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The histidine kinase-related domain of Arabidopsis phytochrome A controls the spectral sensitivity and the subcellular distribution of the photoreceptor

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Phytochrome A (phyA) is the primary photoreceptor for sensing extremely low amounts of light and for mediating various far-red light-induced responses in higher plants. Translocation from the cytosol to the nucleus is an essential step in phyA signal transduction. EID1 is an F-box protein that functions as a negative regulator in far-red light signaling downstream of the phyA in Arabidopsis thaliana. To identify factors involved in EID1-dependent light signal transduction, pools of ethylmethylsulfonate-treated eid1-3 seeds were screened for seedlings that suppress the hypersensitive phenotype of the mutant. The phenotype of the suppressor mutant presented here is caused by a missense mutation in the PHYA gene that leads to an amino acid transition in its histidine kinase-related domain. The novel phyA-402 allele alters the spectral sensitivity and the persistence of far-red-light-induced high irradiance responses. The strong eid1-3 suppressor phenotype of phyA-402 contrasts with the moderate phenotype observed when phyA-402 is introgressed into the wild-type background, which indicates that the mutation mainly alters functions in an EID1-dependent signaling cascade. The mutation specifically inhibits nuclear accumulation of the photoreceptor molecule upon red-light irradiation, even though it still interacts with FHY1 (far-red long hypocotyl 1) and FHL (FHY1-like protein), two factors that are essential for nuclear accumulation of phyA. Degradation of the mutated phyA is unaltered even under light conditions that inhibit its nuclear accumulation, indicating that phyA degradation may occur mostly in the cytoplasm.
Light is an important exogenous factor that is essential for the survival of plants in their natural environment. To sense light quality, intensity, and direction, plants have evolved several classes of photoreceptors, including cryptochromes, phototropins, and phytochromes (Chen et al., 2004; Franklin et al., 2005). All phytochromes utilize covalently attached bilin chromophores, which enable light absorption and reversible photoconversion between two stable forms, Pr and Pfr. Upon absorption of red light, the Pr conformation converts to the far-red-absorbing Pfr conformation, and absorption of far-red light reconverts Pfr back to Pr. PhyA, like most phytochromes, also exhibits dark reversion, in which the Pfr form reverts back to the more stable Pr conformation in the absence of light. Plant phytochromes are synthesized in the Pr conformation in the dark and remain inactive until light absorption, at which point the bioactive Pfr translocates into the nucleus. (Chen et al., 2004; Møller et al., 2002; Rockwell et al., 2006).

Phytochromes comprise a small protein family in all analyzed plant species (Mathews and Sharrock, 1997). In Arabidopsis, five genes have been described, PHYA through PHYE. Four genes, PHYB to PHYE, encode light stable phytochromes that predominantly regulate responses toward strong continuous red light and in white light-grown plants (Chen et al., 2004; Møller et al., 2002; Rockwell et al., 2006). The product of the PHYA gene accumulates to very high levels in darkness and is rapidly degraded in red light (Eichenberg et al., 2000; Hennig et al., 1999). At high levels in the dark, phyA is able to sense extremely low amounts of light and regulate the so-called very low fluence responses (VLFR). Furthermore, phyA triggers far-red-light-dependent high irradiance responses (HIR) (Casal et al., 2003; Chen et al., 2004; Mancinelli, 1994). HIRs have several unique features that cannot be explained on the sole basis of photoconversion between Pr and Pfr conformations. The action spectra of HIRs show a maximum at approximately 720 nm, a wavelength that establishes high levels of Pr and inhibits responses of light stable phytochrome. Furthermore, the magnitude of HIRs depend not only on the duration and fluence rate of far-red light irradiation, but can be halted by short dark periods (Büche et al., 2000; Dieterle et al., 2001, 2005; Mancinelli, 1994; Zhou et al., 2002).
The sub-cellular partitioning of phytochromes in higher plants is regulated by light. The inactive Pr form accumulates in the cytoplasm, whereas the photo-converted Pfr form accumulates in the nucleus (Hisada et al., 2000; Kim et al., 2000; Kircher et al., 1999, 2002; Sakamoto and Nagatani, 1996; Yamaguchi et al., 1999). Nuclear accumulation of phyA is mediated by FHY1 and FHL in Arabidopsis (Hiltbrunner et al., 2005, 2006; Rösler et al., 2007). Plant phytochromes can form at least two different types of nuclear speckles: a rapidly forming, relatively unstable type (early NUS) and a later occurring, more stable type (late NUS). Additionally, phyA can also form sequestered areas of phytochrome (SAP) in the cytoplasm (Bauer et al., 2004; Chen et al., 2003; Dieterle et al., 2005; Kim et al., 2000; Kircher et al., 2002; McCurdy and Pratt, 1986 a, b; Speth et al., 1987).

All phytochromes share a common architecture consisting of an N-terminal photosensory region (NTD), which binds the chromophore, and a C-terminal regulatory domain (CTD; Chen et al., 2004; Rockwell et al., 2006). The NTD of Arabidopsis phyB was sufficient for phytochrome signaling when it was fused to protein domains that enable dimerization and nuclear localization (Matsuhita et al., 2003; Oka et al., 2004). Nevertheless, a multitude of loss-of-function mutants in phyA and phyB clearly demonstrate that the CTD also plays an important role in signal transduction (Wagner and Quail, 1995; Xu et al., 1995; Krall and Reed, 2000; Yanovsky et al., 2002).

The CTDs of all known phytochromes carry a histidine kinase-related domain (HKRD; Schneider-Poetsch et al., 1991; Rockwell et al., 2006) that can be divided into the histidine kinase acceptor (HA) and the ATP-binding kinase (HKin) sub-domains. The HA sub-domain is responsible for dimer formation of two-component signal transducers and normally carries a conserved His residue that functions as an acceptor of the phosphoryl group (Bilwes et al., 1999; Tomomori et al., 1999; West and Stock, 2001; Noack et al., 2007). Prokaryotic phytochromes function as classical two-component histidine kinases that autophosphorylate the dimeric protein partner on a His residue and then transfer this phosphate to an Asp on a receiver protein (Parkinson, 1992; Yeh et al., 1997; Rockwell et al., 2006). In vivo phosphorylation experiments indicate that higher plant phytochromes have shifted to function as Ser/Thr instead of His kinases (Yeh and Lagarias, 1998). Studies with transgenic plants and missense mutants indicate that the HKRD modulates signaling.
output of higher plant phytochromes, but is not essential for their function (Boylan and Quail, 1996; Cherry et al., 1993; Krall and Reed, 2000; Matsuhita et al., 2003; Xu et al., 1995). Nevertheless, little is known about the functional relevance of the HKRD in higher plant phytochromes.

EID1 is an F-box protein that functions as a negative regulator in phyA-specific light signaling (Büche et al., 2000; Dieterle et al., 2001; Zhou et al., 2002; Marrocco et al., 2006). The protein most probably functions as a component of an ubiquitin ligase complex that degrades positively-acting phyA signaling factors. The \textit{eid1-3} mutant has a hypersensitive phenotype under continuous far-red light that is sensed by phyA. Mutant seedlings exhibited a strong reduction in hypocotyl elongation, enhanced cotyledon expansion, enhanced cotyledon opening, and a stimulation under weak far-red light of root growth when compared to wild type.

To identify factors involved in EID1-dependent light signaling, pools of ethylmethylsulfonate-treated \textit{eid1-3} seeds were screened for seedlings with a wild-type-like phenotype under weak far-red light, which normally induces a strong photomorphogenic response in the background line. Lines that exhibited a stable phenotype in a second round of screening were named \textit{rei} for \textit{r}evertants of \textit{eid} 1-3. The phenotype of one of the \textit{rei} mutants is presented here and is caused by a missense mutation in the HKRD of the phyA photoreceptor. This novel phyA allele shifts the action spectrum and persistence of HIR, making it uniquely useful among phyA alleles for understanding transduction mechanisms. YFP fusion protein observations further indicate that the mutation causes an inhibition of nuclear accumulation under light conditions that normally adjust high levels of Pfr in the cytoplasm. Thus, our studies provide new insights into the functional role of the HKRD for the regulation of photomorphogenesis in higher plants.
RESULTS

Isolation of the phyA-402 mutant

To search for genes that are involved in EID1-dependent light signaling, suppressor mutants were screened from pools of ethylmethylsulfonate-treated eid1-3 seeds in a Wassilewskija background that exhibited reduced far-red light-sensitivity compared to the hypersensitive mutant. One of the rei revertant lines was chosen for further analysis because of its strong suppressor phenotype. Under selective light conditions, F2 seedlings of backcrosses with eid1-3 exhibited a clear 3:1 (183:64) segregation for the Eid1-3 phenotype. Thus, the suppressor mutation behaved like a recessive monogenic locus. The mutant was crossed with the eid1-6 allele in a Columbia (Col) background for mapping. A tight linkage was detected to different markers that localize close to the PHYA gene on chromosome 1 (Fig. S1A). This finding strongly suggested that the suppressor phenotype is caused by a mutation in the PHYA gene. This hypothesis was confirmed by experiments with rei eid1-3 mutants that had been transformed with a ProPHYA:PHYA:YFP construct; expression of the phyA-YFP fusion protein in the double mutant reinstated the initial hypersensitive Eid1-3 phenotype (Fig. S1B).

Sequence analysis of phyA in the rei mutant revealed a single C to T transition that leads to the exchange of Leu946 for a Phe residue in the HA sub-domain of the HKRD of PHYA (Fig. 1). The sequence polymorphism was used to create a cleavable amplified polymorphic sequence (CAPS) marker. No recombination was detected between the CAPS marker and the mutation after the analysis of 90 F2 plants that displayed the revertant phenotype.

Both the Leu946 residue and the surrounding sequence are highly conserved throughout plant evolution and could be identified in phytochrome sequences derived from mosses, liverworts, and green algae (Fig. S2). The conserved Leu was only replaced by Val or Ile residues in some sequences belonging to the B and C sub-family of higher plant phytochromes. Sequence alignments with the HA sub-domain of Arabidopsis phyA, a fern phytochrome, and bacterial histidine receptor kinases with known structure exhibited a high
degree of conservation for large aliphatic amino acid residues at the position of the mutated Leu (Fig. 1). When compared to the published structure of the HA sub-domain of the osmoregulator EnvZ and the chemotaxis sensor CheA, the mutation falls into the hydrophobic core that stabilizes the HA loop-structure formed by two α-helices. The new phyA allele was named *phyA-402* according to the rules published by Quail et al. (1994).

### Physiological characterization of *phyA-402*

Seedlings of *phyA-402 eid1-3* double and *phyA-402* single mutants exhibited a phenotype that was nearly indistinguishable from wild-type under the weak far-red light used for screening, whereas *eid1-3* seedlings showed a hypersensitive response (Fig. 2). No difference in seedling phenotypes was observed between wild type, *phyA-402*, *eid1-3*, and *phyA-402 eid1-3* under strong, saturating far-red light (Fig. 2). Thus, *phyA-402* seedlings clearly differ from the *phyA-211* knock-out mutant that does not respond to far-red light. All tested lines exhibited very similar phenotypes under strong continuous red or white light and remained etiolated in darkness (Fig. 2). This data indicates that the *phyA-402* allele encodes for a partially active photoreceptor that alters far-red light signaling.

The *eid1-3* mutant displays a hypersensitive phenotype under continuous far-red light and extremely weak red light (Büche et al., 2000; Dieterle et al., 2001; Marrocco et al., 2006). To analyze whether the chosen revertant line suppresses the hypersensitive Eid1-3 phenotype under both light qualities, fluence rate response curves were measured for hypocotyl elongation and cotyledon opening (Fig. 3). The *phyA-402* mutation clearly suppressed the hypersensitive Eid1-3 phenotype under both light conditions. Interestingly, no differences in red and far-red light sensitivity were observed for inhibition of hypocotyl elongation between *phyA-402 eid1-3*, *phyA-402* and the wild-type (Fig. 3A, B). The *phyA-402* mutation also clearly reduces the effect of *eid1-3* on cotyledon opening under continuous red and far-red light, even though suppression is not complete. The *phyA-402* mutation also exhibited a weak hyposensitivity for cotyledon opening in wild type background under both light qualities tested (Fig. 3C, D).
Up-regulation of anthocyanin accumulation is another far-red HIR in Arabidopsis (Kunkel et al., 1996). The phyA-402 allele also functions as a suppressor of eid1-3 with respect to anthocyanin biosynthesis. The mutated photoreceptor did not cause a reduction of anthocyanin accumulation in an EID1 wild-type or eid1-3 mutant background (Fig. 3E).

The phyA-402 mutation causes a shift in spectral sensitivity and persistence of HIR

Because EID1 functions as an important negative regulator in far-red HIR (Büche et al., 2000; Dieterle et al., 2001, Zhou et al., 2002; Marrocco et al., 2006), it was quite astonishing that wild-type and phyA-402 exhibited nearly identical fluence rate dependencies for inhibition of hypocotyl elongation and for anthocyanin accumulation. To search for more subtle influences of phyA-402 on far-red HIR, additional fluence rate response curves were measured for seedlings irradiated with narrow-banded interference filters, in order to construct an action spectrum for hypocotyl elongation (Fig. S3).

Wild-type seedlings exhibited a typical HIR action spectrum, with maximum light sensitivity at 720 nm (Fig. 4A). The maximum of the phyA-402 action spectrum is shifted from 720 to 716 nm, as light sensitivity is reduced at wavelengths above 716 nm. Thus, the HIR action spectrum of the mutant clearly differs from wild-type.

The release of HIR, and the resulting hypocotyl elongation, normally depends on strong continuous light irradiation and normally stops when dark-phases are interposed (Büche et al., 2000). Seedlings were treated with multiple and strong 2.5 min far-red light pulses that were interrupted by dark-phases of variable lengths. Decay kinetics were followed to estimate the apparent persistence of HIR signals installed by the light pulses.

The persistence of the far-red light signal in wild-type seedlings is about 9.1 min, and the HIR seemed to be nonexistent when dark phases exceeded 30 min (Fig. 4B). The phyA-402 line exhibited a reduction in the apparent half-life of the far-red light signal to about 6.2 min and a complete loss of HIR at about 15 min (Fig. 4B). The residual reduction of hypocotyl elongation remaining at prolonged dark-phases has commonly been interpreted as a VLFR that does not depend on continuous light irradiation (Yanovsky et
al., 1997). The phyA-402 hypocotyls were longer than wild-type when treated with prolonged dark phases, indicating that phyA-402 also alters the VLFR.

Comparable results were obtained for decay kinetics determined for cotyledon opening. The apparent half-life of the far-red light signal was shifted from 8.2 min in the wild-type to 5.6 min in phyA-402 (Fig. 4C). Again a residual difference in cotyledon opening was detected with prolonged dark phases between wild-type and phyA-402, indicative of a reduced VLFR in the mutant.

**The phyA-402 mutation alters neither Pfr dark-reversion nor light-dependent protein degradation**

The observed weak loss-of-function phenotype of phyA-402 might be caused by either a reduced level of the mutated phyA, an increased Pfr degradation rate, or a faster dark-reversion from active Pfr to inactive Pr. To test for putative differences in phyA accumulation, the level of photoreversible phytochrome was measured with a dual wavelength ratio spectrophotometer (ratio-spec) in 4-day-old, dark-grown seedlings. No significant difference ($P < 0.05$) in phyA levels was detected between etiolated wild-type and phyA-402 seedlings (Table I). Comparable results were also obtained by immuno-blot analyses with protein extracts from etiolated seedlings (Fig. 5A).

The phyA protein is typically degraded within the cell upon Pfr formation. No difference in wild-type phyA and phyA-402 protein degradation was detected upon irradiation of 4-day-old, dark-grown seedlings with strong continuous red and far-red light (Fig. 5A, B). The levels of both proteins decreased quite rapidly under continuous red light and fell below the detection level by immuno-blot analysis by 120 min (Fig. 5A). phyA and phyA-402 degradation kinetics under continuous far-red light were followed by ratio-spec measurements. The degradation rate was slower in plants grown under far-red light because of the low Pfr levels caused by photoconversion by far-red light, and measurable amounts of phyA still remained detectable after 12 h of irradiation (Fig. 5B).
Pfr-A also shows dark reversion in many dicotyledonous plant species and some Arabidopsis ecotypes (Eichenberg et al., 2000). Therefore, ratio-spec measurements could be used to follow dark-reversion kinetics by measuring total phyA (P\textsubscript{tot}) and the Pfr/P\textsubscript{tot} ratios (Eichenberg et al., 2000; Dieterle et al., 2005). Four-day-old, etiolated seedlings were treated with a saturating red-light-pulse to induce maximum Pfr levels (~87 %), and then transferred to darkness. Wild-type and phyA-402 seedlings exhibited nearly identical degradation kinetics for P\textsubscript{tot} (Fig. 5C) and Pfr-A (Fig. 5D), with half-lives in the range of 15 min. A weak dark reversion was detectable for wild type and phyA-402 seedlings, which does not significantly contribute to inactivation of active Pfr-A in either line (Fig. 5E). Taken together, these data indicate that the revertant phenotype of phyA-402 is brought about neither by reduced photoreceptor levels nor by increased degradation or dark-reversion rates.

The mutation in the HKRD blocks the nuclear accumulation of phyA upon red-light irradiation

To study the sub-cellular localization of the mutated photoreceptor, Pro\textit{PHYA}:\textit{PHYA}-402:YFP constructs were introduced into phyA-211 loss-of-function mutants. Whereas phyA-211 remained completely etiolated under continuous strong far-red light, transgenic phyA-211 seedlings expressing phyA-402-YFP exhibited a clear photomorphogenic phenotype similar to wild-type and phyA-211 plants expressing wild-type phyA-YFP (Fig. S4). Therefore, the introduced \textit{PHYA}-402:YFP construct can rescue the loss-of-function phenotype of phyA-211 and encodes for a functional photoreceptor molecule. The sub-cellular localization of the mutated YFP fusion protein was compared with a transgenic phyA-211 line that has been transformed with a wild-type Pro\textit{PHYA}:\textit{PHYA}:YFP construct (Kircher et al., 2002). All localization experiments were done with 4-day-old etiolated seedlings.

Similar to published results (Kim et al., 2000; Kircher et al., 2002), the wild-type and mutated versions of the phyA-YFP fusion proteins were exclusively localized in the
cytoplasm in dark-grown Arabidopsis seedlings and did not enter the nucleus (Fig. 6A, A’, B, B’). After irradiation with strong continuous far-red light, a strong, diffuse nuclear YFP fluorescence became visible for lines expressing both phyA-402-YFP and phyA-YFP, while only a weak signal remained in the cytoplasm. Furthermore, so-called late nuclear speckles (lNUS) became visible in the nuclei of many cells with both YFP fusion proteins after prolonged irradiation (Fig. 6C, C’, D, D’).

Compared to the large, stable, and nearly immobile lNUS, early nuclear speckles (eNUS) are slightly smaller, very mobile, and appear quite rapidly upon red or white light irradiation of etiolated seedlings (Bauer et al., 2004; Chen et al., 2003; Dieterle et al., 2005; Kircher et al., 2002). Formation of eNUS can most easily be followed when seedlings are pre-irradiated with 8 to 10 h of far-red light before transfer to white light, because the pre-treatment causes an enrichment of phyA in the nucleus. In the phyA-402-YFP and phyA-YFP expressing-lines, a rapid aggregation of eNUS was observed that started between 2 to 10 minutes after the onset of microscopic light (Fig. 6E, E’, F, F’).

Etiolated seedlings were also transferred to strong red light to study formation of sequestered areas of phyA-YFP and phyA-402-YFP (SAPs). Rapid aggregation of SAP was observed in transgenic phyA-211 lines expressing both wild-type phyA-YFP and phyA-402-YFP starting at about 2 to 5 min after transfer to red light (Fig. 6G, G’, H, H’). SAP disappeared in all tested lines after 30 min of continuous irradiation (Fig. S4).

These microscopic studies demonstrate that phyA-402-YFP exhibits normal cytoplasmic localization in darkness, normal nuclear accumulation under far-red light, and normal eNUS, lNUS, and SAP formation.

Similar to published results (Kim et al. 2000; Kircher et al., 2002), wild-type phyA-YFP became detectable in the nucleus directly after transfer to red light (Fig. 6G, G’) and became almost exclusively localized to the nucleus after 4 h of continuous irradiation. The fluorescence signal was faint due to light induced phyA-YFP degradation (Fig. S4). In striking contrast, phyA-402-YFP signals were almost exclusively observed in the cytoplasm and around the nuclear envelope at all time points analyzed. Nuclear exclusion was observed even at reduced protein levels and without the formation of SAP (Fig. 6H, H’; Fig. S5). This data indicates that the amino acid change in phyA-402 specifically
inhibits nuclear accumulation upon red light irradiation of etiolated seedlings and that the red-light-induced inhibition of phyA-402 nuclear accumulation does not depend on the aggregation of SAP in the cytosol.

To test whether red-light pre-irradiation also blocks far-red light-induced nuclear accumulation of phyA-402-YFP, we applied a light treatment consisting of 20 min of strong red light followed by 20 min of strong far-red light. Etiolated seedlings were irradiated with four cycles of red/far-red light, and the sub-cellular localization of the YFP fusion proteins was analyzed at the end of the last far-red light-pulse.

Strong nuclear YFP fluorescence was observed in pre-irradiated transgenic phyA-211 lines expressing wild-type phyA-YFP, and red light-induced SAP were not detected (Fig. 6I, I’). Thus, phyA-YFP exhibited a sub-cellular localization similar to seedlings treated with continuous far-red light alone (Fig. 6C, C’). In striking contrast, phyA-402-YFP did not become visible inside the nucleus under the red/far-red light pulse treatment, although it does agglomerate at the nuclear envelope to some extent (Fig. 6J, J’). Similar to phyA-YFP, SAP could no longer be observed after the multiple pulse treatment. The data demonstrates that a red light pre-treatment can inhibit far-red light triggered nuclear accumulation of phyA-402-YFP and that the red-light-induced inhibition of phyA-402 nuclear accumulation does not depend on SAP formation.

The Pfr form of the mutated photoreceptor molecule shows normal interaction with FHY1 and FHL

To test whether impaired nuclear accumulation of phyA-402-YFP is caused by a reduced binding to the transport facilitators FHY1 and FHL, an established yeast two-hybrid assay was used (Hiltbrunner et al., 2005). FHY1 or FHL coding sequences were fused to the Gal4-AD and wild-type or mutated PHYA was fused to the Gal4-BD. The constructs were introduced into yeast in combination or with empty two-hybrid vectors as negative controls. To test Pfr-dependent interaction with FHY1 and FHL, the artificial phycocyanobilin
chromophore (PCB) was added to the selective medium and the growth of yeast colonies was followed in darkness or under continuous red and/or far-red light.

Cell growth on selective medium was only observed with yeast strains that carry corresponding \textit{FHY1} or \textit{FHL} and \textit{PHYA} or \textit{PHYA-402} constructs. All transformed yeast strains were able to grow on L-W- medium, which only selects for the presence of two-hybrid vectors. (Fig. 7). Interaction between FHY1 or FHL and the two phyA versions was strictly dependent on the presence of PCB and red light, demonstrating that Pfr is the state that interacts with both FHY1 and FHL. Both wild-type PHYA and mutant PHYA-402 strains grew equally well when combined with either FHY1 or FHL, indicating that the loss of nuclear accumulation under red-light conditions is not likely attributable to a general loss of interaction between the transport facilitators and the Pfr form of phyA-402.

DISCUSSION

This study describes the identification and characterization of a weak loss-of-function phytochrome A allele, \textit{phyA-402}, that suppresses the far-red light hypersensitive phenotype of \textit{eid1-3} and exhibits a strong inhibition of red light-induced nuclear transport in dark-grown seedlings. The phenotype is caused by the mutation L946F at a highly conserved residue in the HA sub-domain of phyA, as determined by mapping analyses, the rescue of the Eid1 hypersensitive phenotype by transformation with a functional \textit{ProPHYA:PHYA:YFP} construct, and by sequencing of the respective gene (Fig. 1, S1, and S2).

Whereas bacterial phytochromes function as light-regulated histidine kinases (Parkinson, 1992; Yeh et al., 1997; Rockwell et al., 2006), results about the functional importance of the HKRD present in higher plant phytochromes are quite ambiguous. A series of phyB mutants in Arabidopsis showed that point mutations in the HATPase-like sub-domain cause phenotypes similar to those of null mutants, whereas a truncation that removes most of the HKRD results in a phyB molecule with partial activity (Krall and Reed, 2000). A truncated oat phyA lacking the final 36 amino acids conferred no
hypersensitive phenotype when expressed in tobacco, indicating that the HKRD is necessary for phyA activity (Cherry et al., 1993). However, site-directed point mutations in conserved motifs of the HKRD of oat phyA did not affect function after transformation into Arabidopsis (Boylan and Quail, 1996). The phyA-105 mutation, located in front of the HA domain, decreases, but does not eliminate, phyA activity (Xu et al., 1995) in a manner similar to phyA-402. Taken together, these findings indicate that the HKRD is not essential for phyA function, but has a modulatory role in phytochrome signaling. This may be the beginning of our understanding of the functional role of the HKRD in signal transduction initiated by higher plant phytochromes.

Several of our observations clearly indicate that the mutation in the HA sub-domain has very severe influences on light signal transduction events related to EID1, which functions as an important negative regulator in phyA-dependent HIR signaling (Büche et al., 2000; Dieterle et al., 2001; Zhou et al., 2002; Marrocco et al., 2006). Firstly, the phyA-402 mutation robustly suppresses the effects of the strong allele eid1-3 under continuous far-red and weak, red light, even though it does not severely affect light sensitivity in the wild-type under the same light conditions (Fig. 2 and 3). Secondly, phyA-402 and eid1 mutations both alter the HIR action spectrum for hypocotyl elongation, though in opposite directions: phyA-402 reduces light sensitivity mainly at longer wavelengths while eid1 mutants increase light sensitivity at shorter wavelengths (Fig. 4A). These are the only mutants that have shifted the action spectrum, as neither the hypersensitive phyA-401 (eid4) allele nor the spa1 mutant (suppressor of phyA-105; Hoecker et al., 1998, 1999) moved the peak, but only altered its overall shape (Dieterle et al., 2001; Zhou et al., 2002). Finally, data obtained after treatment with intermittent dark phases of variable length between far-red light pulses demonstrated that phyA-402 and EID1 also differentially regulate the persistence of HIR signaling: eid1 loss-of-function mutations increase signal persistence to over six times the ~9 min half-life observed in wild-type (Büche et al., 2000; Zhou et al., 2002) while phyA-402 decreases the half-life to ~6 min (Fig. 4B). A unique feature of the eid1 mutant is a shift in the peak of the action spectra of phyA-mediated hypocotyl elongation, from 716 nm to 660 nm, i.e. from the far-red to the red light part of the spectrum (Dieterle et al., 2001; Zhou et al., 2002). The phyA-402 mutant completely
suppresses the increased inhibition of hypocotyl elongation by red light observed in \textit{eid1-3} (Figure 3B). The observed strong reduction in nuclear accumulation under red light might be responsible for the observed strong suppressor phenotype in \textit{phyA-402 eid1-3} double mutants under this light quality, because nuclear accumulation is essential for phytochrome signaling (Matsuhita et al., 2003; Oka et al., 2004; Hiltbrunner et al., 2005, 2006; Rösler et al., 2007). Therefore, inhibition of nuclear accumulation should have a severe impact on responses towards continuous weak red light that are normally inhibited by EID1.

Nevertheless, the most severe effect on the \textit{phyA-402} loss-of-function phenotype has been observed in the far-red light spectrum, and in that very spectrum the \textit{phyA-402} YFP fusion protein did not show detectable alterations in nuclear accumulation. Furthermore, the mutated photoreceptor also strongly suppressed the effect of \textit{eid1-3} under continuous far-red light. This data indicates that the effect of the \textit{phyA-402} mutation cannot solely be explained by an altered nuclear accumulation. The mutation leads to an exchange of a conserved amino acid in a protein domain that is thought to be important for both protein-protein interactions and phosphotransfer in bacterial histidine-kinases, including prokaryotic phytochromes (Bilwes et al., 1999; Rockwell et al., 2006; Tomomori et al., 1999; West and Stock, 2001; Yeh and Lagarias, 1998). Therefore, it is worthwhile to assume that the mutation also alters early steps in phyA-dependent light signal transduction in higher plants.

This assumption is further confirmed by the observed decrease in signal persistence in \textit{phyA-402}. The decrease in signal persistence might look weak, but one has to take into account that full expression of HIR normally depends on continuous far-red light irradiation for several hours (Büche et al., 2001; Mancinelli, 1994; Yanovsky et al., 1997). Therefore, even subtle alterations in signal persistence would accumulate during elongated light treatments. This alteration in signal persistence might also explain the more severe reduction in light sensitivity of \textit{phyA-402} seedlings at wavelengths above 716 nm. At these wavelengths absorption cross sections of Pr are decreasing exponentially, whereas absorption cross sections of Pfr become maximal (Mancinelli, 1994). Thus, lesser amounts of active Pfr will be formed in the first place, and Pfr that does form will be photoconverted back to inactive Pr, leading to reduced stability of the input signal.
Experiments with *spa1* (suppressor of *phyA-105*; Hoecker et al., 1998, 1999) mutants and *eid1-3 spa1* double mutants demonstrated that both of these negative regulators in phyA signaling have different but overlapping functions, and that signal transduction chains controlled by these factors might already branch at the level of the photoreceptor (Zhou et al., 2002). Physiological experiments exhibited a very strong negative influence of *phyA-402* on the hypersensitive Eid1-3 phenotype, whereas the effect is rather weak in wild-type background (Figure 3). This observation indicates that the *phyA-402* mutation has a more severe effect on an EID1-dependent branch of the phyA signaling cascade, but is less important for signaling processes regulated by SPA1 and SPA1-related proteins (Zhu et al., 2008).

Our data further indicate that the mutation in the HKRD does not alter light-induced proteolysis of the photoreceptor, since analyses of degradation kinetics did not exhibit any difference between phyA-402 and wild-type phyA under all light conditions tested (Fig. 5). While red light induces phyA-YFP localization to the nucleus, microscopic studies revealed that nuclear accumulation of phyA-402-YFP is strongly inhibited, although total protein levels appear to be unadulterated (Fig. 6). The lack of differences in red-light-induced proteolysis indicates that either phyA degradation takes place nearly exclusively in the cytoplasm or that cytoplasmic and nuclear degradation counterbalance each other and function with comparable efficiency under this light condition. This finding is in agreement with results obtained in *fhy1 fhl* double mutants, in which phyA degradation remains unaltered even though nuclear accumulation is blocked (Rösler et al., 2007).

The mutation in the HKRD did not lead to detectable changes in speckle formation. Microscopic studies did not reveal any changes in the formation of cytoplasmic SAP, early NUS, or late NUS between wild-type phyA-YFP and phyA-402-YFP (Fig. 6). This finding differs from results obtained with other *phyA* loss-of-function alleles that carry missense mutations in the PAS2 domain and are impaired in NUS formation (Kircher et al., 2002; Yanovsky et al., 2002). The sub-cellular localization of phyA-402 is also different than that of phyA-401-GFP, a hypersensitive version of the photoreceptor that exhibits reduced SAP formation, because of an amino acid transition in the GAF domain (Dieterle et al., 2005).
The most severe effect on subcellular localization has been observed upon red light irradiation of etiolated seedlings, which reveals a defect in the nuclear accumulation of phyA-402-YFP (Fig. 6 and S5). The Pfr form of the mutated photoreceptor is able to interact with FHY1 and FHL in yeast, similar to the wild-type molecule (Fig. 7). Therefore, the observed inhibition of phyA-402-YFP nuclear accumulation is not likely due to the loss of interaction with the two proteins that are essential for nuclear accumulation of phyA (Hiltbrunner et al., 2005, 2006; Rösler et al., 2007). The observed far-red-light induced nuclear accumulation of phyA-402-YFP also argues against a general block in interaction with FHY1, FHL or other components of the nuclear import machinery.

Models for the observed inhibition of nuclear accumulation of phyA-402 have to explain why this effect is most severe under red-light treatments, whereas no differences have been observed under continuous far-red light. The observed block in red-light-induced nuclear accumulation can not fully be explained by reduced affinities to transport facilitators, because such a reduction would become most effective under low Pfr levels adjusted by far-red light, but should be less important at high Pfr levels induced by red light.

One such process might be SAP formation, because it only occurs in etiolated seedling that accumulate high levels of Pr-A, which are then photoconverted nearly completely to Pfr-A upon red-light-irradiation (McCurdy and Pratt, 1986a, b; Speth et al., 1987; Kim et al., 2000; Kircher et al., 1999, 2002; Dieterle et al., 2005). Aggregation of SAP should reduce freely available phyA molecules in the cytoplasm and thus, compete with nuclear transport. However, the observations that phyA-402-YFP remains excluded from the nucleus in cells without detectable SAP argue against a major role of these speckles in the inhibition of nuclear accumulation (Fig. 6I, I’, J, J’, Fig. S5).

Another major difference between red and far-red light-treated seedlings occurs in the ratio of Pfr/P$_{tot}$ in the cell (~0.87 in red light compared to ~0.05 in far-red light). An increased affinity of the Pfr form of phyA-402 toward FHY1 and FHL may result in a block in nuclear accumulation simply due to a reduction in the release from transport facilitators in the nucleus, thus decreasing further import. Deprivation of transport facilitators would be most effective under red light, because both high levels of Pfr are formed and turnover from
Pfr to Pr is low. The release process might be less important under far-red light, because Pfr is efficiently photoconverted back to Pr and thus, sufficient amount of transport facilitator molecules might be available in nuclear transport. However, the proposed delayed-release mechanism can not fully explain why phyA-402-YFP nuclear accumulation is also blocked under repetitive red/far-red light treatments, because far-red light-induced conversion should detach the Pr molecule from its transport facilitators.

An alternative and more complex explanation for the observed effects of phyA-402 on nuclear localization and light responses comprises several aspects of phyA function: the observed Pfr-stimulated autophosphorylation activity of phyA (Yeh and Lagarias, 1998), the dimeric structure of phytochromes (Rockwell et al., 2006), and the nature of the amino acid exchange in the mutant. If phyA autophosphorylation is Pfr-dependent and is caused by a cross-phosphorylation between dimeric subunits in a manner similar to that of bacterial histidine kinases, maximum autophosphorylation of phyA monomers would be reached in red light, because this light quality induces the highest levels of Pfr:Pfr homodimers. In contrast, reduced fractions of both sub-units would be phosphorylated in far-red light, because this light quality induces low levels of Pfr:Pfr homodimers and Pfr:Pr heterodimers. The Leu to Phe conversion in phyA-402 is placed in the hydrophobic core that stabilizes the structure of the HA sub-domain in bacterial two-component histidine kinases (Fig. 1; Bilwes et al., 1999; Tomomori et al., 1999; West and Stock, 2001). Studies with EnvZ established that mutations in the hydrophobic core do not alter cross-phosphorylation of dimeric kinase subunits, but rather effect phosphatase function (Hsing et al., 1998). Even though several lines of evidence clearly prove that higher plant phytochromes do not possess histidine kinase activity and now likely function as Ser/Thr kinases (Yeh and Lagarias, 1998), it is worthwhile to speculate that the phyA-402 mutation in the HKRD affects the phosphorylation state of the photoreceptor molecule. If enhanced phosphorylation on both photoreceptor subunits leads to strong inhibition of nuclear accumulation by enhanced interaction with an unknown retention mechanism, this effect would explain the observed red-light-dependent differences between wild-type phyA and phyA-402.
An enhanced phosphorylation state of phyA-402 explains the moderate reduction in physiological activity of the mutant in the wild-type background, which seems to mimic the effect seen with papp5 mutants (Ryu et al., 2005). The mutation might also reduce the CTD interaction with other positive signaling factors, such as phytochrome interaction factor 3 (Ni et al., 1998) or phytochrome kinase substrate 1 (Fankhauser et al., 1999), in a manner similar to that reported for nucleotide diphosphate kinase 2 (Ryu et al., 2005).

To conclude, the strong Eid1-suppressor phentoype of phyA-402 clearly indicates that the HKRD of phyA has a severe influence on far-red HIR, since EID1 specifically functions as a negative regulator of this response mode, but does not influence VLFR (Zhou et al., 2002). Our findings show that the HKRD modulates the spectral sensitivity and the persistence of phyA-derived signals controlling HIR. Furthermore, experiments with far-red light pulse treatments strongly suggest that the mutated phyA domain is also involved in regulation of VLFR. Our data also demonstrates that the respective protein domain participates in the regulation of the nuclear accumulation of phyA that is essential for photoreceptor function (Hiltbrunner et al., 2005, 2006; Rösler et al., 2007).

MATERIALS AND METHODS

Plant material and mutagenesis

For genetic crossing and physiological analyses, the following ecotypes and photomorphogenic mutants of Arabidopsis thaliana were used: Wassilewskija (WS) wild type, eidl-3 (ecotype WS; Büche et al., 2000; Dieterle et al., 2001), eidl-6 (ecotype Columbia; Zhou et al., 2002), and phyA-211 (ecotype Columbia; Nagatani et al., 1993). Plant propagation and mutagenesis of eidl-3 were done as described by Büche et al. (2000).
Seedling growth, light sources, and screening for mutants

Seeds were sown on four layers of Schleicher & Schüll 595 filter paper circles (Schleicher & Schüll, Dassel, Germany) as described elsewhere (Büche et al., 2000). The standard sowing procedure was followed by a 2-day cold treatment at 6 °C in darkness and a 1-day red light induction of germination at 25 °C before onset of different light treatments for 3 days. Revertant mutants were screened under a weak selective far-red light field (0.3 µmol m⁻² s⁻¹) that induces strong photomorphogenic development in eid1-3 but not in wild type seedlings (Zhou et al., 2002). Standard red light (39 µmol m⁻² s⁻¹; λ(max) = 650 nm) and far-red light fields (20 µmol m⁻² s⁻¹; λ(max) = 730 nm) were used for microscopic studies (Heim and Schäfer, 1982). For all other light treatments, modified Leitz Prado 500-W universal projectors (Leitz, Wetzlar, Germany) were used as light sources with Osram Xenophot longlife lamps (Osram, Berlin, Germany). Red light was obtained by passing the light beam through KG65 filters (λ(max) = 650 nm; Balzers, Liechtenstein). Far-red light treatments were performed with 715-nm DAL interference filters (Schott, Mainz, Germany). To measure fluence rate response curves for the action spectra, light was passed through narrow banded DIL and DEPIL interference filters (Schott).

Determination of hypocotyl elongation, cotyledon opening and anthocyanin content

For hypocotyl length measurements and determination of cotyledon opening, seedlings were spread on water agar and photographed together with a size standard using a Zeiss Stemi SV6 binocular supplemented with a Zeiss AxioCam MRc 5 digital camera (Zeiss, Stuttgart, Germany). Hypocotyl lengths and cotyledon angles were determined using ImageJ software (http://rsb.info.nih.gov/ij/). All data represent the mean ± SEM of at least 40 seedlings analyzed in at least two independent experiments. Anthocyanin was extracted from 50 seedlings, and anthocyanin content was determined spectroscopically as described by Büche et al. (2000). Data represent the mean ± SEM of 5 to 10 independent replicates.
Mapping and isolation of the phyA-402 mutant

Revertant lines were crossed with eid1-6 in a Columbia background for mapping analyses. The phyA-402 mutant was mapped using polymerase chain reaction (PCR)-based simple sequence length polymorphisms and cleavable amplified polymorphic sequence markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994; Table S1). The PHYA gene was amplified as three overlapping fragments, which were subsequently sequenced (Table S1). For detection of the phyA-402 mutation, a derived cleavable amplified polymorphic sequence marker (Neff et al., 1998) was developed using the aa5’_5701 and aa3’_6140 oligonucleotides (Table S1). The PCR product was analyzed for the presence (phyA-402) or absence (wild type) of a BgIII restriction site. To isolate the phyA-402 mutation in a wild type background, the eid1-3 phyA-402 double mutant was crossed with WS wild-type and F1 plants were allowed to self-pollinate to obtain F2 seeds.

Protein extraction and immuno-blotting

Extraction of crude proteins and protein assays were performed as described by Dieterle et al. (2005). Aliquots containing 25 µg of crude protein were separated on SDS-polyacrylamide gels and blotted to polyvinylidifluoride membranes (Millipore, Schwalbach, Germany). Immunodetection of phyA was done using a specific antiserum (Büche et al., 2000; Convance, Freiburg, Germany) and alkaline phosphatase-coupled anti-rabbit or anti-mouse antisera (Bio-Rad, München, Germany).

In vivo spectroscopy

Photoreversible phyA was measured in a dual wavelength ratio spectrophotometer at 5 °C as described by Dieterle et al. (2005). To measure degradation and dark-reversion kinetics,
4-day-old etiolated seedlings were irradiated at 22 °C with different light sources. 715-nm DAL interference filter light was used to study phyA degradation under continuous far-red light. To follow dark reversion, etiolated seedlings were treated with a 2-min red-light pulse (standard red light field) in order to produce the maximum level of Pfr before transfer back to darkness. Curves were fitted using the SigmaPlot 9.0 Regression Wizard.

**Cloning of the PHYA constructs and plant transformation**

Construction of the ProPHYA:PHYA:YFP chimeric gene and the isolation of transgenic lines carrying this gene has been described previously (Kircher et al. 2002). To generate the ProPHYA:phyA-402:YFP fusion, a fragment of the wild type PHYA cDNA was replaced by a mutated fragment amplified by PCR from genomic DNA isolated from phy-402 plants. Arabidopsis transformation was done as described by Clough and Bent (1998). BASTA resistant plants were grown to maturation, selfed, and F2 seeds were tested for a 3:1 segregation of the resistance gene and the rescue of the phyA-211 loss-of-function phenotype. F3 seeds of positive F2 lines were tested for homozygous genetic segregation of both marker genes.

**Epifluorescence and light microscopy**

For epifluorescence and light microscopy, seedlings were transferred to glass slides under dim-green safe light and analyzed with an Axioskop microscope (Zeiss, Oberkochem, Germany). The nuclei were located and searched under dim-green safe light and, except for the analysis of eNUS, only the first pictures taken with a digital Axiocam camera system (Zeiss) are presented. Excitation and detection of the fluorophore YFP was performed with a YFP-specific filter set (AHF Analysentechnik, Tübingen, Germany).
Protein-protein interaction assays

Yeast two-hybrid interaction assays and application of phycocyanobilin chromophores were done as described by Hiltbrunner et al. (2005).

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Results of mapping analyses and rescue of the mutant phenotype with a ProPHYA:PHYA:YFP construct.

**Supplemental Figure S2.** Sequence alignment of plant phytochromes.

**Supplemental Figure S3.** Fluence rate response curves for the inhibition of hypocotyl elongation under continuous light of different wavelengths.

**Supplemental Figure S4.** Phenotype of 4-day-old Columbia wild-type, phyA-211, and rescued phyA-211 lines under strong continuous far-red light.

**Supplemental Figure S5.** Subcellular phyA-YFP and phyA-402-YFP distribution under continuous red light.

**Supplemental Table S1.** Oligonucleotides used for this study.

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FIGURE LEGENDS

**Figure 1.** Location and nature of the phyA-402 missense mutation. Domains of plant phytochromes are indicated and an alignment of the histidine kinase acceptor-like domain (HA) of different histidine kinase-like proteins is shown. Fully conserved, highly conserved, and conserved amino acids are marked with black boxes and dark and light gray shadings, respectively. The following sequences were used for alignment: *Ath*phyA, *Arabidopsis thaliana* phyA (NP_001117256); *Aca*phy2, *Adiantum capillus-veneris* phytochrome 2 (BAA33775); *Eco*EnvZ, *Escherichia coli* osmolarity sensor protein (YP_671367); *Tma*CheA, *Thermotoga maritima* chemotaxis sensor histidine kinase CheA (AAD35784). N, n-terminal domain; PASN, n-terminal PER/ARNT/SIM domain; GAF, domain identified in cGMP-regulated cyclic phosphodiesterases/adenyl cyclases/the bacterial transcription factor FhlA; PHY, phytochrome domain; PAS1, PAS2, two additional PER/ARNT/SIM domains; HKin, ATP-binding histidine kinase-like domain.

**Figure 2.** Phenotype of 4-day-old seedlings of different genotypes grown under different light conditions. The following light treatments were applied: weak cF, weak standard far-red light field (0.3 µmol m⁻² s⁻¹); strong cF, strong standard far-red light field (20 µmol m⁻² s⁻¹); cR, standard red light field (39 µmol m⁻² s⁻¹); cWL, white light (23 µmol m⁻² s⁻¹); cD, darkness.

**Figure 3.** Physiological characterization of light responses in wild type, phyA-402, eid1-3, and eid1-3 phyA-402 seedlings. Measurement of hypocotyl lengths, cotyledon opening and anthocyanin extraction was performed with 4-day-old seedlings. Hypocotyl lengths were determined in relation to the lengths of dark-grown seedlings for each line. Hypocotyl lengths for dark controls were 8.7 ± 0.2 mm for Wassilewskija wild type (WT), 8.4 ± 0.3 mm for phyA-402, 10.0 ± 0.2 mm for eid1-3, and 9.2 ± 0.3 mm for phyA-402 eid1-3 (mean ± SEM). Each data point represents the results of three independent measurements with a total of n = 45 seedlings. Standard error (SE) of the individual measurements were between 0.05 and 0.1. (A) Fluence rate response curve for inhibition of hypocotyl elongation under
continuous far-red light (715-nm DAL filter). (B) Fluence rate response curve for inhibition of hypocotyl elongation under continuous red light (KG65 filter). (C) Fluence rate response curve for cotyledon opening under continuous far-red light (715-nm DAL filter). (D) Fluence rate response curve for cotyledon opening under continuous red light (KG65 filter). (E) Anthocyanin accumulation under strong continuous far-red light (715-nm DAL filter; 6 µmol m\(^{-2}\) s\(^{-1}\)). Error bars = SE.

**Figure 4.** The *phyA-402* mutation alters the spectral sensitivity and persistence of high irradiance responses. (A) Action spectra for hypocotyl elongation in wild type (WT) and *phyA-402* seedlings. Fluence rate response curves were measured at different wavelengths and the fluence rate that led to a 40 % inhibition of elongation when compared with dark controls was determined. The value obtained for wild type at 720 nm (0.2 µmol m\(^{-2}\) s\(^{-1}\)) was set to 1 and the relative photon effectiveness for wild type and *phyA-402* at different wavelengths were calculated accordingly. (B) Signal persistence for inhibition of hypocotyl elongation in wild-type and *phyA-402* seedlings. Wild type and *phyA-402* seedlings were irradiated with multiple far-red light pulses (715-nm DAL filter; 6 µmol m\(^{-2}\) s\(^{-1}\)) for 2.5 min for 3 days after germination induction, varying the duration of the dark phases between light pulses. Curves were fitted with the SigmaPlot 9.0 Regression Wizard (exponential rise to maximum/three parameters). (C) Signal persistence for cotyledon opening in wild-type and *phyA-402* seedlings. Wild-type and *phyA-402* seedlings were treated with multiple far-red light pulses as described in Fig. 3B. Curves were fitted with the SigmaPlot 9.0 Regression Wizard (exponential decay/three parameters). Error bars = SE.

**Figure 5.** Light-induced degradation of wild type and mutant phyA. Four-day-old, etiolated seedlings were exposed to different light treatments, and phyA degradation was followed by immunoblot analysis or spectroscopic measurements. (A) Immunoblot analysis of phyA-degradation under the standard red light field (39 µmol m\(^{-2}\) s\(^{-1}\)). 25 µg of protein was loaded onto a gel, transferred onto a membrane, and detected with phyA-specific antiserum. (B) Degradation kinetics under continuous far-red light (715-nm DAL filter; 6 µmol m\(^{-2}\) s\(^{-1}\)). Levels of total phytochrome (*P*\(_{tot}\)) were given with respect to levels in dark controls.
Data points from two independent time-point experiments are shown. Curves were fitted using the SigmaPlot 9.0 Regression Wizard (exponential decay/three parameters). (C) (D) and (E) show the levels of $P_{tot}$, Pfr, and Pr at different lengths of darkness after a saturating red-light pulse. Relative levels were given with respect to $P_{tot}$ levels in dark controls. Data points from three independent kinetics are shown. Curves were fitted using the SigmaPlot 9.0 Regression Wizard (exponential decay/three parameters for $P_{tot}$ and Pfr; exponential rise to maximum/three parameters for Pr).

**Figure 6.** Subcellular phyA-YFP and phyA-402-YFP distribution under different light conditions. Pro\textit{PHYA:PHYA:YFP} and Pro\textit{PHYA:PHYA-402:YFP} constructs were transformed into the \textit{phyA-211} knock-out line. Four-day-old, dark-grown seedlings expressing phyA-YFP and phyA-402-YFP were either kept in darkness or irradiated with different light programs and analyzed with a YFP specific filter set. DIC (differential interference contrast) images were taken from corresponding cells. nu, nucleus; eNUS, early nuclear speckles; lNUS, late nuclear speckles; SAP, sequestered areas of phytochrome. (A) (A’) (B) (B’) Dark-grown seedlings. (C) (C’) (D) (D’) Etiolated seedlings were irradiated with strong standard far-red light (20 $\mu$mol m$^{-2}$ s$^{-1}$) for 8 h. (E) (E’) (F) (F’) Etiolated seedlings were irradiated with strong standard far-red light (20 $\mu$mol m$^{-2}$ s$^{-1}$) for 8 h and YFP fluorescence was followed after transfer to microscopic white light. (G) (G’) (H) (H’) Etiolated seedlings were irradiated with a saturating red-light pulse and transferred to darkness for 20 min. (I) (I’) (J) (J’) Etiolated seedlings that received four cycles of alternating 20 min red/far-red light (6/20 $\mu$mol m$^{-2}$ s$^{-1}$).

**Figure 7.** Light-regulated interaction between FHY1, FHL, phyA, and phyA-402 in yeast. Yeast (AH109) was transformed with the indicated plasmids. 5 $\mu$l of overnight cultures were spotted onto selective medium (L-W-H-, containing 1 mM 3-aminotriazole) supplemented with 20 $\mu$M PCB. Plates were incubated for 3 days in standard red light (R, 1 $\mu$mol m$^{-2}$ s$^{-1}$), standard far-red light (F, 20 $\mu$mol m$^{-2}$ s$^{-1}$), or in darkness (D). As a control, equal amounts of each overnight culture were spotted onto interaction-selective (L-W-H-)
or non-selective (L-W-) plates without PCB. AD, GAL4 activation domain; BD, GAL4 binding domain.
Table I. Spectroscopically detectable phytochrome amounts in four-day-old etiolated seedlings

| Genotype     | Phytochrome amount (ΔΔΔ x 10^4/mg)⁺ | Number of measurements |
|--------------|-------------------------------------|------------------------|
| wild-type    | 11.7 ± 2.3                          | 11                     |
| phyA-402     | 12.1 ± 2.5                          | 10                     |

⁺Values are means ± SD. P < 0.05 as determined by Student’s T-test.
Figure 1. Location and nature of the phyA-402 missense mutation. Domains of plant phytochromes are indicated and an alignment of the histidine kinase acceptor-like domain (HA) of different histidine kinase-like proteins is shown. Fully conserved, highly conserved, and conserved amino acids are marked with black boxes and dark and light gray shadings, respectively. The following sequences were used for alignment: AthphyA, Arabidopsis thaliana phyA (NP_001117256); Acaphy2, Adiantum capillus-veneris phytochrome 2 (BAA33775); EcoEnvZ, Escherichia coli osmolarity sensor protein (YP_671367); TmaCheA, Thermotoga maritima chemotaxis sensor histidine kinase CheA (AAD35784). N, n-terminal domain; PASN, n-terminal PER/ARNT/SIM domain; GAF, domain identified in cGMP-regulated cyclic phosphodiesterases/adenyl cyclases/the bacterial transcription factor FhlA; PHY, phytochrome domain; PAS1, PAS2, two additional PER/ARNT/SIM domains; HKin, ATP-binding histidine kinase-like domain.
Figure 2. Phenotype of 4-day-old seedlings of different genotypes grown under different light conditions. The following light treatments were applied: weak cF, weak standard far-red light field (0.3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)); strong cF, strong standard far-red light field (20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)); cWL, white light (39 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)); cD, darkness.
Figure 3. Physiological characterization of light responses in wild type, phyA-402, eid1-3, and eid1-3 phyA-402 seedlings. Measurement of hypocotyl lengths, cotyledon opening and anthocyanin extraction was performed with 4-day-old seedlings. Hypocotyl lengths were determined in relation to the lengths of dark-grown seedlings for each line. Hypocotyl lengths for dark controls were 8.7 ± 0.2 mm for Wassilewskija wild type (WT), 8.4 ± 0.3 mm for phyA-402, 10.0 ± 0.2 mm for eid1-3, and 9.2 ± 0.3 mm for phyA-402 eid1-3 (mean ± SEM). Each data point represents the results of three independent measurements with a total of n = 45 seedlings. Standard error (SE) of the individual measurements were between 0.05 and 0.1. (A) Fluence rate response curve for inhibition of hypocotyl elongation under continuous far-red light (715-nm DAL filter). (B) Fluence rate response curve for inhibition of hypocotyl elongation under continuous red light (RG65 filter). (C) Fluence rate response curve for cotyledon opening under continuous far-red light (715-nm DAL filter). (D) Fluence rate response curve for cotyledon opening under continuous red light (RG65 filter). (E) Anthocyanin accumulation under strong continuous far-red light (715-nm DAL filter; 6 μmol m⁻² s⁻¹). Error bars = SE.
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