPH3C2-CBD–dependent immunoprecipitation of NPH1 was not the result of nonspecific interaction with CBD, as a BTB/POZ-containing NPH3-CBD fusion protein did not promote a similar immunoprecipitation response (Fig. 4C). The flavin mononucleotide (FMN)–dependent interaction of NPH1 and NPH3 (Fig. 4C) likely reflects a conformational stabilization of the NPH1 holoprotein in relation to the apoprotein alone, a feature common to many cofactor-containing proteins, including flavin-binding proteins (20).

Together, all of the results presented here indicate that NPH3 interacts with the chromophore-binding portion of NPH1, probably through its coiled-coil region, and that this protein complex is necessary for early phototropic signaling. The biochemical function of NPH3, however, remains unknown. In animal and fungal systems, multimolecular signaling complexes using protein kinases and phosphatases are often assembled around a class of proteins known as adapter or scaffold proteins, which contain multiple protein-protein interaction domains (21). NPH3 contains at least two protein-protein interaction domains, one of which promotes interaction with NPH1, and early phototropic signaling probably occurs through a NPH1–activated phosphorelay (2, 4). Thus, NPH3 may function as an adapter or scaffold protein in plants. Given their homologies to NPH3, other members of the NPH3 family may also function as adapter or scaffold proteins. Protein complexes assembled by the NPH3 family could provide a means of optimizing speed, specificity, and selectivity of early signaling events associated with a variety of physiological responses, while minimizing undesired cross talk with other response pathways. The identification of such a class of proteins could have broad implications for studies of plant signal transduction.

References and Notes

9. A. Motchoulski and E. Liscum, unpublished data.
10. A 6.4-kb Afe I fragment of NPH3, isolated from bacterial artificial chromosome (BAC) clone T314 [22], was used to probe size-selected cDNA libraries [J. J. Kieber, M. Rothenberg, G. Roman, K. A. Feld-
Four Evolutionary Strata on the Human X Chromosome
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Human sex chromosomes evolved from autosomes. Nineteen ancestral autosomal genes persist as differentiated homologs on the X and Y chromosomes. The ages of individual X-Y gene pairs (measured by nucleotide divergence) and the locations of their X members on the X chromosome were found to be highly correlated. Age decreased in stepwise fashion from the distal long arm to the distal short arm in at least four "evolutionary strata." Human sex chromosome evolution was probably punctuated by at least four events, each suppressing X-Y recombination in one stratum, without disturbing gene order on the X chromosome. The first event, which marked the beginnings of X-Y differentiation, occurred about 240 to 320 million years ago, shortly after divergence of the mammalian and avian lineages.

The human X and Y chromosomes, like those of other animals, are thought to have evolved from an ordinary pair of autosomes (J). The pseudoautosomal regions at the termini of the X and Y chromosomes still recombine during meiosis, ensuring X-Y nucleotide sequence identity there. Elsewhere on the X and Y chromosomes, however, X-Y recombination has been suppressed. These nonrecombining regions of the X and Y chromosomes have become highly differentiated during evolution, and only a few X-Y sequence similarities persist within them. These modern X-Y gene pairs are the remaining "fossils" where extensive sequence identity between ancestral X and Y chromosomes once existed. The recent discovery of many X-Y genes has made it possible to examine the entire group to search for patterns of human sex chromosome evolution. Thus far, the human sex chromosomes—the best characterized mammalian sex chromosomes—have been found to contain 19 X-Y gene pairs (2).

We first compared the locations of all 19 pairs of genes on the human X and Y chromosomes (Fig. 1). We determined the relative positions of the X-linked genes through radiation hybrid analysis, in many cases confirming previously published localizations (J). Map positions of the Y-linked homologs were obtained by gene order on the X chromosome. By contrast, on the Y chromosome, the X-Y gene pairs are stratified by age along the length of the Y chromosome. Thus far, the four Ks-defined groups of genes are arranged in an orderly sequence (Fig. 2). X-Y genes are stratified by age along the length of the X chromosome. By contrast, on the Y chromosome, the Ks-defined groups appear to be scrambled (compare Table 1 and Fig. 1).

What might account for the orderly stratification of X-Y genes by age on the human X chromosome? We hypothesize that, during evo-