Overexpression of homologous phytochrome genes in tomato: exploring the limits in photoperception

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Abstract

Transgenic tomato [Lycopersicon esculentum (=Solanum lycopersicum)] lines overexpressing tomato PHYA, PHYB1, or PHYB2, under control of the constitutive double-35S promoter from cauliflower mosaic virus (CaMV) have been generated to test the level of saturation in individual phytochrome-signalling pathways in tomato. Western blot analysis confirmed the elevated phytochrome protein levels in dark-grown seedlings of the respective PHY overexpressing (PHYOE) lines. Exposure to 4 h of red light resulted in a decrease in phytochrome A protein level in the PHYA OE lines, indicating that the chromophore availability is not limiting for assembly into holoprotein and that the excess of phytochrome A protein is also targeted for light-regulated destruction. The elongation and anthocyanin accumulation responses of plants grown under white light, red light, far-red light, and end-of-day far-red light were used for characterization of selected PHYOE lines. In addition, the anthocyanin accumulation response to different fluence rates of red light of 4-d-old dark-grown seedlings was studied. The elevated levels of phyA in the PHYAOE lines had little effect on seedling and adult plant phenotype. Both PHYAOE in the phyA mutant background and PHYB2OE in the double-mutant background rescued the mutant phenotype, proving that expression of the transgene results in biologically active phytochrome. The PHYB1OE lines showed mild effects on the inhibition of stem elongation and anthocyanin accumulation and little or no effect on the red light high irradiance response. By contrast, the PHYB2OE lines showed a strong inhibition of elongation, enhancement of anthocyanin accumulation, and a strong amplification of the red light high irradiance response.

Key words: Far-red light, Lycopersicon esculentum, overexpression, photomorphogenesis, phytochrome, red light.

Introduction

Plants use different photoreceptors to control photomorphogenic responses, and of these the phytochrome family of photoreceptors has been the most extensively characterized. Tomato contains five phytochrome genes, designated PHYA, PHYB1, PHYB2, PHYE, and PHYF (Hauser et al., 1997). The molecular properties of the photoreceptors, encoded by these genes, enable them to perceive and transduce red light (R) and far-red light (FR) signals to downstream cellular components, ultimately leading to modulation of the expression of genes responsible for photomorphogenesis (reviewed in Quail, 2002). Two types of phytochrome can be distinguished: phytochrome protein that is readily degraded in the light and which is abundant in dark (D)-grown seedlings (type I) and phytochrome that is relatively stable in the light (type II). PhyA is of type I and the other phytochromes are of type II. The phytochrome apoproteins (PHYA, PHYB1,
PHYB2, PHYE, and PHYF) are synthesized within the cytosol and assemble autocatalytically with a plastid-derived chromophore to form the phytochrome holoproteins (phyA, phyB1, phyB2, phyB2, phyE, and phyF). Phytochrome holoprotein has two different light-absorbing conformations. The R-absorbing form (Pr) is thought to be inactive, and upon absorption of R is converted to the activated FR-absorbing form (Pfr) (Kendrick and Kronenberg, 1994). General classes of phytochrome responses have been defined: the FR high irradiance response (FR-HIR), the very low fluence response (VLFR), and the R low fluence rate component are all attributed to phyA. The low fluence response (LFR), the end-of-day FR (EODFR) response, the R-HIR component, and the shade-avoidance response (SAR) are all mainly attributed to other phytochromes (Mancinelli, 1994).

Mutants impaired in specific phytochrome genes have been used to study the roles of phytochromes during tomato development (van Tuinen et al., 1995a, b; Kerckhoffs et al., 1997; Weller et al., 2000). From these studies it has been concluded that phyB1 is mainly responsible for mediating the de-etiolation response of seedlings to R as quantified by the inhibition of hypocotyl elongation, enhancement of anthocyanin accumulation, unfolding of the hypocotyl hook, and cotyledon expansion (Kerckhoffs et al., 1997). The contribution of phyB2 to seedling de-etiolation was only seen in the absence of functional phyB1 in the phyB1phyB2 double mutant, suggesting that in a phyB2 mutant the action of other stable phytochromes compensate for the loss in phyB2 signalling (Weller et al., 2000). Studies using different combinations of phytochrome mutations have shown that phyB2 acts redundantly with phyB1 in the SAR (Weller et al., 2000). However, a tomato triple mutant lacking phyA, phyB1, and phyB2 still showed some residual responsiveness to supplementary daytime FR, indicating that at least one of the two remaining phytochromes in tomato may also contribute to the tomato SAR (Weller et al., 2000).

The response to different fluence rates of continuous red light (cR) is primarily regulated by phyA, phyB1, and phyB2. This response has two components: an R low fluence-rate component which is mediated by phyA, but which is co-dependent on the presence of either phyB1 or phyB2, and the R-HIR component, which is mediated largely by phyB1 and only for a small part by phyB2 (Weller et al., 2000). During de-etiolation under cR there is a strong negative effect of phyA on phyB2-mediated anthocyanin accumulation. However, a positive interaction between phyA and phyB1 is seen in the enhancement of anthocyanin synthesis by pretreatment with FR (Weller et al., 2000). Combined, the mutant studies indicate that the visible action of an individual photoreceptor family member may be limited by (i) its own abundance, (ii) the light conditions, and (iii) the capacity of the signal transduction network.

Mutant studies do not provide information about the level of saturation of individual branches of the phytochrome signalling network. Therefore, a transgenic assay based upon overexpression of three tomato PHY genes under control of the constitutive double-35S promoter (PHYOE) was used to explore the limits in phytochrome-mediated photoreception. Ectopic expression of PHYA has mostly been studied in heterologous systems. In the initial studies, the PHYA from oat was used [in tomato, Boylan and Quail (1989); in Arabidopsis, Boylan and Quail (1991), Whitelam et al. (1992), McCormac et al. (1991); in tobacco, Keller et al. (1989), Boylan et al. (1994), Robson et al. (1996); in rice, Clough et al. (1995)]. In these cases, the overexpression of PHYA often resulted in a strong effect on suppression of stem elongation, which depended on the FR conditions (‘conditional dwarfing’). This was explained as an effect of the relative light stability of the monocot phyA, resulting in a more type-II than type-I character of the oat phyA in a heterologous host. The effects of homologous overexpression of PHYA are expected to be more limited, as the degradation machinery of the homologous host more efficiently recognizes the homologous phyA. Indeed, homologous PHYAOE in potato resulted in only a mild ‘conditional dwarfing’ (Heyer et al., 1995; Yanovsky et al., 1998).

There are several reports on homologous PHYB-type overexpression [Arabidopsis in Arabidopsis, Wagner et al. (1991), McCormac et al. (1993), Hennig et al. (1999); tobacco in tobacco, Sharkey et al. (1991), Adam et al. (1996), Fernandez et al. (2005); potato in potato, Jackson et al. (1996)] and numerous reports on heterologous PHYBOE (Arabidopsis in tobacco, Halliday et al. (1997), Fernandez et al. (2005); Arabidopsis in potato, Thiele et al. (1999), Boccalandro et al. (2003); tobacco in chrysanthemum, Zheng et al. (2001); rice in Arabidopsis: McCormac et al. (1993), Halliday et al. (1999)]. In most instances both the homologous and heterologous PHYBOE resulted in an enhanced suppression of hypocotyl and stem elongation. In Arabidopsis, it was shown that PHYBOE modulates the output of the circadian clock (Anderson et al., 1997) and, consequently, PHYBOE can affect flowering time (Halliday et al., 1997). PHYOE experiments have also been used to study the overlap in specific PHY functions. For instance, overexpression of Arabidopsis PHYD and PHYE or rice PHYA in the Arabidopsis phyB mutant all lead to partial complementation of the mutant (Halliday et al., 1999; Sharrock et al., 2003).

Here, the phenotypic effects of overexpression of the tomato PHYA, PHYB1, and PHYB2 on tomato growth and development are presented. In order to distinguish between a lack of phenotypic effect due to saturation of the signalling pathway and a lack of functional phytochrome holoprotein formation (e.g. because of chromophore limitations), the PHYAOE and PHYB2OE were also
tested in the phyA mutant and phyB1phyB2 double-mutant background, respectively. Results are discussed in relation to the actions and interactions of phyA, phyB1, and phyB2 in tomato photomorphogenesis.

Materials and methods

Plant material and growth conditions

Tomato phy mutants and PHYOE lines are all in the tomato [Lycopersicon esculentum (=Solanum lycopersicum)] wild type (WT) cv. MoneyMaker background. The mutant lines used here have been described previously: the phyA-null mutant (phyA-1 [fri1]; van Tuinen et al., 1995a), the phyB1-null mutant (phyB-1 [iri1]; van Tuinen et al., 1995b), the phyB2-null mutant (phyB2-1 [70F]; Weller et al., 2000), and the phyB1phyB2 double mutant (Weller et al., 2000). Seedlings were grown in peat-based compost in trays placed in growth cabinets at constant temperature (25 °C), a relative humidity of 70%, and light conditions as indicated. Plants were watered once a day. The dark control plants were watered under dim green safelight.

Cloning, transformation, and PHYOE line selection

Full-length cDNA clones of the tomato PHYA (Lazarova et al., 1998a), PHYB1 (Lazarova et al., 1998b), and PHYB2 (Kerckhoffs et al., 1999) were fused to the CaMV-2x35S promoter and cloned into the binary vector pKYLX 7 (Schardl et al., 1987) using standard molecular techniques. The binary vectors with the phytochrome cDNA constructs were introduced into Agrobacterium tumefaciens, which was subsequently used for transformation of tomato (McCormick, 1991). Primary transformed tomato T0 shoots were selected on medium containing kanamycin (50 mg l−1). Tetraploid regenerated transformants were grown. Single insert PHYOE lines were identified by segregation analysis of T1 seedlings after germination on medium containing kanamycin. The kanamycin-resistant single-insert seedlings were grown to maturity for collection of T2 seeds. Where applicable, the shorter T1 seedlings were selected to increase the possibility of selecting homozygous plants. The homozygous PHYOE lines were identified by segregation analysis of T2 seed germination on medium containing kanamycin. The homozygous PHYAOE and PHYB2OE lines were crossed with phyA or phyB1phyB2 double-mutant plants, respectively. The F2 progeny was grown on medium with kanamycin under monochromatic FR or R, to help identify plants with PHYOE and plants with mutant backgrounds. Plants homozygous for the phyA or phyB1phyB2 double-mutant background were identified by PCR analysis of genomic DNA, using specific PCR primer sets designed for the identification of WT and mutant phytochrome alleles as described by Weller et al. (2000). Segregation analysis of kanamycin resistance in F3 plants identified the lines homozygous for PHYAOE in the phyA mutant background and PHYB2OE in the phyB1phyB2 double-mutant background.

Protein extraction and phytochrome quantification from immunoblot analysis

Proteins were extracted from whole tomato 4-d-old D-grown seedlings (hypocotyl plus cotyledons) and D-grown seedlings subsequently treated with 4 h R. Plant material was immediately frozen in liquid nitrogen and proteins extracted using the EZ extraction protocol as described by Martínez-García et al. (1999). In addition, a protease inhibitor cocktail (Sigma, St Louis, MO, USA) (10 μl ml−1 EZ buffer) was used to prevent protein degradation during extraction. Protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins (30–300 μg) were size-separated in 7.5% (w/v) SDS-polyacrylamide gels and subsequently electroblotted onto an Immun-Blot PVDF® membrane (Bio-Rad) in 25 mM TRIS-HCl, 192 mM glycine, and 20% (v/v) methanol, pH 8.3. The membranes were blocked in blocking solution containing 1% (w/v) skimmed milk powder in 50 mM TRIS, 150 mM NaCl, and 0.05% (v/v) Tween solution (TBST). For the detection of PHYA, PHYB1, and PHYB2, new PHYA-specific polyclonal antibodies (PAb A/2), PHYB1-specific monoclonal antibodies (MAB B/1), and PHYB2-specific monoclonal antibodies (MAB B/2) were developed (for characterization, see Supplementary data at JXB online). For detection of the total phytochrome pool, the monoclonal antibody MAbPea-25 was used (Cordonnier-Pratt et al., 1986b, b). Western blots were incubated with primary antibody in blocking solution for 65–105 min. After washing four times in TBST, membranes were incubated with the secondary antibody (anti-mouse immunoglobulins for MAb and anti-rabbit immunoglobulins for PAb) IgG-POD, Lumi-lightPLUS Western Blotting Kit (Roche Molecular Biochemicals, Mannheim, Germany) in 1% blocking solution for 30–75 min, and then rinsed four times in TBST. The membrane was then incubated in the Lumi-lightPLUS substrate [Lumi-lightPLUS Western Blotting Kit (Mouse/Rabbit); Roche]. An intensified CCD camera (C2400-77; Hamamatsu Photonics, Hamamatsu, Japan) was used to image the light emission. Intensity of the protein bands on the blot was quantified using the Argus 50 software (Hamamatsu Photonics).

In order to relate PHY levels in the PHYOE lines to that in the WT, western blots with a 1–64-fold dilution series of 300 μg protein extracted from PHYOE D-grown seedlings was analysed with the PHY-specific antibodies.

To test the stability of the ectopically expressed phytochromes in light, the relative PHY level was quantified in extracts of D-grown seedlings and extracts from D-grown seedlings that were subsequently treated with 4 h R. In this series of experiments the signal for PHY in the WT remained below the limits of detection. Therefore, in order to compare the PHY levels in the different PHYOE lines before and after light treatment, the signal of the different PHYOE lines was normalized to that of A/1 D-grown seedlings (mean ± SE, n=5, loaded protein 30 μg in two blots and 300 μg in three blots). The signal of the different PHYB1OE lines was normalized to that of B1/2 D-grown seedlings (mean ± SE, n=4, loaded protein 30 μg in two blots and 300 μg in two blots) and the signal of the different PHYB2OE lines was normalized to that of B2/4 D-grown seedlings (mean ± SE, n=2, loaded protein=30 μg). The results for phyA and phyB1 include biological repeats. In some of these blots, 30 μg protein was loaded to avoid saturation of the phytochrome signal in the PHYOE lines.

White-light experiments

Seeds were sown in trays and transferred to a phytotron with a 16 h WL/8 h D diurnal treatment (WL: 150 μmol m−2 s−1 photosynthetically active radiation) at 25 °C. After 11 d, the hypocotyl length of the seedlings was measured. The seedlings were transferred to individual rock wool blocks, moved to the greenhouse and grown for 4 weeks, after which the plant height and anthocyanin content from young developed leaves (1.0–1.5 cm in length) were measured.

Broad-band red and far-red light experiments

The continuous red light (cR; 600–700 nm) and continuous far-red light (cFR; 700–800 nm) experiments were performed as described by Kerckhoffs et al. (1997). Forty seeds per tray were preincubated in D at 4 °C for 48 h to synchronize germination. Subsequently, seeds were placed in D at 25 °C for 72 h. The irradiation with cR or cFR (both at 3 μmol m−2 s−1) started just before the seedlings emerged through the soil surface. The length of 30–40 hypocotyls was measured after 14 d from sowing. The mean hypocotyl length
of 30–40 seedlings grown in D was also measured. In addition to hypocotyl length, the anthocyanin level was determined in samples of five hypocotyls. The mean values and standard error (SE) of four groups were calculated.

**End-of-day far-red light experiments**

Seeds were sown directly in trays of peat-based compost (40 seeds per tray) and grown for 10 d in a phytotron in a 16 h WL (160 μmol m$^{-2}$ s$^{-1}$)/8 h D cycle at 25 °C, at a relative humidity of 65–70%. On day 10, the plants were transferred to growth cabinets and allowed to adjust to the lower level of WL (120 μmol m$^{-2}$ s$^{-1}$) for 1 d. On the subsequent 18 d the plants received 20 min FR (3 μmol m$^{-2}$ s$^{-1}$) irradiation directly following the daily 16 h WL period (+EODFR), while controls received 16 h WL/8 h D and no FR irradiation (−EODFR). Plant height and anthocyanin content of young leaves (1.0–1.5 cm) were measured at the end of the treatment. From these measurements the mean values ± SE of 35 +EODFR plants and 35 −EODFR plants were calculated.

**Narrow-band fluence rate experiments**

The R and FR fluence rate experiments were performed in a threshold box set-up as previously described by Kerckhoffs et al. (1997). Briefly, 30 seeds of WT and three other transgenic lines were sown separately on four filter papers moistened in germination buffer (10 mM phosphate buffer, pH 7.5, containing 1 mM KNO$_3$) in plastic boxes with transparent lids. Seeds were incubated in D for 90 h (WT seeds) or 114 h (transgenic seeds) at 25°C to synchronize seed germination. Subsequently, seedlings were exposed to 24 h continuous monochromatic R [using an interference filter with a peak transmission at 680 nm (Baird-Atomic, Bedford, PA, USA), 16.6 nm band width at 50% of the maximum transmission] of different fluence rates (Kerckhoffs et al., 1997). The level of anthocyanin was determined at the end of the 24 h light treatment in sets of 10 whole seedlings. The mean values and SE of four independent experiments were calculated.

**Anthocyanin assay**

For determination of anthocyanin accumulation, samples (five whole seedlings, 10 hypocotyls, or a young leaf 1.0–1.5 cm in length) were extracted with 1.2 ml acidified (1% HCl, v/v) methanol as described (1997). Briefly, 30 seeds of WT and three other transgenic lines were selected on kanamycin, resulting in 15 PHYAOE, five PHYB1OE, and 13 PHYB2OE lines with a single locus insertion. Heterozygous and homozygous progeny plants were distinguished from each other by size difference of T$_1$ plants and through segregation analysis of T$_2$ seedlings on medium containing kanamycin. For each transgene, representative homozygous lines were selected for collection of T$_3$ bulk seed batches which were used for the analysis of plants under different physiological conditions (A/1, A/3 for PHYAOE; B1/2, B1/4 for PHYB1OE, and B2/4, B2/8, B2/9 for PHYB2OE). In addition, the A/3 and B2/9 lines were crossed with the phyA mutant and phyB1phyB2 double mutant, respectively. Analyses of the progeny plants identified individual seedlings homozygous for both the phyA or phyB1phyB2 loci and the transgene (A3/phyA and B2/9phyB1phyB2; see Materials and methods).

**Quantification of phytochrome protein level in the PHYOE lines**

For the characterization of phytochrome protein levels in plants, new specific antibodies directed against PHYA, PHYB1, and PHYB2 were used (for characterization of specificity of these antibodies, see Supplementary data at JXB online). For the quantification of PHYA and PHYB1 levels, a dilution series of protein extracts from PHYAOE and PHYAOE plants was made for comparison with the signal in the WT (Fig. 1A, B). The results in Fig. 1A show that extracts from D-grown seedlings from A/1 and A/3 lines have to be diluted approximately 8–16-fold, to obtain a signal similar to that in WT D-grown seedlings.

**Results**

**Generation of tomato PHYAOE, PHYB1OE, and PHYB2OE lines**

For construction of the tomato PHYAOE, PHYB1OE, and PHYB2OE lines, the tomato PHYA, PHYB1, and PHYB2 cDNAs were cloned behind the double CaMV 35S promoter in a binary vector and the chimeric genes were introduced into tomato by Agrobacterium tumefaciens-mediated transformation. Transformed tomato shoots (T$_0$) were selected on kanamycin, resulting in 15 PHYAOE, 12 PHYB1OE, and 33 PHYB2OE independent primary transformants. From each T$_0$ plant, seeds (T$_1$) were harvested, and segregation analysis of T$_1$ seedlings indicated 10 PHYAOE, five PHYB1OE, and 13 PHYB2OE lines with a single locus insertion. Heterozygous and homozygous progeny plants were distinguished from each other by size difference of T$_1$ plants and through segregation analysis of T$_2$ seedlings on medium containing kanamycin. For each transgene, representative homozygous lines were selected for collection of T$_3$ bulk seed batches which were used for the analysis of plants under different physiological conditions (A/1, A/3 for PHYAOE; B1/2, B1/4 for PHYB1OE, and B2/4, B2/8, B2/9 for PHYB2OE). In addition, the A/3 and B2/9 lines were crossed with the phyA mutant and phyB1phyB2 double mutant, respectively. Analyses of the progeny plants identified individual seedlings homozygous for both the phyA or phyB1phyB2 loci and the transgene (A3/phyA and B2/9phyB1phyB2; see Materials and methods).
this indicates that PHYB2 levels in B2/4 and in B2/9 are at least 50–100 times higher than the PHYB2 level in the WT.

Figure 1D–F shows that when D-grown seedlings are treated with 4 h R, the PHYA levels are strongly reduced compared with extracts from D-grown seedlings, when A/3 is expressed in WT or in the phyA mutant background (Fig. 1D). Figure 1E, F shows that the PHYB1 level in the PHYB1OE lines and the PHYB2 level in the PHYB2OE lines (in WT or phyB1phyB2 double-mutant background) do not change significantly when seedlings are exposed to 4 h R. An example of one of the western blots is shown as inserts in Fig. 1D–F.

Characterization of plants grown under white light

For the characterization of the phenotypic effect of PHYOE on plant growth, the hypocotyl length of 11-d-old seedlings and plant height of 8-week-old plants grown under WL conditions (16 h WL/8 h D) were measured. Figure 2A shows that the hypocotyl lengths of A/1, A3, and B1/2 were only mildly reduced, but the hypocotyl lengths of the B1/4 and the PHYB2OE lines were strongly reduced compared with WT. When the plants were 8 weeks old, the PHYA OE in the A/3 line and PHYB1OE in the B1/4 line still had a significant reduction in plant height compared with the WT (Fig. 2B), while the effect of PHYB2OE generally was even more pronounced than at the seedling stage, resulting in up to 50% suppression of stem elongation compared with the WT (Fig. 2B). Figure 2C shows the effect on seedling development of PHYB2OE in the WT and phyB1phyB2 double-mutant background. The pronounced effect of PHYB2OE persisted throughout plant development, resulting in shorter plants with smaller leaves which appeared darker green than those of WT plants (Fig. 2D). Figure 2E shows a cross-section of the hypocotyl of a WT and a B2/4 seedling grown for 11 d under WL, illustrating that anthocyanin accumulation is restricted to the subepidermal cell layer, both in the WT and B2/4, but is enhanced in B2/4 compared with the WT.

Broad-band red and far-red light experiments

For plants grown under WL the effect of PHYOE is modulated by the action of other activated photoreceptors (e.g. cryptochromes; Weller et al., 2001; Lin and Shalitin, 2003). In order specifically to study the action of elevated PHY levels in the PHYOE lines, seedlings from WT, PHYOE lines, and from two phy-mutant lines (phyA and phyB1phyB2), were grown under broad-band cR (600–700 nm).
nm) and cFR (700–800 nm). The inhibition of hypocotyl elongation and the enhancement of anthocyanin accumulation in these lines after 14 d of light treatment were compared with those in the WT. Figure 3G shows the hypocotyl length of D-grown seedlings. The results for the hypocotyl elongation are the mean of measurements of 30–40 seedlings and are expressed as percentage inhibition of hypocotyl elongation relative to D-grown control seedlings (Fig. 3A, C, E). For the anthocyanin content the results are the mean of three independent measurements on groups of five seedlings (Fig. 3B, D, F). When PHYA OE lines are grown under cFR no significant change in the inhibition of hypocotyl elongation compared with WT plants was observed (Fig. 3A). As predicted, the phyA mutant is essentially blind to FR, resulting in reduced inhibition of hypocotyl elongation, and this phenotype is fully rescued when combined with PHYAOE (A/3phyA; Fig. 3A). The anthocyanin content in the seedlings grown under cFR showed a small decrease in accumulation in the A/1 and A/3 lines compared with the WT (Fig. 3B). This figure also shows that in the phyA mutant anthocyanin accumulation is strongly reduced under cFR. This mutant phenotype was rescued by PHYAOE in the phyA mutant background (A/3phyA; Fig. 3B), even leading to values clearly higher than PHYAOE overexpression in the WT background (A/3; Fig. 3B).

Under cR, PHYB1OE had no effect on hypocotyl elongation compared with WT plants (Fig. 3C). However, in the PHYB2OE lines there was a strong effect on inhibition of hypocotyl elongation under cR compared with the WT (80% and 60%, respectively) (Fig. 3E). Under cR, the anthocyanin accumulation in seedlings of the PHYB1OE line B1/2 was similar to that of the WT, but in the B1/4 line was lower than in the WT (Fig. 3D). In the PHYB2OE lines, the anthocyanin level was up to 3-fold higher compared with that in the WT (Fig. 3F). The results in Fig. 3E, F show that, for both hypocotyl elongation and anthocyanin accumulation, the phenotype of the phyB1phyB2 double mutant is rescued by the overexpression of PHYB2 in the B2/9phyB1phyB2 line. For anthocyanin accumulation this even led to values higher than that of PHYB2OE in the WT background (B2/9; Fig. 3F).

Narrow band of red light fluence rate responses

To study the effect of PHYOE under different fluence rates, the response relationship between 24 h cR (680 nm)}
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Fig. 3. Phenotype of PHYOE seedlings grown under cR and cFR. (A, C, E) Percentage inhibition of hypocotyl elongation: (A) WT and PHYA OE seedlings grown under cFR (3 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)). (C) WT and PHYB1 OE; and (E) WT and PHYB2 OE seedlings grown under cR (3 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)). (G) Hypocotyl length (mm) of D-grown seedlings. Values are means ±SE (n=30–40). All measurements were taken 14 d after sowing. (B, D, F) Anthocyanin accumulation under cR or cFR: (B) the anthocyanin content of WT seedlings and PHYA OE seedlings grown under cFR (3 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)); (D) the anthocyanin content of WT and PHYB1 OE or (F) WT and PHYB2 OE seedlings grown under cR (3 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)). All measurements were from groups of five hypocotyls, harvested 14 d after sowing. Results are the means from n=4 ±SE. The experiments were repeated with qualitatively similar results.
of different fluence rates and anthocyanin accumulation in 4-d-old D-grown PHYOE seedlings was determined. The results in Fig. 4A show that overexpression of A/3 in the phyA mutant background (A/3phyA line) could rescue the phyA mutant phenotype by restoring the low fluence rate component to a level similar to that in the WT. This suggests that the effective phyA level resulting from PHYAOE in the A/3 line is similar to the phyA level in the WT. However, the same PHYAOE of A/3 in the WT background resulted in a slight decrease rather than an increase of the low fluence rate component compared with the WT (Fig. 4A).

Characterization of the response in the PHYB1OE lines shows that the R-HIR was little affected compared with the WT (Fig. 4B). Overexpression of PHYB1 also had no effect on the low fluence rate component in the B1/2 line, but resulted in a small reduction of the low fluence rate component in line B1/4 (Fig. 4B). Figure 4C shows that both the low fluence rate and R-HIR components are absent in the phyB1phyB2 double mutant. A strong amplification of the R-HIR was observed when the B2/9 transgene is expressed in the WT background. When the B2/9 transgene is expressed in the phyB1phyB2 double-mutant background (B2/9phyB1phyB2) the results show a response that is even stronger than that of B2/9 compared with the WT (Fig. 4C). This indicates that the expression of PHYB2 in the B2/9 line rescues the lack of both a functional phyB1 and phyB2 in the phyB1phyB2 double mutant. A strong amplification of the R-HIR was observed in both PHYB2OE lines (Fig. 4C). The results in Fig. 4C show a shift of the WT R-HIR response to lower fluence rates in the PHYB2OE lines.

Responses of wild-type and PHYB2OE lines to end-of-day FR

A classical phytochrome response can be characterized by a response to an R pulse which can be suppressed when followed by an FR pulse. Similarly, a response specific for activated phytochrome may be characterized by the inhibitory effect of an FR treatment given at the end of the day period (EODFR; Smith, 1982). Therefore, the R/FR reversibility of the response of B2/9, which showed a strong phenotype both in the normal and the phyB1phyB2 double-mutant background, was tested. The elongation and anthocyanin accumulation response in WT and B2/9 plants was measured after 18 d with or without EODFR treatment. The results in Fig. 5A show that the difference in plant height between non-treated and treated plants was less in the phyB1phyB2 double mutant than in the WT control, confirming previous observations that phyB1 and phyB2 are involved in this response to EODFR treatment. In the PHYB2OE lines B2/9 and B2/9phyB1phyB2, this response to the EODFR treatment was larger than in the WT control. Figure 5B shows that the anthocyanin levels are strongly reduced in plants treated
with EODFR. Again, in the PHYB2OE lines the difference in anthocyanin content between treated and non-treated plants was larger than in the WT control. Combined, the results are consistent with a higher level of functional phytochrome (Pfr) in the PHYB2OE lines B2/9 and B2/9phyB1phyB2 than in the WT.

Discussion

Elevated levels of biologically active phytochrome in tomato seedlings

Tomato plants with overexpression of the homologous tomato PHYA, PHYB1, and PHYB2 genes were constructed. Protein analysis indicated that relative PHY expression levels were indeed increased 8–16-fold (PHYAOE), 32-fold (PHYB1OE), and 50–100-fold (PHYB2OE) (Fig. 1; see Supplementary data at JXB online). The detection of phytochrome protein on western blots does not distinguish between ectopically produced phy apoprotein and biologically active holoprotein. However, the fact that the excess of PHYA in D-grown seedlings in the A/3 line is light labile (Fig. 1D) suggests that this PHYA does contain a chromophore, as only phyA holoprotein is light labile (Kendrick and Kronenberg, 1994). Moreover, the PHYAOE in A/3phyA rescued the phyA mutant phenotype, indicating that in this mutant background at least part of the detected PHYA is biologically active and assembles into holoprotein (Figs 1D, 3A, B, 4A).

Two lines of evidence indicate that the effect on growth and development of PHYB1OE or PHYB2OE tomato plants is from light-activated phytochromes (Pfr): (i) the hypocotyl phenotype in these lines is strictly light dependent, since the elevated levels of phyB1 or phyB2 have no effect in the D-grown seedlings (Fig. 3G); and (ii) the EODFR treatment (which converts 95% of Pfr to Pr at the onset of the night period; Kendrick and Kronenberg, 1994) of the B2/9 transgene in both the WT and the phyB1phyB2 double-mutant background largely eliminates the suppression of elongation and the enhancement of anthocyanin accumulation, indicating that the phenotypic effect of PHYB2OE can be reversed by FR treatment (Fig. 5). Moreover the fact that the B2/9 transgene rescues the phyB1phyB2 double-mutant background largely eliminates the suppression of elongation and the enhancement of anthocyanin accumulation, indicating that the phenotypic effect of PHYB2OE can be reversed by FR treatment (Fig. 5). Moreover the fact that the B2/9 transgene rescues the phyB1phyB2 double-mutant phenotype (B2/9phyB1phyB2; Figs 1F, 2C, 3E, 4C, 5) also indicates that PHYB2 assembles into biologically active phyB2.

Homologous PHYAOE has little effect on plant growth and development

Despite the estimated 8–16-fold increase of PHYA in A/1, A/3, and A/3phyA lines, only a mild enhancement of suppression of hypocotyl elongation was observed in these lines under WL and cFR (Figs 2A, 3A, B, 4A). This either suggests that in WT phyA levels are already saturated for the elongation responses or that the ectopic homologous phyA is rapidly degraded in light. A negative effect of phyA on anthocyanin accumulation has been shown before in phyA mutant studies (Kerckhoffs et al., 1997). Indeed the anthocyanin accumulation response was further reduced in the PHYAOE line A/3, both under FR (Fig. 3A) and low fluence rates of R (Fig. 4A). However, this additional dominant negative effect of PHYAOE seems to be threshold dependent as the effect is visible in A/3 but not in A/3phyA (Fig. 3B) or in hemizygous A/3/WT (results not shown). The reduced anthocyanin accumulation in the PHYAOE plants could be the indirect result of cross-regulation between PHYA and PHYB1 or PHYB2. However, the threshold dependency could also
point to a titration by ectopically produced phyA of chromophores or phytochrome-interacting proteins, reducing the signalling capacity of the remaining phytochromes. The dominant negative effect of PHYA OE was only observed for anthocyanin accumulation, which in many studies involving PHYA OE has not been quantified. Because of the higher stability of monocot phyA in dicots, overexpression of monocot PHYA in dicot hosts may actually result in higher levels of phyA than with homologous (dicot) PHYA OE. However, in the tomato plants with oat PHYA OE, anthocyanin accumulation was enhanced and not suppressed under WL (Boylan and Quail, 1998). In the Arabidopsis and tobacco plants with oat PHYA OE the anthocyanin accumulation was not quantified [Arabidopsis, Boylan and Quail (1991), McCormac et al. (1991), Whitelam et al. (1992); tobacco, Keller et al. (1989), Boylan et al. (1994), Robson et al. (1996), Halliday et al. (1999)].

The action of PHYB2 OE may require the co-action of other phytochromes

Although PHYB1 OE and PHYB2 OE resulted in a similar increase in the total phytochrome pool (tested with MAb Pea-25; results not shown), the effect of PHYB2 OE on plant growth and pigmentation was shown to be stronger than that of PHYB1 OE (Fig. 2B, D). The phenotypic effects are caused by active Pfr because they are largely eliminated by the 20 min EODFR treatment (Fig. 5). Because 20 min of FR treatment converts approximately 95% of all Pfr to Pr (Kendrick and Kronenberg, 1994), the remaining PfrphyB2 from PHYB2 OE in B2/9 (approximately 100× WT phyB2 levels; see Supplementary data at JXB online) is predicted to be still more than 5-fold higher than PfrphyB2 levels from endogenous PHYB2 expression in plants without the FR treatment. However, these remaining elevated levels of PfrphyB2 after FR treatment apparently are not effective in suppression of hypocotyl elongation or anthocyanin accumulation (Fig. 5), suggesting that the other activated phytochromes may be required for the full effect of PHYB2 OE.

Predicting the effects of PHYOE

The present results show that effects of PHYOE are not easily inferred from phy-mutant studies. For instance, the PHYOE studies presented here indicate a more dominant role for phyB2 than phyB1 in photomorphogenesis, while phy-mutant studies indicate a more dominant role for phyB1 than for phyB2 (van Tuinen et al., 1995a). Mutant analysis has also indicated that the phyA-dependent low fluence rate response component depends on the presence of either phyB1 or phyB2 (Weller et al., 2000). However, the increase of phyB1 in the B1/4 line results in an actual reduction of this response (Fig. 4B), suggesting a negative interaction between phyA and phyB1 at elevated levels of phyB1. This may be similar to the negative interaction between PHYBOE on the action of phyA in Arabidopsis (Casal et al., 2000). Also, from phy-mutant studies it could be inferred that PHYA OE amplifies the response to low fluence rates and that the response to higher fluence rates of R are amplified by PHYB1 OE and less so by PHYB2 OE (Weller et al., 2000). However, the present results show that PHYA OE actually lowers the response at low R fluence rates, while it is PHYB2 OE and not PHYB1 OE that causes a large increase in sensitivity to R.

PHYOE effects are limited by competence of individual cell types

The differential effects of PHYB1 OE and PHYB2 OE in tomato could be related to the differences in the expression pattern of the CaMV-35S promoter and the endogenous PHYB1 and PHYB2 genes. Although the CaMV-35S promoter is active in all cells, it is preferentially expressed in the vascular bundles. Indeed, expression driven by the 35S promoter was shown to be more effective than PHYAOE in mesophyll cells or under control of the ubiquitin promoter (Jordan et al., 1995). The PHYB1 and PHYB2 genes are expressed at a similar level in most tissues (Hauser et al., 1997), but no detailed information on the cellular expression pattern of tomato PHYB1 or PHYB2 is available. The 35S promoter lacks light or circadian clock regulation, and the disruption of the specific phase difference between the endogenous PHYB1 and PHYB2 activity in leaves (Hauser et al., 1998) may contribute to the different effects of PHYB1 OE and PHYB2 OE. Analysis of tissue-specific and cellular expression patterns of PHYB from Arabidopsis, tobacco, and potato have shown that PHYBs are expressed throughout the seedling [Arabidopsis, Somers and Quail (1995), Goosey et al. (1997); potato, Heyer and Gatz (1992a, b); tobacco, Adam et al. (1996)]. The PHYD of Arabidopsis is expressed in the same tissues as PHYB, but its expression is more limited to the vascular bundles (Goosey et al., 1997). Neither the tomato PHYB1 nor the PHYB2 is orthologous with either Arabidopsis PHYB or PHYD (Mathews et al., 1995; Pratt, 1995). By contrast to the effect of PHYBOE in Arabidopsis, the PHYDOE had very little effect on plant growth (Sharrock et al., 2003). Therefore, the effects of PHYB1 OE in tomato are more similar to those of PHYDOE in Arabidopsis and the effects of PHYB2 OE in tomato are more similar to those of PHYBOE in Arabidopsis.
the WT (Fig. 2E). Future studies of the lines produced in these studies will be aimed at analysis of the effects of PHYOE on the shade-avoidance response and fruit yield under various greenhouse conditions.

Supplementary data
A description of the production and characterization of the PHYA-, PHYB1-, and PHYB2-specific antibodies and quantification of relative expression levels in the PHYOE lines is available at JXB online.

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References


