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The Expression of Light-Regulated Genes in the High-Pigment-1 Mutant of Tomato[1]

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Three light-regulated genes, chlorophyll *a/b*-binding protein (*CAB*), ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, and chalcone synthase (*CHS*), are demonstrated to be up-regulated in the high-pigment-1 (*hp-1*) mutant of tomato (*Lycopersicon esculentum* Mill.) compared with wild type (WT). However, the pattern of up-regulation of the three genes depends on the light conditions, stage of development, and tissue studied. Compared with WT, the *hp-1* mutant showed higher *CAB* gene expression in the dark after a single red-light pulse and in the pericarp of immature fruits. However, in vegetative tissues of light-grown seedlings and adult plants, *CAB* mRNA accumulation did not differ between WT and the *hp-1* mutant. The ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit mRNA accumulated to a higher level in the *hp-1* mutant than WT under all light conditions and tissues studied, whereas *CHS* gene expression was up-regulated in de-etiolated vegetative *hp-1*-mutant tissues only. The *CAB* and *CHS* genes were shown to be phytochrome regulated and both phytochrome A and B1 play a role in *CAB* gene expression. These observations support the hypothesis that the HP-1 protein plays a general repressive role in phytochrome signal transduction.

Light controls many aspects of plant morphogenesis and provides energy for photosynthesis. Different regions of the spectrum are perceived by different photoreceptor molecules: the B photoreceptors, the UV photoreceptors, and the R-/FR-sensitive phytochromes. Phytochromes were physiologically identified 50 years ago, and in the last two decades different phytochrome types have been purified and cloned from several plant species. Mutants deficient in specific phytochrome family members have been isolated from several species: e.g. Arabidopsis (for review, see Smith, 1995), tomato (*Lycopersicon esculentum* Mill.) (van Tuinen et al., 1995a, 1995b), and pea (Weller et al., 1995). These mutants are excellent tools for studying the functions of the different members of the phytochrome family.

Although there is information about photoperception by phytochromes, little is known about the signal transduction pathways linking these receptors with gene expression. Several approaches have been used to study phytochrome signal transduction pathways. First, using microinjection, Neuhaus et al. (1993) identified several molecules that participate in phytochrome-signal transduction. The existence of two separate pathways was proposed: the cGMP-mediated pathway that leads to the regulation of *CHS* genes, and the Ca^{2+}-calmodulin mediated pathway that regulates the expression of *CAB* and *RBCS* genes. In both pathways the signal is transduced from phytochrome via a heterotrimeric G protein, and subsequently the existence of a reciprocal control mechanism between the pathways has been demonstrated (Bowler et al., 1994a, 1994b).

A second approach to identify and characterize components and regulators of phytochrome-signal transduction pathways is the isolation and characterization of mutants with altered light responses. Constitutive-response mutants such as constitutive photomorphogenesis (*cop*), de-etiolated (*det*), and fusca (*fus*) in Arabidopsis (for review, see Wei and Deng, 1996), light-independent (*lip*) in pea (Frances et al., 1992), and *hyp2* in tobacco (Traas et al., 1995) are excellent tools for these studies. These mutants fail to exhibit the characteristics of dark-grown seedlings and show reduced elongation and expanded leaves. Some also accumulate anthocyanin in the dark. The cloning of the corresponding genes from Arabidopsis allowed the biochemical characterization of the affected gene products and provided information about the possible role and function of these components in phytochrome-signal transduction (Wei and Deng, 1996). In addition, mutants in genes affecting phyA and B signaling have been reported (Whitelam et al., 1993; Ahmad and Cashmore, 1996; Wagner et al., 1997; Hoecker et al., 1998).

A third approach to identify the components of phytochrome-signal transduction is to screen directly for mutants altered in the regulation of particular light-regulated genes. Li et al. (1994, 1995) isolated Arabidopsis mutants altered in the regulation of *CAB* gene expression. These mutants were named *doc* (for dark overexpression of *CAB*) and *cue1* (for *CAB* underexpressed). The *doc* mutation affects the expression of *CAB* genes and the *cue1* mutation affects the expression of both *CAB* and *RBCS* genes. The expression of *CHS* genes was neither modified in *doc* nor in *cue1* mutant plants. In contrast, the increased *CHS* expres-

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Abbreviations: B, blue light; *CAB*, chlorophyll *a/b*-binding protein; *CHS*, chalcone synthase; FR, far-red light; R, red light; *RBCS*, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; VLFR, very low fluence response; WL, white light; WT, wild type.

sion (*icx1)* mutant of Arabidopsis shows enhanced induction of *CHS* gene expression by light, but no alteration in the level of *CAB* transcript accumulation (Jackson et al., 1995).

In this paper we examine a putative phytochrome signal transduction mutant of tomato, the *hp-1* (high-pigment-1) mutant. This monogenic recessive *hp-1* mutant was first identified in 1917 (Reynard, 1956) and exhibits higher anthocyanin content, shorter hypocotyl (Kerr, 1965; Mochizuki and Kamimura, 1985; Peters et al., 1989), and darker green foliage (Jarret et al., 1984) and fruits (Thompson, 1962) when compared with WT. The *HP-1* gene has been recently mapped to chromosome 2 (Yen et al., 1997). Soressi (1975) identified a recessive *hp-2* mutant, which is phenotypically similar but nonallelic to *hp-1* and maps to chromosome 1 (van Tuinen et al., 1997). Attempts to isolate Arabidopsis counterparts of the tomato *hp* mutants have been reported (Ichikawa et al., 1996), but await detailed analysis.

Although the nature of the *hp* mutations is still unclear, detailed physiological characterization of the *hp-1* mutant provided a valuable insight into phytochrome signal transduction processes. The *hp-1* mutant has high levels of anthocyanin and reduced height of light-grown seedlings (Peters et al., 1992a; Kerckhoffs et al., 1997a). Furthermore, the photoinduction of several enzymes in biochemical pathways: Phe ammonia lyase (Goud et al., 1991), nitrate reductase, nitrite reductase, and amylase (Goud and Sharma, 1994), are amplified in the *hp-1* mutant compared with WT. All of these features have been shown previously to be phytochrome regulated, and therefore, it was concluded that the *hp-1* mutant shows exaggerated phytochrome responses (Kerckhoffs and Kendrick, 1997). The apparent phenocopying of the *hp-1* mutant's phenotype and immature fruit color as a result of phyA overexpression in tomato (Boylan and Quail, 1989) is consistent with this idea. However, in vivo spectrophotometric and immunochemical analysis failed to provide evidence that the *hp-1* mutant is a photoreceptor mutant (Peters et al., 1992b; Kerckhoffs et al., 1997a). Therefore, it was proposed that the *hp-1* mutation is associated with an amplification step in the phytochrome-transduction chain (Peters et al., 1992b; Kerckhoffs et al., 1997a; Kerckhoffs and Kendrick, 1997). This conclusion is supported by the recent observation using specific phytochrome family-member-deficient mutants, that it is phyA and phyB1 that play a dominant role in the seedling anthocyanin response (Kerckhoffs et al., 1997b). In the phytochrome-amplification model, phytochrome responses are envisaged to be under the constraint of the *HP-1* gene product. Both B and the *hp-1* mutation appear to be able to relieve this constraint (Peters et al., 1989, 1992b).

The dark-green immature fruit color of the *hp-1* mutant compared with WT is due to higher chlorophyll levels (Sanders et al., 1975; Kerckhoffs, 1996) and the mature *hp-1*-mutant fruits have a higher lycopene and carotene content and increased levels of ascorbic acid than those of WT (Thompson, 1962). Recently, the plastid copy number in the hypocotyls and the Suc and flavonoid contents of ripe fruits have been reported to be elevated in the *hp-1* mutant compared with WT (Yen et al., 1997).

In this paper we characterize the effect of the *hp-1* mutation on *CAB*, *RBCS,* and *CHS* gene expression at different developmental stages using the most extreme allele available (*hp-1*^{w}).

MATERIALS AND METHODS

Plant Material and Growth Conditions

The tomato (*Lycopersicon esculentum* Mill.) genotypes used in the experiments were *hp-1*^{w} (Peters et al., 1989); *hp-1*^{w},*fri*^{1} (far-red light insensitive), deficient in phyA (Kerckhoffs et al., 1997b); *hp-1*^{w},*tri*^{1} (temporarily red-light insensitive), deficient in phyB1 (Kerckhoffs et al., 1997b) in the genetic backgrounds MoneyMaker (MM) or breeding line GT.

For the experiments with seedlings, seeds were surface sterilized for 3 min in a 1% (v/v) dilution of commercial bleach and rinsed for 5 min in Milli-Q water (Milli-RO 8 water purification system, Millipore). Seeds were sown at noon on 0.6% (w/v) agar medium containing 0.46 g L^{-1} Murashige-Skoog basal salts (Murashige-Skoog, 1962) in plastic tissue culture containers (Plantcon, Flow Laboratories Inc., McLean, VA) and germinated in a FR-6113A growth chamber (Koito, Tokyo, Japan) at 25°C. To germinate seedlings in absolute darkness, tissue culture containers were wrapped in aluminum foil, put in a black velvet sack, and grown in a dark room at 25°C. In the light-pulse experiments R (27 μmol m^{-2} s^{-1}) was obtained from FL20SRF fluorescent tubes (National, Osaka, Japan) filtered through a red, plastic filter (Shinkolite A no. 102, Mitsubishi Rayon Corp., Tokyo, Japan) and FR (33 μmol m^{-2} s^{-1}) from FL20S–FR74 fluorescent tubes (Toshiba) wrapped with one layer of Polycolor no. 22 and one layer of Polycolor no. 72 film (Tokyo Butai Shomei Co., Tokyo, Japan). B (11 μmol m^{-2} s^{-1}) was obtained from FL20S.B fluorescent tubes (Toshiba). WL-grown seedlings were germinated in 16-h WL (120 μmol m^{-2} s^{-1} PAR) 8-h dark cycles at 25°C. WL was obtained from FL20SD SDL fluorescent tubes (National).

For the experiments with adult plants and fruits, seeds were sown in the greenhouse in a 4:1 vermiculite/granular-clay-based compost mixture. After 1 month plants were transplanted to pots (19 cm [diameter] × 15 cm [height] for vegetative tissues of adult plants and 27.1 cm × 28.6 cm for fruits) containing 2:1 vermiculite/granular-clay based compost mixture and transferred to a phytotron KG-206HL-D (Koito) with 16-h WL (250 μmol m^{-2} s^{-1} PAR) 8-h dark cycles at 25°C. Vegetative plant material was harvested 2 months after sowing and frozen in liquid nitrogen. The frozen material was stored in a −135°C freezer until use. After the first fruit(s) on a plant became red, all fruits of that particular plant were harvested. Harvest was always at noon because of diurnal mRNA fluctuations of *CAB* genes in tomato fruits (Piechulla and Gruissem, 1987). Directly after harvest a picture was taken of the fruits from one plant. The age, diameter, length, and weight of each

fruit were measured and samples were taken for the chlorophyll assay. The remaining material was separated into pericarp (the outer wall of the pericarp including the epidermis) and the inner section (radial and inner wall of the pericarp, placental tissue, and locular cavity with seeds), and frozen in liquid nitrogen. The frozen material was stored in a −135°C freezer until use.

Chlorophyll Assay

To determine the chlorophyll content in the fruits, samples were taken from the equator of the fruits using an 11-mm cork borer. From this sample the pericarp and about 5 mm of the inner section (for definition, see above) directly bordering the pericarp were cut. For each fruit the chlorophyll was extracted from one pericarp and one inner-section disc. The fruit discs were placed in 15-mL tubes (Falcon) and incubated in darkness for at least 48 h at 65°C in DMSO (after the work of Hiscox and Israelstam, 1979). Samples were re-extracted with DMSO until no extra chlorophyll could be extracted, and the samples were always kept in the dark. When the samples were cooled to room temperature, A_{649} and A_{665} were determined spectrophotometrically. Chlorophyll *a* and *b* were calculated on a gram fresh weight basis, using the equations for ethanol published by Lichtenthaler and Wellburn (1983).

Anthocyanin Assay

Anthocyanin was extracted from cotyledons and hypocotyls of seedlings with 0.6 mL of acidified (0.3% HCl, v/v) methanol for 48 h. The extraction was carried out by shaking the samples in darkness at room temperature for 48 h. At the end of the extraction 0.45 mL of water and 1.2 mL of chloroform were added. Samples were vortexed and centrifuged for 20 min at 4500*g*. The A_{535} of the upper anthocyanin-containing phase was determined spectrophotometrically (DU650, Beckman).

RNA Gel-Blot Analysis

Total RNA was isolated by a modification of the method of Loening (1969) and was previously described by Peters and Silverthorne (1995). RNA was electrophoresed in 1% (w/v) agarose gels containing Mops buffer (20 mM Mops, 1 mM EDTA, and 5 mM sodium acetate, pH 7.0) and 6.7% (v/v) formaldehyde. Gels were soaked in distilled water to remove the formaldehyde (three changes of 20 min each) and visualized by staining with ethidium bromide (1 μg/mL) prior to blotting onto Hybond N^+ membranes (Amersham). Protocol no. 4 of the VacuGene XL blotting system (Pharmacia LKB Biotechnology, Bromma, Sweden) was used for vacuum transfer of RNA.

The blots were prehybridized overnight at 42°C in 50% (v/v) formamide, 5× Denhardt's reagent (1× Denhardt's reagent is 0.02% [w/v] Ficoll Type 400 Sigma, 0.02% [w/v] PVP, and 0.02% [w/v] bovine albumin Fraction V, Sigma), 0.1% (w/v) SDS, 5× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), and 50 μg/mL salmon sperm DNA (0.5 mL per cm^2 blot).

The coding region of the tomato *CAB-1* (Pichersky et al., 1985) and *RBCS-2* (Pichersky et al., 1986) and *CHS1* (O'Neill et al., 1990) genes were used to synthesize DNA probes by random priming using the Rediprime DNA labeling system (Amersham). To remove unincorporated nucleotides 1 μL of 10% SDS and 2 μL of denatured salmon sperm (10 mg/mL) were added to the 50-μL reaction mix in an ultra-free microcentrifuge tube (Ultrafree-C3 TGC, Nihon Millipore Ltd., Tokyo, Japan) and spun at room temperature (5 min, 5000*g*). After washing the labeled DNA with 100 μL of sterile water, the probe was recovered from the upper part of the Millipore tube, denatured (by boiling for 5 min), and put on ice until use. For hybridization, the appropriate probe (specific activity 0.5 dpm/μg) was added to the hybridization buffer (0.1 mL of prehybridization buffer per cm^2 blot). Hybridizations were carried out overnight at 42°C. As a loading control each blot was rehybridized with a 17-base oligonucleotide complementary to the 18S rRNA (Gallo-Meager et al., 1992). The oligonucleotide was labeled by phosphorylation with T4 polynucleotide kinase and the hybridization was carried out as described by Gallo-Meager et al. (1992).

Washes of nylon membranes (Hybond N^+, Amersham) were performed in 2× SSC, 0.1% SDS (3 × 10 min at room temperature), and 0.1× SSC, 0.1% SDS (2 times for 30 min at 65°C). The signals were visualized and quantitated with a phosphor imager (Fujix BAS 2000, Fuji, Japan).

RESULTS

Effect of a Single R Pulse on *CAB* and *RBCS* Gene Expression

In tomato seedlings the expression of *CAB* genes is controlled by phytochrome (Sharrock et al., 1988; Wehmeyer et al., 1990). Wehmeyer et al. (1990) showed that *CAB* mRNA accumulation reached a maximum 4 h after a R pulse. In contrast to *CAB* mRNA, they could not easily demonstrate phytochrome regulation of *RBCS* mRNA. To determine whether the *CAB* and *RBCS* gene expression in the *hp-1*^{w}-mutant seedlings is controlled by phytochrome, we first studied the kinetics of expression of both genes after a single R pulse. To this end, 4-d-old etiolated seedlings were irradiated with a 10-min saturating R pulse and returned to the dark. Samples were collected immediately (0 h) and at 1, 2, 4, 6, and 8 h after the R pulse. Control seedlings were maintained in continuous darkness and harvested simultaneously with the seedlings harvested immediately after the R pulse. Very low levels of *CAB* gene expression were detectable in dark-grown WT seedlings (Fig. 1). In contrast to WT, the *hp-1*^{w}-mutant seedlings exhibited a substantial level of *CAB* gene expression in the dark. A R pulse induced *CAB* mRNA accumulation in both WT and *hp-1*^{w}-mutant seedlings and maximum expression occurred 4 h after the light-pulse treatment. However, at this time point the *hp-1*^{w} mutant accumulated more transcript than WT. The *RBCS* mRNA accumulation in dark-grown *hp-1*^{w}-mutant seedlings was about 3-fold higher than the levels observed in WT. Although a R pulse slightly induced *RBCS*

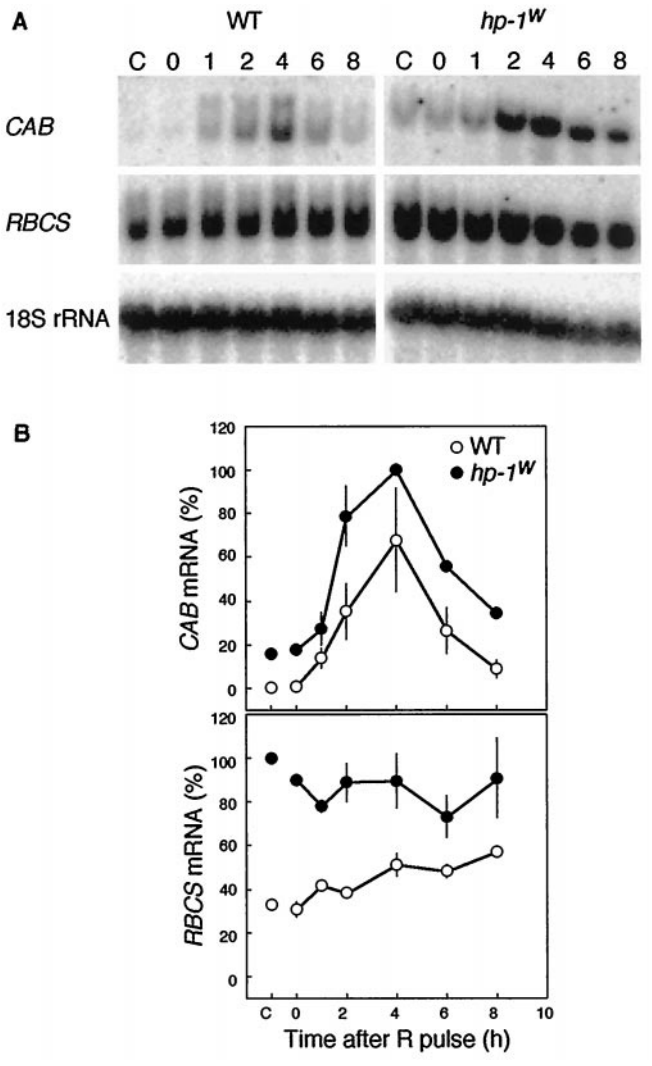

Figure 1. Effect of a 10-min R pulse on the *CAB* and *RBCS* mRNA abundance in etiolated 4-d-old WT and *hp-1*^{w}-mutant tomato seedlings. A, For the RNA blots shown, RNA was extracted directly (0 h), 1, 2, 4, 6, and 8 h after onset of the R pulse. The control (lanes C) represents the *CAB* mRNA amount in seedlings that did not receive a R pulse but were kept in continuous darkness. As a loading control the blots were probed with an 18S rRNA probe. B, The *CAB* and *RBCS* mRNA abundance was quantified using a phosphor imager and the mean values (±SE) are shown for *CAB* and *RBCS*, respectively. A value of 100% on the ordinate represents the maximum steady-state mRNA detected within the experiment.

gene expression in WT seedlings, no induction was observed in *hp-1*^{w}-mutant seedlings.

Phytochrome Regulation of *CAB* Gene Expression in Photomorphogenic Mutants

Figure 1 showed that in *hp-1*^{w}-mutant seedlings *CAB* but not *RBCS* gene expression could be induced by a single R pulse. Therefore, we limited our experiment to study the involvement of phytochrome in light-regulated gene expression to *CAB* gene expression. Since various photomorphogenic mutants of tomato are available, we used phyA-deficient, *hp-1*^{w},*fri*^{1}, and phyB1-deficient, *hp-1*^{w},*tri*^{1}, double mutants in addition to the *hp-1*^{w} mutant and WT. All genotypes were grown in continuous darkness for 4 d and exposed to a 10-min R pulse, 15-min FR pulse, or 10-min R pulse followed by a 15-min FR pulse. Control seedlings were kept in continuous darkness during the experiment. Whole seedlings were harvested 4 h after the light pulse(s) when maximum response occurs (Fig. 1).

In WT, *CAB* mRNA accumulation was induced by a 10-min R pulse and could be partially reversed by a FR pulse (Fig. 2). This incomplete reversal can be explained by a partial escape from FR reversibility during the R pretreatment. The response after FR alone probably reflects the VLFR component of *CAB* mRNA accumulation (Sharrock et al., 1988). Recently, a similar VLFR component of *CAB* gene expression was shown for Arabidopsis (Hamazato et al., 1997). In agreement with the data in Figure 1, a substantial level of *CAB* gene expression was detected in dark-grown *hp-1*^{w}-mutant seedlings (Fig. 2). Due to the *hp-1*^{w}

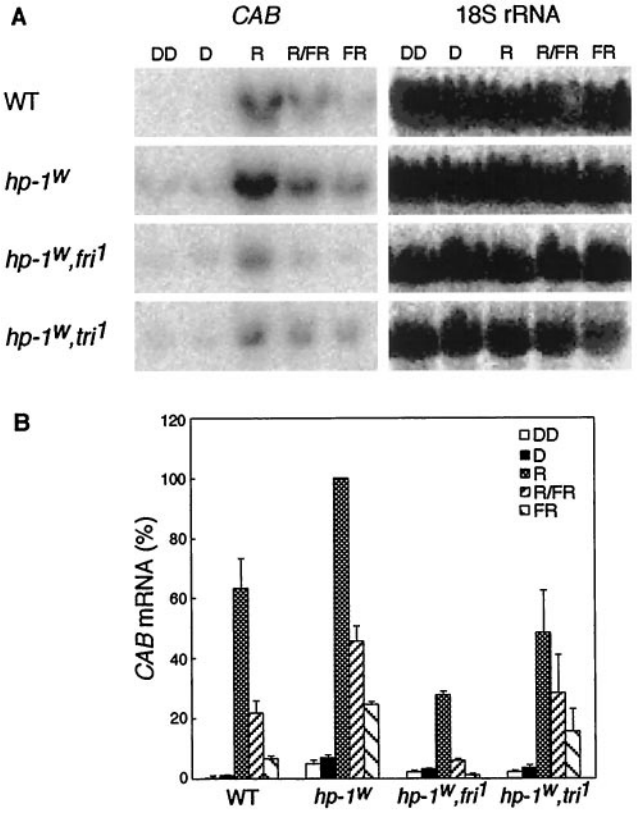

Figure 2. Effect of no light pulse (lanes D), a 10-min R, 15-min FR, and 10-min R followed by 15-min FR pulse (R/FR) on the *CAB* mRNA abundance in etiolated 4-d-old tomato seedlings. The genotypes used were WT, *hp-1*^{w} mutant, *hp-1*^{w},*fri*^{1}, and *hp-1*^{w},*tri*^{1} double mutants. A, For the RNA gel blots shown, RNA was isolated 4 h after the light pulse(s) and probed with a *CAB* cDNA probe. As a loading control the blots were probed with an 18S rRNA probe. B, The *CAB* mRNA abundance was quantified using a phosphor imager and the mean values (±SE) are shown. A value of 100% on the ordinate represents the maximum steady-state mRNA detected within the experiment.

mutation, the *hp-1*^{w},*fri*^{1} and *hp-1*^{w},*tri*^{1} double mutants also showed *CAB* gene expression in the dark, although at a reduced level compared with the *hp-1*^{w} monogenic mutant. To account for the possible effect of harvest under green safe light on *CAB* gene expression, the seedlings were also harvested in total darkness (in Fig. 2, DD) and compared with samples harvested under green safe light (Fig. 2, D). Figure 2 shows that when seedlings were harvested in complete darkness, the *hp-1*^{w} mutation resulted in significant *CAB* mRNA accumulation. Moreover, the *hp-1*^{w} mutant exhibited a higher response to all light-pulse treatments. The phyA-deficient *hp-1*^{w},*fri*^{1} double mutant exhibited approximately 30% of the *CAB* gene expression induced by a single R pulse in the *hp-1*^{w} mutant. A similar reduction in *CAB* mRNA accumulation could be seen when the *fri*^{1} mutant was compared with WT (data not shown). This implies a role for phyA in the low fluence response, either directly or indirectly. The effect of FR and R/FR treatments are markedly reduced in the *hp-1*^{w},*fri*^{1} mutant compared with the *hp-1*^{w} mutant, which verifies the role of phyA in the VLFR proposed previously (Casal et al., 1994; Botto et al., 1996; Shinomura et al., 1996). In the phyB1-deficient *hp-1*^{w},*tri*^{1} double mutant, the *CAB* gene expression induced by a R pulse was also reduced (about 50%) compared with the *hp-1*^{w} mutant. This clearly indicates that phyB1 also plays a role in the regulation of *CAB* gene expression.

Phytochrome Regulation of *CHS* Gene Expression

Although a single R pulse could induce *CAB* gene expression (Figs. 1 and 2), the same light treatment was ineffective for the induction of *CHS* gene expression (data not shown). Therefore, an irradiation schedule used by Peters et al. (1992b), which is efficient in inducing phytochrome regulation of anthocyanin biosynthesis, was applied to determine if phytochrome regulates *CHS* gene expression. The WT and *hp-1*^{w}-mutant seedlings were grown in darkness for 4 d and exposed to a 12-h R or B pretreatment followed by no pulse, a 10-min R pulse, 15-min FR pulse, or a 10-min R pulse followed by a 15-min FR pulse. Control seedlings were kept in darkness during the experiment. Figure 3 shows that neither WT nor *hp-1*^{w}-mutant seedlings accumulated detectable levels of *CHS* mRNA when grown in complete darkness. In seedlings that were given R and B pretreatments, a R pulse induced high levels of *CHS* expression in the WT. This effect could be reversed by FR. In the *hp-1*^{w} mutant R and B pretreatments followed by a R pulse resulted in a significant amplification of *CHS* gene expression compared with WT, and a FR pulse could not reverse the level of expression to the same extent as in the WT. In summary, the total level of response is enhanced in the *hp-1*^{w} mutant compared with WT for all treatments. Thus, by using light pretreatments, *CHS* gene expression was shown to be regulated by phytochrome in tomato. Moreover, the phytochrome-induced, R/FR reversible, *CHS* mRNA accumulation response is significantly enhanced in the *hp-1*^{w} mutant compared with WT after R pretreatment, but not significantly affected after B pretreatment. This indicates that B alone can enhance the phytochrome response in WT to an amount similar to that in the *hp-1*^{w} mutant.

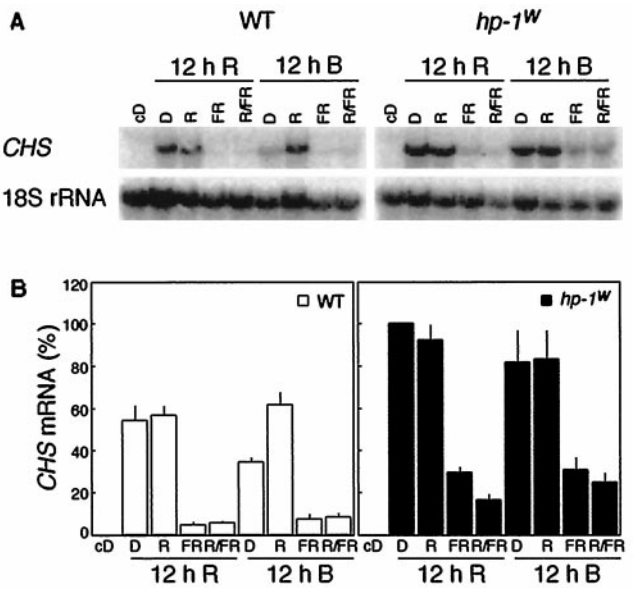

Figure 3. Effect of a 12-h R or B pretreatment terminated with no light pulse (lanes D), a 10-min R, 15-min FR, and 10-min R followed by 15-min FR pulse (R/FR) on the *CHS* mRNA abundance in etiolated 4-d-old WT and *hp-1*^{w}-mutant seedlings of tomato. A, For the RNA gel blots shown, RNA was isolated 4 h after the light pulse(s) and probed with a *CHS1* cDNA probe. As a loading control the blots were probed with an 18S rRNA probe. B, The *CHS* mRNA abundance was quantified using a phosphor imager and the mean values (±SE) are shown. A value of 100% on the ordinate represents the maximum steady-state mRNA detected within the experiment.

Circadian Rhythm of *CAB* Gene Expression

Accumulation of *CAB* mRNA shows a circadian rhythm in tomato with a maximum level of expression at noon (Kellman et al., 1993). A difference in the circadian rhythm of *CAB* mRNA accumulation of WT and *hp-1*^{w}-mutant plants could result in misleading conclusions when studying tomato plants grown in light/dark cycles. Therefore, we studied the effect of the *hp-1*^{w} mutation on the circadian rhythm. Seedlings were grown in 16-h WL, 8-h dark cycles (lights on at 6 AM; light off at 10 PM) and transferred to continuous darkness at 8 AM of d 5 (Fig. 4, WL/D → D/D). Control seedlings were kept in WL/D cycles and showed diurnal oscillations (Fig. 4, WL/D). Whole seedlings were harvested every 4 h for 3 d, starting at 8 AM on the 4th d after sowing. No differences in the diurnal oscillations of *CAB* gene expression of WT and *hp-1*^{w}-mutant seedlings were observed (Fig. 4, WL/D). The brief, 2-h exposure to WL before the transfer to continuous darkness resulted in a phase shift, and the subsequent peak of *CAB* gene expression occurred at 8 AM instead of at noon in both WT and *hp-1*^{w} mutant (Fig. 4, WL/D → D/D). Although a slight difference in dampening of the *CAB* gene expression between WT and *hp-1*^{w}-mutant seedlings may exist, the pattern and quantitative level of *CAB* mRNA accumulation in the two genotypes is very similar.

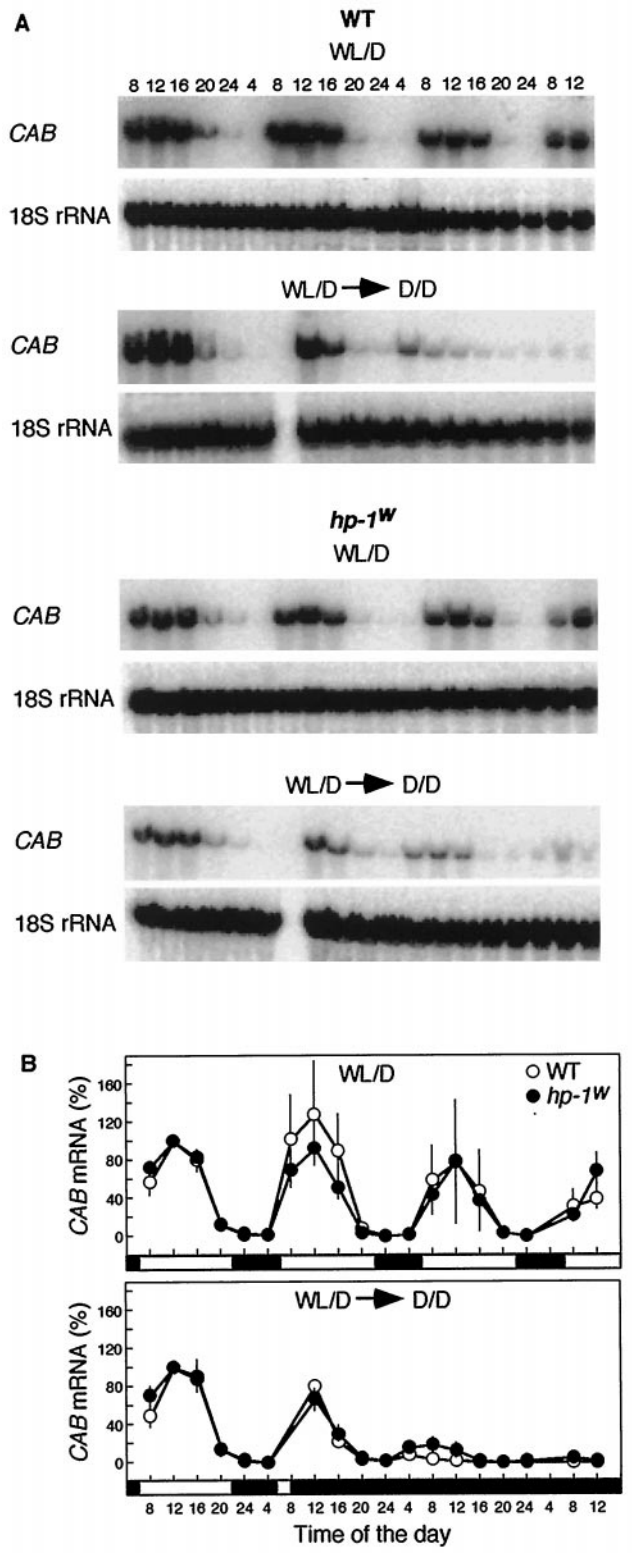

Figure 4. Circadian rhythmic *CAB* mRNA accumulation in WT and *hp-1*^{w}-mutant seedlings of tomato. A, For the RNA gel blots shown, seedlings were grown in 16-h WL (WL, 6 AM–10 PM), 8-h dark (lanes D, 10 PM–6 AM) cycles. To describe the diurnal *CAB* transcript oscillations (WL/D), samples were collected every 4 h starting with 4-d-old seedlings at 8 AM. To study the circadian rhythm of *CAB* genes (WL/D → D/D), seedlings were transferred to darkness on d 5 at 8 AM. As a loading control the blots were probed with an 18S rRNA probe. B, The *CAB* mRNA abundance was quantified using a phosphor imager and the mean values (±SE) are shown. A value of 100% on the ordinate represents the mRNA abundance detected on d 4 at noon.

Organ-Specific Expression of *CAB*, *RBCS*, and *CHS* Genes

At least one of the light-regulated genes studied, the *RBCS* gene family, exhibits organ-specific expression in tomato (Sugita and Gruissem, 1987; Wanner and Gruissem, 1991). Since the *hp-1*^{w}-mutant seedlings showed elevated responses for the genes studied, it is important to compare the organ-specific expression patterns of these genes in seedlings and adult plants of the WT and *hp-1*^{w} mutant. Such a comparison can answer the question of whether more mRNA accumulates in the same organs or whether the distribution patterns also change due to the *hp-1*^{w} mutation. Seedlings were grown in either continuous darkness or in 16-h WL, 8-h dark cycles for 4 d. The *CAB*, *RBCS*, and *CHS* mRNA accumulation in cotyledons and hypocotyls is shown in Figure 5. None of the genes were expressed in the roots (data not shown). The patterns of mRNA accumulation in *hp-1*^{w}-mutant seedlings differ from that of WT in several aspects. *CAB* mRNA in dark-grown hypocotyls and cotyledons and *RBCS* mRNA in dark-grown hypocotyls accumulated to a much higher level in the *hp-1*^{w} mutant than WT (Fig. 5). Moreover, a higher *CHS* mRNA accumulation was observed in WL/D-grown *hp-1*^{w}-mutant seedlings compared with WT. The *CHS* mRNA accumulation was significantly increased in cotyledons of the *hp-1*^{w} mutant compared with WT (Fig. 5), which correlates well with the anthocyanin accumulation data for the WT and *hp-1*^{w} mutant (A_{535} per three cotyledon pairs was 0.065 ± 0.004 and 0.449 ± 0.015 for WT and *hp-1*^{w} mutant, respectively; A_{535} per three hypocotyls was 0.323 ± 0.022 and 0.600 ± 0.038 for WT and *hp-1*^{w} mutant, respectively). The *RBCS* gene expression was always higher in the *hp-1*^{w} mutant, irrespective of the light conditions under which the plants were grown (Fig. 5). As in the R and B pretreatment experiment (Fig. 3), no *CHS* mRNA accumulation was observed in the dark. Accumulation of *CHS* mRNA reached a higher level in WL/D-grown *hp-1*^{w}-mutant seedlings than in WT. In contrast to *RBCS* and *CHS*, *CAB* gene expression was only higher in seedlings grown in darkness.

To investigate the *CAB*, *RBCS*, and *CHS* mRNA levels in the leaves, stems, and roots of adult plants, plants were grown under 16-h WL, 8-h dark cycles for 8 weeks. None of the genes were expressed in the roots of adult WT and *hp-1*^{w}-mutant plants (Fig. 6). As in 4-d-old, WL/D-grown seedlings (Fig. 5), the *CAB* mRNA accumulation in adult plant parts was not higher in the *hp-1*^{w} mutant than WT. However, both *RBCS* and *CHS* gene expression were significantly higher in stems of the *hp-1*^{w} mutant compared with WT. These data on the organ-specific expression of light-regulated genes in seedling and adult plants show that the three genes studied are differentially affected by the *hp-1*^{w} mutation.

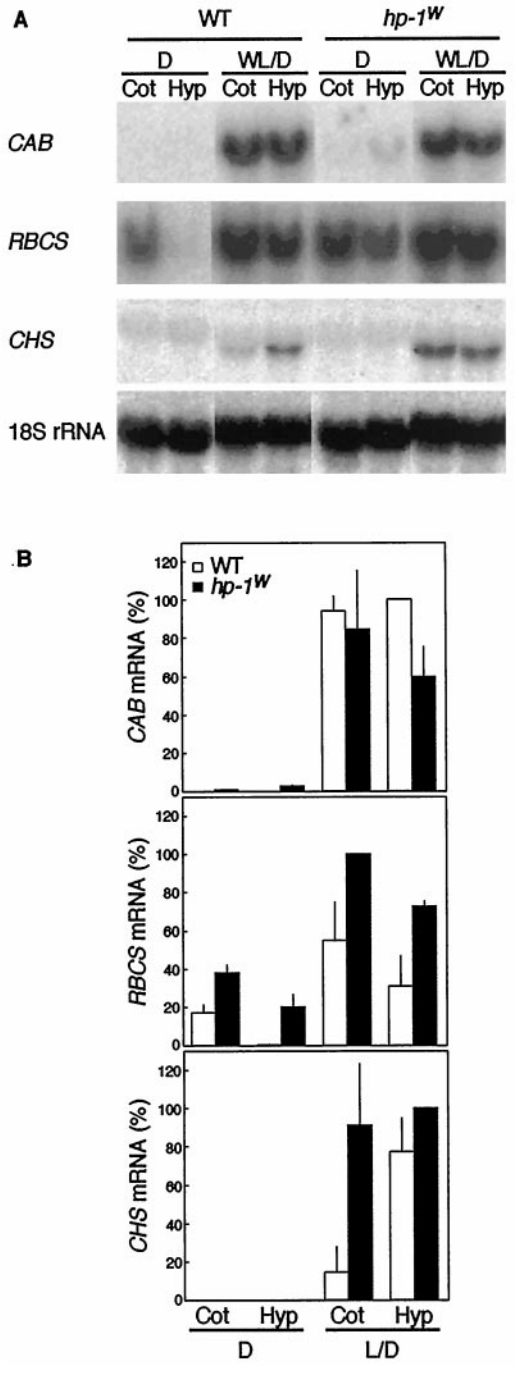

Figure 5. *CAB, RBCS,* and *CHS* mRNA abundance in cotyledons (lanes Cot) and hypocotyls (lanes Hyp) of 4-d-old WT and *hp-1*^{w}-mutant seedlings of tomato. A, For the RNA gel blots shown, RNA was isolated from seedlings grown in dark (D) or 16-h WL, 8-h dark cycles (WL/D). As a loading control the blots were probed with a 18S rRNA probe. B, The *CAB, RBCS,* and *CHS* mRNA abundance was quantified using a phosphor imager and the mean values (±SE) are shown. A value of 100% on the ordinate represents the maximum steady-state mRNA detected within the experiment.

To investigate *CAB* and *RBCS* gene expression and chlorophyll content during fruit development, we analyzed fruits at seven different physiological ages. The levels of *CAB* and *RBCS* expression in the pericarp were found to be highest in young fruits and they both gradually declined during fruit development (Fig. 7A). The expression level of both *CAB* and *RBCS* was amplified in the *hp-1*^{w}-mutant fruits compared with those of WT. The increased level of *CAB* and *RBCS* expression in the pericarp correlates well with its approximate 5-fold higher chlorophyll content (25-d-old fruits contain 100 and 25 μg chlorophyll g^{-1} fresh weight in the *hp-1*^{w} mutant and WT, respectively; Fig. 7B).

DISCUSSION

The results presented indicate that *CAB*, *RBCS,* and *CHS* gene expression is up-regulated in the *hp-1*^{w} mutant compared with WT. Two of these genes, *CAB* and *CHS*, were shown to be phytochrome regulated in the *hp-1*^{w} mutant (Figs. 2 and 3). However, their pattern of up-regulation is different and dependent on the stage of development and tissue studied. For instance, if we had only investigated gene expression in WL-grown seedlings, we would have only seen up-regulation of *RBCS* and *CHS*, but not *CAB*, compared with WT in the *hp-1*^{w} mutant (Figs. 4–6). In other words, in WL-grown seedlings, *CAB* gene expression appears to be saturated and is comparable in WL-grown WT and *hp-1*^{w}-mutant seedlings (Figs. 4–6). In contrast, dark-grown *hp-1*^{w}-mutant seedlings accumulate higher levels of *CAB* (and *RBCS*) transcripts than WT. In dark-grown seedlings of *hp-1,* up-regulation of enzyme activity for Phe ammonia lyase (Goud et al., 1991), nitrate reductase, nitrite reductase, and amylase (Goud and Sharma, 1994) have been previously reported. All of these enzymes have been shown to be phytochrome regulated (Goud et al., 1991; Goud and Sharma, 1994). Taken together, these findings indicate that the *hp-1* mutation causes changes in the dark at the molecular level. Unlike the dark "de-etiolated" Arabidopsis mutants such as *cop*, *det,* and *fus*, there are no visible differences between dark-grown WT and *hp-1*-mutant seedlings. Therefore, the *hp-1* mutation appears to affect only a subset of responses regulated by *cop*, *det,* and *fus* genes. The *CAB* mRNA measured in dark-grown *hp-1*^{w}-mutant seedlings could be due to an amplification of the response to the residual Pfr available in the seeds, and probably reflects a phyA-mediated VLFR, which is very difficult to test experimentally. The results with the phyA-deficient *hp-1*^{w}*,fri*^{1} double mutant suggest that this is the case.

R induction of *CAB* was similar in the *hp-1*^{w}-mutant and WT seedlings (Fig. 1) and confirms the observation of Wehmeyer et al. (1990). However, the difference between *hp-1*^{w} and WT observed in darkness was retained. The *RBCS* gene expression level was always higher in *hp-1*^{w} seedlings than those of WT, regardless of the conditions under which they were grown. In fact, the high *RBCS* gene expression in the dark could not be further enhanced by a single pulse of R, whereas in the WT a gradual elevation of expression was observed (Fig. 1), which never attained the level in *hp-1*^{w}. We can therefore conclude that the *hp-1*^{w} mutation affects both *CAB* and *RBCS* expression (Figs. 1, 2, 5, and 6). Studies involving *doc* and *cue1* mutants suggested that different biochemical pathways downstream of phy-

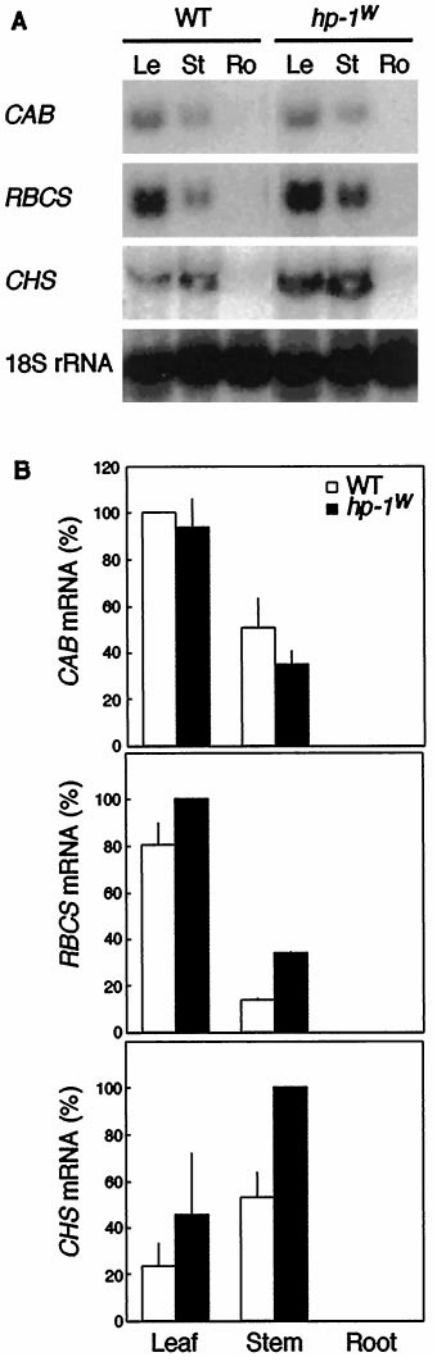

Figure 6. Abundance of *CAB*, *RBCS*, and *CHS* mRNA in young leaves (lanes Le), stems (lanes St), and roots (lanes Ro) of 8-month-old WT and *hp-1*^{w}-mutant tomato plants. A, For the RNA gel blots shown, RNA was isolated from adult plants grown in 16-h WL, 8-h dark cycles. As a loading control the blots were probed with a 18S rRNA probe. B, The *CAB*, *RBCS*, and *CHS* mRNA abundance was quantified using a phosphor imager and the mean values (±SE) are shown. A value of 100% on the ordinate represents the maximum steady-state mRNA detected within the experiment.

tochrome regulate *CAB* and *RBCS* gene expression (Li et al., 1994, 1995). Therefore, the differential effect of the *hp-1* mutation on the expression of *CAB* and *RBCS* genes may be related to the differences in the role of HP-1 in these pathways.

The *hp-1*-mutant has high anthocyanin levels in both seedlings and adult plants (Kerckhoffs et al., 1997a) and increased flavonoid accumulation in ripe fruits (Yen et al., 1997). The *CHS* transcript accumulation of the enzyme that is the first committed step of flavonoid biosynthesis also shows a higher level in the *hp-1* mutant than WT (Figs. 3, 5, and 6). Since no *CHS* gene expression could be observed in dark-grown or single pulse-treated seedlings, a 12-h R or B pretreatment irradiation schedule known to be effective in anthocyanin production (Peters et al., 1989) was used to study *CHS* gene expression (Fig. 3). Irrespective of whether a R or B pretreatment was given, significantly higher levels of *CHS* transcripts accumulated in the *hp-1*^{w}-mutant seedlings compared with WT (Fig. 3). These *CHS* mRNA accumulation data correlate reasonably well with the anthocyanin accumulation data under the same irradiation conditions (Peters et al., 1989). When WL-grown seedlings were examined, we also found a strong correlation between *CHS* abundance and anthocyanin content (results given in text and Fig. 5).

The expression of *CAB* and *RBCS* genes decreased with increasing fruit age in both WT and *hp-1*^{w}-mutant fruits (Fig. 7). In the pericarp these data show a positive correlation with the chlorophyll data (data not shown). The pericarp of green fruits is known to be photosynthetically active, and this activity decreases during chloroplast/chromoplast differentiation (Piechulla and Gruissem, 1987). Earlier work of Piechulla et al. (1985) showed that mRNA for photosynthetic polypeptides disappear during fruit ripening. These changes of mRNA levels correlated with alterations that occur at the photosynthesis and polypeptide level (Piechulla and Gruissem, 1987). Work of Meehan et al. (1996) using Arabidopsis transgenics that expressed a *CAB* promotor fused to a GUS reporter gene shows that GUS activities were positively correlated with chlorophyll content and cell size. Therefore, the transcription of nuclear genes for chloroplast components could be modulated by chloroplast numbers, which increase with cell size.

Our results support the proposal that the *hp-1* mutation amplifies phytochrome responses (for review, see Kerckhoffs and Kendrick, 1997): the R induction of both *CAB* and *CHS* gene expression was higher in the *hp-1* mutant compared with WT and was shown to be mediated by phytochrome (Figs. 2 and 3). Since the R induction of *CAB* gene expression is considerably lower in the phyA-deficient, *hp-1*^{w},*fri*^{1} mutant than the *hp-1*^{w} mutant (Fig. 2), phyA appears to modulate the magnitude of the low fluence response. Phytochrome-mediated *CAB* gene expression in tomato has a VLFR component (Sharrock et al., 1988) and this response, as indicated by the level induced by FR alone, is much reduced in the *hp-1*^{w},*fri*^{1} mutant compared with the *hp-1*^{w} mutant (Fig. 2). These data support the conclusion that phyA mediates the VLFR (Casal et al., 1994; Botto et al., 1996; Shinomura et al., 1996). In addition, phyB1 plays a role in *CAB* gene expression. The induction of *CAB* transcript accumulation is reduced in the phyB1-deficient, *hp-1*^{w},*tri*^{1} mutant compared with the *hp-1*^{w} mu-

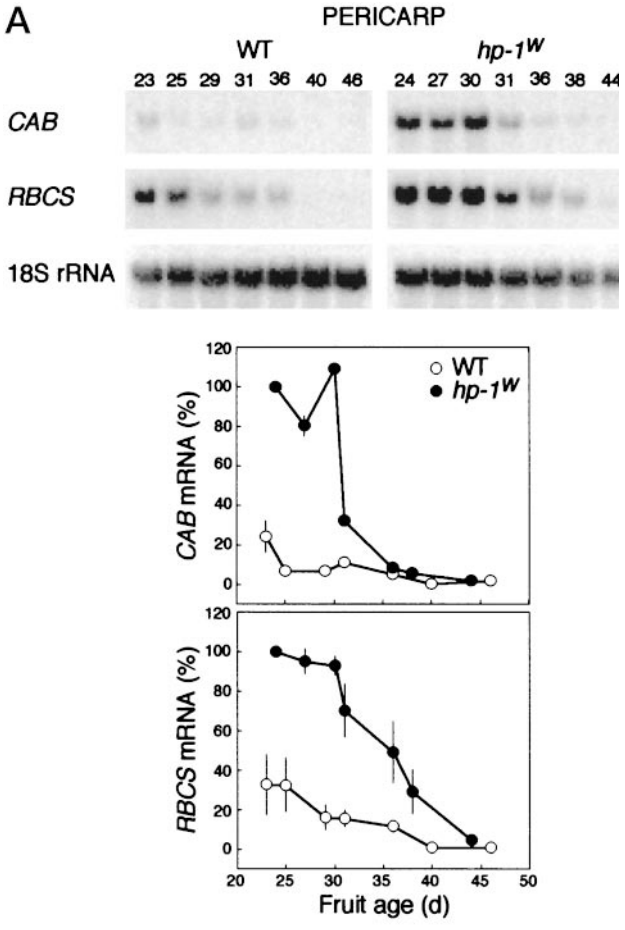

Figure 7. A, The *CAB* and *RBCS* mRNA accumulation in the pericarp of the *hp-1*^{w}-mutant and WT tomato fruit during fruit ripening. For both *CAB* and *RBCS* mRNA accumulation, the amount of mRNA in the pericarp of the youngest *hp-1*^{w}-mutant fruit was set at 100%. As a loading control the blots were probed with a 18S rRNA probe. B, A truss of WT (left) and *hp-1*^{w}-mutant (right) immature tomato fruits. The fruits of the *hp-1*^{w} mutant are darker green and have an elongated shape when compared with WT.

tant (Fig. 2). Thus, both phyA and phyB1 play a role in *CAB* gene expression in tomato. Reed et al. (1994) and Hamazato et al. (1997) came to a similar conclusion when they studied *phyA* and *phyB* mutants in Arabidopsis.

The data presented support the hypothesis that the HP-1 protein has a repressive role in phytochrome-signal transduction. The pattern of up-regulation observed for *CAB*, *RBCS*, and *CHS* gene expression depends on the light conditions, stage of development, and tissue studied. To date, *hp*-like mutations have not been described in other plant species. The dark phenotype of the *hp-1* mutant is more subtle compared with de-etiolated mutants such as *cop*, *det*, and *fus*. Considering the higher levels of anthocyanin responses and *CHS* mRNA accumulation, the tomato *hp-1* mutant is somewhat similar to the *icx1* mutant of Arabidopsis (Jackson et al., 1995). The major difference between the two mutations is that, whereas the *icx1* mutation affects only the signal transduction processes leading to the regulation of *CHS* expression, the tomato *hp-1* mutation also affects the expression of genes (*CAB* and *RBCS*) encoding proteins for the photosynthetic apparatus. This suggests that the *hp-1* mutation acts on an upstream signal transduction event(s) that leads to the altered pattern of gene expression. Therefore, HP-1 is proposed to be a fundamental phytochrome signal transduction regulator, and the cloning of its gene and molecular characterization is eagerly awaited.

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