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The jasmonate-free rice mutant *hebiba* is affected in the response of phyA′/phyA″ pools and protochlorophyllide biosynthesis to far-red light

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Phytochrome (phy) A in its two native isoforms (phyA′ and phyA″) and the active (Pchlide^{655}) and inactive (Pchlide^{633}) protochlorophyllides were investigated by low-temperature fluorescence spectroscopy in the tips of rice (*Oryza sativa* L. *Japonica* cv Nihonmasari) coleoptiles from wild type (WT) and the jasmonate-deficient mutant *hebiba*. The seedlings were either grown in the dark or under pulsed (FRp) or continuous (FRc) far-red light ($\lambda_a \geq 720$ nm) of equal fluences. In the dark, the mutant had a long mesocotyl and a short coleoptile, whereas the situation was reversed under FR: short mesocotyl and long coleoptile, suggesting that the effect is mediated by phyA. Under these conditions the WT displayed a short coleoptile and emergence of the first leaf. In the dark, the spectroscopic and photochemical properties of phyA, its content and the proportion of its two pools, phyA′ and phyA″, were virtually identical between WT and *hebiba*. However, the total content of protochlorophyllides was higher in the mutant. Upon illumination with FRc, [phyA] declined in the WT and the ratio between phyA′ and phyA″ shifted towards phyA″. In *hebiba*, the light-induced decline of [phyA] was less pronounced and the ratio between phyA′ and phyA″ did not shift. Moreover, in the WT, FRp stimulated the biosynthesis of Pchlide^{655}, whereas FRc was inhibiting. In contrast, in the mutant, both FRp and FRc stimulated the synthesis of Pchlide^{655}. This means that FRc caused the opposite effect in *hebiba*. This difference correlates with a slower photodestruction of primarily the light-labile phyA′ pool in *hebiba*.

Introduction

One of the dominating themes in current phytochrome (phy) research is the structural and functional heterogeneity of the phys. A number of phys, products of different *PHY* genes, were discovered, with phyA and phyB as major members of the family.[1] PhyB, the 'classical' phy, mediates the red light (R)-induced/far-red light (FR) reversible photoresponses in the low fluence response (LFR) range, whereas phyA accounts for the very-low fluence (VLFR) and high-irradiance (HIR) responses that are most efficiently triggered by FR.[2] Both phys are active throughout the whole plant life cycle, but their function is often complementary and sometimes even antagonistic. PhyA, in particular, participates in promotion and inhibition of seed germination, FR-induced de-etiolation, promotion of flowering and resetting of the circadian clock. It modifies gravitropic and phototropic sensitivity and also modulates the activity of phyB and other minor phys.

It is generally believed that the complex functions of phyA are accomplished by one and the same homogeneous pigment species, whereby different regions of the molecule are responsible for the different modes of the photoresponses, VLFR and HIR.[3] However, a mounting body of evidence suggests that phyA itself is heterogeneous and that this heterogeneity may account, at least partially, for the complexity of its action. Two post-translationally modified phyA populations were discovered in monocots and dicots and termed phyA′ and phyA″.[4] These populations differ in photochemical and spectroscopic properties, in content and distribution between different organs and tissues, and in their light stability. The exact structural differences between these two phyA species are not completely understood. Experiments on transgenic plants over-expressing full-length oat phyA or mutant phyA with deletions at the N-terminus suggest that a stretch between amino-acid residues 6 and 69 could be involved in the modification. The two phyA pools probably differ in this yet unknown post-translational modification and/or in the intracellular distribution of the pigment: phyA′ appears to be soluble, whereas phyA″ is either bound to membranes or to a protein lattice. From correlations between changes of phyA′ *versus* phyA″ content and the phenotype of *phyA* mutants and transgenic plants it can be inferred that the light-labile phyA′ pool is probably responsible for de-etiolation whereas the relatively light-stable phyA″ pool might function throughout the entire life cycle of the plant. In addition, phyA″ might also modify (suppress) the action of phyA′. It has been proposed that the balance between both phyA pools could provide a mechanism for the fine-tuning of phyA-action (see ref. 4 for review).

The physiological responses to phyA are likely to involve changes in the hormonal status of the plant. Hormones might affect the state and functions of the pigment, a possibility that so far has been mostly neglected. Mutants deficient in hormone synthesis, signaling or responses are valuable tools to address this issue. For instance, the rice mutant *hebiba* [5] is deficient in jasmonic acid and shows a sign reversal in the light response of growth. Whereas coleoptile growth is elevated in the dark and efficiently inhibited by R (where phyB dominates the responses[2]), mutant coleoptiles are arrested in growth as long as they remain in the dark, but expand rapidly upon illumination.

Here we report that the inversion of the light response in *hebiba* is also induced by irradiation with FR (expected to act predominantly through phyA[2,4]). In connection with this response we find that both pools of phyA (phyA′ and phyA″) behave normally in *hebiba* as long as the coleoptiles are kept in the dark but not upon exposure to FR. In the WT, total phyA content (P_{tot}) declines accompanied by a considerable shift in the equilibrium towards phyA″, whereas in the mutant this decline was much less pronounced and the phyA′/phyA″ ratio remained constant. We also observed an altered response of active protochlorophyllide (Pchlide^{655}) to continuous FR as

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Pchlide^{655} decreased in the WT mutant and increased in the mutant. This sign-reversal of the protochlorophyllide response is discussed in the context of the altered light-response of the two pools of native phyA.

Materials and methods

WT rice (*Oryza sativa* L. *Japonica* cv Nihonmasari) and the jasmonate-deficient *hebiba* mutant[5] isolated from the same background were used in the experiments. The seedlings were raised for 5 days at 27 °C on floating meshes on tap water in complete darkness (D) or under FR ($\lambda_a \geq 720$ nm). The light source was a 100 W tungsten lamp in combination with filters KS-19 + FS-7 (thickness 3 mm, Optical Glass Plant, Krasnogorsk, Russia) and provided a fluence rate of approx. 0.1 W m^{-2}. There were two illumination regimes at equal total fluence: (1) 7 min pulsed light and 53 min D (FRp) and (2) continuous FR (FRc). Since homozygous mutants are male-sterile,[5] seed material from heterozygous plants was used, which segregated into approximately 75% of seedlings with a WT phenotype and 25% exhibiting the mutant phenotype (see below). Coleoptile tips (the apical 3 mm) were harvested for the measurement. From 3 to 6 tips were attached to a Plexiglas sample holder using a water–glycerol mixture (50 : 50%) and frozen at 85 K in a cryostat in a transparent Duwar flask. All the manipulations were carried out under green safelight.

Low-temperature (85 K) fluorescence emission spectra of phy (λ_e = 633 nm) and protochlorophyllide (λ_e = 450 nm) were measured in the coleoptile tissue as described previously[6,7] using a custom-built spectrofluorimeter based on two double-grating monochromators of the DFS-12 and DFS-24 types (LOMO, Leningrad, USSR). Briefly, the spectrum was initially recorded from the sample frozen in D when phy is in its R-absorbing form, Pr, (state 0) using a very weak measuring beam at 633 nm favorable for phy measurements. The source of the excitation light was a He–Ne laser, 1 mW, in combination with a monochromator MDR-2 to cut-off the pumping light, and the intensity was reduced by about 50-fold with neutral filters, such that the excitation light did not induce photochemical changes. Subsequently, the sample was illuminated at 85 K with the full light of the laser to convert Pr into lumi-R, the first photoproduct that is stable at low temperatures. The second spectrum of the sample was recorded for this state 1 when Pr is in the photoequilibrium with lumi-R. A third spectrum was recorded from the same sample upon monochromatic excitation at 450 nm to measure active (Pchlide^{655}) and inactive (Pchlide^{633}) protochlorophyllides.

The raw spectra were corrected for background fluorescence to obtain the real spectra of phy and protochlorophyllide. As reference spectra for background fluorescence, spectra obtained from the base of the primary root were used because this tissue is virtually free of phy and protochlorophyllide. These reference spectra were recorded at 85 K upon excitation at 633 nm (for phy) and 450 nm (for protochlorophyllide) and subtracted from the respective raw spectra. It was shown earlier with the use *phyA phyB* mutants of *Arabidopsis*[8] and pea[9] that the spectra of old roots at their base (or of old shoots at their base), after saturating R converting Pr into Pfr, were very close to the emission spectra of etiolated stems of the double mutants. These spectra did not reveal emission bands belonging to phy, suggesting that the emission in the spectra of respective WT belonged to phyA and phyB and that the input of the minor phys (phyC–phyE) in the total phy fluorescence is negligible and can be ignored.

The corrected (difference) phy spectra provided information on the spectroscopic and photochemical properties of the pigment, in particular, position of the peak, λ_{max}, half-band width, $\Delta\lambda$, of the spectrum, and extent of the Pr $\rightarrow$ lumi-R conversion to reach a photoequilibrium at 85 K, $\gamma_1 = (F_0 - F_1)/F_0$, where F_0 and F_1 are the intensities of the Pr emission in the maximum in state 0 and state 1, respectively. The fluorescence intensity in the Pr maximum in state 0, related to the intensity of the background fluorescence at 660 nm where the input of the Pr fluorescence is negligible, were used as measure of total phy content, [P_{tot}], in relative units. The experimental parameter γ_1 provided information on the relative content of the two phenomenological phy types: Pr′ which comprises phyA′ (Pr′ = phyA′) and Pr″ which consists of phyA″ and phyB (Pr″ = phyA″ + phyB) (see ref. 4). Phenomenologically, the two Pr species in rice are characterized by the individual γ_1 values of approx. 0.49 ± 0.03 for Pr′ and 0 for Pr″.[6] Both [P_{tot}] and the Pr′/Pr″ ratio allow to estimate the content of phyA′ and phyA″ in a given sample in relative units. These estimates are possible, when the input of phyB is ignored—it contributes to less than 10% of [P_{tot}] and by Western analysis we do not observe differences between WT and *hebiba* mutant in terms of phyB levels.[10] The content of Pchlide^{655} and Pchlide^{633} was determined proceeding from the intensities in their respective emission maxima after the spectra deconvolution as described in ref. 11. From 4 to 7 independent samples were used to determine the parameters listed above and the standard error (±SE) usually did not exceed ≈10%. The spectra were not corrected for the spectral sensitivity of the fluorimeter. The noise of the spectra registration was below 3–5%. The spectra were interpolated manually.

Results

Growth responses

To assess the physiological effect of our lighting regime, we checked the morphology of the seedlings (Fig. 1). *In the dark*, the mutant seedlings had a long mesocotyl, and a short curved coleoptile, quite reverse to the situation in the WT, where the mesocotyl was short and the coleoptile long. *Pulsed FR illumination (FRp)* induced emergence of the first leaf in approximately half of the WT plants. Under the same conditions, the mutant displayed a relative decrease of the mesocotyl length, whereas the coleoptile was still very similar to that of dark-grown *hebiba* seedlings. Finally, under *continuous FR illumination (FRc)*, all the WT seedlings had the first leaf released from the coleoptile,

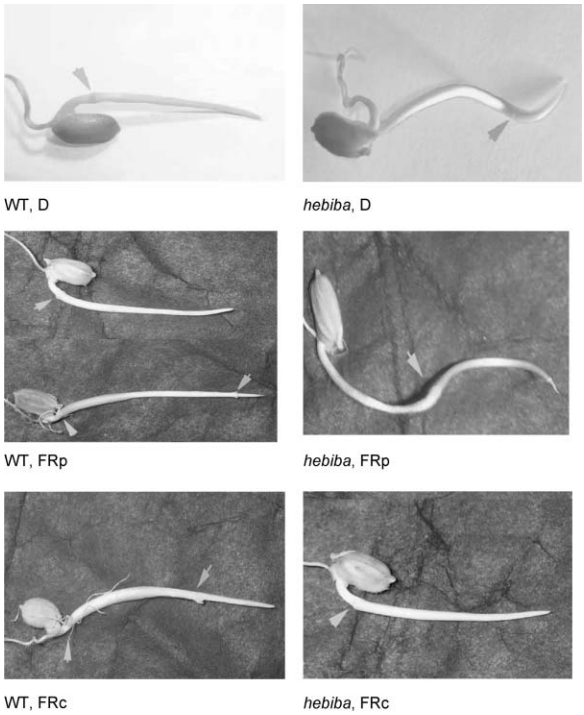

Fig. 1 Seedling phenotypes of WT and *hebiba* mutant after growth for 5 days in complete darkness (D) or under pulsed (FRp) or continuous (FRc) FR ($\lambda_a \geq 720$ nm) of equal total fluence. The position of the coleoptilar node and the coleoptile tip (in seedlings, where the primary leaves have already emerged) are indicated by arrows.

whereas in *hebiba* the coleoptiles were closed and elongated, thus resembling dark-grown seedlings of the WT.

Phytochrome

In dark grown seedlings, practically no difference in the spectroscopic and photochemical characteristics of phy in the WT and the mutant was detected (Fig. 2a). Position ($\lambda_{max} = 685$ nm) and half-band width ($\Delta\lambda = 24$ nm) were the same in both lines, as well as the extent of the Pr → lumi-R photoconversion. The total phy content, the proportion of phyA′ and phyA″ and their concentration in the mutant were virtually the same as in the WT (Fig. 3).

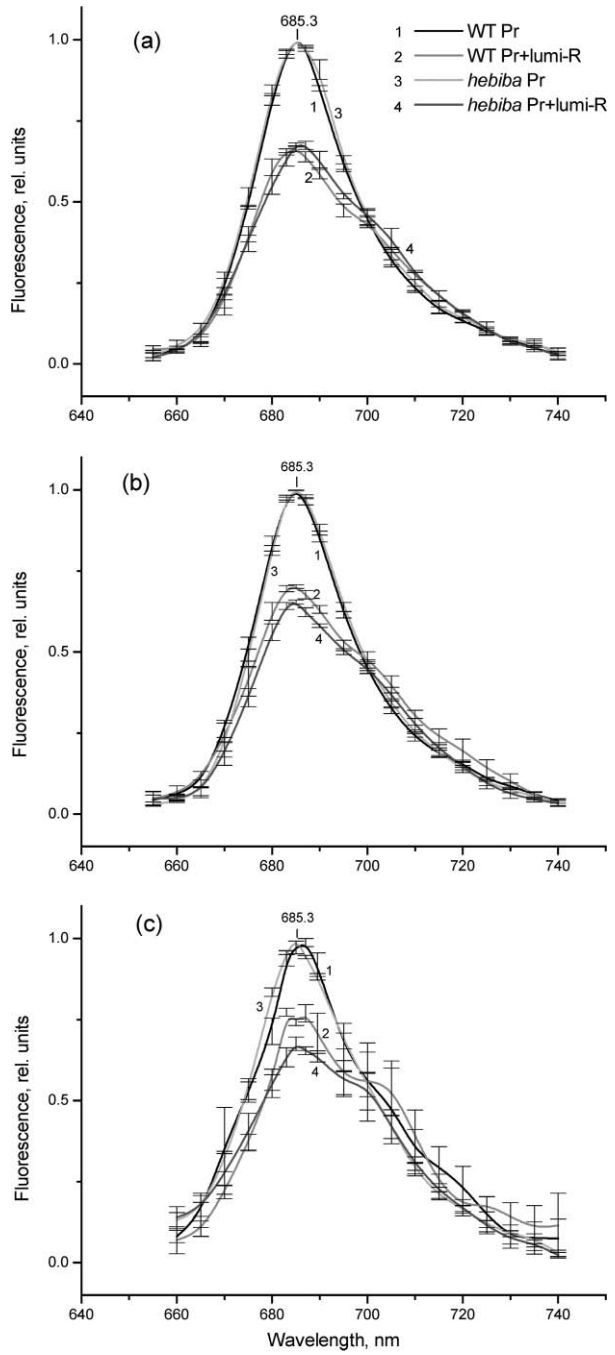

Fig. 2 Low-temperature (85 K) fluorescence emission spectra ($\lambda_e = 633$ nm) of phytochrome in coleoptile tips of 5 day old seedlings of WT (1,2) and *hebiba* mutant (3,4) grown in the dark (a) and under pulsed, FRp (b) and continuous, FRc (c) far-red illumination ($\lambda_a \geq 720$ nm) of equal total fluence. 1,3 sample frozen in the dark at 85 K when all the pigment is in its R absorbing form, Pr (state 0); 2,4 the same sample as 1,3 but after saturating illumination with R ($\lambda_a = 633$ nm) at 85 K partially converting Pr into lumi-R, the first photoproduct stable at low temperatures (state 1, photoequilibrium between Pr and lumi-R). The spectra were obtained from the raw data (not shown) by correction for the background fluorescence as described in Materials and methods and normalized to 1 in the maximum. The curves are an average of 4–7 spectra, error bars show the standard deviation (SD). The spectra were not corrected for spectral sensitivity of the instrument.

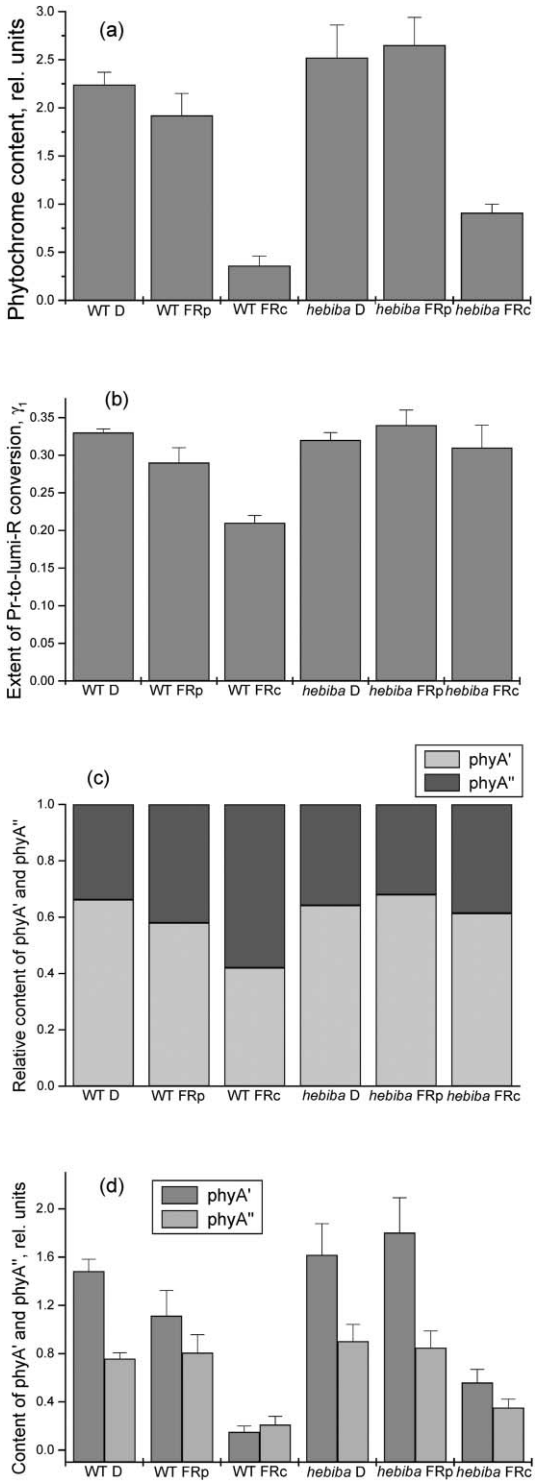

Fig. 3 Total phytochrome A content (a), extent of the Pr → lumi-R photoconversion at 85 K (b), proportion of the two phyA pools (phyA′ and phyA″) (c) and their content (d) in coleoptile tips of WT and *hebiba* mutant. The seedlings were grown for 5 days in complete darkness (D) or under pulsed (FRp) or continuous (FRc) FR ($\lambda_a \geq 720$ nm) of equal total fluence. The input of phyB, which is less than 10%, is ignored in these evaluations.

In seedlings grown under FRp, the parameters of phy differed between the WT and the mutant. Although phy was spectroscopically very similar ($\lambda_{max} = 685$ nm and $\Delta\lambda = 24$ nm) (Fig. 2b),

γ_1 was lower in the WT as compared to the mutant (Fig. 3). Pronounced differences were observed also in the content of total phyA and of its species: in *hebiba*, [P_{tot}], [phyA′] and [phyA″] were significantly higher than in WT. In general, upon FRp illumination a considerable decline in the total phy content and a shift in the phyA′/phyA″ equilibrium towards phyA″ was found in the WT, whereas *hebiba* showed almost no changes in [P_{tot}] and in the proportion of the two phyA species (Fig. 3).

Even more pronounced changes in the phy state were found in *seedlings grown under FRc*. The position and the shape of the spectra of phyA in the Pr form in the WT and the mutant were essentially similar (λ_{max} = 685 nm and $\Delta\lambda$ = 26 nm) (Fig. 2c). However, the extent of the Pr → lumi-R conversion (γ_1) dropped further down in the WT whereas in *hebiba* it remained practically unchanged as compared with dark-grown seedlings (Fig. 3). Seedlings of both lines revealed a considerable decline in [P_{tot}] upon FRc, although it was more pronounced in the WT. This decline was followed by further lowering of the phyA′/phyA″ proportion in the WT whereas in the mutant it remained essentially unchanged (Fig. 3).

Protochlorophyllide

In the dark, *hebiba* accumulates much higher protochlorophyllide both in the active and inactive forms than the WT, but the ratio [Pchlide^{655}]/[Pchlide^{633}] remains unaffected (Figs. 4 and 5).

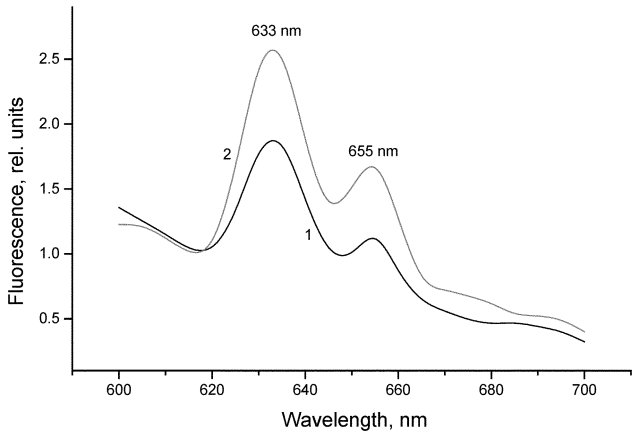

Fig. 4 Low-temperature (85 K) fluorescence emission spectra (λ_e = 450 nm) of protochlorophyllide in its active (Pchlide^{655}) and inactive (Pchlide^{633}) form in WT (1) and *hebiba* mutant (2), grown in the dark. The row spectra which were not corrected for the background fluorescence and for the spectral sensitivity of the instrument.

Upon FRp we observed a stimulation of the synthesis for both Pchlide^{655} and Pchlide^{633} by approximately 1.5-fold in the WT whereas in the mutant it remained practically unchanged.

A quite different picture was observed in *seedlings grown under FRc*. In the WT, a 3-fold inhibition of the synthesis of the active protochlorophyllide, Pchlide^{655}, was detected, whereas a considerable activation of the synthesis (1.6-fold) was observed in *hebiba* (Fig. 5a). A somewhat different situation was found with regard to the content of inactive Pchlide^{633}. In the WT, it remained similar to the levels observed in the dark, whereas in *hebiba* it grew approx. 1.4-fold (Fig. 5b).

Discussion

Dark-grown seedlings

The phenotype of the mutant seedlings was different of that of the WT (Fig. 1) in agreement with earlier observations.[5] However, the fluorescence and photochemical parameters of phyA in etiolated seedlings of both the WT and *hebiba* lines were found to be identical (Fig. 2) as well as its total content and proportion of the phyA subpopulations (Fig. 3). The fact that the balance between phyA′ and phyA″ does not change in the mutant indicates that the post-translational modification of phyA (possibly a phosphorylation) supposed to be responsible for the difference between phyA′ and phyA″[12] is not affected in *hebiba*. The level of protochlorophyllide was, however, considerably higher in the mutant (Fig. 4 and 5). Since the mutant is deficient in jasmonate,[5] these findings would be consistent with a role of jasmonate in the synthesis of protochlorophyllide whereas it does not considerably affect the state of phyA in the dark.

Seedlings grown under FRp

Upon FR illumination, the difference between mutant and WT became manifest not only in the phenotype (Fig. 1) and in the protochlorphyllide content (Fig. 5) but also in the status of phyA (Fig. 2 and 3). However, the direction and extent of the changes depend on the mode of illumination: FRp or FRc. FRp brought about a small shift in the phyA′/phyA″ balance towards the latter in the WT, with no considerable changes in the mutant. The relatively weak changes in the phyA state under FRp (which is expected to trigger VLFR[13]) indicate that the photodestruction of phyA and the regulation of phyA synthesis are unlikely to follow the VLFR response mode. However, FRp treatment proves to be inductive both for Pchlide^{655} and Pchlide^{633} biosynthesis in the WT, whereas in the mutant the active form of protochlorophyllide did not respond. Thus,

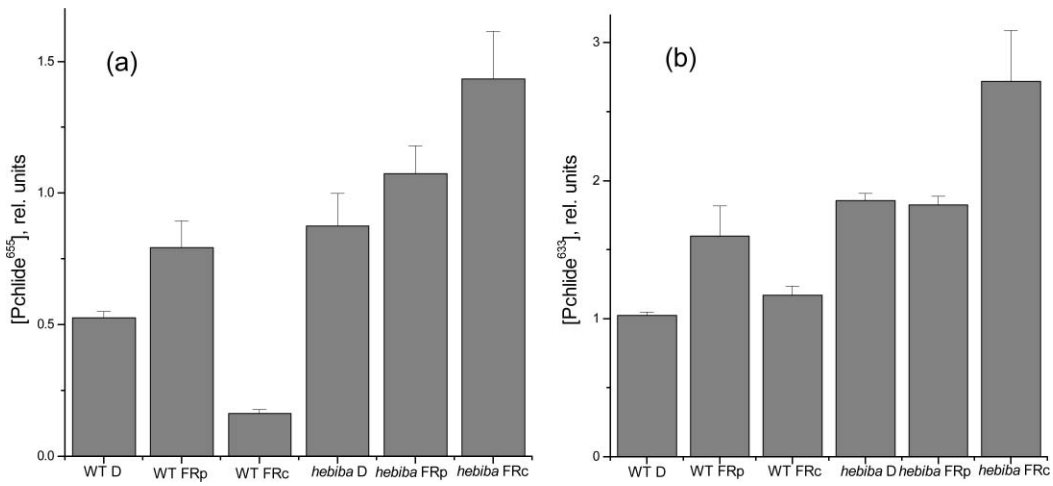

Fig. 5 Active (Pchlide^{655}) (a) and inactive (Pchlide^{633}) (b) protochlorophyllide content in WT and *hebiba* mutant. For details refer to the legend of Fig. 3.

protochlorophyllide falls under the most sensitive targets for the VLFR but seems to be not completely triggered in the mutant.

Seedlings grown under FRc

For the same total fluence, FRc proves to be much more effective than FRp and reveals profound differences between the WT and the mutant (Fig. 1). Although spectroscopically phyA in the mutant was very close to that in the WT (Fig. 2) phyA seems to be much more stable in the mutant (Fig. 3). In the WT, this decline is accompanied by a shift in the phyA′/phyA″ ratio in favor of phyA″. In contrast, there is practically no change in these parameters for the mutant. In agreement with ref. 14, these differences are interpreted to result from the interaction of two processes: (i) autoregulation of phyA synthesis by phyA itself, which should proceed without changes in the phyA′/phyA″ ratio, and (ii) light-induced destruction of phyA primarily in the phyA′ form. In the mutant, photodestruction is slowed down primarily, whereas in the WT, autoregulation and photodestruction contribute roughly to the same extent to the light-induced disappearance of phyA. FRc is more efficiently triggering changes of [phyA′] as compared to FRp although the total fluence is kept constant. This suggests that the HIR rather than VLFR mode of phyA action is responsible for the changes of [phyA′].

FRc drastically reduces the content of active protochlorophyllide in the WT (Fig. 5), in line with the effect found in *Arabidopsis*, tomato and barley.[15–17] The content of the inactive form was, however, practically unaffected. A quite different situation is observed in the *hebiba* mutant: instead of inhibition, FRc induced the synthesis of both protochlorophyllides. The sign of the FR effect on the biosynthesis of active protochlorphyllide had been shown to depend on plant species and plant tissues and to be mediated by phyA′.[11] Here we show that it also depends on the genotype (WT *versus* a mutant). Interestingly, not only protochlorophyllide synthesis, but also growth responds inversely to FR in the mutant (see above and Fig. 1).

The sign of the active protochlorophyllide regulation was found to depend on the irradiation protocol. FRp stimulated the synthesis both in WT and mutant. FRc inhibited the synthesis of the active form in the WT and stimulated it in the mutant. The same tendency of the FRp and FRc effects was followed by inactive protochlorophyllide, Pchlide^{633} (Fig. 5). As far as we know, the dependence of the sign of the effect on the mode of action of the FR (VLFR or HIR) on the protochlorophyllide biosynthesis was demonstrated for the first time.

The *hebiba* mutant is deficient in jasmonate.[5] This deficiency is already present in the dark, but becomes especially manifest in response to continuous light (both R and FR), when jasmonate synthesis is strongly induced in the WT. This strongly suggests that the light effects mediated by phyA depend on the hormonal status of the plant. It remains to be elucidated whether the changes in the relative content of the two native pools of phyA can account not only for the extent but also for the alterations in the sign of the light reactions in *hebiba*. Future work will be directed to understand the role of jasmonate in the photodestruction of the highly photolabile pool of phyA′.

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