EID1, an F-box protein involved in phytochrome A-specific light signaling

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To perceive red and far-red light, plants have evolved specific photoreceptors called phytochromes. Even though the spectral properties of all phytochromes are very similar, they show a distinct mode of action. Here we describe EID1, a negatively acting component of the signaling cascade that shifts the responsiveness of the phytochrome A (phyA) signaling system associated with hypocotyl elongation from red to far-red wavelengths. EID1 is a novel nuclear F-box protein that contains a leucine zipper whose integrity is necessary for its biological function. EID1 most probably acts by targeting activated components of the phyA signaling pathway to ubiquitin-dependent proteolysis.

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Plants are sessile organisms that are forced to adapt to a given environment. Therefore, compared with animals, plants have achieved an enormous developmental plasticity that makes possible very flexible responses to diverse exogenic factors. Among these exogenic factors, light is probably one of the dominating signals that controls gene expression and development throughout all phases of the plant life cycle (Neff et al. 2000). To perceive red and far-red light, plants use the phytochrome family of photoreceptors that comprises five members [phytochromes A–E (phyA)] in Arabidopsis. All phytochromes can exist in two different conformers: the red light-absorbing, biologically inactive Pr form and the far-red light-absorbing, biologically active Pfr form. Because both forms can be photoconverted, phytochromes can function as red/far-red photoreversible light switches when exposed to pulses of red and far-red light (Mancinelli 1994; Neff et al. 2000).

Even though the spectral properties of all phytochromes are very similar (Eichenberg et al. 2000), they show a distinct mode of action. The products of the PHYB, PHYC, PHYD, and PHYE genes remain light stable, with the PHYB gene showing the most expression. As expected, photoreponses triggered by the light-stable phytochromes show their highest sensitivity in red light and follow the classical red/far-red photoreversibility [Neff et al. 2000]. In contrast, phyA accumulates to a high level in the dark and its Pfr form is rapidly degraded. PhyA is responsible for the so-called very low fluence responses and exhibits its highest effects under strong continuous far-red light that induces the so-called high-irradiance responses [HIRs]. The HIR effect is very important for the regulation of plant development in canopy shade, but it shows many aspects that are difficult to explain by the spectral properties of a photoreversible phytochrome alone. First, the action spectrum of HIR reveals a maximum at ∼720 nm, at which wavelength the responses of the light-stable phytochromes become completely inhibited. Furthermore, the extent of the HIR depends on photon fluence rates instead of photon fluences. Thus, the respective signaling cascade has not only to sense the total amount of photons that reach the tissue, but must also be able to detect the amount of photons that reaches the plant in a given time (Mancinelli 1994; Neff et al. 2000).

Several approaches have been undertaken to identify components of the phytochrome signaling cascade. Pharmacological and microinjection studies indicate an involvement of heterotrimeric G proteins, cGMP, and calcium/calmodulin in phytochrome signaling (Neuhaus et al. 1993; Bowler et al. 1994). Yeast two-hybrid screenings revealed several components, including PIF3, PKS1, and NDPK2 (Neff et al. 2000), that interact with both phyA and phyB. PIF3 is a basic helix-loop-helix [bHLH] protein that can bind to G-box DNA motifs present in many light-regulated promoters (Martinez-Garcia et al. 2000).

Genetic approaches have resulted in the isolation of Arabidopsis mutants, which exhibit different light-dependent phenotypes [Hardtke and Deng 2000; Neff et al. 2000]. Until now, at least eight independent mutants have been identified that are defective in phyA-dependent responses. So far, FAR1, FIN219, PAT1, HFR1, REP1, and RSF1 have been characterized at the molecular level (Hudson et al. 1999; Bolle et al. 2000; Fairchild et al. 2000; Hsieh et al. 2000; Soh et al. 2000; Spiegelman et al. 2000). HFR1, REP1, and RSF1 are allelic and code for a bHLH protein similar to PIF3. The biochemical function of the other proteins is not defined. In contrast to the large number of loss-of-function mutants, only two mutants, spa1 and eid1, show enhanced phyA-dependent responses (Hoecker et al. 1999; Büche et al. 2000) and thus most probably encode for negatively acting components of phyA signaling. SPA1 contains WD40 repeats and exhibits some weak homology with protein kinases, but its biochemical function also remains to be elucidated.

In an attempt to screen for new phyA-specific mutants, we have isolated the recessive eid1 alleles that show a strictly phyA-dependent phenotype and an alteration in all analyzed HIRs (Büche et al. 2000). The eid1 mutation results in an extremely enhanced light sensitivity and an increased temporal persistence of the HIR, which indicates an important role in photon fluence rate measurement. Thus, EID1 seems to play a key role as a repressor in HIR.
signaling. Here we describe the cloning and the functional characterization of EID1. EID1 is a nuclear localized F-box protein that shifts the responsiveness of the phyA signaling system associated with hypocotyl elongation from red to far-red wavelengths. It most probably acts by targeting activated components of the phyA signaling cascade to ubiquitin-dependent proteolysis.

Results

Cloning of the EID1 gene

To clone the EID1 gene, we followed two different strategies: screening for new eid1 alleles in T-DNA lines and positional cloning. The increased light sensitivity observed in eid1 has been used to screen for new alleles. Under the applied low fluence rates of continuous red and far-red light, eid1 mutants exhibited a strong photomorphogenic development with open cotyledons and a reduced hypocotyl growth, whereas wild-type seedlings remained nearly etiolated (Fig. 1A,B). This phenotype was strictly light dependent because all of the mutant lines exhibited an etiolated phenotype in darkness, with elongated hypocotyls and closed cotyledons on an apical hook (Fig. 1C). One T-DNA tagged line (eid1-4) belonging to the eid1 complementation group was used to clone flanking genomic sequences by inverse PCR. The genomic fragments belong to a gene located at about 55 kbp from the lumi

Figure 1. Phenotype of 4-d-old wild type, eid1 mutant, and transgenic seedlings under different light conditions. (WS) Wassilewskija wild type; (Col) Columbia wild type. eid1-1 and eid1-2 were isolated from the phyB-5 mutant in the Landsberg erecta (Ler) background. The eid1-5, eid1-4, and eid1-5 alleles were derived from a WS background and the eid1-6 from a Col background. Scale bars, 3 mm. (A) Seedlings grown under the weak red-light field used for screening. (B) Seedlings grown under the weak far-red light field used for screening. (C) Etiolated seedlings grown in darkness. (D) Phenotype of wild-type, eid1-3, and rescued eid1-3 lines under the weak red light used for screening. The phenotype of independent transgenic lines transformed with a 3SS-promoter–EID1-ORF–nos-terminator construct [P3SS] or a genomic EID1 fragment [gene] is shown.

gene on chromosome 2. This close proximity to the lumi

nidependens gene fits well with the data of the fine mapping because no recombinants with the respective marker were detectable after the analyses of 732 chromatids. Each of the six eid1 alleles isolated so far showed a mutation in an intronless open reading frame (ORF) having a length of 1008 bp (Fig. 2A). Northern blots revealed one band of −1350 nucleotides, corresponding to the size of the ORF of the intronless EID1 gene and the longest isolated EID1 EST (data not shown).

For complementation analysis, the strong eid1-3 mutant was transformed with a 3SS-promoter–EID1-ORF–nos-terminator construct and a genomic fragment that includes the putative promoter and the terminator of EID1. Transgenic eid1-3 lines exhibited a clearly reduced light sensitivity and they appear like wild-type seedlings under selective light conditions (Fig. 1D). Thus, the EID1-ORF and the genomic fragment were sufficient to repress the hypersensitive phenotype in the eid1-3 mutant. Therefore, we conclude that the respective DNA sequence codes for the EID1 gene product.

EID1 is a novel type of F-box protein

The deduced EID1 protein can be subdivided into at least five different domains [Fig. 2A]. The most N-terminal domain shows homology with F-box proteins [Fig. 2B]. SCF [Skp1, Cdc53, and F-box] proteins are the major components of the so-called SCF complexes that function as ubiquitin ligases. SCF complexes are involved in the proteasome-dependent degradation of many regulatory proteins in yeast, animals, and plants [Craig and Tyers 1999].

A leucine zipper pattern is adjacent to the F-box motif [Fig. 2A]. Leucine zipper domains facilitate the homo- or heterodimerization of polypeptides [Busch and Sassone-Corsi 1990], and they are present in different families of transcription factors that also play an important role in the light signaling of plants [Hardtke and Deng 2000; Nell et al. 2000]. The functional significance of the respective domain in EID1 is underlined by the mutation in the weak eid1-2 allele, which results in a glycin to cysteine transition immediately in front of the second leucine residue of the zipper pattern. To our knowledge, EID1 is the first F-box protein reported to contain a leucine zipper that seems to be necessary for its function.

The C-terminal part of EID1 has no significant homology with known proteins. It contains a highly acidic domain followed by a basic domain. The functional importance of the C-terminal part is emphasized by the phenotype of the eid1-3, eid1-5, and eid1-6 alleles that show structural alterations or deletions in this area [Fig. 2A].

EID1 interacts with ASK1 and ASK2

The F-box protein function of EID1 is further underlined by the results of a yeast two-hybrid screening by using the full-length polypeptide fused to the GAL4 binding domain as bait. From 534 positive clones, ~95% were identical to ASK1 and one clone was identified as being ASK2. ASK1 and ASK2 are two Arabidopsis homologs [Arabidopsis SKP1-like] of the yeast Skp1 protein [Gray et al. 1999].
TodemonstratethattheF-boxdomainofEID1mediates interactionwithASK1andASK2,twodifferentmutated constructsweretested. FortheEID1/H9004Fconstruct,thefirst 54aminoacidscontainingtheF-boxdomainweredeleted, andfortheEID1P10Amutant, thehighlyconservedproline aminoacidatposition10waschangedtoanalanineresidue. BothmutationsabolishedtheinteractionofEID1 withASK1andASK2inatwo-hybridassay(Fig.3A).

The resultsofthetwohybridassayscouldevetrifiedby pull-downexperimentsbyusingfusionproteinsofGST to wild-typeEID1oritsmutatedforms. TheGSTfusion proteinswereexpressedinEscherichia coli andboundto Glutathione-S-Sepharose, [35S]methionine-labeledASK1and ASK2polypeptideswereaddedandbindingwasanalyzed by SDS-PAGE andautoradiography. ASK1and ASK2 showed a strong interactionwiththechimeric GST–EID1polypeptide [Fig. 3B]. The interaction was clearly reduced or abolishedwiththe mutated GST–EID1F and GST–EID1P10A fusion proteins. No interactionswere detectedwithGST, theemptyGlutathione-S-Sepharose, andthelabeled Luciferaseas negativecontrols.

EID1is localized in the nucleus

Translational fusions to green fluorescent protein (GFP) were used to investigate the subcellular localizationofEID1. In transientlytransformedparsley protoplasts, EID1–GFP appeared homogeneouslydistributed in the nucleus in dark- and light-treatedprotoplasts (Fig. 4A). In contrast, cytoplasmic localizationbecame detectablewiththeEID1mNLS–GFPproteinfour whichthe first two basicamino acids in the basic domain were replaced by serine and glutamate residues [Fig. 4B]. This result clearly demonstrates that the basic domain in EID1 is a functional nuclear localization sequence (NLS).

EID1 is necessary for a far-red light shift in the phyA-dependent regulation of hypocotyl elongation

The eid1mutants exhibited an extremely enhanced sensitivity in a broad-banded red light field as long as phyA was not completely degraded at higher fluence rates. Analysis of eid1 phyA double mutants further confirmed that the observed hypersensitivity in red and far-red light is strictly phyA-dependent (Bu¨che et al. 2000). To test for the spectral sensitivity in greater detail, we determined action spectra for hypocotyl elongation in phyB-5 and phyB-5eid1-1 mutants under low lightfluence rates. phyB-5mutants were used to exclude responses in the red-light region of the electromagnetic spectrum that are predominantly regulated byphyBin Arabidopsis. A typical HIR action spectrum with a maximum at 716 nm was obtained for phyB-5seedlings [Fig. 5A]. In contrast, the action spectrum with the phyB-5 eid1-1mutant was completely altered [Fig. 5B]. Even though the eid1mutant exhibited a 23-fold increase in light sensitivity at 716 nm, the maximum light response was detectable around 670 nm, where it exhibited a 1170-fold higher value for relative photon effectiveness. The shape of the phyB-5 eid1-1 action spectrum is very similar to the absorption cross-section of the inactive Prform of the photoreceptor and thus, to the cross-section expected for the formation of the physiologically active Pfr form [Fig. 5B].

Discussion

This report describes the identificationof the EID1 gene, a negatively acting component that plays an important role in phyA-specific regulation of plant photomorphogenesis. The intronlessgene was isolated by fine mapping and T-
DNA tagging. The identity of the gene was verified by the presence of mutations in the coding region of all six independent \textit{eid1} alleles and by complementation of the \textit{Eid1} phenotype. The apparent length of the \textit{EID1} transcript in Northern blots fitted to the expected size of the longest EST.

Because the spectral properties of the different types of phytochromes are almost identical (Eichenberg et al. 2000), they should exhibit their highest physiological responses in red light that leads to the formation of a high Pfr level. However, phyA clearly differs from all other phytochrome types because it is most effective under strong far-red light and loses its activity under continuous red light. We could demonstrate that, in the absence of \textit{EID1}, the light-labile phyA can function as a red-light photoreceptor under continuous irradiation similar to the light-stable types of phytochrome. To our knowledge, this is the first example that a component of a signaling cascade leads to a spectral shift in the sensitivity of a photoreceptor. Usually in the eye and all known other systems the spectral diversity of photoreceptor molecules is obtained by structural modifications that result in an altered absorption spectra. To allow efficient perception of far-red light, plants followed an alternate strategy. They suppress the signal following irradiation with high fluence rates of red light by phyA degradation (B"uche et al. 2000) and use \textit{EID1} to suppress phyA responses at low fluence rates.

In this context it is highly interesting that \textit{EID1} encodes an F-box protein that interacts with the \textit{Arabidopsis} Skp1 homologs ASK1 and ASK2. Thus, \textit{EID1} is most probably a component of an SCF complex. SCF complexes function as ubiquitin ligases in different signaling pathways, whereby the F-box component has been proposed to provide substrate specificity (Craig and Tyers 1999). Because the level and the degradation of phyA was not altered in \textit{eid1} mutants, a direct function in the proteolysis of the photoreceptor can be excluded (B"uche et al. 2000). \textit{EID1} most probably acts by targeting other positively acting components of the phyA signaling pathway to degradation. Good candidates would be positively acting, nuclear localized components of the phyA signaling pathway such as FAR1 and the bHLH transcription factor HFR1 (Hudson et al. 1999; Fairchild et al. 2000). In most systems, the F-box proteins exclusively interact with the phosphorylated forms of their target proteins. Because protein phosphorylation most probably also plays an important role in phytochrome signaling (Fankhauser et al. 1999; Hardtke and Deng 2000; Watson 2000), it is worthwhile to speculate that \textit{EID1} only interacts with its targets after a Pfr-dependent modification. If this modification also leads to a functional activation, this mechanism would result in an antagonism between degradation and activity. \textit{EID1}-dependent proteolysis might eliminate all activated signaling intermediates at low Pfr amounts formed under weak light. Under strong

![Figure 3](image-url)
EI1, an F-box protein in light signaling

Figure 5. Action spectra for hypocotyle elongation in phyB-5 and phyB-5 eid1-1 seedlings. Fluence rate response curves were measured at different wavelengths and the fluence rate that led to 40% inhibition compared with dark controls was determined. The value obtained for phyB-5 at 716 nm (0.12 nmol/m2/sec) was set to 1 and the relative photon effectiveness for phyB-5 and phyB-5 eid1-1 at different wavelengths was calculated. The determined values are symbolized by squares or circles connected with a bold line. Photoconversion cross-sections are plotted as thin lines by using published data for purified oat phytochrome [Mancinelli et al. 1994]. (A) Action spectra for phyB-5. (B) Action spectra for phyB-5 eid1-1.

Materials and methods

Plant material, light conditions, and characterization of mutants

Seeds of the Wassilewskaja wild type were mutagenized by imbibition in ethyl methanesulfonate as described by Büche et al. (2000). T-DNA lines were obtained directly from INRA or from the Nottingham Arabidopsis Stock Center. For light experiments, seedlings were grown on paper for 4 d. For screenings, standard light fields were used and fluence rates were adjusted to 15 nmol/m2/sec with red light and 140 nmol/m2/sec with far-red light [Büche et al. 2000]. Action spectroscopy was performed as described by Schäfer and Fukshansky (1984). To obtain monomeric phyA, far-red light, DIL and DEPIL interference filters (Schott, Mainz, Germany) together with modulated Leitz Prado 500-W projectors [Leitz, Wetzlar, Germany] and Osram Xenophot Longlife lamps (Osram, Berlin, Germany) were used. Fluence rates were measured by using a J16 photometer and a J6512 radiant energy probe (Tektronix, Beaverton, USA).

New hypersensitive mutants were crossed with eid1-3 to test for complementation. T-DNA flanking sequences in eid1-4 were isolated by inverse PCR by using the primers 5′-CCACCTGCATATAGCCGAC GGTATGCC-3′ and 5′-TAAGGGATACCACTCGATGGAAGC-3′ that anneal to the T-DNA of the INRA lines [Bouchet et al. 1993]. To check for mutations in the EID1 gene, we amplified genomic DNA by PCR by using two different primer pairs: 5′-AAGCTTAAAGACATTTCCCG GTTACCGC-3′ together with 5′-GTTTGGATACCGACCTTGCTAC GTTCCTA-3′ [EID1-N primer], and 5′-GCGGCTTTGGTTCTCCTG GTCTCCTCCC-3′ [EID1-C primer] together with 5′-TCTGCTCAGAA AGTAAGAAGGAGGTC-3′. DNA fragments were sequenced directly. Mapping was done as described by Konieczny and Ausubel (1993). For DNA extraction we used a DNeasy plant mini kit (QIAGEN).

Construction of mutated EID1 fragments

To create the basal construct, we amplified the EID1 coding region from genomic DNA by PCR by using the 5′ BarnHI-NcoI primer (5′-TTGGATCATCGGGGCAATTCTCCTCTCTTC-3′) and the 3′ SalI primer (5′-AGAT GTCAAAGTGGAGCTGGAGAAGAATGAAAG-3′). The BarnHI–SalI fragment was cloned into the pluebscriptSK (Stratagene) vector and sequenced. All further mutations were introduced by PCR and the subcloned fragments were sequenced again. The mutated F-box constructs were obtained by using the EID1-N primer together with the oligonucleotide 5′-ATCCATGCGAAATTCTCCTCTCTTC-3′ and the SalI–NcoI restriction sites [Koncz et al. 1994]. For the rescue fragment, the EID1 coding region was amplified by using two different primer pairs: 5′-AAGCTTAAAGACATTTCCCG GTTACCGC-3′ together with 5′-GTTTGGATACCGACCTTGCTAC GTTCCTA-3′ [EID1-N primer], and 5′-GCGGCTTTGGTTCTCCTG GTCTCCTCCC-3′ [EID1-C primer] together with 5′-TCTGCTCAGAA AGTAAGAAGGAGGTC-3′. DNA fragments were sequenced directly. Mapping was done as described by Konieczny and Ausubel (1993). For DNA extraction we used a DNeasy plant mini kit (QIAGEN).

Plant transformation

To create the 35S-promoter–EID1-α- terminator construct, we cloned the basal EID1 fragment into the pPCV812 plant transformation vector by using internal BarnHI and SalI restriction sites [Konieczny et al. 1994]. For the rescue with the EID1 genomic construct, a 4.2-kbp NsiI fragment obtained from the BAC clone T14P8 was ligated into the PstI site of the pluebscriptSK vector. The genomic fragment was isolated by using SstI and EcoRV sites, and it was subcloned into the pGPTV vector. The oligonucleotide 5′-CGAGATCC TCCCGGTATGCGACATTCTCCTGATCC-3′ spanning an internal PstI site was used together with the Smal primer to change the amino acids in the basic domain (arginine 197 to serine, and lysine 198 to glutamate) to get the EID1MON1 construct.

For yeast transformation, yeast strain pJ69-4A was first transformed with pGBKT7–EID1 as a bait followed by transformation with a lambda-DNA library [Lohmann et al. 1999]. The ASK1 and ASK2 coding regions were amplified by PCR as BarnHI–PstI cloning and cloned into the yeast vector pGADT7 (Clontech). These constructs were used for further analysis of growth on selective media and of β-galactosidase activity [Lohmann et al. 1999] together with EID1, EID1MON1, and EID1MON2 constructs that were subcloned into the yeast two-hybrid vector pGBK7.
GST pull-down experiments

The EID1 constructs were subcloned into the GST gene fusion vector pGEX-4T-1 (Amersham Pharmacia). GST, GST-EID1, GST-EID1CT, and GST-EID1CTAA were expressed in E. coli M15 [pREP4]. Soluble extracts were applied to 1 mL of glutathione-Sepharose (Sigma) and washed two times with extraction buffer (10 mM Na2HPO4, 1.8 mM KH2PO4, 0.14 M NaCl at pH 7.5). Equal aliquots of 35S-labeled ASK1 or ASK2 were added to 60 µL aliquots of these matrices, as well as to the empty Sepharose matrix. All samples were filled up with extraction buffer to a total volume of 200 µL and incubated for 90 min at 4°C. The matrices were washed four times with 500 µL of extraction buffer, then the bound proteins were eluted with 50 µL of elution buffer (50 mM Tris-HCl at pH 8.0, 10 mM reduced glutathione) for 10 min at 25°C. Forty microliters of the eluate were mixed with 10 µL of 5x SDS-sample buffer and incubated for 2 min at 95°C. Twenty microliters of the eluate and 0.5 µL of the 35S-labeled proteins were size-fractionated on an SDS-PAGE to visualize the labeled ASK proteins by autoradiography. 35S-labeled ASK1 or ASK2 were obtained by using their pGADT7 constructs together with a TNT Quick Coupled Transcription/Translation System (Promega) and 35S-methionine (Amersham Pharmacia).

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References


