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ORIGINAL ARTICLE

Phytochrome A requires jasmonate for photodestruction

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Abstract The plant photoreceptor phytochrome is organised in a small gene family with phytochrome A (phyA) being unique, because it is specifically degraded upon activation by light. This so called photodestruction is thought to be important for dynamic aspects of sensing such as measuring day length or shading by competitors. Signal-triggered proteolytic degradation has emerged as central element of signal crosstalk in plants during recent years, but many of the molecular players are still unknown. We therefore analyzed a jasmonate (JA)-deficient rice mutant, *hebiba*, that in several aspects resembles a mutant affected in photomorphogenesis. In this mutant, the photodestruction of phyA is delayed as shown by in vivo spectroscopy and Western blot analysis. Application of methyl-JA (MeJA) can rescue the delayed phyA photodestruction in the mutant in a time- and dose-dependent manner. Light regulation of phyA transcripts thought to be under control of stable phytochrome B (phyB) is still functional. The delayed photodestruction is accompanied by an elevated sensitivity of phytochrome-dependent growth responses to red and far-red light.

Keywords Coleoptile · Jasmonate · Photodestruction · Phytochrome · Rice (*Oryza sativa* L.)

Abbreviations

Pr	Red-light-absorbing form of phytochrome
Pfr	Far-red light-absorbing form of phytochrome
phyA (phyB)	Phytochrome A (B)
PHYA	PhyA gene
phyA	*PHYA* mutant
B	Blue light (450 nm)
R	Red light (660 nm)
FR	Far-red light (730 nm)

Introduction

Plant growth and development are greatly influenced by environmental light that is perceived by several photoreceptors (Nagy and Schäfer 2002; Wada et al. 2005). Among these photoreceptors, the phytochromes are unique by a reversible photoconversion of their absorption spectra between a red light (R)-absorbing form, Pr, and a far-red

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light (FR)-absorbing form, P_{fr} (Butler et al. 1959). The classical operational criterion for the participation of phytochromes has been the photoreversibility of the physiological response by far-red light following an inductive pulse of red light, assuming that P_{fr} is the only active form. An increasing number of observations, however, showed that this classical dogma did not hold for many cases where the amount of spectrophotometrically measurable P_{fr} was not correlated with the amplitude of the physiological response (Furuya 1993).

The discovery that phytochrome constitutes a gene family (Sharrock and Quail 1989) opened new approaches to solve this problem. Using phytochrome A(*phyA*)- and phytochrome B(*phyB*)-null mutants of *Arabidopsis*, it became evident that the responses to continuous far-red irradiation result from phyA, whereas the responses to continuous red light originate from phyB (Quail et al. 1995). Since its discovery, the abundance and state of phytochrome have been mainly determined spectrophotometrically in vivo and in vitro (Butler et al. 1959). However, in the meantime the different members of the phytochrome family can be discriminated by immunochemical techniques (Hirschfield et al. 1998). Phytochrome A in the P_r form is synthesized in the dark and, once exposed to light, is transformed to the P_{fr} form, which converts back to P_r in the dark (dark reversion) or is degraded by a reaction that is called photodestruction (Clough and Vierstra 1997). Phytochromes other than phyA (phyB–E) are synthesized constitutively, irrespectively of environmental light condition, and do not decay upon irradiation with light so that they are terminologically separated as 'stable' from the 'labile' phyA-phytochromes (Furuya 1993). Inactivation of phyB is mediated by absorption of far-red light (Rockwell et al. 2006), by dark reversion (Sweere et al. 2001), and by partial phyB degradation in prolonged continuous red light (Khanna et al. 2007). Light-mediated responses in plants can be classified into three different modes according to their energy requirement: low-fluence response (LFR), very-low-fluence response (VLFR), and far-red dependent high-irradiance response (FR-HIR). PhyA is the only phytochrome involved in all of these responses (Kneissl et al. 2008), while other phytochromes only contribute to LFR. Therefore, regulation of the abundance of phyA is of great importance for the plant.

Specific proteolysis of signaling proteins has emerged as central element of plant signaling. This includes the response to phytohormones such as auxin (Gray et al. 2001), ethylene (Guo and Ecker 2003; Potuschak et al. 2003), jasmonate (Thines et al. 2007; Chini et al. 2007), or gibberellins (Sasaki et al. 2003), and involves specific interactions of ubiquitin-E3-ligases with the 26S-proteasome (Frugis and Chua 2002). In addition, phyA signaling has been shown to be linked to ubiquitin-dependent proteolysis through the F-box protein EID1 (Büche et al. 2000; Dieterle et al. 2001). Since different signaling pathways compete for common elements regulating the targeting of this proteolysis, there is complex and extensive cross-talk between different signals such as light, auxin, or jasmonate (Schwechheimer et al. 2002). From this model it is expected that inhibition of one of these signaling pathways should alter the processing of the other signals (Nemhauser 2008).

In fact, there is experimental evidence supporting a cross-talk between light and jasmonate. When we analyzed the rice mutant *hebiba* that had been isolated from a screen of mutants altered in their response to red light, we found that this mutant is not able to produce jasmonates (Riemann et al. 2003). During early development, the *hebiba* mutant displayed a light-phenotype that represented a perfect phenocopy of wild type seedlings grown in complete darkness. The growth of rice seedlings is efficiently inhibited by even minute pulses of light. Classical studies have shown that the light signal is perceived by the phytochrome system, whereas blue light receptors seem to be of minor importance (Pjon and Furuya 1967). Thus, the photobiology of rice seems to be simpler as compared to dicot seedlings. Nevertheless, the dynamics of the phytochrome system has been preserved in its central aspects including the photodestruction of phyA (Schäfer et al. 1975).

To understand, why a jasmonate-deficient rice mutant exhibits altered responses to phytochrome, we compared the dynamics of phytochrome in the *hebiba* mutant to that in wild type seedlings. We observed that the photodestruction of phyA is delayed in the mutant. This delay can be rescued by application of exogenous methyl-JA in a time- and dose-dependent manner. In contrast to photodestruction of phyA, light regulation of phyA transcripts, thought to be under control of stable phyB, is still functional in the mutant. The delayed photodestruction is accompanied by an elevated sensitivity of phytochrome-dependent growth responses to red and far-red light. To our knowledge, this is the first time that a hormonal signal has been shown to regulate the dynamical properties of a plant photoreceptor.

Materials and methods

Plant material

The *hebiba* mutant was obtained in a *japonica* background (*Oryza sativa* L. cv 'Nihonmasari') and has been propagated independently in three sites in Northern Italy (Almo Semi, Mortara) and Japan (Hokuriku Experimental Station, Niigata, and National Institute of Agrobiological Sciences, Tsukuba). The mutant is male sterile, such that it has to be maintained through self-pollination of heterozygotic plants.

For each plant, small seed aliquots were checked separately to define the genotype of the population. An isogenic sister line homozygous for the wild type allele was used as reference throughout the study. The *phyA* mutant (Takano et al. 2001) was obtained in the two *japonica* cultivars 'Nipponbare NC-5' and 'Akita-komachi A2'.

Light sources and plant cultivation

The light sources for red light (RL, 660 nm), far-red light (FR, 730 nm), and green safelight (G, 550 nm) used in the photobiological studies are described in detail in Heim and Schäfer (1982). All light measurements were performed using a Tektronic-J16 photoradiometer (Tektronix, Beaverton, OR, USA). If not stated otherwise, the seedlings were raised at 25°C in photobiological darkness (using black boxes, black cloth, and isolated dark chambers) on floating meshes as described in Nick et al. (1994). Under these conditions, germination was higher than 97% and seedling length among the population varied by less than 5%. For JA experiments seedlings were raised in agripots (Kirin Brewery Ltd, Tokyo, Japan) on 0.6% water–agar in sterile conditions. They were raised in complete darkness at 28°C. Seedlings were irradiated as described in Takano et al. (2005) using diode panels emitting red light (Model LED-R; Eyela, Tokyo, Japan), far-red light (Model LED-FR; EYELA), and blue light (Model LED-B; Eyela), respectively. The FR emitting panel was supplemented with a filter box containing one layer of acryl cutoff filter foil (KYOWALITE PG, SP-60-3K 202, thickness 2 mm; Kyowa Gas Chemical, Tokyo, Japan). The light intensity was set to 20 µmol m^{-2} s^{-1} in each experiment. For JA treatment, we used 450 µM MeJA (Wako Pure Chemical Co., Osaka, Japan) which was diluted with water and 1 µl ml^{-1} Tween 20 (Sigma). The mock control consisted in an aqueous solution of 1 µl ml^{-1} Tween 20 without MeJA. The solutions were administered to the seedlings by spraying seedlings raised in agripots followed by incubation for the respective time intervals.

Spectroscopy in planta and in vitro

All manipulations except the irradiation itself were performed under a dim green safelight (550 nm, 60 nmol m^{-2} s^{-1}) to avoid phytochrome activation. For spectroscopy in planta, ten coleoptile tips for each assay (apical 10 mm) were harvested on ice directly after irradiation, transferred into a precooled measuring cuvette and pressed carefully by means of a pestle to the bottom of the cuvette, such that it was covered by a continuous layer of tissue. The fresh weight of the sample was determined by weighing the cuvette before and after loading and was determined a second time at the end of the experiment. Photoreversibility at 660 and 730 nm was determined by a dual-wavelength ratiospectrometer (Gross et al. 1984) at 4°C using cycles of 40 s of red light followed by 40 s of far-red light. Each experiment was repeated 3–5 times in independent time series performed at different days. For spectroscopy in vitro, hundred coleoptile tips were harvested and subjected to a partial phytochrome purification following the protocol of Nagatani et al. (1991). Phytochrome difference spectra were determined and quantified at 10°C using a diode-array spectrophotometer (HP8452A, Hewlett Packard, Frankfurt, Germany) according to Kunkel et al. (1993) using cycles of 45 s of red light, a dark interval of 10 s and a far-red pulse of 90 s.

Antibodies

A panel of monoclonal antibodies from mouse was tested that had been raised against phyA from *Arabidopsis* (Shinomura et al. 1996), phyA from pea (Nagatani et al. 1984), phyA from rye (Nagatani et al. 1987), and phyB from tobacco (Shinomura et al. 1998). The specificity of the signals obtained by Western blot was verified by using the *phyA* mutant (Takano et al. 2001) as negative control. None of the antibodies raised against phyA from *Arabidopsis* or pea recognized any signals in rice extracts (data not shown), whereas the antibodies mAR07 and mAR08 raised against phyA from rye (Nagatani et al. 1987) could be used to detect rice phyA with identical results (Fig. 2a). Whereas antibodies raised against phyA from dicotyledonous plants failed to recognize their monocot homologue, the antibody mBT04 raised against phyB from tobacco could be used to detect phyB in rice extracts (Fig. 2b).

Western blot analysis

About 30 coleoptile tips (the apical 10 mm) were harvested into liquid nitrogen under green safelight and ground to a fine powder. Total protein extracts were obtained and quantified as described in detail in Nick et al. (2000). Equal loading of lanes was verified by staining equally loaded parallels with gelcode blue stain (Pierce, Rockford, IL, USA) according to the manual of the producer. Proteins were transferred to nitrocellulose according to Nagatani et al. (1991), and the membranes probed with the primary antibodies (see above), and a secondary anti-mouse antibody conjugated to alkaline phosphatase (ProtoBlot AP System, Promega, Tokyo, Japan) as described in Nagatani et al. (1991). To quantify the signal, the blot membranes were scanned (LS-1000, Nikon, Tokyo, Japan), and the signals quantified relative to an internal standard by the ONE-D-Scan software (Scanalytics, CSPI, Billerica, MA, USA). In order to exclude non-linearities that might be caused by saturation of the signal, each sample was loaded in a

dilution series (factor ½) and the linearity of the signals was verified by linear regression. The correlation coefficients were found to be higher than 0.9. Each time course was repeated at least 3 times at different days. Western blot analysis related to the examination of the effect of JA on phyA degradation was performed as described in Takano et al. (2001).

Northern analysis and sequencing

About 30 coleoptile tips (the apical 10 mm) were harvested into liquid nitrogen under green safelight and ground to a fine powder. RNA was extracted and quantified according to Ehmann et al. (1991), separated by electrophoresis in a 1% agarose–formaldehyde gel and transferred by capillary blotting using 10 × SSC buffer to a positively charged nylon membrane (Boehringer, Mannheim, Germany). The correct loading of the different lanes was verified by staining with ethidium bromide and recording the blot on a UV-screen and subsequently quantifying the signal for the two subunits of the rRNA. The membranes were probed with a digoxigenin-labeled probe that was specific for rice PHYA and the signal was visualized by chemoluminescence according to protocol of the producer (Boehringer). The template was then amplified from this cDNA in a PCR reaction, where uridine nucleotides were replaced by digoxigenin-conjugated uridine. The primers were placed in positions 15 and 696 of the *phyA* coding sequence, respectively. The signals were recorded on X-ray film (Fuji RX New, Fujii Photo Film Co., Tokyo, Japan) and quantified as described for the Western blots. Again, the linearity of the signal was verified by dilution series. All time courses were repeated twice at different days. The coding sequences for *phyA* and *phyB* were isolated from etiolated coleoptiles of the isogenic wild type and *hebiba* plants by RT-PCR using the synthetic oligonucleotide primers described in detail in Eichenberg et al. (1999). The sequence was compared to published sequences for rice *phyA* and *phyB* (Kay et al. 1989; Dehesh et al. 1991), but the sequences were identical with exception of a few base exchanges that were cultivar specific and observed in both the coding sequences from isogenic wild type and mutant.

Microscopy

Coleoptile tips were cryosectioned, and transverse sections of 8 µm thickness were subjected to immunofluorescence staining of phyA as described in detail in Hisada et al. (2000). The phytochrome signal was visualized using the monoclonal antibodies mAR07 and mAR08 (Nagatani et al. 1987) as undiluted hybridoma supernatant, and an anti-mouse IgG antibody conjugated to fluorescein-isothiocyanate (Amersham Japan, Tokyo, Japan) in a dilution of 1:20. The samples were viewed under a epifluorescence microscope (Olympus Optical, AX70, Tokyo, Japan) using a specific FITC-filter set (filter set U-MNIB, Olympus Optical). For each experiment, about 10 slides comprising around 100 individual sections were used. The whole series was completed for both, the mAR07 and the mAR08 antibody with the same result. Each time course was repeated twice at different days.

Auxin analysis

Endogenous auxin levels were quantified as described in detail in Riemann et al. (2003).

Results

Photodestruction of phyA is delayed in the *hebiba* mutant

Our previous work had demonstrated that, in the jasmonate-deficient mutant *hebiba*, the growth responses to both red light and far-red light were affected (Riemann et al. 2003 and unpublished data). We therefore investigated the dynamic properties of phyA and measured photodestruction during continuous irradiation with red light. In coleoptiles of the wild type, the photoreversible change of absorbance ($\Delta(\Delta A)$) as measure for phytochrome activity decreased rapidly with a half life of about 50 min (Fig. 1). The behavior of ($\Delta(\Delta A)$) in the mutant was strikingly different. After a transient rapid increase, it decreased very slowly with a half life of about 230 min (Fig. 1). To see whether the transient initial peak was caused by shifts in the spectral properties of the photoreceptor, phytochrome was purified prior and subsequent to an irradiation with 15 min of red light. However, the measured difference spectra did not reveal any significant changes in the spectral properties between wild type and mutant nor in the amount of extractable photoreversibility per fresh weight (data not shown). When the localization of the phyA apoprotein (PHYA) was followed by immunofluorescence during the response to continuous red light, a redistribution of the signal was observed in coleoptiles of both, wild type and *hebiba* mutant. Prior to irradiation, the signal was found predominantly in the cytoplasm of both epidermal cells of wild type and *hebiba* mutant coleoptile (Supplementary Fig. 1a–d). This signal was observed to decrease progressively with the time of irradiation in both wild type and *hebiba* mutant (Supplementary Fig. 1e–h). During degradation, the phyA apoprotein was sequestered into speckles. These speckles were more abundant in *hebiba* (Supplementary Fig. 1g) as compared to the wild type (Supplementary Fig. 1f).

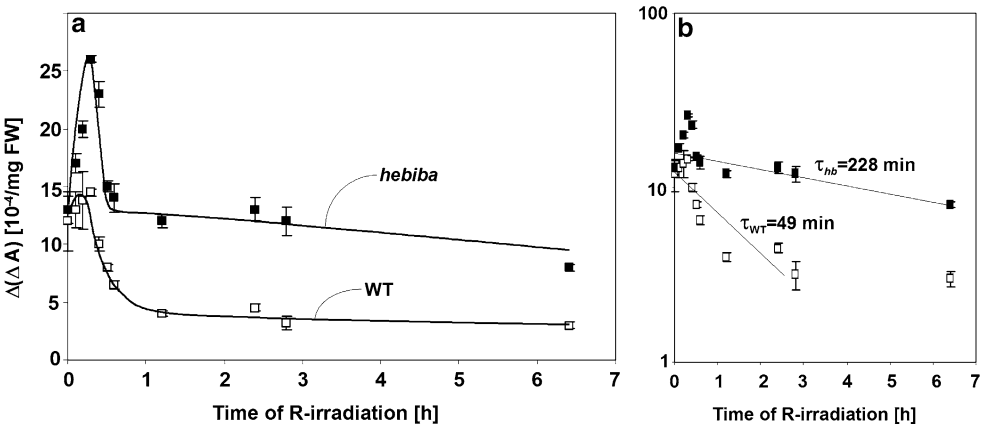

Fig. 1 Time course of phytochrome photoreversibility in wild type (WT) and *hebiba* seedlings. **a** Seedlings were grown in continuous red light for the times indicated. Subsequently difference spectra of were measured as described above. (Δ(ΔA) for each time point is shown. **b** The half life time of phytochrome in WT (τ_{WT}) and *hebiba* (τ_{hb}) was determined based on the slope of the semi-logarithmical function

The light-induced destruction of phyA was then confirmed by Western analysis using monoclonal antibodies mAR07 and mAR08 that had been raised against rye phyA (Nagatani et al. 1987). Figure 2a demonstrates the specificity of the signal using verification by a *phyA* null mutant (Takano et al. 2001) as negative control. The abundance of phyB was assessed in *hebiba* and the *phyA* null mutant using monoclonal antibodies raised against phyB from tobacco (Shinomura et al. 1998). However, phyB was found to be expressed at the same levels as compared to the wild type (Fig. 2b), and throughout this study the expression of phyB was observed to be constitutive in both wild type and *hebiba* mutant (data not shown). This contrasts with phyA that disappeared progressively under continuous irradiation for both red (Fig. 2c) and far-red light (Fig. 2d) in the wild type, whereas it persisted longer in the mutant. Since the abundance of phyA in etiolated coleoptiles of *hebiba* was somewhat higher than in the wild type, the changes of phyA abundance were quantified by density measurements. Dilution series of mutant extracts were calibrated against the wild type and the density of the bands quantified relative to the dark value of phyA in the wild type (Fig. 2e, f). Consistently with the spectroscopical observation (Fig. 1a), phyA disappeared in red light with a half life of about 50 min in the wild type, whereas in the mutant the half life was about 110 min. For far-red light, the half life was around 9 h for the wild type, 14 h for the mutant. The differences in the residual levels of phyA were especially conspicuous for prolonged irradiation. Even after almost 2 weeks of strong white light (Fig. 2g), the phyA protein was still abundant in extracts from the mutant, whereas not a trace of phyA could be detected in extracts from wild type plants. When coleoptiles were irradiated for 1 or 2 days with continuous far-red light (Fig. 2h), mutant samples that had been diluted four times still yielded a much stronger signal than undiluted samples from the wild type. Thus, the photodestruction of phyA is drastically impaired in the jasmonate-deficient *hebiba* mutant.

Regulation of phyA transcripts remains functional in *hebiba*

To test, whether the mutant was affected in a response dependent on the stable phyB, we analyzed the regulation of phyA transcripts in red light for wild type and *hebiba*. Transcripts of phyA decreased in response to red light in both wild type and mutant (Fig. 3a). However, the mutant accumulated somewhat higher amounts of phyA transcripts in the dark. As in the Western analysis, the signals were calibrated by dilution series against the dark signal observed for the wild type (Fig. 3b). Although phyA transcripts accumulate to about the twofold level in etiolated mutant coleoptiles, the velocity of down-regulation was not significantly different with half times of 22 min for the wild type and 30 min for the mutant. Thus, regulation of *phyA* transcripts was found to proceed normally in the *hebiba* mutant. Again, the abundance of *phyB*-transcripts was found to be constant, independently of light treatment or the presence of the *hebiba* mutation (data not shown).

Exogenous MeJA rescues delayed phyA degradation in *hebiba*

The aberrant photomorphogenetic response of the jasmonate-deficient *hebiba* mutant can be rescued by exogenous MeJA (Riemann et al. 2003). We tested therefore, whether application of exogenous JA can reconstitute a normal degradation of phyA in the mutant. Etiolated seedlings were sprayed either with a solution containing 450 μM MeJA or with a mock solution containing the solvent, but no MeJA. The treated seedlings were then irradiated with continuous red light (20 μmol m^{-2} s^{-1}) for different time intervals, and proteins were extracted and probed by Western blotting for the abundance of phyA and α-tubulin (as reference) for each sample. For the wild type (Fig. 4a), the abundance of phyA was observed to decrease rapidly, irrespectively of whether the seedlings had been treated with MeJA or subjected to the mock treatment. In the mutant, upon mock

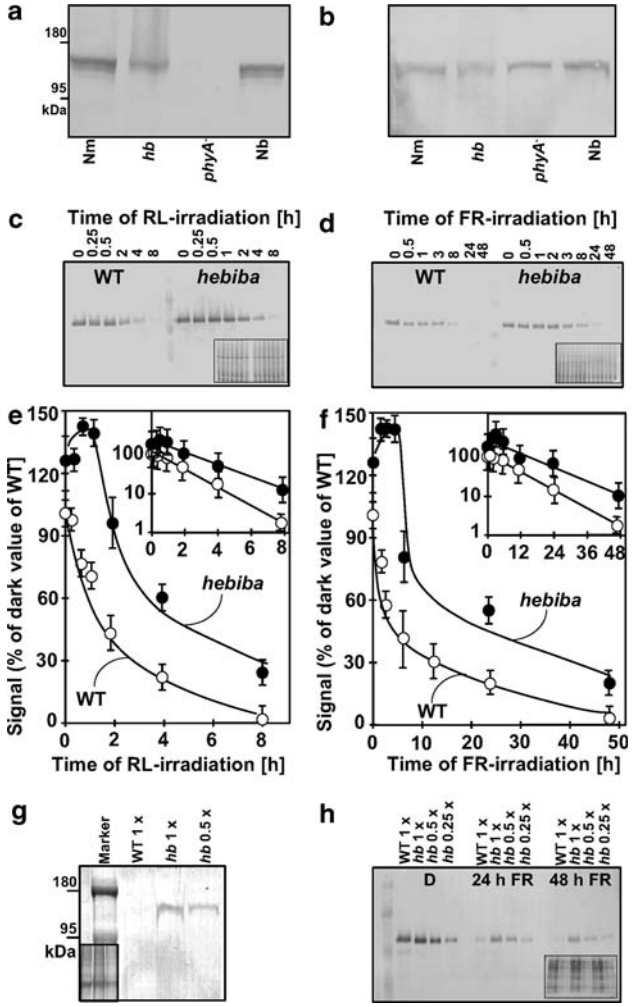

Fig. 2 Degradation of phyA in coleoptiles of wild type and *hebiba* during irradiation with red and far-red light followed by western blotting. Specificity of the monoclonal antibodies used for the detection of phyA (**a**) and phyB (**b**) from rice. Fifty micrograms of total protein extract were loaded per lane and challenged either with mAR07 (raised against phyA from rye) or with mBT04 (raised against phyB from tobacco). *Nm* cultivar Nihonmasari (background for the *hebiba* mutant), *hb hebiba*, *phyA phyA* mutant, *Nb* cultivar Nipponbare (background for the *phyA mutant*). Time course of phyA photodestruction in continuous red light (**c**, **e**) and continuous far-red light (**d**, **f**). Representative blots are shown in **c** and **d**, the quantification of the phyA signals in **e** and **f**, respectively. The insets show the time courses plotted in a semilogarithmic scale for the determination of the time constants. **g**, **h** Dilution series for hebiba in comparison to the respective wild type samples after 12.5 days of strong white light (8,500 Lx m^{-2}) (**g**) or 24 and 48 h of continuous far-red light (**h**). Ten microgram of total protein were loaded per lane in **c**, **d**, 100 µg (or 50 µg, respectively) in **g**, 10 µg (or 5 or 2.5 µg, respectively) in **h**

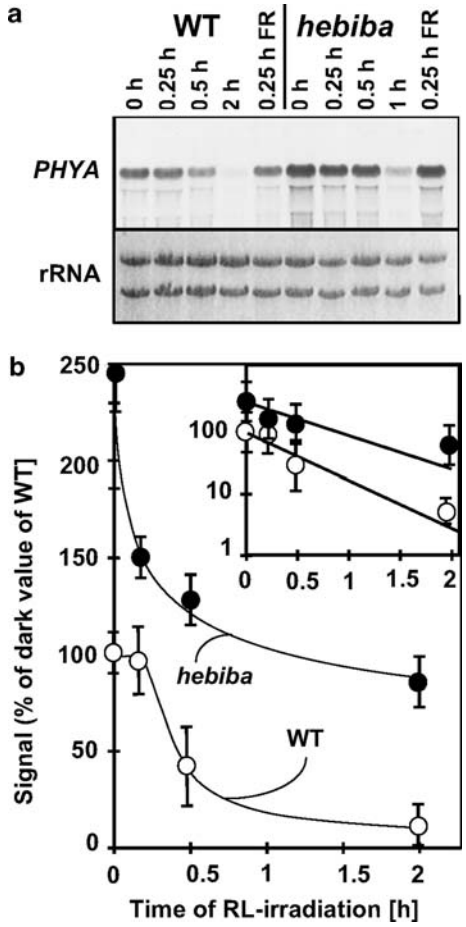

Fig. 3 Regulation of *phyA* mRNA in coleoptiles of wild type and *hebiba* examined by northern blotting. **a** Representative northern blot showing the time course of the steady-state levels of *phyA* transcript in continuous red light (RL) or after 15 min of far-red light (FR) as compared to the rRNA levels observed in the same samples. **b** Quantification of the Northern analysis. The inset shows the time course plotted in a semi-logarithmic scale for the determination of time constants

treatment, the decrease of phyA was found to be slower. This was most prominent at 6 hours after the onset of irradiation, when in the wild type only a residual phyA signal could be observed (Fig. 4a), whereas in the mutant the abundance of phyA was still relatively high (Fig. 4b). However, when the mutant was treated with MeJA, the phyA was found to be much lower than in the mock control dropping to the levels present in the wild type at this time point (Fig. 4b). To verify, whether MeJA can reduce the abundance of the phyA apoprotein under continuous red light, the 6-h time point was recorded in three independent experimental series for both wild type (Fig. 4c) and mutant (Fig. 4d). Again, in the wild type, the abundance of phyA was not significantly altered by MeJA as compared to the mock control (Fig. 4c). However, in the mutant, the phyA signal was persistently reduced in the sample treated by MeJA in relation to the signal observed in the mock control (Fig. 4d). We tested a couple of different MeJA concentrations and time points (data not shown), and observed that lower concentrations of MeJA are not sufficient, and that the difference between mock control and MeJA sample in relation to the overall abundance of the phyA signal was optimal for 6 h of irradition. Thus, the red light dependent

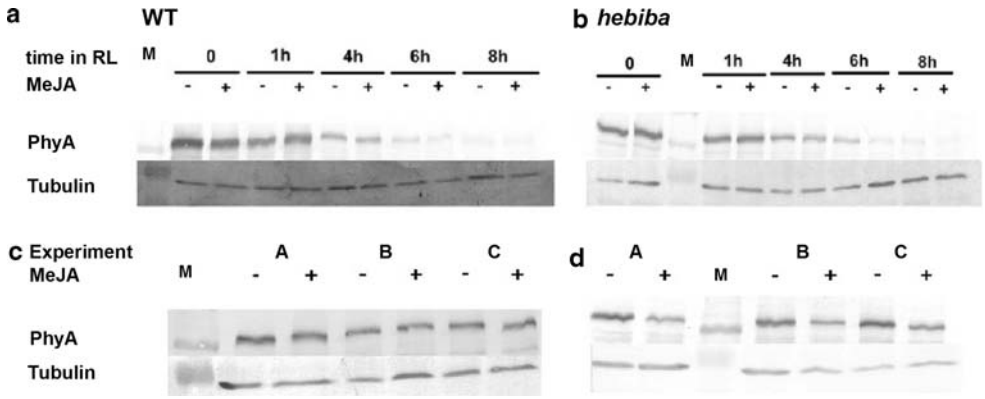

Fig. 4 Level of phyA in WT and *hebiba* seedlings in dependency on red light and JA as determined by western blotting. Seedlings of WT (**a**) and *hebiba* (**b**) were irradiated with continous red light (20 μmol m^{-2} s^{-1}) for the period indicated. They were either treated with a solution containing 450 μM MeJA (indicated by '+') or with a mock solution without MeJA (indicated by '−'). PhyA levels were detected with a monoclonal phyA antibody. As a loading control tubulin was detected on the same blot. *Lane M* molecular weight markers. The MeJA treatment was repeated three times (**a**–**c**) for WT (**c**) and *hebiba* (**d**) using a constant red light irradiation period of 6 h

decrease of phyA apoprotein in the mutant can be reconstituted by addition of exogenous MeJA.

Wavelength-dependent elevated sensitivity of light responses in *hebiba*

As shown previously, *hebiba* coleoptiles display an inverse growth response to light (Riemann et al. 2003). When phytochrome is activated in etiolated seedlings at the onset of coleoptile elongation, this inhibits growth in the wild type, whereas in the mutant, growth is stimulated. We used this light response to test for potential physiological consequences of the delayed photodestruction in *hebiba*. Therefore, we recorded fluence response curves for red light, far-red light, and blue light (Fig. 5). Irrespective of light quality we observed that, in the wild type, for increasing fluence, growth became progressively inhibited, whereas it was stimulated in the mutant. For red light, both light responses became detectable from a threshold of about 100 μmol m^{-2} (Fig. 5a). However, for far-red light (Fig. 5b) and blue light (Fig. 5c), the mutant response became manifest at lower fluence rates as compared to the wild type. In *hebiba*, growth became stimulated from 100 μmol m^{-2} far-red or blue light, whereas the (negative) growth response of the wild type required between 10 and 100 mmol m^{-2} to become significantly different from etiolated growth. Measurements of epidermal cell length in etiolated and irradiated coleoptiles showed that the growth response was caused by changes in cell elongation rather than in cell number (data not shown). Thus, whereas the fluence-rate threshold of wild type and mutant to red light is comparable, the mutant response is shifted at least two orders of magnitude to lower fluence rates in far-red and blue light.

Auxin levels in wild type and *hebiba* seedlings decrease in response to cFR

We had observed previously that the biosynthesis of jasmonate is induced in continuous red light, and that this response is absent in *hebiba* (Riemann et al. 2003). At the same time, auxin content was decreasing in response to red light. To test, whether the increase in residual P_{fr} in the mutant pronounced under continous far-red light, would be correlated with an increased auxin content, we followed auxin content during irradiation with cFR. Qualitatively, the regulation of auxin content was the same as in red light irradiated seedlings. For both wild type and mutant, auxin levels were decreasing in response to irradiation with continuous far-red light (Table 1). Both, the absolute values as well as the relative change after 2 h of irradiation, were lower in the mutant as compared to the wild type. This means that the increase in residual P_{fr} in the mutant is not correlated with an increased auxin content. In contrast, auxin content is decreased in the mutant.

Discussion

At first glance, *hebiba* resembles a photomorphogenetic mutant. It was obtained from a screen for impaired phytochrome-induced inhibition of coleoptile growth. In contrast to the wild type, where growth is blocked completely by irradiation with red or far-red light, seedlings homozygous for the *hebiba* mutation exhibit fully expanded, long and slender coleoptiles that are perfect phenocopies of dark grown wild type coleoptiles (Riemann et al. 2003). This impression is supported by the phenotype of adult mutants that are of light-green color, show a reduced ratio

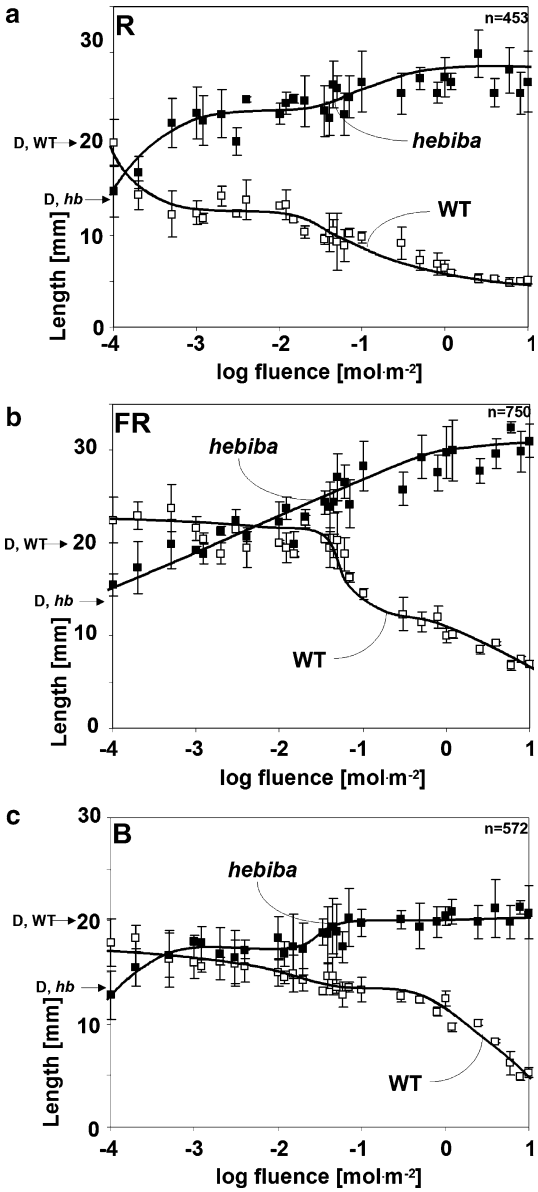

Fig. 5 Fluence-response curves of coleoptile length of WT and *hebiba* for red (**a**), far-red (**b**), and blue light (**c**). Seedlings were grown for 3.5 days in darkness, irradiated, and then returned to the dark until the completion of coleoptile elongation at day 6 of cultivation

of chlorophyll *a* to chlorophyll *b*, hypertrophic elongation, and flower earlier.

Hence *hebiba* shows a unique response, opposite to any predicted phyA action. The elongation response of coleoptiles is enhanced mediated by phyA. This probably holds true for leaves as well, because phyA protein is abundant in *hebiba* plants which have been grown in white light, while no phyA was detectable in wild type plants grown in the same conditions (Fig. 2g).

However, the *hebiba* mutant has a clear phenotype in the dark such as short coleoptiles and hypertrophic elongation of the mesocotyl or increased abundance of phyA protein

Table 1 Content of auxin after irradiation with far-red light (FR)

Time in FR	WT	Mean (%)	*hebiba*	Mean (%)
0	73.9 ± 6.7	100	48.2 ± 5.2	100
120	41.6 ± 7.8	56.3	24.0 ± 3.8	49.8

In the left column the incubation time of seedlings in far-red light (20 μmol m^{-2} s^{-1}) is indicated in minutes. The content of auxin in coleoptiles is shown in pmol per g fresh weight. For the control value (0 min FR) 26 independent experiments were averaged, the data for 120 min far-red light consist of 12 (wild type) and 10 (*hebiba*) independent experiments. In each experiment auxin from 40 to 50 seedlings was extracted. The relative values are given in relation to the initial value at time 0

(Fig. 2) and transcript (Fig. 3), which is clear evidence that the HEBIBA gene product can function independently of phytochrome signaling. Moreover, the spectroscopical properties of phyA seem to be normal, the coding sequences of the *PHYA* and *PHYB* genes do not reveal any differences between wild type and mutant (data not shown). *Hebiba* is therefore not a photomorphogenetic mutant in *sensu strictu*, but affected in a pathway that is active in the dark and modulated by light. In fact, it could be demonstrated further that JA is synthesized in etiolated wild type seedlings and induced by irradiation, whereas it is completely absent in mutant seedlings (Riemann et al. 2003). By application of exogenous JA and its precursor OPDA it was possible to restore a normal light-growth response in the mutant—under these conditions, the mutant seedlings were indistinguishable from the wild type. Moreover, the male sterility of the mutant could be overcome by spraying MeJA during floral comitment. In the present work, we show that also the photodestruction of the phyA protein that is impaired in *hebiba*, can be rescued by MeJA. Thus, JA is sufficient to complement all tested aspects of the mutant phenotype. This means that this phenotype can be attributed to jasmonate deficiency. In a forthcoming publication we can show that the mutation maps to a gene in the jasmonate synthesis pathway.

When photoreversibility was followed over time under continuous red light, a strong, transient peak of (Δ(ΔA)) was observed in the *hebiba* mutant (Fig. 1). In the wild type, this peak was detectable, but not very pronounced. Classical studies on photodestruction have described such small, but significant, transient increases in photoreversibility (Marmé et al. 1971), however, the molecular base of this phenomenon is still unknown. PhyA has been shown to undergo sequestration in a couple of systems (for instance, Pratt 1994), and when we followed the intracellular distribution of the phyA protein by immunofluorescence in coleoptile tips of rice we observed that the initially homogenous, cytoplasmic distribution of the phyA protein was rapidly replaced by sequestered particles (Supplemental Fig. 1). The frequency of these sequestered particles in

the mutant is significantly increased. When an initially homogenous distribution of an absorbing molecule undergoes redistribution into a heterogenous pattern, absorbance can change due to a so called sieve effect (Fukshansky 1978). However, the amplitude of this effect is far too small to account for the stronger peak in the mutant.

In contrast to phyA, the behavior of phyB seems to be normal in the mutant. The regulation of phyA transcription under red light (Fig. 3) is essentially normal (ignoring a slightly increased dark level of phyA transcripts). In addition, the threshold of a growth response to continuous red light (mediated by phy A and phyB redundantly) is comparable between wild type and mutant, whereas under far-red and blue light it is lowered by about two orders of magnitude in *hebiba*. In other words: the sensitivity of growth (sensitivity used in the strict sense, as dose-dependency, not to be mixed up with the responsivity, which describes the amplitude of the response) is elevated in the mutant in far-red and blue light, but not in red light. This indicates alterations in light sensing that are related to the activity of phyA (and phyC), but not to the stable phyB. In fact, the photodestruction of phyA was observed to be retarded in the mutant as shown by in vivo spectroscopy (Fig. 1), and by Western blot studies (Fig. 2).

The phenotype of hypertrophic elongation by impaired photodestruction leaving non-negligible residual amounts of active phyA under conditions, where they would be completely eliminated in the wild type (Fig. 2g). In fact, when photodestruction is reestablished in the mutant seedlings by treatment with exogenous MeJA (Fig. 4), the light response of mutant coleoptiles is completely normalized (Riemann et al. 2003). Such a complementation assay, however, is difficult to conduct in adult plants, because timing and concentration of jasmonate application is hard to optimize.

There are three possible scenarios to link JA-deficiency, impaired phyA photodesctruction, and elevated cell elongation.

In the first scenario, the elevated content of residual phyA (as consequence of JA-deficiency) would lead to an induction of auxin synthesis culminating in stimulated cell growth. If the biosynthesis of auxin were stimulated in response to high residual levels of P_{fr}, endogenous auxin content should be elevated in *hebiba* under continuous far-red light. However, our data (Table 1) show that, in the mutant, there is even less auxin extracted after irradiation with far-red light (Table 1). Therefore, scenario 1 can be ruled out.

In the second scenario, the elevated residual levels of phyA and the increased cell elongation could occur independently of auxin synthesis by an increase of auxin responsiveness. Both, auxin and jasmonate signaling compete for AXR1, one subunit of the ubiquitin-activating enzyme, as limiting factor (Tiryaki and Staswick 2002; Nick 2006). Hence, when one of the signals is knocked out, which is the case for the jasmonate pathway in *hebiba*, the responsiveness of the other pathway will be amplified. In fact, a downregulation of auxin responsiveness in response to gravitropic stimulation has been shown to be correlated with an increase of jasmonate content (Gutjahr et al. 2005). In addition, the growth response of segments excised from mutant coleoptiles to exogenous auxin is strongly enhanced as compared to the wild type (Riemann et al. 2003).

In the third scenario impaired phyA destruction and elevated growth occur in parallel, but are not causally linked. Both of them are modulated by jasmonate, but through independent pathways. Evidence for this scenario derives from experiments, in which oat phyA was overexpressed in rice. Although the amount of phyA in the transgenic lines was increased and was shown to be physiologically functional, light-grown plants did not show differences on the phenotypic level (Clough and Vierstra 1997). Thus, when the levels or active phyA are increased independently of the jasmonate pathway, this per se does not enhance cell elongation. In contrast, the opposite effect was observed, when oat phyA was overexpressed in tobacco. While transgenic plants were reaching more or less the same height as wild type plant when raised at low canopy densities, stem elongation was significantly reduced at high canopy densities, a phenomenon termed "proximity-conditional dwarfing" by the authors suggesting that the increased level of active phyA leads to an inhibition of stem elongation (Robson et al. 1996).

We conclude from the present data that jasmonates are necessary for the efficient photodestruction of phyA. In the *hebiba* mutant, the failure to produce jasmonates results in elevated levels of residual phyA in its P_{fr} form, which becomes especially pronounced under continuous far-red light. The stimulated growth in the mutant is not a direct consequence of the elevated P_{fr} levels (scenario 3), nor caused by an induction of auxin synthesis (scenario 1). We therefore hypothesize that it is the absence of light-induced jasmonate that, in the mutant, results in an increased auxin responsiveness of growth. Growth stimulation and elevated P_{fr} levels would therefore be parallel phenomena that are not directly linked. Future work will be directed to identify the *hebiba* mutation and to test, how it interferes with auxin signaling.

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