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Altered Phytochrome Regulation of Greening in an *aurea* Mutant of Tomato[1]

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ABSTRACT

A brief pulse of red light accelerates chlorophyll accumulation upon subsequent transfer of dark-grown tomato (*Lycopersicon esculentum*) seedlings to continuous white light. Such potentiation of greening was compared in wild type and an *aurea* mutant W616. This mutant has been the subject of recent studies of phytochrome phototransduction; its dark-grown seedlings are deficient in phytochrome, and light-grown plants have yellow-green leaves. The rate of greening was slower in the mutant, but the extent (relative to the dark control) of potentiation by the red pulse was similar to that in the wild type. In the wild type, the fluence-response curve for potentiation of greening indicates substantial components in the VLF (very low fluence) and LF (low fluence) ranges. Far-red light could only partially reverse the effect of red. In the *aurea* mutant, only red light in the LF range was effective, and the effect of red was completely reversed by far-red light. When grown in total darkness, *aurea* seedlings are also deficient in photoconvertible PChl(ide). Upon transfer to white light, the *aurea* mutant was defective in both the abundance and light regulation of the light-harvesting chlorophyll *a*/*b* binding polypeptide(s) [LHC(II)]. The results are consistent with the VLF response in greening being mediated by phytochrome. Furthermore, the data support the hypothesis that light modulates LHC(II) levels through its control of the synthesis of both chlorophyll and its LHC(II) apoproteins. Some, but not all, aspects of the *aurea* phenotype can be accounted for by the deficiency in photoreception by phytochrome.

Photoresponse mutants in several higher plants are being studied with the aim of elucidating the mechanism of action of phytochrome, cryptochrome, and other photoreceptors in photomorphogenesis (2). Mutations could affect either the photoreceptor(s) or the transduction chain linking the photoevents to a measurable and relevant response. Mutations at the *au*[2] locus in tomato give rise to a defective phenotype that fits well what would be expected from a phytochrome-deficient mutant. Chl content, seed germination, anthocyanin accumulation and hypocotyl elongation were altered (11). The *au* mutant line W616 has been used in several recent studies to ask questions about phytochrome action (1, 11, 16, 17, 21). The *au* mutant does show phytochrome responses attributable to the low level of stable phytochrome present in light-grown plants, such as promotion of hypocotyl elongation by end-of-day far-red light (1). The light-stable phytochrome pool is presumably little affected by the *au* mutation (1). Dark-grown *au* seedlings contain less than 5% of the wild-type level of phytochrome, as shown by spectrophotometric and immunochemical methods (16, 17). The phytochrome deficiency in *au* probably results from instability of the apoprotein. The evidence for this is the presence of hybridizable and *in vitro* translatable phytochrome mRNA sequences at normal levels in the mutant (21).

Blue light promotes accumulation of several nuclear-encoded mRNAs encoding plastid proteins in wild-type seedlings, but is inactive in *au* seedlings (16). The finding that blue light did not induce certain mRNAs in phytochrome-deficient seedlings is evidence that some gene product(s) that the *au* lacks are required for this blue light response. A likely candidate for such a gene product is phytochrome itself (16). Another question that can be addressed is whether VLF responses are indeed phytochrome mediated, as has been postulated from spectral and physiological data (4). The VLF threshold is near the theoretical limit of sensitivity (a few molecules of Pfr per cell), so that a significant reduction in phytochrome concentration should cause the VLF to vanish.

A pulse of red light increased the abundance of LHC(II) mRNA in tomato; the small effect that could be detected in the *au* was completely reversed by FR, evidence that the VLF is indeed lacking in the mutant, as predicted (21). The overall low levels of LHC(II) mRNA in the *au* mutant (21), though, precluded quantitative photobiological studies. We therefore investigated, in mutant W616 and its isogenic wild type, the effect of a red pulse on the rate of Chl accumulation upon transfer of dark-grown seedlings to continuous white light. Such phytochrome-mediated potentiation of rapid greening has been observed in all angiosperms studied so far (for example, 5, 10, 20, 22), and provides a sensitive assay for VLF and LF phytochrome function (5, 10, 20).

Light controls the development of the photosynthetic apparatus via at least two photoreceptors, phytochrome and protochlorophyllide (8). The abundance of Chl was closely correlated with that of one of its major apoproteins, LHC(II), during greening of wild type and several Chl-deficient mutants of maize (9). The *au* mutation in tomato apparently affects the levels of both phytochrome and Chl (11). Hence, we compared the abundance of LHC(II) polypeptides following different light treatments in wild type and mutant, to deter-

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[2] Abbreviations: *au*, *aurea* locus mutant, genotype au^w/au^w; LHC(II), light-harvesting Chl *a*/*b* binding polypeptide(s) of photosystem II; LF, low-fluence phytochrome response; VLF, very low fluence phytochrome response.

mine the influence of this dual defect on accumulation of the LHC(II).

MATERIALS AND METHODS

Plant Material

Seeds of *Lycopersicon esculentum* cv Moneymaker and the *aurea* mutant line W616 (au^w/au^w, line kindly provided by Dr. M. Koornneef, Wageningen) were obtained from ripe fruits of greenhouse-grown plants, treated with 0.3% hypochlorite for 20 min, and allowed to dry. Seeds were treated with 0.1% hypochlorite for 30 min and then imbibed in deionized water for 4 h. Prior to imbibition, a small hole was made in the coat of each seed. Seeds were sown in vermiculite soaked with 0.1 mM gibberellic acid A_3 (Sigma). The combination of these two methods of enhancing germination (1, 21) led to germination rates of at least 80% in wild type and mutant. The addition of gibberellin slightly decreased the greening rate as compared to controls germinated on deionized water alone, but did not affect the extent of potentiation of greening by a red pulse (data not shown). Seedlings were grown for 6.5 d in a temperature-controlled darkroom at 24 to 25°C in 10 × 10 × 10 cm plastic boxes that were placed, open, in large covered plastic boxes to which about 500 mL water was added. The large boxes were wrapped in black cloth. In some seedlings (of both wild type and mutant) the cotyledons remained within the seed coat even at age 6.5 d; such seedlings were either discarded, or the seed coats were removed during the first part of long (100 s or more) irradiations.

Light Treatments

For red irradiations, the light source was a 150 W tungsten halogen slide projector lamp. The projector was equipped with heat filters and a long pass filter (RG610; Schott, Mainz). The fluence rate was reduced as required with neutral density filters (Schott). For far-red irradiations, a second projector equipped with a far-red pass heat filter (coating number 116, Schott) and far-red bandpass filter (7-69, Corning) was used instead. Red photon fluence rates were measured with a quantum radiometer (LI-185B, Lambda Instruments, Lincoln, NB); neutral density filter combinations were calibrated with a photomultiplier (R375, Hamamatsu). Far-red light was measured with a thermopile (number 7103, Oriel) and Keithley 197 digital multimeter, and corrected for IR as radiation passed by a Corning 7-56 800 nm long pass filter. The thermopile was calibrated against the quantum radiometer with red light.

Measurement of Chl Accumulation

Seedlings were transferred to a growth chamber (LabLine) at 24 to 25°C, 80% RH, under continuous white light (100 μmol m^{-2} s^{-1} photosynthetically active radiation) from fluorescent tubes (cool-white). Cotyledons were harvested (about 15/assay), weighed and put into glass vials. Total Chl was measured by extraction of intact tissue (13): *N,N*-dimethylformamide (Frutarom, Haifa) was added (1 mL per 20 mg fresh weight), the vials were kept in the dark at 4°C for 2 d, and the fluorescence read at 673 nm (the peak of the emission spectrum) with a Perkin-Elmer MPF-44B spectrofluorimeter. The excitation wavelength was 620 nm. Fluorescence was linear with A_{664} up to 0.2 OD, well above the range used. The absorption spectra (data not shown) of the extracts showed no indication of any shoulder arising from Chl *b* at 647 nm. From the absorption coefficients (13), we calculated a factor converting fluorescence units directly to total Chl per g fresh weight.

Immunoblotting

About 150 cotyledon pairs (0.6 g fresh weight) were ground in a glass-glass homogenizer in 2.4 mL of 100 mM Tris HCl (pH 8.0), 5 mM β-mercaptoethanol, 1 mM PMSF, and 1 mM benzamidine. The extract was filtered through 35 μm monofilament nylon mesh and centrifuged 10 min at 12,000 rpm in a Sorvall Microspin 24S. The pellet was sonicated 30 s in Laemmli sample buffer, boiled 1 min, centrifuged 5 min at 12,000 rpm, and the solubilized proteins separated on a 10 to 20% SDS-PAGE gradient gel. Immunoblotting methods were as described (14): the separated polypeptides were blotted to nitrocellulose (Schleicher and Schull, BA85) at 400 mA for 2.5 h. Blocking of nonspecific binding sites was with 3% bovine serum albumin (Fraction V, Sigma), 0.05% Tween 20, 25 mM Tris HCl (pH 7.5), and 140 mM NaCl for 24 h at 25°C. The blots were incubated with a 1:1700 dilution of the antibody (raised against spinach LHC(II) in rabbits [12]) overnight at 25°C. The antibody was detected on the blots by autoradiography following 1.5 h incubation at 25°C with ^{125}I-Protein A (77 μCi/μg, New England Nuclear).

In Vivo Spectrophotometry

Difference spectra were recorded with a single-beam spectrophotometer designed for samples with high scatter (6). The source for the measuring beam is a 100 W tungsten halogen lamp (model 66170 housing, model 68735 power supply; Oriel, Stratford, CT), with a monochromator (model 77264, Oriel) equipped with slits passing 1 nm bandwidth, and step motor wavelength drive (model 77325). The measuring beam reaches the sample (vertical cuvette) via a fiber optic cable. The detector is a red-sensitive photomultiplier (Hamamatsu R375) with a high voltage power supply (model 215, Bertan, Hicksville, NY). The anode photocurrent is converted to voltage by an operational amplifier (AD515JH, Analog Devices, Norwood, MA), processed by an AD755N log amplifier, digitized by an analog-digital converter (DASH-8, MetraByte, Taunton, MA) and stored by a personal computer (Spring XT-compatible, 20 mB hard disk). The computer also controls the wavelength scan via the digital output port of the DASH-8 board and an Oriel 17992 step motor control unit. Actinic light from the projector source described above reached the sample through one branch of the (bifurcated) fiber optic cable.

RESULTS AND DISCUSSION

A brief pulse of red light given to dark-grown tomato seedlings accelerated greening upon transfer to continuous

white light (Fig. 1), as observed previously for other angiosperms. A 4 h interval between the inductive pulse and transfer to white light was optimal for expression of the effect (data not shown). The time courses of Chl accumulation were quite different in the *aurea* mutant W616 and its isogenic wild type, cv Moneymaker. The mutant is Chl-deficient (11), and, as expected, greened more slowly than the wild type. Furthermore, cotyledons of the mutant accumulated little Chl during the first hours, in contrast to the wild type, which showed only a short lag period even in seedlings that did not receive an inductive red pulse (Fig. 1). We chose 3 and 6 h after transfer to white light as convenient times to assay induction by the red pulse in the wild type and mutant, respectively. Despite the lower levels of Chl, the enhancement by the red pulse was almost as large (twofold at 6 h) in the *aurea* seedlings as in the wild type (twofold at 3 h). This result was surprising in view of the low levels of spectrophotometrically and immunochemically detectable phytochrome in dark-grown *aurea* seedlings (17). We therefore carried out fluence-response and far-red reversal experiments to see whether the behavior of the mutant could be explained by phytochrome.

The fluence-response curve for potentiation of greening in the wild type was biphasic (Fig. 2), as observed previously for barley (5) and pea (10, 20). The data are consistent with a combined VLF and LF response. The half-saturation value for the VLF could not be determined by the least squares fit; more data at very low fluences would be needed. The *aurea* mutant, on the other hand, responded only in the LF range (Fig. 2). The *aurea* mutant contains at most a few percent of the normal phytochrome concentration (17). The threshold for the VLF approaches the theoretical limit of a few molecules of Pfr per cell, so the lack of VLF in the mutant is consistent with its low phytochrome levels.

A further prediction from this reasoning is that far-red light, which produces a photostationary state of at most about 3%

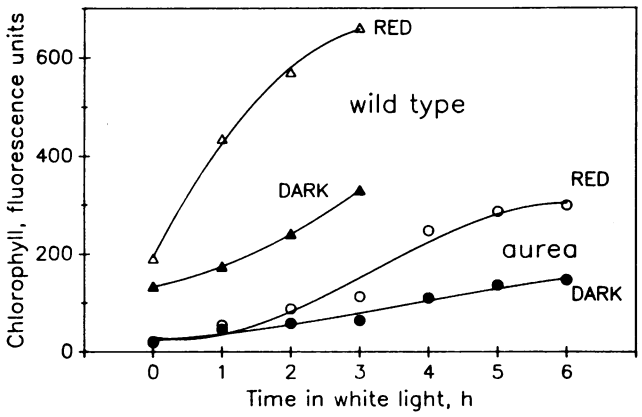

Figure 1. Time course of Chl accumulation in cotyledons of wild type and *aurea* tomato seedlings. Cotyledons were harvested at different times after transfer to continuous white light (100 μmol m^{-2} s^{-1}) for Chl determination. Red pulse: 100 s, 50 μmol m^{-2} s^{-1} given at 4 h prior to transfer. Symbols represent means of four experiments; average range of the data was 17%. 1000 fluorescence units (excitation, 620 nm; emission 673 nm) correspond to 59 μg Chl/g fresh weight.

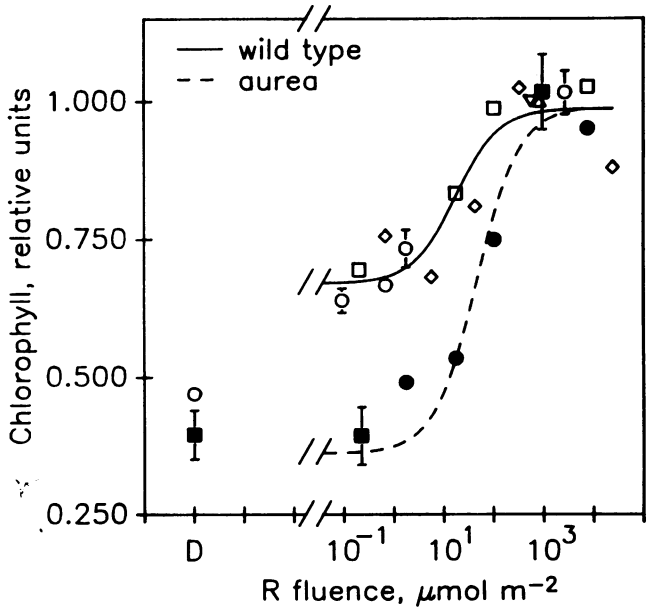

Figure 2. Fluence response curve for potentiation of rapid greening by a pulse of red light. The fluence of the inductive red pulse was varied, with a constant exposure time of 100s, as indicated; otherwise treatments were as in Figure 1. Cotyledons of the wild type were harvested after 3 h in white light, while those of the *aurea* mutant were harvested after 6 h in white light. Means of 2 to 11 replicate extracts per point. Points with error bars (SEM) indicate means of at least four replicates from 2 to 4 experiments. The curves are least squares fits to monophasic or biphasic hyperbolic saturation curves (method: from Press *et al.* [19], modified by D. Durant). For the wild type, the biphasic fit (shown here) had a χ^2 value of 0.456, while the monophasic fit gave a value of 1.69. For the mutant, the monophasic fit (shown here) gave a χ^2 of 4.39, while the biphasic fit gave a value of 5.98; in the biphasic fit, the very low fluence component was almost undetectable.

Pfr, should be effective in the wild type but not in the mutant. In the wild type, a FR pulse (Fig. 3) was about as effective as a R pulse in the VLF range (Fig. 2). The *aurea* mutant did not respond to FR (Fig. 3). We also tested reversal of the effect of R by FR. Preliminary experiments with the wild type (data not shown) indicated little reversal of the effect of R by a subsequent irradiation with FR. We therefore applied FR as a background illumination, before and during exposure to the R pulse. In this way, the effect of R could be reversed to approximately the level produced by FR alone (or a R pulse in the VLF range, for the wild type). In the *aurea* mutant, reversal by FR was nearly complete (Fig. 3).

The behavior of the mutant is, in general, consistent with its low (light-labile, 1) phytochrome levels. Some features of the *au* phenotype, though, cannot be explained on this basis alone. Even at time zero (transfer to white light) in Figure 1 there was more Chl, per unit fresh weight, in wild type than mutant cotyledons. This difference was found both in dark controls and in red-treated seedlings. The cotyledons were harvested in white light, so that the 'zero time in white light' (Fig. 1) value should represent Chl photoconverted from PChl(ide) during harvesting. To check this assumption, *in vivo* difference spectra (red minus dark) were recorded for

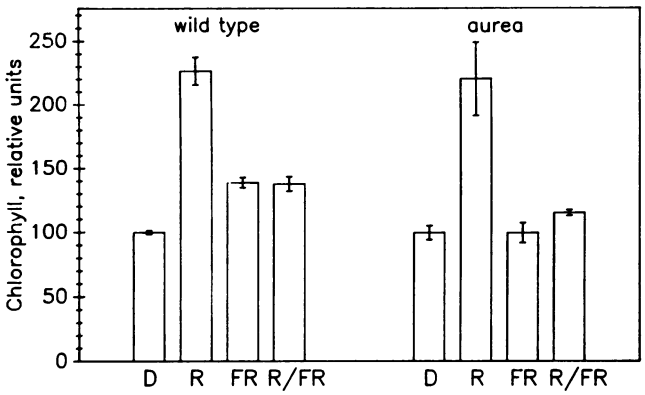

Figure 3. Far-red reversal of potentiation of rapid greening by a red light pulse. FR was applied as pulse of 200 s duration at 95 μmol m^{-2} s^{-1} which began 20 s before the start of the red pulse (100 s at 30 μmol $^{-2}$ s^{-1}). Means and SEM of at least six replicates from two to three experiments.

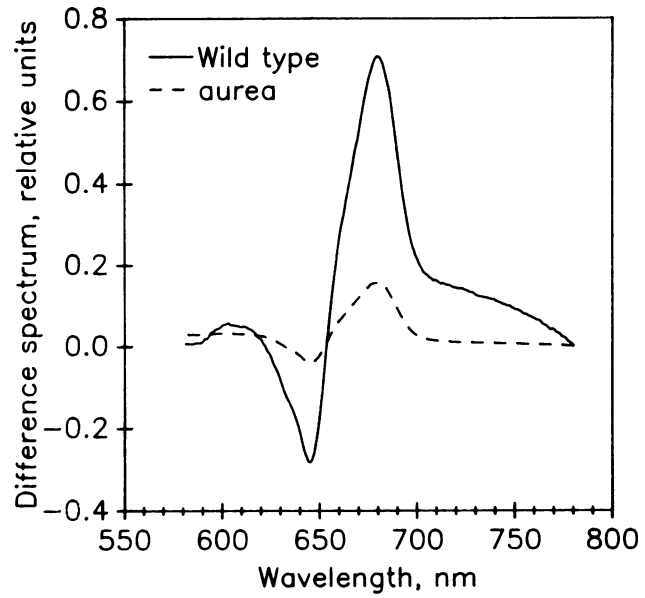

Figure 4. Protochlorophyll(ide) phototransformation in *aurea* tomato seedlings. Seedlings were grown in total darkness, cotyledons were harvested under dim green light and gently packed into the bottom of the vertical cuvette. Difference spectra were recorded at 4°C. A saturating red pulse, 60 s at 400 μmol m^{-2} s^{-1}, was given between the two scans for each spectrum. The amount of Chl per g fresh weight was determined independently, from fluorescence of extracts of the same samples in DMF, and the *in vivo* difference spectra were then corrected for the effective optical path length. Curves are means of two experiments. The signal is plotted relative to the peak-to-peak magnitude of the PChl-to-Chl difference spectrum of the wild type (average value, 0.15 A).

cotyledons of seedlings grown in total darkness (Fig. 4). The shapes of the spectra are nearly identical in wild type and mutant. The relative peak-to-peak values of the PChl to Chl difference spectrum in the two strains agree well with the zero time in white light value in Figure 1. It is thus apparent that, on a per gram fresh weight basis, the mutant produces less photoconvertible PChl(ide) in the dark than does the wild type. Light-labile phytochrome is reduced at least 20-fold in the *au* mutant (2, 17), while PChl was reduced only four- to sixfold (Figs. 1 and 4). The dark-control seedlings received no light at all, so the low PChl levels in the mutant cannot be related in any way to regulation by Pfr. Another aspect of the *au* phenotype that cannot be easily explained on the basis of phytochrome alone is the low level of LHC(II) mRNA in dark-grown seedlings of the mutant (21).

Phytochrome regulation of the mature LHC(II) protein probably results from control of LHC(II) mRNA levels and of the rate of accumulation of Chl (8). The level of LHC(II) polypeptides is light-regulated in several plants (7, 9, 12, 18); phytochrome control was reported for cucumber and tomato (7, 18). A pulse of red light given 4 h prior to transfer of wild-type tomato seedlings to white light strongly increased the level of immunochemically detectable LHC(II) polypeptides (Fig. 5). The level of LHC(II), as well as the extent of induction by the red pulse, was lower in the *au* mutant. Densitometric scans of (various exposures of) autoradiograms like the one shown in Figure 5 were used to estimate the relative increase in LHC(II) caused by the red pulse in the two strains. This induction ratio was 3.0 for the wild type and 1.3 for the mutant (average of two experiments). In the wild type, the red pulse thus increased the LHC(II) polypeptide levels by at least the same factor as it increased Chl. In the mutant, Chl levels in the red-pretreated cotyledons were twofold higher than in the dark controls (Figs. 1–3), with only a slight increase in LHC(II) (Fig. 5). The *aurea* mutation thus almost completely eliminated the enhancement of LHC(II) polypeptide levels by the red pulse. The result for the wild type is in agreement with the rather general observation that LHC(II) and Chl levels are closely coupled (9, for example). In contrast, the *aurea* mutant shows uncoupling of Chl and LHC(II) levels.

The molecular basis for the pleiotropic nature of the defect in the *au* strain is not clear. The low level of protochlorophyllide in dark-grown *au* seedlings may result in part from a paucity of (pro)thylakoid membranes, and this can be checked with other plastid marker proteins and ultrastructural studies of the cotyledons of etiolated seedlings. The *au* etioplasts cannot be drastically defective, because greening was normal under low light (16). One might speculate that the mutant is defective in some aspects of the signal (3, 9, 15) postulated to coordinate plastid and nuclear gene expression.

The *aurea* mutant is a valuable tool for exploring the multiple levels of control involved in chloroplast development. The defect in this mutant has pleiotropic aspects that may complicate the interpretation of some experiments: if the *au* mutant is used to test the involvement of phytochrome in a response, one tacitly assumes that all the defects in the *au* can be directly attributed to the lack of phytochrome. Biochemical characterization of the defect, which does not appear to be in a structural gene encoding phytochrome (21), will be essential for further progress.

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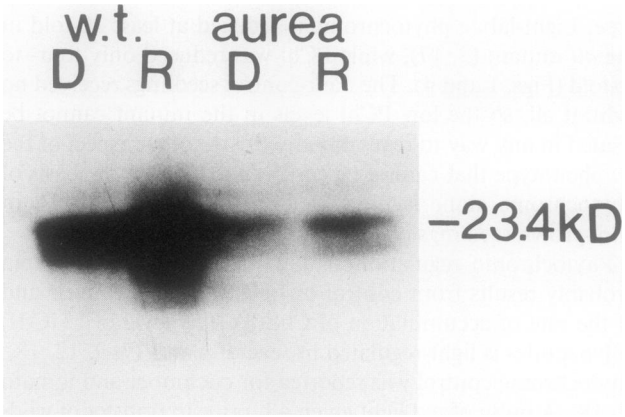

Figure 5. Photocontrol of LHC(II) levels in wild type and *aurea* tomato seedlings. Fifty μg protein of the pellet fraction (see "Materials and Methods) of cotyledon extracts were loaded on each lane of a 10 to 20% gradient SDS-PAGE gel and LHC(II) polypeptides were detected by immunoblotting and autoradiography. The pellet fraction accounted for 9.6% of total protein in the wild type and 12.1% in the mutant (average of four samples each), and the yield of total protein per g fresh weight was also about the same in wild type and mutant. The mol wt of the largest LHC(II) polypeptide was calculated from the migration of prestained markers (BRL, 14.3–200 kD) transferred to the nitrocellulose. D, no inductive red pulse; R, 100 s 50 μmol m^{-2} s^{-1} red light 4 h prior to transfer to continuous white light. Cotyledons of *aurea* were harvested 6 h after transfer to white light; for the wild type, harvest was at 3 h (see Fig. 1).

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