A Dominant Mutation in the Pea *PHYA* Gene Confers Enhanced Responses to Light and Impairs the Light-Dependent Degradation of Phytochrome A[1]

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Phytochrome A (phyA) is an important photoreceptor controlling many processes throughout the plant life cycle. It is unique within the phytochrome family for its ability to mediate photomorphogenic responses to continuous far-red light and for the strong photocontrol of its transcript level and protein stability. Here we describe a dominant mutant of garden pea (*Pisum sativum*) that displays dramatically enhanced responses to light, early photoperiod-independent flowering, and impaired photodestruction of phyA. The mutant carries a single base substitution in the *PHYA* gene that is genetically inseparable from the mutant phenotype. This substitution is predicted to direct the replacement of a conserved Ala in an N-terminal region of PHYA that is highly divergent between phyA and other phytochromes. This result identifies a region of the phyA photoreceptor molecule that may play an important role in its fate after photoconversion.

Isolation and characterization of mutants has been an integral part of recent progress in understanding the signaling pathways that connect phytochrome, cryptochrome, and phototropin photoreceptors to downstream responses (Lin, 2002; Nagy and Schäfer, 2002). This genetic approach is most advanced for analysis of the phytochrome A (phyA) photoreceptor, due to its distinctive role in mediating responses to continuous far-red light (FR), and several phyA-specific signaling components have now been identified at the molecular level in Arabidopsis. They include both nuclear (HFR1, FAR1, and LAF1) and cytosolic (FIN219 and PAT1) enhancers and nuclear repressors (SPA1 and EID1) of phyA responses (Nagy and Schäfer, 2002; Wang and Deng, 2003).

In addition, it is becoming increasingly clear that phyA dynamics are an important factor controlling the extent of phyA signaling. Although all Arabidopsis phytochromes appear to be regulated at both the transcript and the protein level (Clack et al., 1994; Sharrock and Clack, 2002), this regulation is most dramatic for phyA. The *PHYA* genes in most species so far examined are expressed at a high level in darkness and are strongly down-regulated by light (Quail, 1991). Conserved positive elements within the *PHYA* promoter are required for the high level of expression in darkness, while a conserved negative element is likely to play an important role in the light-induced transcriptional repression (Bruce et al., 1991). The phyA protein has a number of different fates after light absorption and conversion to its FR-absorbing Pfr form. It is subject to rapid proteolytic degradation and can also undergo reversion to its relatively more stable red light (R)-absorbing Pr form. In addition, phyA in dark-grown plants is present mainly in the cytoplasm and relocalizes rapidly to the nucleus following irradiation with R or FR (Nagy and Schäfer, 2002). PhyA has been shown to interact physically with nuclear proteins that are essential for normal phyA signaling (Fairchild et al., 2000; Zhu et al., 2000), suggesting that nuclear localization of phyA is required for its activity. The importance of subcellular location for the processes of degradation and reversion is currently not clear.

The overall level of physiologically active phyA in the cell is therefore subject to complex regulation at several different levels. These processes depend partly on features of the phyA gene or protein itself, but are also likely to involve other as-yet-unidentified cellular components. For example, deletion and domain-swapping experiments have defined regions of the phyA molecule that are necessary for its degradation (Wagner et al., 1996; Clough et al., 1999). Also, dark reversion can be impaired and light-induced degradation accelerated by certain single residue substitutions within the Arabidopsis PHYA protein (Eichenberg et al., 2000). In contrast, the rate of dark

[1] This work was supported by the Australian Research Council (grants to J.B.R. and J.L.W. and Australian postgraduate awards to J.L.W. and S.L.B.) and by the Russian Foundation for the Fundamental Investigations (grant no. 02–04–49516 to V.A.S.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.036103.

reversion differs in the Landsberg *erecta* and RLD ecotypes despite the fact that they have an identical *PHYA* sequence (Eichenberg et al., 2000).

Loss-of-function mutations were initially important in establishing the physiological role of phyA (Dehesh et al., 1993; Nagatani et al., 1993; Whitelam et al., 1993) but have also been used to identify regions of the phyA molecule that are important for its photochemical characteristics, its signaling interactions, or for directing its localization and fate within the cell. Many different substitution mutations of phyA are now known, and several have been ascribed a specific role in phyA signaling on the basis of a loss-of-function phenotype under continuous FR without conspicuous effect on the level of phyA (Quail et al., 1995; Xu et al., 1995). These signaling or regulatory mutations initially appeared to cluster in certain regions, notably a 160 amino acid segment in the C-terminal domain (residues 699–858) that contains two Per-Arnt-Sim-like (PAS) domains and an additional short conserved sequence often referred to as the Quail box (Quail et al., 1995; Song, 1999; Taylor and Zhulin, 1999). However, a significant number are also located outside this region (Quail et al., 1995; Xu et al., 1995). Most of the regulatory mutants reported for phyA are recessive and have a loss-of-function phenotype, although a dominant loss-of-function mutant has been described (Fry et al., 2002). No hypermorphic phyA mutants have yet been described, although site-directed mutagenesis of several Ser residues in the extreme N terminus of rice (*Oryza sativa*) phyA has been shown to confer increased biological activity in transgenic tobacco (*Nicotiana tabacum*; Stockhaus et al., 1992).

As part of ongoing studies of photomorphogenesis in pea (*Pisum sativum*), we screened ethylmethanesulfonate-mutagenized pea seedlings under FR to identify mutants deficient in functional phyA or showing altered phyA responses (Weller et al., 1997a). In this paper, we report on the characterization of a novel dominant *phyA* mutation conferring enhanced light responses. We also confirm that the previously described phyA-deficient mutants *fun1-1* and *fun1-2* (Weller et al., 1997a) carry lesions in the *PHYA* gene.

RESULTS

Isolation of AF05, a Dominant Mutant Showing Enhanced Deetiolation under R and FR

Screening of ethyl methanesulfonate-mutagenized M_2 populations of pea seedlings under continuous FR identified a number of putative mutants with altered deetiolation phenotypes. One mutant, AF05, showed an enhanced deetiolation phenotype and was clearly distinguishable from wild-type plants on the basis of short internodes and an advanced state of leaf development. This phenotype was very similar to that of the pea *cop1-1* mutant (Sullivan and Gray, 2000) grown under the same conditions. Figure 1A shows a comparison of the seedling phenotypes of a plant homozygous for the AF05 mutation and the *cop1-1* mutant grown under continuous FR. The AF05 mutant was also identified when M_2 plants from the same M_1 family were screened under R, and under these conditions the effects of the mutation on elongation and leaf expansion were equally as dramatic as under FR. R conditions were predominantly used for subsequent genetic analysis of the mutant, as seedlings grown under R survived better after transfer to normal glasshouse conditions than those grown in FR.

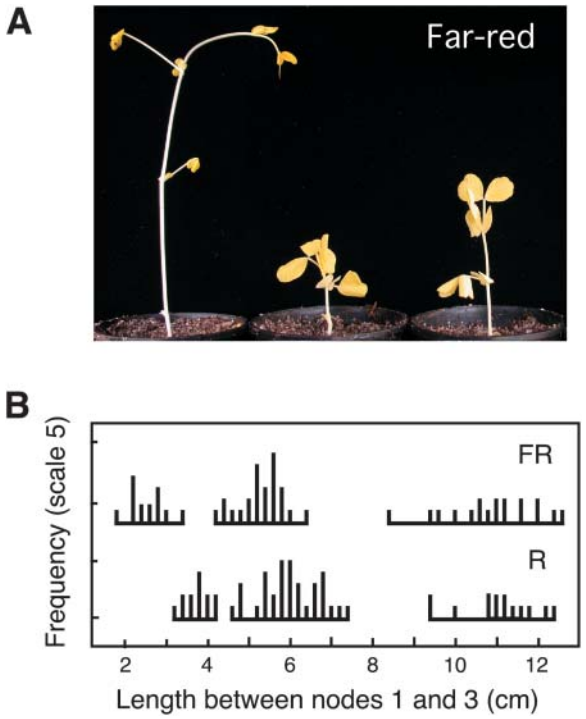

Figure 1. Inheritance of the enhanced response to FR in the AF05 mutant. A, Twelve-day-old wild-type, homozygous AF05 mutant, and *cop1-1* mutant seedlings grown from sowing under continuous FR (10 μmol m^{-2} s^{-1}). B, Segregation for internode length in the F_2 progeny of a cross between AF05 and wild-type cv Torsdag grown under continuous FR or R.

The M_3 progeny of the original AF05 mutant segregated into three distinct phenotypic classes under both R and FR, corresponding to wild type, the original AF05 mutant, and a class of slightly shorter plants. This segregation was in accordance with a 1:2:1 ratio and suggested that the original AF05 mutant was heterozygous for a dominant mutation. This pattern of inheritance was confirmed in the M_4 generation and in the F_2 of a cross between wild type cv Torsdag and a dwarf, putative homozygous dominant mutant M_3 segregant (Fig. 1B). The experiments described below used seed bulked from homozygous mutant plants identified in the M_4 progeny of the original heterozygous mutant, or in the F_3 of the cross described above. The AF05 designation is used hereafter to refer to the homozygous dominant mutant.

The degree of dominance for the AF05 mutant allele calculated on the basis of stem length between nodes 1

and 3 was approximately 35% under both R and FR conditions. Interestingly, the degree of dominance of the mutant allele appeared to vary with development of the plant. Under FR, the AF05 allele was almost completely dominant in controlling elongation of the first internode, but in subsequent internodes, approached codominance with the wild-type allele (data not shown). In contrast to the stem elongation phenotype, the AF05 mutant allele was essentially fully dominant in the control of leaf expansion under both R and FR (data not shown).

The Seedling Phenotype of the AF05 Mutant Is Light-Dependent

In order to examine further the similarity of the AF05 mutant to *cop1*, homozygous AF05 mutant seedlings were grown under monochromatic FR, R, blue light (B), white light (W), and in darkness. In contrast to the *cop1-1* mutant, which displayed a deetiolated morphology when grown in complete darkness, AF05 mutant plants showed a dark-grown phenotype not substantially different from wild-type plants (Fig. 2A), indicating that the AF05 mutant phenotype is essentially light-dependent and is thus quite unlike the *cop1-1* mutant. A small difference between wild type and AF05 plants grown in the dark was usually observed (Fig. 2A, note the slightly larger apical bud and more open apical hook in the AF05 seedling). We consistently observe similar small differences in elongation and apical bud morphology between wild-type and photoreceptor mutant pea seedlings grown in darkness (e.g. wild type/*fun1* in Fig. 3), and similar effects have occasionally been reported for photoreceptor mutants in other species. One explanation is that in some cases there may be a low level of phyA activity in darkness (dark current), which may be amplified by the AF05 mutation. Another possibility is that phyA signaling can be initiated by other stimuli possibly present in our growth cabinets, such as locally elevated ethylene levels or low-level vibration. Figure 2B shows that the reduced elongation and increased leaf expansion of AF05 plants is also strongly expressed under B, as well as under R and FR. Under W, elongation of AF05 seedlings is also substantially reduced relative to wild type, but leaf expansion is inhibited. This does not represent incomplete deetiolation since AF05 leaflets are fully open and darker green than wild type (not shown). The results in Figure 2C show that under lower W irradiance (as under monochromatic R and B), AF05 mutant leaflets are significantly larger than wild type, implying that an active inhibition of leaflet expansion is occurring in the mutant under higher irradiances.

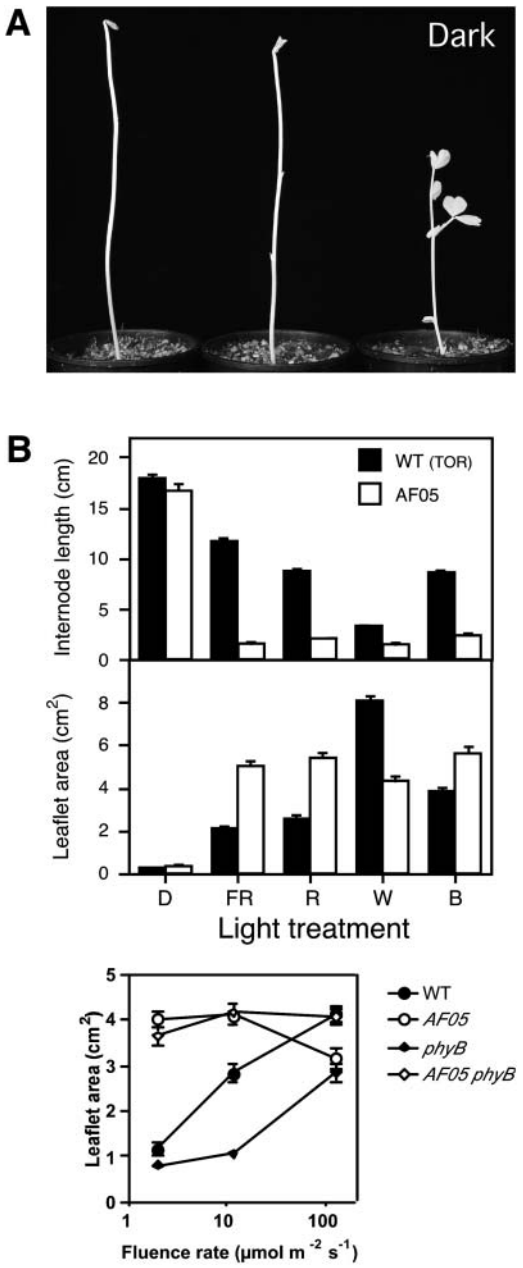

Figure 2. Light-dependent phenotype of the AF05 mutant. A, Twelve-day-old dark-grown seedlings. B, Stem elongation and leaflet expansion in seedlings grown under continuous FR (10 μmol m^{-2} s^{-1}), R (10 μmol m^{-2} s^{-1}), B (10 μmol m^{-2} s^{-1}), W (120 μmol m^{-2} s^{-1}), or in darkness (D). C, Irradiance response for leaflet expansion under W. Stem elongation was measured as the length between nodes 1 and 3. Leaflet area was estimated as length × breadth of a single leaflet from the first true foliage leaf (at node 3). Values ± SE, n = 8 to 12.

The AF05 Mutation Is Closely Linked to the *FUN1* Locus

We next attempted to examine the effects of phyA and phyB deficiency on the AF05 phenotype by crossing AF05 into the *fun1-1* and *phyB-5* mutant backgrounds, which are null for phyA and phyB, respectively (Weller et al., 1997a, 2001). When grown under R, the F_2 of the AF05 × *phyB-5* cross segregated into four phenotypic classes (AF05, AF05 heterozygotes, wild type, and *phyB*) with observed numbers of

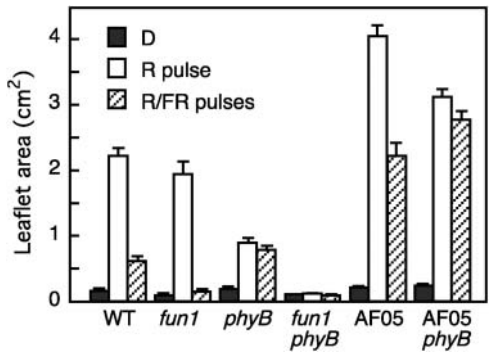

Figure 3. Effect of the AF05 mutation on leaflet expansion in response to R pulses. Seedlings were given a saturating pulse of R (5 min, 10 μmol m^{-2} s^{-1}) or R followed by FR (10 min, 10 μmol m^{-2} s^{-1}) at 4-h intervals, or grown in complete darkness (D). Leaflet area was estimated as length $\times$ breadth of a single leaflet from the first true foliage leaf (at node 3). Values $\pm$ SE, $n = 10$ to 12. The *fun1* and *phyB* mutants used (*fun1-1* and *phyB-5*) are null for phyA and phyB apoprotein, respectively.

14, 27, 20, and 3, respectively. These numbers showed a close fit to a 3:6:6:1 ratio ($\chi^2 = 1.63$, $P > 0.3$) and suggested that pure-breeding AF05 *phyB* double-mutant plants had a phenotype similar to wild type under these conditions. This was confirmed in the F_3, where AF05 *phyB* plants were readily identified as shorter segregants in F_3 progeny from some *phyB* F_2 segregants. However, the F_2 of the AF05 $\times$ *fun1-1* cross segregated into only 2 classes, and no recombinant (i.e. wild type) plants were found in a population of over 2,000 plants. This result demonstrated a very close linkage between AF05 and the *FUN1* locus and provides an estimate of the maximum linkage distance between AF05 and *FUN1* of less than 0.1 cM.

The AF05 Phenotype Represents an Enhanced Response to PhyA

Because it was not possible to examine the dependence of AF05 on phyA genetically, we instead addressed the question by examining the deetiolation response of the AF05 mutant to R and FR light pulses. A consensus has emerged from study of Arabidopsis, tomato, and pea phytochrome mutants showing that phyB-dependent responses require higher fluences/fluence rates of R and are reversible by FR, whereas phyA-dependent responses may be induced by very low fluences/fluence rates of R or FR and are not reversible by FR (Reed et al., 1994; Weller et al., 1995, 1997a; Shinomura et al., 1996; Kerckhoffs et al., 1997). The results in Figure 3 show that leaflet expansion in AF05 was promoted to a much greater extent than in wild type by exposure to brief saturating R pulses given at 4-h intervals. However, the size of the FR-reversible component of this response in the AF05 mutant was similar to that in wild type and phyA-deficient *fun1-1* seedlings, and the increased effectiveness of the R pulses was largely due to an enhancement of the nonreversible component (Fig. 3). It therefore appears that AF05 does not affect phyB action and may specifically enhance responses controlled by phyA. This was subsequently confirmed using an AF05 *phyB* double mutant. Removal of phyB in the AF05 background essentially eliminated the FR-reversible component but did not substantially alter the enhanced, non-FR reversible component (Fig. 3).

The AF05 Phenotype Is Associated with an Elevated Level of PhyA

The apparently specific enhancement of phyA responses in the AF05 mutant could conceivably result from a mutation affecting *PHYA* transcription, PHYA protein stability, or inherent activity of the phyA molecule. We initially examined the characteristics of the phyA protein by in vivo spectrophotometry. This is a simple way of quantifying spectrally active phyA, because more than 95% of spectrophotometrically detectable phytochrome in etiolated pea seedlings is phyA (Weller et al., 1997a). Figure 4A shows that the phytochrome difference spectrum for phytochrome (predominantly phyA) in dark-grown AF05 seedlings did not differ from that in wild type. When dark-grown wild-type seedlings were transferred to continuous R at 25°C, the amount of photoreversible phytochrome declined with a half-life of approximately 84 min (Fig. 4B). Dark-grown AF05 seedlings had marginally lower levels of photoreversible phytochrome than wild type but showed a much lower rate of phytochrome degradation under R, with a half-life of 154 min. As a consequence, the AF05 mutant had 2.5 times the amount of phytochrome (3.0 ± 0.1 units) as wild type (1.2 ± 0.1 units) after 6 h exposure to R. Time points beyond 6 h were not tested due to interference from chlorophyll.

Low temperature fluorescence emission spectroscopy also revealed a lower rate of phyA degradation in AF05 mutant seedlings transferred to continuous R (Fig. 4C). This effect was also seen in a *phyB* mutant background, showing that the increase in phytochrome content of the AF05 mutant is due to a higher level of phyA and not of phyB.

The elevated level of phyA after light exposure could result from either an impaired ability to down-regulate *PHYA* transcription or from a defect in degradation. However, the phyA transcript level was very similar in dark-grown wild-type and AF05 seedlings and showed a very similar down-regulation following transfer to R (Fig. 4D). This result suggests that AF05 specifically affects the level of PHYA protein. The *PHYA* transcript level in seedlings of the phyA apoprotein null mutant *fun1-1* was substantially lower than in wild type, but showed a relatively normal down-regulation in response to R (Fig. 4D), consistent with a previous report that the repression of Arabidopsis *PHYA* expression under R is mediated mainly by phyB (Cantón and Quail, 1999).

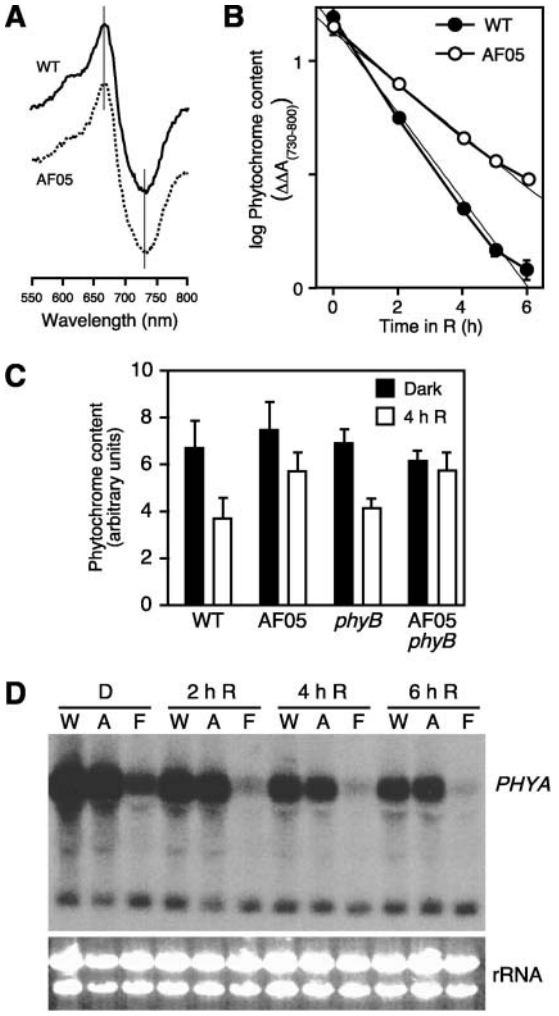

Figure 4. Effect of the AF05 mutation on phyA properties. A, In vivo difference spectra for phytochrome in dark-grown wild-type and AF05 seedlings. B, Depletion of phytochrome after transfer of 7-d-old etiolated seedlings to continuous R (10 μmol m^{-2} s^{-1}) at 25°C, monitored by in vivo spectrophotometry. Values ± SE, $n = 3$. The experiment was repeated twice with similar results. C, Depletion of phytochrome after transfer of 7-d-old etiolated seedlings to continuous R (approximately 1 μmol m^{-2} s^{-1} at 25°C), monitored by in situ low-temperature fluorescence spectroscopy. Values ± SE, $n = 5$ to 10. D, Down-regulation of *PHYA* transcript levels after transfer of 7-d-old etiolated seedlings to continuous R (10 μmol m^{-2} s^{-1}) at 25°C. (W, wild-type; A, AF05 mutant; F, *fun1-1* mutant).

The AF05 Mutant Carries a Missense Mutation in the *PHYA* Gene

The fact that *FUN1* and *PHYA* map to the same region of pea linkage group II (Weller, 1997; Pavy et al., 1998) and the existence of *fun1* mutants that are deficient in both phyA protein (Weller et al., 1997a) and mRNA (Fig. 4D) strongly suggest that these two loci are in fact the same gene. This was confirmed by sequencing the *PHYA* gene from wild-type cv Torsdag and the two independent phyA-deficient *fun1* mutants previously described by Weller et al. (1997a). Single nucleotide substitutions were identified in both mutant alleles, introducing premature stop codons in the place of W531 (TGG-TGA) in *fun1-1* and Q59 (CAA-TAA) in *fun1-2* (Fig. 5A). These mutations introduced restriction polymorphisms that were used to verify cosegregation of the mutant allele with the phyA-deficient phenotype. The *fun1-1* and *fun1-2* mutants have therefore been renamed *phyA-1* and *phyA-2*.

The close linkage of the AF05 mutation to *PHYA*, and the phyA-specific phenotype of the AF05 mutant suggested that the AF05 mutation might also be located in the *PHYA* gene. Sequencing of *PHYA* from AF05 revealed a single C to T substitution, at position 581 in the *PHYA* cDNA (Fig. 5A). No other differences were found between AF05 and cv Torsdag in a region of 6 kb including the entire *PHYA* gene and 1.7 kb 5′ to the transcription start site of the most abundant *PHYA* transcript (Sato, 1988). On the basis of these results, the AF05 mutant has been renamed *phyA-3D*. The *phyA-3D* mutation is predicted to direct a relatively conservative Ala to Val substitution at position 194 in the PHYA amino acid sequence. A194 is perfectly conserved in all available full-length higher plant PHYA sequences and is located within a region that is highly divergent between PHYA and PHYB (Fig. 5B).

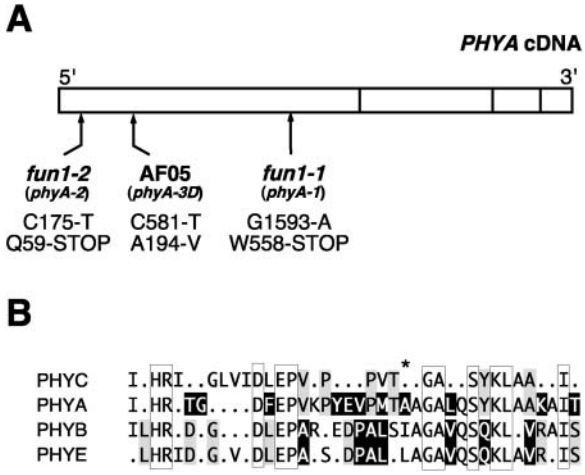

Figure 5. Molecular lesions in pea *phyA* mutants. A, Location and nature of mutations in the pea *PHYA* cDNA. B, Alignment of consensus amino acid sequences for each phytochrome type in the region surrounding the residue substituted in the *phyA-3D* mutant (A194; marked with asterisk). These consensus sequences are derived from alignments of all available full-length dicot sequences for each phytochrome type and show only residues that are invariant for each phytochrome type. This region spans amino acids 170 to 209 in the pea PHYA apoprotein, which correspond to positions 219 to 259 in the alignment of Mathews et al. (1995). Residues perfectly conserved across all full-length sequences are boxed. Residues perfectly conserved and uniquely present in phyA or phyB/E are shown in black. Other residues perfectly conserved in phyA or phyB/E are shaded.

The *PhyA-3D* Mutant Shows Early, Photoperiod Insensitive Flowering

Figure 6 shows that *phyA-3D* mutant plants grown under a 24-h photoperiod (8 h of daylight with a 16-h extension with weak incandescent light) did not differ substantially from wild type in terms of node of flower initiation, time to first open flower, or the total number of reproductive nodes. However, in short-day (SD) conditions (8 h of daylight), *phyA-3D* plants flowered earlier and at a lower node and produced substantially fewer reproductive nodes than wild-type plants. The AF05 mutant was therefore effectively day-neutral, flowering and senescing early under noninductive conditions. This phenotype is similar to that of the previously described day-neutral mutants *sn*, *dne,* and *ppd* (Weller et al., 1997b) and is the converse of that seen for the *phyA-1* null mutant, which flowers late in inductive photoperiods (Weller et al., 1997a). Figure 6 also shows that mature *phyA-3D* plants were substantially shorter than wild-type plants under either long day (LD) or SD.

DISCUSSION

We have identified a dominant, gain-of-function pea mutant that shows enhanced phyA responses and carries a single amino acid substitution in a conserved N-terminal domain of the phyA apoprotein. The mutant also maintains a higher level of phyA than wild type after transfer of etiolated seedlings to R, suggesting that it may be impaired in the light-induced degradation of phyA. Without transgenic complementation experiments, it remains formally possible that the mutant phenotype might result from a different mutation in a gene adjacent to *PHYA*. However, the tight linkage between the mutant phenotype and the *PHYA* gene, and the absence of other mutations in over 6 kb of genomic DNA spanning the PHYA coding region, strongly support the *phyA3-D* mutation as the cause of the mutant phenotype.

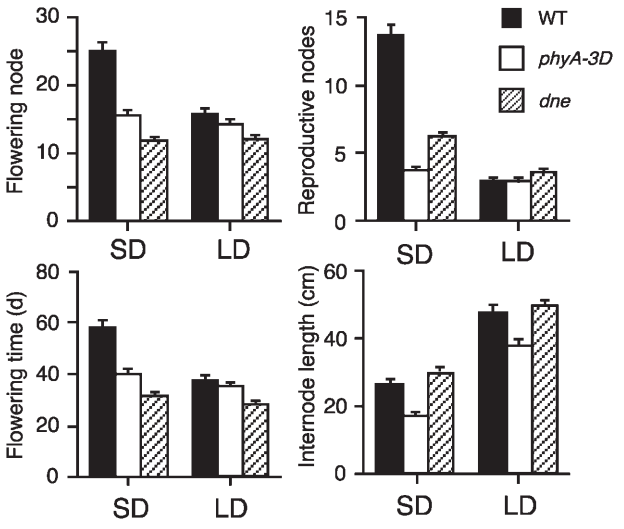

Figure 6. Photoperiod response of the *phyA-3D* mutant. Plants were grown from sowing in an 8-h photoperiod of natural daylight either with (LD) or without (SD) a 16-h extension of light from an incandescent source (10 μmol m^{-2} s^{-1}). Internode length was measured between nodes 1 and 9. All values ± SE, n = 6 to 8.

The *PhyA-3D* Phenotype Can Be Explained by Impaired Degradation of PhyA

There are several different ways that a mutation within the phyA coding sequence might result in increased phyA activity. The mutation could interfere with the photochemistry or the initial signaling reactions of phyA or could increase the level of phyA by impairing its light-induced degradation. The first two possibilities have not yet been rigorously tested. However, the mutant phyA appears to possess normal spectroscopic properties and is identical to wild-type phyA at least with respect to the photochemistry of the initial photoreaction and the Pr-Pfr conversion (V.A. Sineshchekov, unpublished data). The most direct interpretation of our results is therefore that the mutation affects the level of active phyA. We monitored the level of active phyA during deetiolation using two different spectrophotometric techniques and in both cases found that phyA levels remained significantly higher in the mutant than in wild type over the first few hours after exposure of dark-grown plants to R (Fig. 4). In the apparent absence of any effect of the mutation on *PHYA* transcript level during this period (Fig. 4), it is likely that this lower degradation is reflected in an elevated steady state level of phyA in fully deetiolated mutant plants. Transgenic *PHYA* overexpression in several species has shown that strong enhancement of phyA responses requires only a 2- to 3-fold increase in the level of PHYA and further increases in phyA level have little additional effect (Boylan and Quail, 1991; Jordan et al., 1995). This suggests that the similarly increased level of phyA in the *phyA-3D* mutant may be sufficient to explain the mutant phenotype.

The *PhyA-3D* Mutant Differs from Other PhyA Signaling Mutants

Among known phyA signaling mutants in Arabidopsis, several have been shown to interfere with phyA protein dynamics, and of these, two have been characterized in some detail. Eichenberg et al. (2000) describe a recessive loss-of-function mutation (*phyA-106*) that results in an acceleration of phyA photodestruction. However, it is not yet clear whether the effect on degradation is sufficient to explain the mutant phenotype, which is very similar to that of a null mutant (Xu et al., 1995). The *phyA-106* mutant carries a single substitution (C716Y) that lies in the core of the C-terminal signaling region defined by Quail et al. (1995). This region has been implicated in

the binding and activation of phy-associated proteins like PIF3 and NDPK2 (Choi et al., 1999; Ni et al., 1999), and it is thus possible that the *phyA-106* phenotype might result in part from an impaired interaction of the mutant phyA with signaling partners. However, since both this specific Cys residue and the surrounding region are also highly conserved in phyB, it seems unlikely that the primary effect of the mutation is impairment of a phyA-specific process. This is further supported by the fact that substitution of the adjacent conserved Ala residue impairs signaling of phyB (Wagner and Quail, 1995).

Another recessive loss-of-function mutation affecting phyA dynamics in Arabidopsis was recently identified by Maloof et al. (2001) as a naturally occurring M548T polymorphism in the Lm-2 ecotype. In contrast to *phyA-106,* this mutation results in a complete loss of light-induced PHYA degradation, a reduction in autophosphorylation activity, and subtly altered photochemical properties of the photoreceptor. The substituted residue is in this case located in the hinge region between the N- and C-terminal domains. Relatively few mutations have been mapped to this region, although a G564E substitution in phyB (corresponding to G533 in phyA) has been reported to confer increased responsiveness (Kretsch et al., 2000).

The A194V substitution in the pea *phyA-3D* mutant thus clearly differs from both of these previously identified mutations in several respects. Like the Arabidopsis M548T mutation, the A194V mutation impairs the destruction of phyA but seems to slow it down rather than blocking it completely. In addition, the observed effects of the pea *phyA-3D* mutation on the level of phyA appear sufficient to explain the hypermorphic phenotype, whereas the loss-of-function phenotype of the Arabidopsis M548T mutant clearly cannot be explained by its elevated phyA content, which is presumably a secondary consequence of impaired signaling.

The *PhyA3-D* Mutation Identifies a Residue Critical for Normal PhyA Degradation

The predicted amino acid substitution A194V in the *phyA-3D* mutant is located in a region that shows a high degree of conservation among available full-length PHYA sequences. Figure 5B shows a comparison of consensus sequences for PHYA, PHYB, PHYC, and PHYE phytochromes in the region of the mutation. These consensus sequences were derived from alignments of all available full-length dicot sequences for each phytochrome type. Within a 40-amino acid region between residues 170 and 210, 11 residues are perfectly conserved across all phytochromes. Of the remaining 29 residues, 17 (including A194) are perfectly conserved in phyA but not present in phyB or phyE, and of these 11 are unique to phyA. In the same region, 14 residues are conserved in phyB/D/E but not present in phyA, and of these 7 are unique to phyB/D/E. Thus, the region shows strong sequence conservation in both phyA-type and in phyB-type phytochromes but is highly divergent between the two types, consistent with a role in specifying a functional difference between the two.

The location of the *phyA-3D* mutation within the N-terminal half of phyA is also consistent with previous domain swapping and deletion studies that located the determinants of phyA degradation within a 74-kD region of phyA between residues 70 and 598 (Wagner et al., 1996; Clough et al., 1999). Six loss-of-function regulatory mutations have been identified within this region (Quail et al., 1995; Xu et al., 1995) in addition to the M548T natural variant described by Maloof et al. (2001). The effects of these mutations on PHYA degradation have not been reported, except for one (*phyA-107*) that shows normal kinetics (Eichenberg et al., 2000). Further mutational or transgenic dissection of this region has so far not been pursued. A small motif within this region, the so-called PEST sequence (residues 323–360), has been suggested as a possible determinant of phyA degradation based on the observation that it is present in short-lived proteins and is preferentially exposed in the Pfr form of phyA (Quail, 1991), but there is no direct experimental support for this proposal. The identification of the *phyA-3D* mutation provides evidence for the involvement of a specific residue in phyA degradation and raises the possibility that the surrounding highly conserved region of the phyA molecule may also be important for this process.

Alternative Explanations for the Elevated PhyA Level in the *PhyA-3D* Mutant

Like Arabidopsis phyA, pea phyA is translocated from cytoplasm to nucleus following exposure to R or FR (Kircher et al., 1999; Hisada et al., 2000; Kim et al., 2000). Recent evidence from nuclear and cytosolic targeting suggests that phyB is only active in the nucleus and not in the cytosol (Huq et al., 2003; Matsushita et al., 2003), and it is probable, although not yet proven, that the same is true for phyA. In addition, phyA is apparently lost from the cytosol in etiolated Arabidopsis and tobacco seedlings transferred to continuous R without appreciable accumulation in nucleus (Kim et al., 2000), suggesting that degradation is mainly occurring in the cytosol. These studies have led to the proposal that the activity of phyA relates mainly to the level of phyA in the nucleus, which reflects a balance between nuclear import and cytosolic turnover (Nagy and Schäfer, 2002).

One interpretation of the elevated phyA level in the *phyA-3D* mutant is therefore that the mutation specifically interferes with recognition of the Pfr form of phyA by the degradation machinery. The mechanism of Pfr-specific PHYA destruction is still unknown, but there is evidence for the involvement of the ubiquitin/26S proteasome pathway. The loss of Pfr has been shown in several species to coincide both temporally

and spatially with the appearance of ubiquitin-phy conjugates (Clough and Vierstra, 1997). In addition, deletion studies have shown that a small C-terminal region of phyA (residues 1,118–1,123) is essential both for ubiquitination and degradation of phyA to proceed (Clough et al., 1999). Specificity for protein degradation by the ubiquitin/26S proteasome pathway is thought to be determined by the interaction between the target protein and an E3 ubiquitin ligase. These proteins comprise a large and diverse family in Arabidopsis, with over 1,200 members in 5 distinct classes (Vierstra, 2003). Several proteins affecting light signaling have E3 ligase homology and/or activity, including one (EID1) that specifically affects phyA responses (Dieterle et al., 2001), but an E3 ligase that specifically affects the phyA protein has not yet been identified. Another possibility is that the mutation could enhance the nuclear import of phyA, resulting in a smaller pool of phyA in the cytosol available for degradation. Work is now under way to test the effect of the corresponding A194V substitution in Arabidopsis PHYA with respect to both degradation and subcellular localization.

The *PhyA-3D* Mutation also Affects Responsiveness to Photoperiod

Mature *phyA-3D* mutant plants are dwarf and show early, photoperiod-independent flowering (Fig. 6). This is consistent with the important role identified for pea phyA in mature plants (Weller et al., 1997a) and implies that the phyA degradation defect identified in mutant seedlings persists throughout the life of the plant. Arabidopsis plants overexpressing phyA also show early flowering and a suppression of the photoperiod response (Bagnall et al., 1995). In this respect, the *phyA-3D* mutation could be considered analogous to the cryptochrome 2 (*CRY2*) allele present in the Arabidopsis Cvi accession, which confers enhanced stability to the CRY2 protein under SD and a genetically dominant early-flowering phenotype (El-Assal et al., 2001). The roles of phyA and cry2 in the photoperiodic control of flowering are complex, but they are thought to promote flowering in LD mainly by stabilizing the CO protein late in the day, thus enhancing the induction of first open flower expression by CO (Yanovsky and Kay, 2003; Valverde et al., 2004). It is possible that an elevated level of phyA could confer an early-flowering phenotype by stabilizing CO also during SD.

The early-flowering, day-neutral phenotype of the *phyA-3D* mutant is also similar to previously described day-neutral pea mutants *sn*, *dne,* and *ppd*, which have all been shown to be deficient in a graft-transmissible inhibitor of flowering (Weller et al., 1997b). PhyA plays an important role in down-regulating this inhibitor under LD (Weller et al., 1997a). Although the graft-transmissibility of the *phyA-3D* mutant phenotype in SD has not yet been tested, the phenotype is consistent with a reduced level of flower inhibitor, and under SD conditions *phyA-3D* rootstocks would be expected to show a reduced ability to inhibit flowering in grafted scions.

Another characteristic of phyA overexpression is the suppression of responses to shading and neighbor proximity. Normally, the increased shading and/or lateral reflection of FR within a canopy causes an increase in the proportion of FR in the incident light and reduces phyB-mediated inhibition of stem and petiole elongation (Ballaré, 1999). In wild-type plants this response is antagonized by inhibitory effects of FR mediated through phyA (McCormac et al., 1992; Weller et al., 2001). Overexpression of phyA can further increase the inhibition of elongation by FR, to the extent that the phyB-mediated shade- and neighbor-avoidance responses can be suppressed and even reversed (McCormac et al., 1992; Robson et al., 1996; Shlumukov et al., 2001). Higher levels of phyA activity may thus enable plants to be grown at greater densities while avoiding detrimental effects of shading (Robson et al., 1996). The similarity of the pea *phyA-3D* mutant to phyA-overexpressing lines suggests that it may be possible to achieve a specific enhancement of phyA activity by mutagenesis and selection alone, without resort to genetic modification and its associated controversy.

MATERIALS AND METHODS

Plant Material

The AF05 mutant was selected during screening under FR of an M_2 population of ethyl methanesulfonate-mutagenized seedlings of pea (*Pisum sativum*) cv Torsdag (Weller et al., 1997a). The *phyA-1*, *phyA-2*, *phyB-5*, and *dne* mutants have been described previously (King and Murfet, 1985; Weller et al., 1995, 1997a, 2001).

Growth Conditions and Plant Husbandry

Three different growing environments were used for the experiments described below. Plants used for spectrophotometry (Fig. 4) and for pulse experiments (Fig. 3) were grown in growth cabinets at 25°C. These plants were grown in water-saturated, drained vermiculite. All other plants were grown in the phytotron or in growth cabinets, in a 1:1 mix of dolerite chips and vermiculite topped with potting mix. For mutant screening, seedling deetiolation experiments, and segregant selection, plants were grown in growth cabinets at 20°C. In the Hobart phytotron, plants received an 8-h photoperiod of natural daylight in a glasshouse before transfer to night compartments maintained at 16°C where they received either 16 h of darkness or 16 h of light from an incandescent source. Standard monochromatic light sources were employed as previously described (Weller et al., 1997a). All plants grown to maturity received nutrient solution once weekly. Lateral shoots were regularly excised from all plants grown in flowering experiments. Only main shoots were scored, and counting of nodes began with the cotyledons as node 0. Stem length was measured as the length between two nodes *x* and *y*.

Spectrophotometric Measurements

Growth and harvest of plant material and spectrophotometric measurements of phytochrome were carried out according to previously described methods (Weller et al., 1995; Sineshchekov et al., 1999).

RNA Extraction and Analysis

Total RNA was extracted (from 2.5 g of fine ground powder) from 7-d-old seedlings using RNAse free TLES buffer (100 mM Tris HCL pH 8, 100 mM LiCl,

10 mM EDTA, 1% SDS) and a series of citrate-equilibrated phenol/chloroform/iso-amylalcohol, then precipitated in 4-M LiCl and resuspended and washed in 70% ethanol (Verwoerd et al., 1989). Five micrograms of total RNA was electrophoresed through a 1% agarose gel (containing 9% formaldehyde) as described by Schultz et al. (2001) and transferred onto Gene Screen Plus (Perkin Elmer, Boston) hybridization transfer membrane. A 995-bp fragment of the pea PHYA cDNA (corresponding to amino acids 683–1014 in the PHYA protein) was labeled with ^{32}P following the DecaLabel DNA labeling kit (MBI Fermentas, Burlington, Canada). The blots and probe were left to hybridize overnight at 42°C. Membranes were washed in a series of 2 × SSC/0.1% SDS followed by 0.2 × SSC/0.1% SDS before being autoradiographed for 1 to 2 d at −70°C on Biomax MS (Kodak, Rochester, NY).

DNA Extraction and Sequencing

Genomic DNA was extracted from 2-week-old W-grown seedlings of wild-type cv Torsdag and the *fun1-1*, *fun1-2*, and AF05 mutants following the protocol of Ellis (1994). Five overlapping fragments spanning nucleotides 25 to 6,249 of the 6,264-bp pea *PHYA* genomic sequence (Sato, 1988) were amplified by PCR using the following primer pairs: phyAF1 (5′-TTCAAATTCATGACATCCGACT-3′)/phyAR1 (5′-ATGCGTTGGATAATGGGAAA-3′), phyAF2 (5′-ATACGATGACATGGCATCACTATCATTCAG-3′)/phyAR2 (5′-TACAATACCTAAGGGTGCATCTCGCATCAA-3′), phyAF3 (5′-AGTTTCTGGCTCAAGTGTTT-3′)/phyAR3 (5′-ATCCCCATGTGTTTTTATCTC-3′), phyAF4 (5′-CTGTTGTGTGATATGTTGATGCGAGATGCA-3′)/phyAR4 (5′-GCTGTGATGGATTGGAAGAAAGACACTATT-3′) and phyAF5 (5′-AAATTCCCATGCACCCATAG-3′)/phyAR5 (5′-GTTCCTACCCCAGATTTGA-3′). Sequencing primers were designed from the published sequence at intervals of approximately 350 bp on both strands, and direct sequencing of PCR products was performed using DTCS chemistry and a CEQ8000 automated sequencer (Beckman Coulter, Fullerton, CA).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY688953.

ACKNOWLEDGMENTS

We thank Ian Cummings and Tracey Jackson for assistance with plant husbandry, Dick Kendrick for access to spectrophotometer and growth cabinet facilities at RIKEN (Japan), and Noel Ellis for help with linkage analysis.

Received November 11, 2003; returned for revision April 14, 2004; accepted April 29, 2004.

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