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Identification of photo-inactive phytochrome A in etiolated seedlings and photo-active phytochrome B in green leaves of the *aurea* mutant of tomato

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Summary

The contents of spectrophotometrically measurable phytochrome A (PhyA) and phytochrome B (PhyB) and the corresponding immunochemically detectable apoproteins (PHYA and PHYB) were examined in dark- and light-grown tissues of the *aurea* mutant of tomato and its wild-type (WT). The amount of PHYA in etiolated *aurea* seedlings was found to be about 20% of that in the WT; this PHYA showed no photoreversible changes in absorbance, no downregulation of the level of PHYA in light-grown seedlings, and no differential proteolysis of Pr and Pfr species *in vitro* which was seen in the case of the WT. By contrast, the amount of PHYB in *aurea* seedlings was not significantly different from that in WT seedlings. Phytochrome isolated from green leaves of the *aurea* mutant and purified by ion-exchange chromatography showed a red/far-red reversible spectral change, and its elution profile during chromatography was essentially similar to that of PHYB. The results indicate that *aurea* is a mutant that is deficient in photoactive PhyA at the etiolated stage, when it contains a spectrally inactive PHYA. However, the mutant contains spectrally active PhyB in its green tissue as does the WT.

Introduction

The development of plants is associated with considerable plasticity in response to a variety of environmental stimuli, in particular light. The perception of light by plants is mediated by a small number of photoreceptors, which include UVB, blue-UVA photoreceptors, and phytochrome (Shropshire and Mohr, 1983). Of these photoreceptors, only the red/far-red reversible phytochrome (Phy), a biliprotein of approximately 120 kDa, has been identified and characterized (Thomas and Johnson, 1991).

Although Phy has been considered, in principle, to be a single molecular species, it has long been known that some responses of plants to red/far-red light cannot be explained in terms of a single species of Phy (see review by Furuya, 1989). The Phy that is observable in dark-grown (etiolated) plants is short lived in the light, while many responses remain photoreversible for long periods of time, a phenomenon that suggests the existence of a light-stable form of Phy. Such a light-stable Phy, or type II Phy (Phy II), when isolated from green tissue, was found to have an amino acid sequence distinct from that of the light-labile, or type I Phy (Phy I) (Abe *et al.*, 1989). It was reported at about the same time (Sharrock and Quail, 1989) that, in *Arabidopsis*, there are five phytochrome genes, *phyA–phyE*, that encode different Phy apoproteins (PHY); three of the genes have been characterized: *phyA* encodes PHY I, which is detectable at very high levels in etiolated tissue. The dominant Phy II is analogous to the product of *phyB*. In light-grown *Arabidopsis* the relative molar abundance of PHYA, PHYB and PHYC is 5:20:1 (Somers *et al.*, 1991).

Since phytochromes control a wide variety of molecular, cellular and developmental responses, it is clearly important to characterize their individual or common functions. Several plants with Phy-related mutations have been isolated (see review by Kendrick and Nagatani, 1991). These mutants, of which the *aurea* (*au*) mutant of tomato is a good example, have become essential for efforts at characterizing phytochromes, and they may prove to be important in the analysis of the signal transduction(s) pathways associated with phytochromes.

The *au* mutant of tomato has been the subject of multiple studies. It was isolated as a non-germinating, gibberellin-responsive plant. It has a long hypocotyl, yellow-green leaves, reduced levels of anthocyanin, and defective and delayed development of chloroplasts (Koornneef *et al.*, 1985). No Phy is detectable either spectrophotometrically (Koornneef *et al.*, 1985) or immunochemically (Parks *et al.*, 1987) in etiolated *au* tissue. However, *phy* mRNA is present, and its translation *in vitro* yields a PHY that is indistinguishable from that of the WT during PAGE (Sharrock *et al.*, 1988). Contrary to the deficiency in responses to Phy in etiolated seedlings, light-grown *au* plants respond to end-of-day far-red light with an enhancement in the rate of stem elongation (Adamse *et al.*, 1988) and several other developmental reactions

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(López-Juez *et al.*, 1990). The *au* plants also respond to high-irradiance supplementary far-red light during the photoperiod, a strong shade-light signal (Whitelam and Smith, 1991). The plant's response to end-of-day far-red light appears complementary to the lack of response of an *lh* mutant of cucumber, deficient in light-stable Phy (López-Juez *et al.*, 1992). Phy is spectrally detectable in green leaves of the *au* mutant (López-Juez *et al.*, 1990) at levels close to those in the WT on an equal protein basis. It has then been speculated that the *au* mutant contains normal Phy II, which would be responsible for the end-of-day light effects. With respect to responses at the molecular level, the expression of the *Cab* genes, encoding chloroplast light-harvesting apoproteins, in etiolated *au* seedlings in response to light is reduced (Sharrock *et al.*, 1988). Similarly, expression of genes for nitrate and nitrite reductases is also reduced (Becker *et al.*, 1992), at least after long periods of etiolation. Nevertheless, the effect of the *au* mutation on the production of anthocyanin in etiolated seedlings can be partly overcome by the Phy-sensitizing, *hp* mutation (Adamse *et al.*, 1989). Exposure to blue light also sensitizes the *au* mutant to display normal, Phy-induced expression of four chloroplast genes (Oelmüller and Kendrick, 1991). The expression of genes for nitrate and nitrite reductase is also normal in *au* mutant plants grown in white light (Becker *et al.*, 1992).

The molecular basis of the *au* mutation is not known. Other mutants have been isolated that are specifically defective in one molecular species of Phy. They include PhyB-deficient *Arabidopsis* (Nagatani *et al.*, 1991; Reed *et al.*, 1993; Somers *et al.*, 1991), PhyA-deficient *Arabidopsis* (Nagatani *et al.*, 1993; Parks and Quail, 1993), PhyB-deficient cucumber (López-Juez *et al.*, 1992) and *Brassica* (Devlin *et al.*, 1992) and one Phy II-deficient *Sorghum* (Childs *et al.*, 1992). Since the *au* mutant is the focus of studies on the action of molecular species of Phy, it is important to characterize the Phy species that it contains, and to determine whether they vary at different stages of development. The purpose of this study was to examine the presence of PhyA and PhyB in the *au* mutant, by spectrophotometry and by the use of specific antibodies (López-Juez *et al.*, 1992; Nagatani *et al.*, 1984, 1987).

Results

PHYA and PHYB can be detected in etiolated au *seedlings*

Using specific antibodies, we detected PHYA in extracts of soluble protein obtained from etiolated tissues of both WT and *au* seedlings. The amount of PHYA in the *au* mutant was lower than in the WT (Figure 1). By contrast, identical quantities of PHYB were detected in the etiolated *au* and WT seedlings. Various monoclonal antibodies specific for different epitopes of PHYA (mAP14, mAP20, and mAP21) gave results similar to those obtained with mAP5, although the sensitivity of recognition was different. Similarly, PHYB-specific antibodies, namely mAT2 and mAT5, gave similar results to those obtained with mAT1. From an experiment with a mixture of PHYA- and PHYB-specific antibodies, it became clear that the two sets of monoclonal antibodies recognized different polypeptides with approximate molecular masses of 117 kDa and 114 kDa, respectively (Figure 2). This difference in molecular masses indicates that there was no cross-reactivity among the antibodies against PHYA and PHYB used in this study.

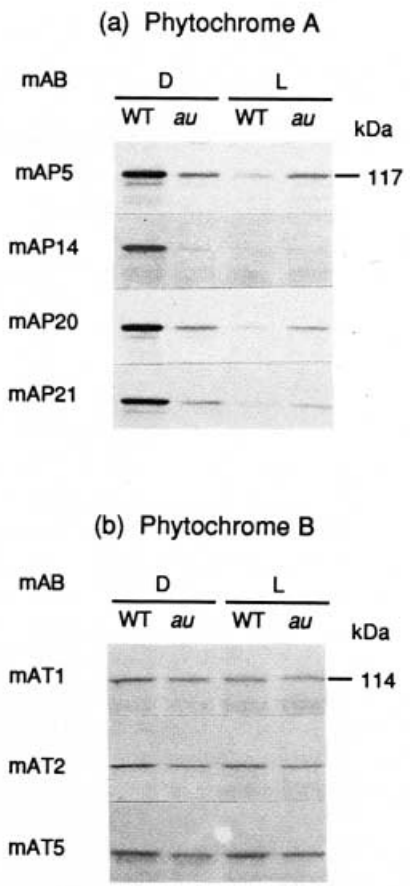

Figure 1. Detection of PHYA and PHYB, using different monoclonal antibodies, in light- and dark-grown tomato seedlings.
(a) The blots were probed with different monoclonal antibodies raised against Phy from etiolated pea. The relative specificity of different monoclonal antibodies was arbitrarily calculated on the basis of the reciprocal of the time needed to achieve a specific intensity of bands on the immunoblot, and these values are given in parentheses: mAP5(100); mAP20(50); mAP21(50); and mAP14(10).
(b) The blots were probed with different monoclonal antibodies raised against a C-terminal fragment of the product of the tobacco *phyB* gene.

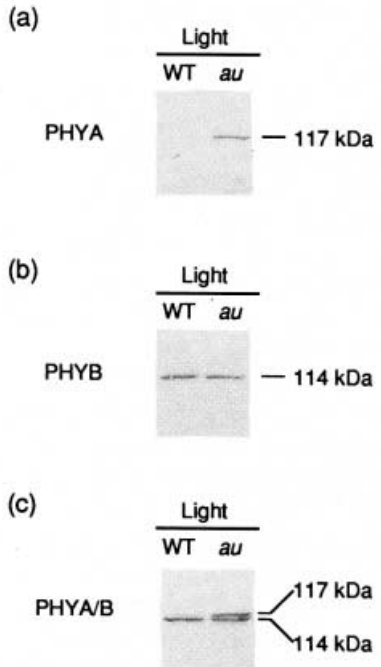

Figure 2. Simultaneous detection of PHYA and PHYB polypeptides with a mixture of mAP20 and mAT5 antibodies.
Extracts from light-grown *au* and WT seedlings were probed with mAP5 (a), mAT5 (b) and a mixture of mAP20 and mAT5 (c) antibodies. The PHYA polypeptide (a) can be clearly distinguished from PHYB (b) in the blot generated with the mixture of antibodies (c) by its characteristic low level in light-grown WT seedlings, as compared with that in *au* seedlings.

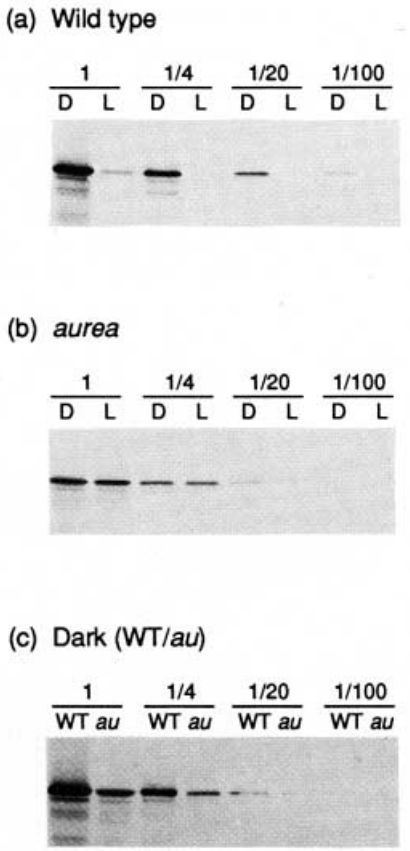

Figure 3. Comparison of amounts of PHYA in WT and *au* seedlings of tomato, detected with mAP5 antibody.
(a) Serial dilution of PHYA from dark- and light-grown WT seedlings, showing the extent of the light-mediated down-regulation of the abundance of PhyA in WT seedlings.
(b) Serial dilution of PHYA from dark- and light-grown *au* seedlings, demonstrating the absence of light-mediated downregulation of the abundance of PhyA in *au* seedlings.
(c) Serial dilution of PHYA from dark-grown WT and *au* seedlings, demonstrating that the abundance of PhyA in *au* seedlings is approximately 20% of that in the WT.

PHYA in etiolated au *seedlings is spectrally inactive*

We examined the spectral actvity of PHYA by analyzing its downregulation by light, and the changes in its conformation *in vitro*, as revealed by endogenous proteolysis. The data in Figures 1 and 2 showed that, in the WT, the concentration of PHYB was not affected by growth in continuous light. In the case of PHYA, the decrease in its concentration in light-grown WT seedlings confirmed that it can be photoconverted to a light-labile form and, therefore, is spectrally active (Figure 3a). By contrast, even though the *au* mutant does contain immunoreactive PHYA, this protein is not degraded by light, an observation that suggests that the PHYA in the *au* mutant is not spectrally active (Figure 3b).

The relative amounts of PHYA in *au* and WT seedlings were analyzed by serial dilution. The concentration of PHYA in etiolated tissue of the *au* mutant was about 20% of that in the WT (Figure 3c). The low level of PHYA in *au* seedlings was not the result of poor extractability of PHYA since direct extraction of lyophilized powders of seedlings that had been grown in the dark and in the light, by boiling in SDS buffer, yielded the same results (data not shown).

We also performed spectroscopic measurements on etiolated seedlings *in vivo* as described in Nagatani *et al.* (1993). In the *au* mutant, PHYA showed no spectral activity in samples of etiolated seedlings (an increment of less than 0.001 in the difference in absorbance (ΔA) between 660 nm and 730 nm, or one Phy spectral unit). A similar sample of WT seedlings had a spectral activity equivalent to 40 Phy spectral units. Furthermore, we failed to detect any Phy-related signal even in a soluble fraction prepared from etiolated *au* seedlings and enriched 20-fold by concentration with ammonium sulphate (data not shown).

PhyA exhibits differential susceptibility to proteolysis by endogenous proteases in its Pr and Pfr forms (Parks *et al.*, 1989; Vierstra and Quail, 1982). This property was exploited as a tool to determine whether the PHYA polypeptide in *au* seedlings is spectrally active. Figure 4 shows that, while PhyA in the WT was resistant to proteolysis in the Pfr form, the polypeptide from the *au* mutant

was degraded without any distinct light-triggered changes in conformation. It is evident from these data that PHYA in *au* etiolated seedlings has no spectral activity either *in vitro* or *in vivo*.

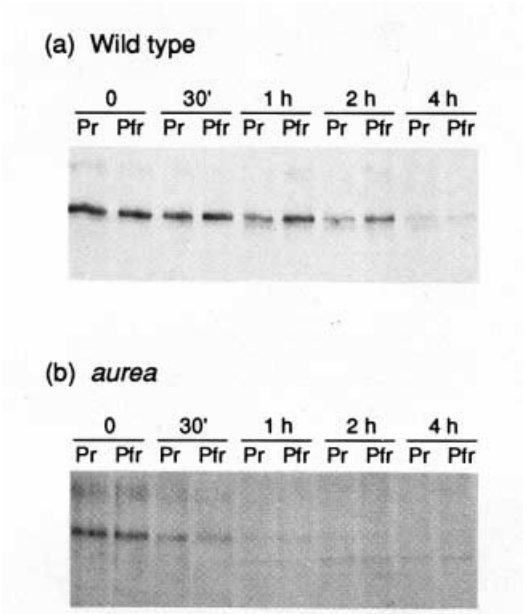

Figure 4. Proteolysis *in vitro* of PhyA in crude extracts from etiolated WT (a) and *au* (b) seedlings of tomato, monitored with mAP5 antibody.
Crude extracts of seedlings were divided into two portions which were irradiated with red or far-red light on ice for 5 min and then were incubated at 25°C in darkness. Aliquots were withdrawn at the times indicated and were mixed with an equal volume of sample buffer. After boiling, they were subjected to electrophoresis and immunoblotting.

PhyB in green leaves of the au *mutant is a spectrally active protein*

Since the PHY that was detectable immunochemically in etiolated *au* seedlings lacked spectral activity, we attempted next to determine which Phy species cause the photoreversible spectral change that can be detected in the green tissue of the *au* plants. Figure 5 shows the chromatographic profile of Phy isolated from leaves of greenhouse-grown 6-week-old tomato plants, after extraction and fractionation on a column of DEAE–Sepharose. The peak fractions were concentrated to a very small volume (500 μl) and were analyzed for PHYA and PHYB by immunoblotting. The immunoblot analysis revealed that PHYB eluted slightly earlier than PHYA from the ion-exchange column. Spectrophotometric analysis indicated that the spectral activity of the various fractions was closely correlated with the elution profile of PHYB, as detected by immunoblotting. However, a contribution by other species of Phy to this spectral activity cannot be ruled out (the limit of detection of the spectral activity was around one unit in the experiment for which results are shown in Figure 5). These results support the hypothesis that spectrally active Phy from green tissue is mainly PhyB, in both the WT and *au* mutant plants, while other species of Phy, if any are present, make only small contributions to the spectral activity.

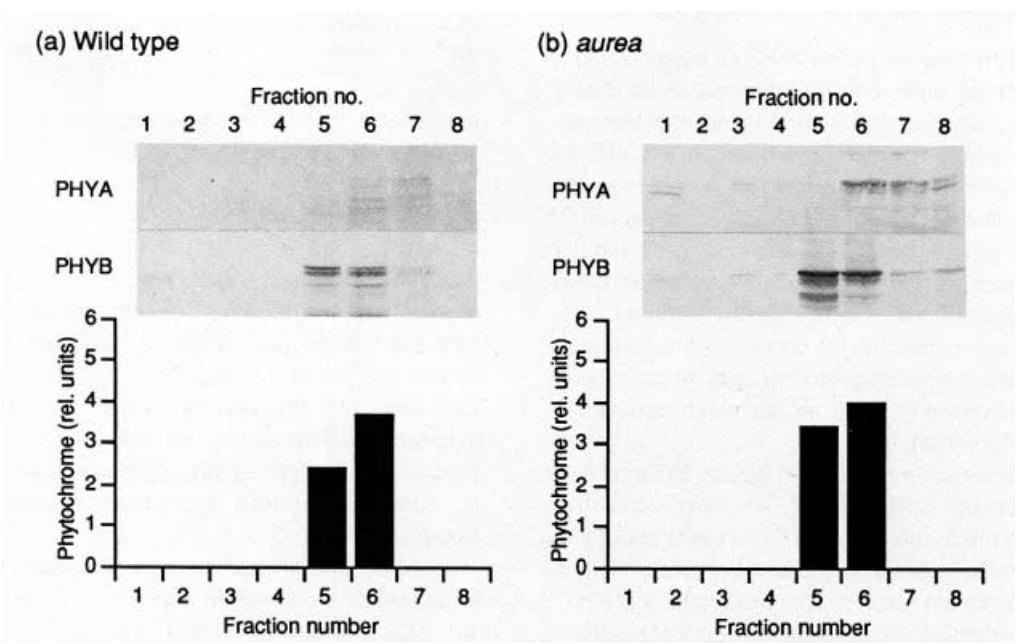

Figure 5. Profile of the purification of Phy from WT (a) and *au* (b) green leaves on a DEAE–Sepharose column.
One relative unit of Phy is equal to a $\Delta(\Delta A)$ value of 0.001 (see also text) and corresponds approximately to the limit of detection. The photographs show the immunoblot profiles of the PHYA (with mAP5 antibody) and PHYB (with mAT1 antibody) polypeptides. In view of the very low amount of the PHYA polypeptide in green tissues, the blots for PHYA were developed for three to four times longer than blots for PHYB. Fractions 1 and 2 represent the protein eluted in unbound fractions, while fractions 3–8 were collected around the peak of elution of Phy from the DEAE–Sepharose column.

Discussion

The results obtained in this study demonstrate that, even though PHYA can be detected in the *au* mutant of tomato, it is spectrally inactive in etiolated seedlings. The absence of the downregulation of levels of PHYA by light in the etiolated *au* seedlings clearly indicates that PHYA is present in a spectrally inactive form (Figures 1 and 3, some data not shown). In the experiments that involved endogenous proteolysis, PHYA did not respond to light signals in crude extracts of the *au* mutant and disappeared without providing any indication as to whether it was present in the Pr or the Pfr form in the crude sample (Figure 4). However, it remains unclear whether or not PhyA is spectrally active in the light-grown tissue of the *au* mutant since we were unable to detect spectral activity of PhyA even in the WT probably due to limitations of the assay method used (Figure 5). It is noteworthy in this context that in mature WT plants dark adaptation results in the reaccumulation of PHYA, which is degraded after return to the light. In contrast, dark-adapted *au* plants do not reaccumulate PHYA, and it is therefore not possible to examine Pfr-dependent PHYA destruction in light-grown plants (data not shown).

In a previous study, no PHY at all was detected in tomato *au* seedlings (Parks *et al.*, 1987). By contrast, we have demonstrated that the *au* mutant of tomato does contain PHYA at a level equal to about 20% of that in the etiolated WT (Figure 3). A mutant in the Moneymaker cultivar of tomato was used in the previous study (Parks *et al.*, 1987), whereas we used the Ailsa Craig cultivar in the present study. However, the discrepancy between results cannot be due to differences between cultivars, since we have obtained results similar to those in Figures 1 and 3 using seedlings of the Moneymaker cultivar also (data not shown). Neither can the discrepancy be due to cross-reactivity with PHYB of our antibody against PHYA. The double staining of PHYA and PHYB, using a mixture of antibodies, on the same blot showed that the antibodies used in the present study did not cross-react with other species of PHY in the extracts (Figure 2). It is likely that the discrepancy stems from the fact that a monoclonal antibody against Phy from oat was used in the study by Parks *et al.* (1987). It is possible that their antibody raised against Phy from a monocot reacts poorly with PHYA from tomato, as compared with our mAP antibodies, that were raised against Phy from a dicot (Nagatani *et al.*, 1984, 1987).

The concentration of PHYB appeared to be equal in the light- and dark-grown seedlings of both the WT and the *au* mutant (Figures 1 and 2). Since no spectral activity could be assigned to PHYA in etiolated seedlings of the *au* mutant (Figures 1, 3 and 4; Parks *et al.*, 1987), it was of interest to determine whether PHYB in the *au* mutant had spectral activity. In view of the large amount of tissue required for such a test, we resorted to the partial purification of Phy from the green leaves of mature *au* plants. The profile of the elution of spectrally active Phy from the DEAE–Sepharose column was the same as the profile of elution of PHYB, monitored with specific antibodies (Figure 5). Thus, we suggest that PHYB in the *au* mutant is spectrally active, at least in mature plants.

Although the genes for phytochrome in tomato have not been characterized in detail yet, an initial study has identified at least four such *phy* genes (Hauser and Pratt, 1991), suggesting the existence of multiple species of Phy in tomato. Thus, it is possible that, in addition to PhyB, other stable phytochromes, namely PhyC or PhyD, also contribute to the spectral activity in the green tissue. In the absence of monoclonal antibodies against these phytochromes, the precise contribution of the spectral activity by different species of Phy in the chromatographic profile discussed above is not known. However, it is noteworthy in this context that PHYB is the predominant species of Phy in light-grown seedlings of pea (Abe *et al.*, 1989) and *Arabidopsis* (Somers *et al.*, 1991).

The present results show that the *au* mutant is a PhyA-deficient mutant which contains a spectrally inactive PHYA at the etiolated-seedling stage, whereas PhyB is spectrally active in green leaves. These findings are consistent with the physiological observation that the *au* mutant shows its most dramatic phenotype during its transition from an etiolated to a light-grown seedling (Ken-Dror and Horwitz, 1990; Koornneef *et al.*, 1985; Oelmüller and Kendrick, 1991; Sharrock *et al.*, 1988), at the stage at which the amount of PhyA is high in the WT. However, the molecular basis of the *au* mutation, which leads to reduced amounts of PHYA and the absence of spectral activity of PhyA in etiolated tissues, remains unclear.

There is no evidence that the PhyA-deficiency in the *au* mutant is caused by a reduction in the level of *phyA* mRNA, because the WT and the *au* mutant contain equal amounts of translatable mRNA of *phy*, identified by hybridization to a zucchini *phyA* probe (Sharrock *et al.*, 1988). It remains possible that *au* is a Phy-chromophore deficient mutant, resembling *Arabidopsis hy1*, *hy2* and probably *hy6* (Parks and Quail, 1991). Since phytochromobilin and chlorophyll share many steps in their respective biosynthetic pathways, a mutation affecting chromophore biosynthesis in the *au* mutant would also explain the reduced level of protochlorophyll in etiolated *au* seedlings (Ken-Dror and Horwitz, 1990) and contribute to the yellow-green color. However, the reduction in the amount of PHYA is atypical when compared with levels of PHYA in other reported chromophore mutants of *Arabidopsis*. In the latter mutants, the deficiency in the availability of chromophore does not lead to a reduction in the abundance of the PHYA polypeptide (Chory *et al.*, 1989; Parks

and Quail, 1991; Parks *et al.*, 1989). Alternatively, the *au* mutation might be a mutation in the *phyA* gene which results in a non-functional PhyA. However, the evidence does not support this possibility. The apparent homolog of the *phyA* gene in tomato maps to a chromosome location distinct from the *au* locus (Sharrock, personal communication). Secondly, the phenotype of *au* is quite different from that of putative *phyA* mutants of *Arabidopsis* (Nagatani *et al.*, 1993; Parks and Quail, 1993). Thus, we cannot exclude the possibility that the *au* mutation affects multiple species of Phy, including PhyA, at the etiolated stage only. Further molecular and genetic analysis of the au mutant are required if we are to identify the basis of this mutation.

Experimental procedures

Cultivation of seedlings

Seeds of the *au* mutant of tomato (*Lycopersicon esculentum*) cv. Ailsa Craig and the nearly isogenic WT (Koornneef *et al.*, 1985) were obtained from Dr M. Koornneef. The mutation, obtained initially in the Moneymaker cultivar, was subsequently introduced into the Ailsa Craig cultivar. Seeds were first washed with 70% ethanol and briefly (2–3 min) incubated with a 1% solution of commercial bleach, and then they were washed thoroughly with distilled water. Seeds were sown on an agar-solidified medium that contained 0.6% (w/v) agar in 1/10 inorganic MS medium (Murashige and Skoog, 1962) in plant growth boxes obtained from Flow Laboratories (McLean, UK). Seedlings were raised at 25°C either in darkness or under continuous red- and blue-enriched white light from fluorescent tubes (FLS 20 BRF; Toshiba, Tokyo), which provided irradiance of 100 μmol photons m^{-2} sec^{-1}, between 400 and 700 nm (185-B quantum meter; Li-Cor, Lincoln, USA), in a growth cabinet. Entire shoots were harvested from 3-day-old seedlings for the estimation of the amount of Phy *in vivo*. The harvested samples were frozen in liquid nitrogen and stored at −70°C before analysis of Phy. Tomato plants were also raised in pots in a greenhouse under cycle of approximately 12 h of daylight and 12 h of darkness. The leaves were harvested at day time from 6- to 8-week old plants and, after freezing in liquid nitrogen, they were lyophilized and stored at 4°C in a cold room.

Extraction of protein for immunoblotting

Protein from frozen seedlings was extracted for detection of PHY as described by López-Juez *et al.* (1992), with modifications. The frozen tissue was homogenized in extraction buffer that contained 100 mM Tris–HCl (pH 8.3), 50% (v/v) ethylene glycol, 140 mM ammonium sulfate, 4 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide, 10% (g g $tissue^{-1}$) insoluble polyvinylpyrrolidone, 56 mM 2-mercaptoethanol and 10 mM EDTA at 4°C in a microfuge tube, using a custom built homogenizer fitting the tube. The homogenate was centrifuged at −5°C for 15 min at 14 000 ***g*** in a microfuge. The supernatant was mixed directly with 2× SDS–PAGE sample buffer (Laemmli, 1970) and heated at 100°C for 4 min. Some of the sample was then analyzed by SDS–PAGE (Laemmli, 1970) and the remainder was stored at −20°C for further analysis.

Immunoblotting

Immunoblotting was performed as described elsewhere (López-Juez *et al.*, 1992). Unless otherwise stated, the blots were probed for PHYA and PHYB with mAP5 (Nagatani *et al.*, 1984) and mAT1 (López-Juez *et al.*, 1992) antibodies, respectively. Other mAP (Nagatani *et al.*, 1987) and mAT (López-Juez *et al.*, 1992) antibodies have been previously characterized.

Extraction of Phy

Purification was carried out using a procedure based on that of Grimm and Rüdiger (1986) with modifications. All steps were carried out under green safe lights at 4°C. The lyophilized tissue was homogenized in the same extraction buffer outlined above, for immunoblotting (1 g per 20 ml buffer) except that the concentration of ethylene glycol was 25% (v/v) and sodium bisulphite (to 10 mM) and a few drops of Antifoam A (Sigma Chemical, St. Louis, MO) were freshly added to the buffer. The buffer was added to the lyophilized tissue as a semifrozen slush before the beginning of homogenization in a Physcotron homogenizer (Niti-on, Tokyo). The homogenate was centrifuged at −5°C at 36 000 ***g*** for 15 min and the supernatant was filtered through a single layer of cheesecloth. The supernatant was mixed with polyethylenimine (0.1% v/v) and, after stirring for 1 min, was centrifuged as above. The clear supernatant was then irradiated with red light on ice for 5 min. Iodoacetamide and phenylmethysulfonyl fluoride were added to the extract at several steps during the purification. The sample was then mixed with 0.72 volumes of a saturated solution of ammonium sulfate (pH 7.8) and centrifuged again. The pellets were solubilized in 5–8 ml of 10 mM potassium phosphate buffer that contained the above protease inhibitors and 1 mM EDTA on a reciprocal shaker. The solubilized pellets were centrifuged to remove undissolved proteins and desalted by gel filtration on Econopack columns (Bio-Rad, Richmond, CA). Glycerol was added to the eluate to a concentration of 10% (v/v) before freezing of the eluate in liquid nitrogen and storage at −70°C. The DEAE–Sepharose (Pharmacia, Uppsala, Sweden) chromatography was performed essentially as previously described (López-Juez *et al.*, 1990), with a gradient of 0–0.5 M KCl in 10 mM K-phosphate buffer that contained 1 mM EDTA (400 ml at a flow rate of 4 ml min^{-1}) employed for the binding and elution of Phy. Three fractions of 12 ml each were combined, mixed with 9 g of solid ammonium sulfate which was dissolved by shaking on a reciprocal shaker, and protein was pelleted by centrifugation. The protein was solubilized in 500 μl of 10 mM potassium phosphate buffer and the concentration of Phy was measured spectrophotometrically with a dual-wavelength spectrophotometer (model U-3410, Hitachi, Tokyo). An aliquot was mixed with an equal volume of sample buffer and, after boiling, was subjected to electrophoresis and immunoblotting. Sources of red and far-red light and green safe lights have been described elsewhere (Nagatani *et al.*, 1989).

Endogenous proteolysis

Endogenous proteolysis was carried out basically as described by Parks *et al.* (1989). Etiolated seedlings were irradiated for 5 min on ice with red light and were homogenized in 3-(*N*-morpholino)propanesulfonic acid buffer, pH 7.8, that contained 50% (v/v) ethylene glycol, 5 mM EDTA and 56 mM 2-mercaptoethanol. The homogenate was clarified by centrifugation at 0°C. The supernatant was split into two equal portions which were then

irradiated on ice with either red or far-red light. Aliquots were removed for the 'zero time point' measurements. The samples were then transferred to a water bath at 25°C in darkness to allow the action of proteases in the extract, and aliquots were withdrawn at the times indicated, mixed with 2× SDS–PAGE sample buffer, heated, and used for immunoblotting.

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