Phytochrome A plays a major role in early seedling development by triggering the transition from etiolated growth to greening. Seedlings germinated under constant far-red (FR) light show a partially de-etiolated phenotype that is not seen in phyA mutants. This phytochrome A specific response was used to screen a population of T-DNA mutagenized Arabidopsis seedlings. One mutant line, pat3 (phytochrome A signal transduction), showed no inhibition of hypocotyl elongation under FR light conditions and no FR-induced killing response, contained a T-DNA insertion in a 609-bp ORF. The recessive mutation co-segregated with the T-DNA resistance marker and could be allelic to fhy1. A 2,248-bp genomic fragment of the PAT3 locus can complement the pat3 mutant phenotype. PAT3 transcript peaked 3 d after germination and was downregulated by light. PAT3 has no significant homology to any known protein and shows no preferential cellular localization. The protein can activate transcription in yeast when fused to the GAL4 DNA-binding domain. Our results show that PAT3 is a positive regulator of phytochrome A signal transduction.

Key words: Arabidopsis — FHY1 — Hypocotyl elongation — PAT3 — Phytochrome A — Signal transduction.

Abbreviations: B, blue; D, darkness; FR, far red; GFP, green fluorescent protein; HIR, high irradiance response; NES, nuclear export signal; NLS, nuclear localization signal; phyA, phytochrome A; POR, protochlorophyllide oxidoreductase; R, red; SSLP, simple sequence length polymorphism; UAS, upstream activator sequence; W, white; WT, wild type; RACE, rapid amplification of cDNA ends; ORF, open reading frame.

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number AF424739 (PAT3 cDNA).

Introduction

Light is not only the essential energy source for plants but also serves as an important environmental signal for plant development. Seeds buried in soil contain only limited energy resources. Consequently, germinated seedlings are compelled to reach light as efficiently as possible by adapting a slender, leafless growth with an apical hook (etiolation). Upon exposure to light, the growth program is radically changed: hypocotyl extension is inhibited, the hypocotyl hook opens, the cotyledons unfold and proplastids develop into mature chloroplasts (Kendrick and Kronenberg 1993, Smith 2000). The signal for de-etiolation is mainly perceived by the phytochrome photoreceptor family (Quail et al. 1995, Whitelam and Devlin 1997). In Arabidopsis, five members of the phytochrome family have been identified, PHYA to PHYE (Sharrock and Quail 1989, Clack et al. 1994) which all share high amino acid homology but are differentially regulated at the transcriptional and post-transcriptional levels (Quail et al. 1995, Canton and Quail 1999).

Among the phytochrome family members, phytochrome A (phyA) is quite abundant in darkness but its transcription is rapidly down regulated upon exposure to light and the protein is degraded to a small pool (Quail 1994). As the prevalent phytochrome in etiolated seedlings, it plays a key role in sensing light to trigger germination and de-etiolation. Phytochrome A-deficient mutants are insensitive to far-red (FR) light and show an etiolated phenotype under these conditions, whereas wild-type (WT) plants undergo partial de-etiolation (Whitelam et al. 1993). Moreover, downregulation of protochlorophyllide oxidoreductase A (PORA) in WT seedlings by FR light blocks plastid maturation (Runge et al. 1996) rendering these FR-treated seedlings sensitive to subsequent irradiation with white light (“FR induced killing”; Barnes et al. 1996a). As phyA is the only photoreceptor that can be activated by FR light, these specific morphological phenotypes have been used to identify mutants deficient in phyA signaling. Although several phyA-specific mutants (fhy1, fhy3, fin2, spa1, far1, fin219, pat1, eid1, hfr1/rep1/rsf1, laf1, laf6; Whitelam et al. 1993, Soh et al. 1998, Hoecker et al. 1998, Hudson et al. 1999, Hsieh et al. 2000, Bolle et al. 2000, Büche et al. 2000, Fairchild et al. 2000, Spiegelman et al. 2000, Soh et al. 2000, Fankhauser and Chory 2000, Møller et al. 2001, Ballesteros et al. 2001) have been identified so far, the roles of their gene products, if known, and their role in the different aspects of phyA signaling are just beginning to be unraveled.
Further downstream in the signaling cascade, the class of the pleiotropic cop/det/fus mutants has been identified (reviewed in Schwechheimer and Deng 2000, Hardtke and Deng 2000) but their encoded gene products have not yet been functionally linked to phytochrome. As these mutants show photomorphogenesis in darkness, their gene products likely act as repressors of the de-etiolation pathway. However, molecules directly linking light perception by phytochrome to the regulation of these repressors have not yet been identified. It has been shown that phytochrome A and B can translocate to the nucleus after light perception (Kircher et al. 1999, Hisada et al. 2000, Yamaguchi et al. 1999, Kim et al. 2000) where they are able to interact with transcription factors as such as PIF3 (Ni et al. 1998). A single FR pulse is sufficient for phyA to translocate to the nucleus. As phyA itself does not contain a known nuclear localization motif, its translocation might depend on other molecules or a larger protein complex. The diversity in subcellular localizations amongst all characterized signaling components (PIF3, FAR1, REP1 LAF1: nuclear and FIN219, PKS1, PAT1: cytoplasmic) underlines the suggested distinct functions of phytochrome in the cytoplasm and nucleus (Smith 2000). Here, we describe the identification and characterization of PAT3, a positive regulator of the phyA signaling pathway, which is distributed in the cytoplasm as well as in the nucleus.

**Results**

**Mutant screen and physiological analysis of pat3**

Arabidopsis seedlings from a T-DNA mutagenized population (Weigel et al. 2000) were screened after 3 d of growth on sucrose-free MS medium under constant FR light (5 μmol m⁻² s⁻¹). Seedlings showing a long hypocotyl and resistance to “FR-induced killing” were selected for further analysis (Barnes et al. 1996a, Bolle et al. 2000). Several putative mutants were recovered and here we describe the characterization of one of them, called pat3 (pat: phytochromeA signal transduction). The elongated hypocotyl phenotype of pat3 was only observed under FR light but not under any other light conditions tested such as red (R), blue (B) and white (W) light (Fig. 1), indicating that the mutant phenotype is specific to the phytochrome A signal transduction pathway. The phenotype was almost as severe as that of the phyA null mutant with respect to inhibition of hypocotyl elongation, cotyledon expansion and resistance to “FR-induced killing”. PHYA protein levels, on the other hand, were similar to WT levels as determined by Western blot analysis (Fig. 2). As the phenotype was specific for FR light, but not observed under R light conditions, a defect in the chromophore biosynthesis is unlikely.

Backcrosses with Columbia WT generated an F1 progeny that showed a WT phenotype under FR conditions, indicating
that pat3 is a recessive mutation (Fig. 1). The mutant phenotype co-segregated among the F2 progeny with the BASTA selectable marker linked to the T-DNA. Crosses with fhy1 (Whitelam et al. 1993) showed no segregation in the F2 generation suggesting that pat3 is allelic to fhy1, although we cannot exclude that the two mutations might be tightly linked. Coarse positional mapping was performed to verify that the mutation indeed map to the bottom arm of chromosome II. The mutation was mapped close to marker BIO2 on chromosome II.

**Cloning of the PAT3 gene**

Sequences flanking the T-DNA insertion were cloned by plasmid rescue utilizing digested and re-ligated genomic DNA. Sequence analysis revealed that the T-DNA was inserted into the ORF At2g37680 (GenBank, Lin et al. 1999), thereby substituting approximately 800 bp. Results from 3’ and 5’ rapid amplification of cDNA ends (RACE) reactions with the WT cDNA using primers within the ORF showed, however, that the transcribed region was much smaller than the one predicted by GenBank (Lin et al. 1999). In the GenBank annotation, At2g37680 contains one 3,695-bp ORF containing six small and widely spaced exons. Our RACE results, however, showed that At2g37680 contains two genes, one of which is PAT3. PAT3 consists of three exons among which exon II contains most of the coding sequence (Fig. 3A). The insertion resulted in the deletion of 785 bp including the 5’ region of PAT3, exon I, and a part of exon II (Fig. 3A).

**Fig. 3** Genomic structure of PAT3 and its encoded protein. (A) A schematic diagram showing the genomic structures of the PAT3 gene. EI, EII, and EIII represent the three exons of PAT3. The T-DNA substituted the sequences indicated, which included EI and part of the 5’ end of EII. (B) The deduced amino acid sequence of PAT3. Putative nuclear localizing signal and nuclear export signal are in bold letters and putative phosphorylation sites are underlined. (C) Amino acid sequence comparison between PAT3 and a related protein encoded by PAT3 homo (At2g02200) in the Arabidopsis genome. Identical amino acids are shown in white letters on black background. Sequences were aligned to give maximum homology. The PAT3 cDNA sequence was deposited in GENBANK (AF424739).

**Fig. 4** Expression of PAT3 under different light conditions and during seedling development. Seedlings were grown in darkness (D) or in W, B, R, and FR light. In the bottom panel, WT seeds were kept in the cold for 4 d (stratification) and then exposed to light for 24, 72, and 120 h. Each lane contained 5 μg total RNA. Northern blots were hybridized with a PAT3 cDNA probe. The 25S RNA band as stained by ethidium bromide was shown as a loading control. The arrowhead on the fhy1 panel indicates the position of the PAT3 transcript.
Positive factor PAT3 in phytochrome A signaling

A databank search (Blast, Altschul et al. 1990) revealed a close homolog (30% amino acid identity) on Chromosome V (At5g02200) of Arabidopsis. This shows similarity in sequence and in the location of the putative NLS and NES of PAT3. Interestingly, a stretch of 17 amino acids at the C-terminus is also highly conserved (Fig. 3C).

To further confirm that the phenotype was due to a disruption of the PAT3 gene, we transformed pat3 with a 2,248-bp long genomic fragment, beginning 688 bp 5’ of the ATG start codon of PAT3 and ending 542 bp 3’ of the TAA stop codon, using the binary vector VIP96 which contains a kanamycin resistance marker (van der Krol and Chua 1991). An empty vector was used as a negative control. Two independent transformation experiments were carried out with the PAT3 genomic clones. T1 transformants were analyzed under FR light conditions and 25 seedlings that showed WT-like hypocotyl length were randomly picked. All these seedlings were found to be resistant to kanamycin, indicating that they were indeed transformed with the vector carrying the PAT3 gene. By contrast, kanamycin-resistant seedlings carrying the empty vector displayed the pat3 mutant phenotype (Fig. 1B). This confirms that the disruption of this ORF causes the mutant phenotype.

Expression pattern of PAT3

Northern blot analysis of PAT3 expression was performed on total RNA prepared from seedlings treated either with FR, R, W or B light for 3 d. As expression levels of PAT3 are very low, RNA probes were used for hybridization. Expression was detected in etiolated seedlings and seedlings grown in FR light. Reduced levels of PAT3 transcript was found in seedlings exposed to B and FR light but no expression was detected in seedlings grown under W or R light conditions (Fig. 4). These results suggest that PAT3 expression is negatively regulated by light in a similar way as PHYA (Quail et al. 1995). In the phyA mutant, PAT3 expression level was overall higher than in WT but it continued to be down regulated by W and R light, and even by B light (Fig. 4). As expected, no transcript could be detected in the pat3 mutant supporting the contention that it is a null. On the other hand, very low levels of a slightly larger transcript were detectable in the fhy1 mutant.

To analyze PAT3 expression during early seedling development, RNA of dark-grown seedlings was extracted at various time points. Northern analysis showed that PAT3 expression was induced 3 d after germination and then declined. No transcript was detected in seeds and prior to germination.

Localization of PAT3::GFP fusion protein in onion epidermis

C-terminal fusion constructs of full length PAT3 protein with GFP were transiently expressed in onion epidermis cells to investigate the intracellular distribution of PAT3. The PAT3::GFP fusion protein was found in the nucleus as well as in the cytoplasm. No obvious difference in the distribution was found after incubation in D or under W light conditions 12 h after bombardment (Fig. 5). The overall fluorescence intensity of PAT3::GFP was lower than that of GFP alone. No specific change in localization could be detected in onion epidermis cells which had been transferred from darkness to W, R or FR light (data not shown).

PAT3 can activate transcription

PAT3 was fused to the GAL4 DNA binding domain in the Matchmaker two hybrid system (Clontech, CA, U.S.A.) to test its ability to transactivate transcription in a heterologous system. Interestingly, this protein was able to induce transcription from a GAL1 upstream activator sequence (UAS) resulting in growth on media lacking histidine and a significantly elevated α-galactosidase activity as compared to the control (Fig. 6).
Discussion

Here we describe the isolation and characterization of pat3, a null mutant specifically impaired in phyA signaling. In FR light, pat3 resembles phyA in having long hypocotyls, closed cotyledons and being resistant to FR-induced killing. By contrast, pat3 is indistinguishable from WT under all other light conditions tested. The T-DNA insertion is tightly linked to the mutant phenotype as seen by the co-segregation in the F2 progeny of the cross pat3 × WT. T-DNA plasmid rescue from pat3 genomic DNA and RACE analysis on the At37680 locus in WT plants revealed two genes in the Genbank-predicted ORF. The T-DNA integrated in the upstream part of the second gene (PAT3). This event was apparently associated with a deletion of approximately 800 bp. Transformation of pat3 with a 2,248-bp genomic fragment, containing the PAT3 gene and 1,230 bp surrounding sequences, complemented the mutation. Therefore, we conclude that PAT3 is the gene responsible for the observed phenotype. The disruption of the 5′ end of the PAT3 gene resulted in a null mutation which is also evident from the inability to detect its transcript in pat3.

Because the recessive mutation leads to a disruption of phyA signaling, we conclude that PAT3 is a positive regulatory component in phyA signal transduction. Only few mutants with a similar phenotype to phyA have been isolated in genetic screens, among which are fhy1, fhy3 and pat1. PAT1 encodes a member of the GRAS protein family and in the semidominant pat1 mutant expression of a truncated PAT1 blocks phyA signaling (Bolle et al. 2000). The genes disrupted in fhy1 and fhy3 have not been cloned so far (Whitelam et al. 1993). To test for possible allelism, we crossed pat3 to both these mutants. As there was no segregation in the F2 plants of the cross with fhy1, it is likely that pat3 is allelic to fhy1. Under FR light, fhy1 also has a strong hypocotyl phenotype similar to phyA null mutant and FIHY1 has been proposed as a negative regulator of phyA signaling (Bolle et al. 2000). The acidic nature of PAT3 (Pim er al. 1998) and yeast transcription activators (Schwechheimer et al. 1998) corresponds with the described acidic properties for some plant transcription factors which have been shown to function in yeast (Xie et al. 2000, Rossini et al. 2001, Schwechheimer et al. 1998). The acidic nature of PAT3 (P of 4.3) corresponds with the described acidic properties for some plant (Schwechheimer et al. 1998) and yeast transcription activators (Ma and Ptashne 1987). The PH3 homolog (PH3homo) shares not only the NLS and NES signals with PAT3 but also the C-terminal 17 amino acids. The function of this part of the protein remains to be elucidated. Despite an overall identity of 30% between these two proteins, they likely execute their functions in different pathways, as PH3homo apparently cannot substitute for PAT3 in phyA signaling. Although the pat3 mutant was identified by an FR screen, the function of PAT3 in the natural environment will most probably be connected with direct signaling for the transition from etiolated growth to growth in light (Smith 1993). As phyA cycles between Pr and Pfr continuously under high irradiance response (HIR) FR conditions (Shinomura et al. 2000), its signaling can be assumed to be much stronger under continuous FR light than in natural conditions where phyA has its major role at the precise moment when the seedlings break through the soil and perceive light for the first time. Therefore, under laboratory HIR conditions, disruptions in the signal flow from photoreceptor to downstream developmental regulators have a much stronger phenotype when compared to growth under the natural environment, as seen in pat3.
Material and Methods

Plant materials
A collection of T-DNA insertion lines generated as described by Weigel et al. (2000) was used for genetic screens. A phyA mutant in A. thaliana Columbia background (phyA 211) and fhy1 in Landsberg erecta background (kindly provided by Gary Whitelam, University of Leicester, U.K.) were used as controls. WT refers to ecotype Columbia unless otherwise noted.

Growth conditions
Seeds were surface sterilized and sown on Murashige and Skoog (MS) agar (Gibco BRL) plates without sucrose. Seeds were incubated for 4 d in the dark at 4°C for stratification. After a white (W) light pulse of 1 h, plates were kept at 25°C in the dark for 24 h and then transferred to the appropriate light conditions for 4 d. Individual lines were re-screened as described in Moller et al. (2001). To determine hypocotyl lengths 50 seedlings grown under each light condition were measured. For the kinetics of PAT3 expression during germination, samples were taken before sterilization (dry seeds), after stratification and at 24, 72 and 120 h after the white light pulse. Light sources were as described in Bolle et al. (2000) and blue light was provided by fluorescent light tubes (Osmar Sylvania, Danvers, MA, U.S.A.) filtered through Plexiglas 470±20 nm (WestLake Plastics, Lenni, PA, U.S.A.). Fluence rates for the various light treatments were as follows: FR 5 μmol m⁻² s⁻¹, R 20 μmol m⁻² s⁻¹, B 20 μmol m⁻² s⁻¹ and W 20 μmol m⁻² s⁻¹.

PHYA extraction and immunoblotting
Seedlings were grown in darkness for 4 d, ground in liquid nitrogen and the resulting powder was resuspended in SDS sample buffer followed by SDS-PAGE analysis (Sambrook et al. 1989). Western blotting with the polyclonal anti-phytochrome A antibody ARF3 was performed after Halliday et al. (1999).

Genetic analysis
Mutant plants of pat3 were crossed with WT Columbia plants and the resulting F1 progeny was analyzed for hypocotyl lengths under FR light. The F2 progeny showed co-segregation of the mutant phenotype with the BASTA resistance marker (T-DNA associated, Weigel et al. 2000). For positional mapping pat3 was crossed with ecotype Landsberg erecta and plants displaying the FR phenotype in the F2 generation were selected for molecular analysis. From 30 F2 generation mutant plants, DNA was isolated with the Plant DNA Mini Kit (Qiagen, CA, U.S.A.) following the instructions of the manufacturer. The SSLP markers T16F6, T8018 and BIO2 (Bell and Ecker 1994) were used for coarse positional mapping of PAT3.

Isolation of PAT3 genomic locus and cDNA
To identify sequences flanking the T-DNA flanking sequences, 500 ng of pat3 genomic DNA was digested with BamHI and re-ligated in 200 μl with T4 DNA ligase (New England Biolabs). After phenol/chloroform purification and ethanol precipitation the DNA was resuspended in 50 μl water. Two μl DNA solution were used to transform competent XL-10 E. coli cells (Stratagene). Plasmid DNA was recovered (plasmid miniprep kit, Qiagen, CA, U.S.A.) from transformed colonies. Plasmids containing the T-DNA and contiguous genomic sequences were sequenced with the oligonucleotide LB6 (5'-CGACTTGCTGCCCCGAGAA) to determine the sequences flanking the T-DNA border. The obtained sequences were compared to sequences in the Arabidopsis genome by BLAST (Altschul et al. 1990). To determine the size of the PAT3 transcript, RACE analysis was performed using an Arabidopsis cDNA library generated with the Marathon cDNA Amplification Kit (Clontech, CA, U.S.A.) and gene-specific primers MZ178 (5'-AACAAATGTTTGGTTCACAGGAGG) and MZ180 (5'-GGAAAGTGGTTGACAAAGAAGG). Fragments obtained by PCR with TakaraEx (Panvera, WI, U.S.A.) were cloned into the pGEM T-Easy vector (Promega, WI, U.S.A.) and sequenced. For complementation analysis, a 2,248-bp long DNA fragment, beginning 686 bp upstream of the PAT3 ATG start codon and ending 542 bp downstream of the TAA stop codon, was amplified from genomic DNA with oligonucleotides MZ194 (5'-AGGACCTGTTGACCAAGCTAAATATTTTCTCTG) and MZ195 (5'-GAATTCTAGAAAATCTTGGATCTTGG). The PAT3 genomic DNA was cloned as a PvuII–EcoRI fragment into the binary vector VIP96 containing a kanamycin-resistance gene (van der Krol and Chua 1991). Agrobacterium strain ABI carrying VIP96 with the complementation construct or the empty vector alone was used to transform pat3 mutant plants by vacuum infiltration (Clough and Bent 1998). T1 seeds were screened for short hypocotyls in FR light. Twenty-five seedlings were randomly picked, measured and transferred to MS medium with sucrose and kanamycin. Plates were kept in darkness for 1 d and then transferred to low intensity W light (0.5 μmol m⁻² s⁻¹) for 5 d to rescue the seedlings from FR-induced killing and to test for presence of the T-DNA.

Northern blot analysis
Approximately 200 mg plant material was ground in liquid nitrogen and the RNA extracted using the Plant RNA Extraction kit (Qiagen, CA, U.S.A.). Northern blot analysis was performed as described in Sambrook et al. (1989). For each sample, 5 μg total RNA was loaded. 32P-labeled probes were generated from PAT3 cDNA with the Maxiscript kit (Ambion, TX, U.S.A.) and hybridization was carried out using Ultrahyb solution (Ambion, TX, U.S.A.) at 65°C overnight. Blots were washed once with 2× SSC 0.1% SDS at 65°C and two times with 0.1× SSC 0.1% SDS at 65°C.

Intracellular localization of GFP and PAT3::GFP fusion proteins
For intracellular localization studies, PAT3 was fused at its carboxyl (C)-terminus to green fluorescent protein (GFP). The PAT3 cDNA was amplified with oligonucleotides MZ145 (5'-CTAGCTCGAGAATTCATGGAAGTGAATCAGGAAATCAA) and MZ164 (5'-CTAGGCTTGAGGCGACACCATACAGGATGTAGAGTTTG). The resulting fragment was digested with XhoI and KpnI and ligated into XhoI–KpnI-digested pGFP (Kost et al. 1998). As a control pGFP alone was used. Gold particles with a diameter of 1.6 μm were coated with the constructs and bombarded into onion epidermal cells using a helium gas driven particle accelerator (PDS-1000/He; BIO-RAD, Hercules, CA, U.S.A.) operated at a pressure of 1,100 psi and a chamber vacuum of 25 inches of mercury. Onion petals were kept at room temperature in darkness for 24 h before they were examined for GFP fluorescence with an Axioskop microscope (Zeiss, Thornwood, NY, U.S.A.).

Yeast expression assay
The full length PAT3 coding sequence was amplified with oligonucleotides MZ196 (5'-CATATGGCTGAATGCGATTGCTATG) and MZ197 (5'-CCCCGGCTACAGCATTAGCGTGAGAATCGTGAGATGGT). This fragment was cloned into a notI fragment fragment into the yeast expression vectors pGBK7 and pGADT7 of the Matchmaker3 kit (Clontech, CA, U.S.A.) and cloned in-frame as a NotI–XhoI fragment into the yeast expression vector pGBK7 and pGADT7. The yeast strain AH109 (Clontech, CA, U.S.A.) were made competent following the conditions described by the manufacturer. Transformed yeast cells were selected on synthetic drop-out media without histidine, adenine and either leucine or tryptophan for the pGADT7-PAT3 or the pGBK7-PAT3 construct, respectively. The α-galactosidase activity assay was performed and quantified as described by the manufacturer.
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References


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