Plant Physiol. (1995) 109: 53–61

# Function of Phytochrome A in Potato Plants as Revealed through the Study of Transgenic Plants[1]

**Arnd G. Heyer, David Mozley, Volker Landschütze, Brian Thomas, and Christiane Gatz***

Institut für Genbiologische Forschung GmbH, Ihnestr. 63, 14195 Berlin, Germany (A.G.H., V.L.); Department of Molecular Biology, Horticulture Research International, Worthing Road, Littlehampton, West Sussex, BN7 6LP, United Kingdom (D.M., B.T.); and Institut für Genetik, Universität Bielefeld, 33501 Bielefeld, Germany (C.G.)

**We have generated transgenic potato plants (*Solanum tuberosum*) containing the potato phytochrome protein encoded by the *PHYA* gene cDNA (phyA) in sense or antisense orientation under the control of the 35S cauliflower mosaic virus promoter. Plants with increased and decreased phyA levels were analyzed. When grown under white light, development and growth of sprouts and plants were barely distinguishable from wild type. Under continuous far-red light, stem extension, leaf expansion, and hook opening of sprouts were accelerated in phyA overexpressors and delayed in antisense plants. Sprouts with reduced phyA levels were less sensitive to red light with regard to stem extension and expression of the small subunit genes for ribulose bisphosphate carboxylase. Under low red light:far-red light ratios, increased phyA levels reduced the stem extension component of the shade-avoidance response, whereas decreased levels led to an increase in the response.**

Many different aspects of plant growth and development are influenced by light, which is sensed both quantitatively and qualitatively (Salisbury and Ross, 1985). Understanding how the range and complexity of the different responses is brought about requires knowledge of the roles of the photoreceptor molecules in the plant tissue. R and FR are sensed by the phytochrome system, a family of related chromoproteins (reviewed by Quail, 1991; Furuya, 1993; Whitelam and Harberd, 1994). In Arabidopsis five *PHY* genes have been identified that are between 50 and 80% identical with each other at the amino acid level (Sharrock and Quail, 1989; Clack et al., 1994). The best-characterized members are *PHYA* and *PHYB*, which can be distinguished by different light stabilities of the encoded proteins (Somers et al., 1991). PhyA accumulates in the dark and is rapidly depleted upon conversion to the labile Pfr form. In contrast, light-stable phyB is expressed at low but relatively consistent levels in light- and dark-grown plants. It has been shown for etiolated Arabidopsis seedlings that phyA is 25 to 50 times more abundant than phyB. In green tissues, however, both proteins are present in about equimolar amounts (Somers et al., 1991).

The availability of Arabidopsis mutants specifically defective in either *PHYA* (Dehesh et al., 1993; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993) or *PHYB* (Nagatani et al., 1991a; Reed et al., 1993, 1994) has allowed the study of the function of each of the two photoreceptors individually. Because of its relatively high concentration in etiolated seedlings, phyA can mediate high sensitivity to FR. At equilibrium, the Pfr form is converted back into the Pr form by FR and evades the light-induced destruction. Under these conditions, 2% of the total phytochrome pool is present in the biologically active form, mediating the FR-HIR (Mancinelli, 1980; Smith and Whitelam, 1990). These characteristics enable phyA to promote de-etiolation of plants under shade conditions. In contrast, phyB seems to play a major role in regulating de-etiolation, growth, and development under R and W. However, it is not clear to what extent the results from Arabidopsis represent general principles or special cases.

We are interested in defining the roles of the different phytochromes in potato (*Solanum tuberosum*) as an example of an important crop species. Potato differs from Arabidopsis, tobacco, and tomato in that propagation is primarily vegetative through sprouts growing out of tubers. Therefore, the de-etiolation process might follow different variants of phytochrome control as compared to the situation in seedlings. Because potato is not easily amenable to genetic analysis, our approach has been to modify the levels of the endogenous phytochromes by genetic transformation using both overexpression and antisense technologies.

Whereas plants with reduced phyA levels due to antisense constructs have not yet been reported, overexpression of cloned *PHYA* cDNAs in heterologous plants has been performed by several laboratories. In all of these cases, phytochromes from monocotyledonous species have been used (Kay et al., 1989; Keller et al., 1989; Boylan and Quail, 1989, 1991). Transgenic seedlings show an increased sensitivity to FR for the inhibition of hypocotyl elongation

[1] Supported by the Bundesministerium für Forschung und Technologie BCT 0389 "Molekulare und Zellbiologische Untersuchungen an höheren Pflanzen und Pilzen" (A.H., V.L., C.G.), the European Communities BIOTECH Programme, as part of the Project of Technological Priority (D.M.), and the Biotechnology and Biological Science Research Council, 1993 to 1996 (B.T.).

* Corresponding author; e-mail cgatz@genetik.uni-bielefeld.de; fax 49-521-106-5626.

Abbreviations: *CAB*, gene for Chl *a*/*b*-binding protein; CaMV, cauliflower mosaic virus; FR, far-red light; HIR, high-irradiance response; nt, nucleotide; phyA (phyB), phytochrome proteins encoded by the *PHYA* (*PHYB*) gene; R, red light; *RBCS*, gene for ribulose bisphosphate carboxylase, small subunit; W, white light; wt, wild type.

(McCormac et al., 1992b), which corresponds well with the lack of FR sensitivity displayed by phyA-deficient Arabidopsis seedlings. In green plants, overexpression of phyA leads to pronounced growth inhibition under low R:FR ratios (McCormac et al., 1991, 1992b). This effect, which overrides the wt shade-avoidance response (increased growth under low R:FR ratios), has been suggested to be due to retention of the FR-HIR mediated by unusually high levels of phyA in the light-grown transgenic plants.

However, there are several photoresponses of these transgenic plants that are not in agreement with the suggestion that the function of phyA is mainly to detect FR and that R is detected by phyB. Seedlings of transgenic plants show increased sensitivity to R, and the phenotype of de-etiolated plants (reduced height, increased pigmentation, increased branching, reduced leaf senescence) is strongly affected under W in the absence of FR. The reason why the introduced monocotyledonous phyA protein mimics the phyB pool is not yet known: it could be its structure, which cannot be specifically recognized in a heterologous background, its increased stability, its increased abundance, or a combination of all three aspects.

Here we analyze the effect of overexpression of endogenous phyA, thus avoiding the complication of the increased half-life or different primary structure of the transgenic gene product. We also analyze the effect of decreasing phyA levels in the same system to identify processes normally regulated through phyA.

## MATERIALS AND METHODS

### Construction of Transgenic Plant Lines

Plasmid pMm1.4 (Fig. 1) contains the 3.6-kb *PHYA* cDNA (Heyer and Gatz, 1992a) inserted into the *Kpn*I site of pUC18. To avoid a potential regulatory control mediated by the small open reading frame between nts 50 and 88, the *Hin*dIII site located 19 bp upstream from the ATG of the *PHYA* open reading frame was chosen for further subcloning. First, the 408-bp *Hin*dIII/*Eco*RV fragment (nts 135–542) was subcloned into pBR322 cut with *Hin*dIII and *Eco*RV, resulting in pBR322phyA-N. The residual coding region was cloned as an *Eco*RV/*Sal*I fragment into pBR322 phyA-N, yielding pBR322phyA. The *PHYA* insert was cut out by *Cla*I(partial)/*Sal*I and inserted into pBINAR cut with *Sma*I, yielding pBINPS. pBINAR is BIN19 containing a CaMV 35S promoter and an *octopine synthase* polyadenylation signal, separated by multiple cloning sites (Höfgen and Willmitzer, 1990).

Two different *PHYA* fragments were used for the antisense construct: the 3.6-kb *Kpn*I insert of pMm1.4 and the internal *Eco*RI fragment from nt 1152 to nt 1934. Both fragments were cloned into pBINAR, cut with *Kpn*I (pBINAP) or *Sma*I (pBINAS), respectively. Transfer of plasmid DNA to *Agrobacterium tumefaciens* pGV2260 (Deblaere et al., 1985) was performed according to the method of Höfgen and Willmitzer (1988). Transformation of potato (*Solanum tuberosum*, cv Désirée) plants was performed as described by Rocha-Sosa et al. (1989).

### Northern Blot Analysis

Poly(A)$^+$ RNA from total RNA (Logemann et al., 1987) was extracted using the Dynabeads protocol from Dynal (Oslo, Norway). Northern blot analysis was performed as described by Eckes et al. (1986). DNA fragments were labeled using the Multi-Prime kit from Amersham. To generate sensespecific transcripts, the 780-bp *Eco*RI fragment was inserted into pSK (Stratagene), yielding pSK-780. pSK-780 was transcribed in the presence of [$^{32}$P]UTP using the

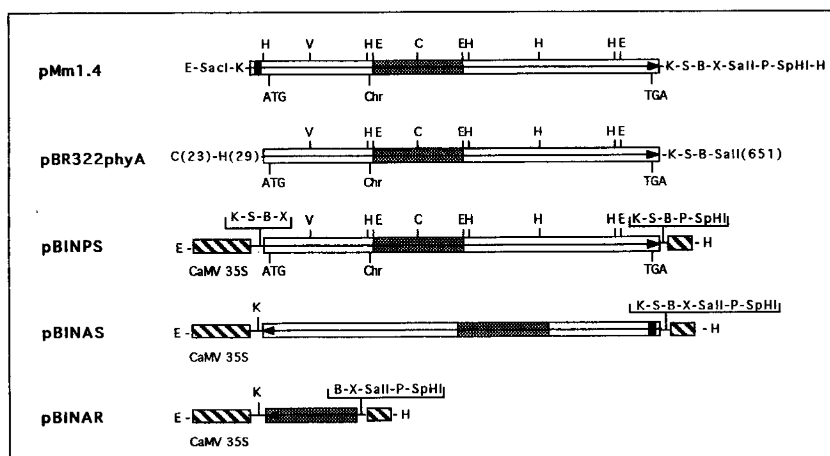


**Figure 1.** Construction of chimeric phytochrome genes. pMm1.4 contains the *PHYA* cDNA, including an upstream, small open reading frame (black box). pBR322 contains the *PHYA* cDNA without the small open reading frame between the *Hin*dIII site at position 29 of pBR322 and the *Sal*I site at position 651 (Nos. are in brackets). pBINPS contains the *PHYA* open reading frame between the CaMV 35S promoter and the *octopine synthase* polyadenylation signal (hatched box at the 3′ end). pBINAS contains the full-length *PHYA* cDNA between the CaMV 35S promoter and the polyadenylation signal but in the antisense orientation. pBINAR contains the 780-bp *Eco*RI fragment (gray box) in the BINAR expression cassette. E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; V, *Eco*RV; C, *Cla*I; S, *Sma*I; B, *Bam*HI; X, *Xba*I; P, *Pst*I; chr, chromophore. The arrow denotes the orientation.

SP6/T7 in vitro transcription kit from Boehringer Mannheim. Hybridization was performed as described by Sharrock et al. (1988), and filters were washed in 2× SSC, 0.5% SDS at 65°C.

## Western Blot Analysis

Western blot analysis using the monoclonal antibody (P25) generated against pea phyA (Cordonnier et al., 1986) was performed as described by Heyer and Gatz (1992b). For the western blot analysis shown in Figure 4B a different set of antibodies was used. Recombinant phytochrome fragments (phyA, amino acids 456–593; phyB, amino acids 970-1105) were expressed as fusion proteins with the maltose-binding protein (New England Biolabs). Gel-purified peptides were used to raise rabbit polyclonal antibodies. C52 is a rabbit polyclonal antiserum raised against the peptide VSRNLLRLMNGDVRHC, which represents a conserved region of the C terminus of *Avena* phyA. Surprisingly, this antibody recognizes light-stable phytochrome but not light-labile phytochrome (i.e. phyA) in potato. For light-grown plant material (protein blots probed with C52 and anti-phyB), western blotting was carried out on extracts in which the phytochrome content was enriched by partial purification. Fresh green material, ground in liquid nitrogen, was mixed with extraction buffer [25% ethylene glycol, 50 mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 5 mM $Na_4EDTA$] and was adjusted to 25 mM $Na_2S_2O_5$, pH 8.5, and 2 mM PMSF just prior to use at a ratio of 1:1 (g fresh weight:mL of extraction buffer). The brei was mixed with 5 mL of 10% polyethyleneimine per 100 mL of extract, incubated for 10 min, and centrifuged at 27,000*g* for 30 min. The supernatant was adjusted to 4 mM PMSF, and 25 g of $(NH_4)_2SO_4$ were added per 100 mL of polyethyleneimine supernatant. The supernatant was stirred for 10 min and centrifuged at 27,000*g* for 30 min. The pellet was resuspended in 5 mL of extraction buffer minus $(NH_4)_2SO_4$ per 100 g of fresh tissue and clarified at 48,000*g* for 20 min. The green extract was diluted with 2× sample buffer (Laemmli, 1970), heated at 100°C for 10 min, and then centrifuged at 16,000*g* for 5 min, and the supernatant was subjected to SDS-PAGE (Laemmli, 1970). Etiolated tissue from sprouts (extracts probed with anti-phyA) was extracted directly into hot sample buffer, heated at 100°C for 10 min, and then centrifuged at 16,000*g* for 5 min. Immunochemical detection after electrophoresis and electroblotting was performed using alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulins.

## Spectrophotometric Phytochrome Assay

Total spectrally active phytochrome levels were determined by adding the change in *A* of Pr during irradiation with R ($\Delta A_{660}$) and of Pfr ($\Delta A_{730}$) during irradiation with FR [$\Delta(\Delta A)_{660-730}$] after alternating saturating irradiations at 4°C (Pratt, 1983). A Perkin-Elmer Cetus 557 dual-wavelength spectrophotometer fitted with a custom-built automatic radiation unit producing actinic light at 660 and 730 nm, half bandwidth 10 nm, was used.

## Light Treatments

For experiments on sprout growth under different light qualities, tubers were germinated in soil in darkness and transferred to R or FR or maintained in darkness. Measurements began when shoots were first visible after penetrating the soil surface. Shoot length was measured daily under a dim-green safelight. All experiments were carried out at 25°C. R was obtained from 20-W Grolux tubes (Thorn EMI, Middlesex, UK) filtered through red Perspex to give a fluence rate of 30 $\mu$mol m$^{-2}$ s$^{-1}$. FR was from 20-W FR tubes (Sylvania F20T12/232) filtered through blue Perspex and orange cenemoid no. 58 filters (Rank Strand Electric, London, UK) to give a fluence rate of 12 $\mu$mol m$^{-2}$ s$^{-1}$ between 700 and 780 nm. The spectral output from the light sources was shown by Muleo et al. (1994). For growth-cabinet experiments tubers were planted in soil in 13-cm-diameter pots. Plants were grown in Saxcil growth cabinets (R.K. Saxton, Ltd., Bradbury, Cheshire, UK) at 40 W m$^{-2}$ PAR (400–700 nm) for 128 d at 23°C and 70% RH and were given nutrient solution once per week. Plant height was measured at regular intervals, starting when shoots were at least 5 cm long, which was taken as time zero. Light was supplied by white fluorescent lamps alone or supplemented with 80-W FR tubes (Sylvania F80T12/232) to give the following irradiance ratios at 660 nm:730 nm (R:FR): high, 9.7; medium, 0.7; and low, 0.27 (Fig. 2). Nominal Pfr/total phytochrome values were calculated from measured spectral irradiance data and published photoconversion cross-sections for *Avena* phytochrome (Mancinelli, 1988). They were 0.84 (high), 0.74 (medium), and 0.63 (low). Spectrophotometric photoequilibrium measurements were carried out on etiolated mung bean (*Phaseolus aureus*) hypocotyl hooks after exposing them on ice in the cabinets for 10 min, taking Pfr/total phytochrome in saturating R to be 0.85. Values were 0.83 (high), 0.69 (medium), and 0.6 (low).

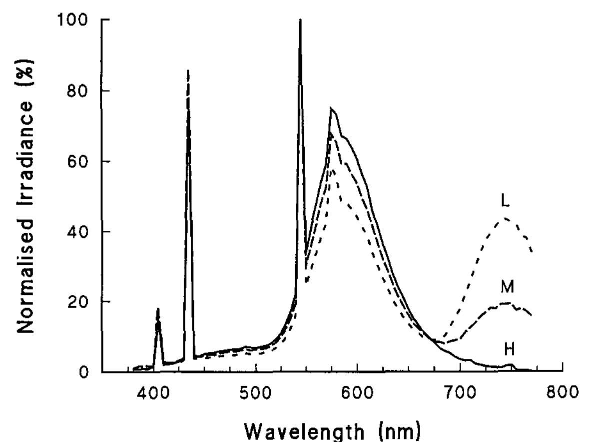


**Figure 2.** Spectral distribution of light sources in Saxcil growth cabinet experiments. Measurements in $\mu$mol m$^{-2}$ nm$^{-1}$ were made at 10-nm intervals using an Optronics model 740 automated spectroradiometer (Glen Spectra, Ltd., Stanmore, Middlesex, UK) with a fiber probe and using a 5-nm monochromator slit width. Spectra were normalized against the irradiance at 545 nm.

## RESULTS

### Analysis of Phytochrome Levels in Transgenic Plants

To obtain transgenic potato plants with increased phyA levels, we cloned the *PHYA* coding region into a binary vector containing the CaMV 35S promoter, yielding pBINPS (Fig. 1). To obtain plants with reduced phyA levels, two plasmids were constructed containing inserts in the antisense orientation behind the CaMV 35S promoter: pBINAP, which contains the full-length 3500-bp cDNA, and pBINAS, which contains an internal 780-bp fragment (Fig. 1). Transformation of potato leaf discs with pBINPS yielded only four kanamycin-resistant shoots. Using an equivalent amount of tissue, our transformation protocol routinely gives at least 20 to 30 transgenic plants, which was also the case when the constructs pBINAS and pBINAP were used.

Northern blot analysis of poly(A)$^+$ RNA from the green BINPS plants showed that three lines (PS2, PS3, and PS4) expressed higher levels of *PHYA* mRNA as compared to untransformed control plants (Fig. 3A). PS1 showed less *PHYA* mRNA, a phenomenon that might be due to co-suppression (Flavell, 1994). The three potential overexpressors were further characterized by western blot analysis (Fig. 4A). Cuttings of tissue culture material were incubated in the dark for 4 d and either irradiated for 1 h with R followed by 2 h of darkness or left in darkness for the whole experiment. Figure 4A shows that considerably more immunodetectable phytochrome is present in re-etiolated transgenics compared to control plants. Loss of the polypeptide occurred after a saturating pulse of R, indicating that the transgenic phyA is linked to the chromophore, allowing photoconversion to the light-labile Pfr form. PS2 and PS4 seemed to be the highest expressing lines and were thus chosen for further analysis. Because of low tuber yields and erratic germination of BINPS plants, etiolated material was limited. Spectrophotometric analysis of dark-grown sprouts was conducted for PS4 only and indicated an approximate doubling of photoreversible phytochrome in PS4 (Table I). An independent study of PS2 plants using low-temperature fluorescence spectroscopy (V. Sineshchekow, personal communication) confirmed that PS2 also contained twice the amount of phyA compared to wt plants.

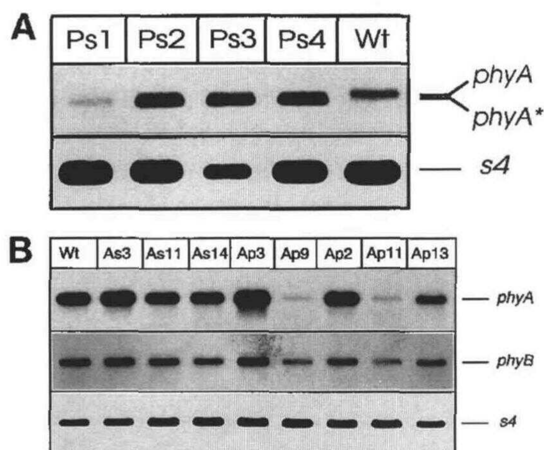


**Figure 3.** Northern blot analysis of transgenic plants. A, Analysis of overexpressors. Poly(A)$^+$ RNA (1 $\mu$g) from green leaves of wt and four BINPS plants was probed with a phytochrome fragment (top) and with a probe for the ribosomal protein S4 (Devi et al., 1989; bottom) to show that equivalent amounts of RNA were loaded. The transgenic mRNA runs slightly higher than the endogenous RNA because it lacks part of the 5′ untranslated leader. B, Analysis of antisense plants. Poly(A)$^+$ RNA (1 $\mu$g) from etiolated sprouts of wt and selected BINAS and BINAP plants was first probed with a sense-specific *PHYA* probe (top), with a *PHYB* fragment (middle), and finally with an *S4* probe (bottom).

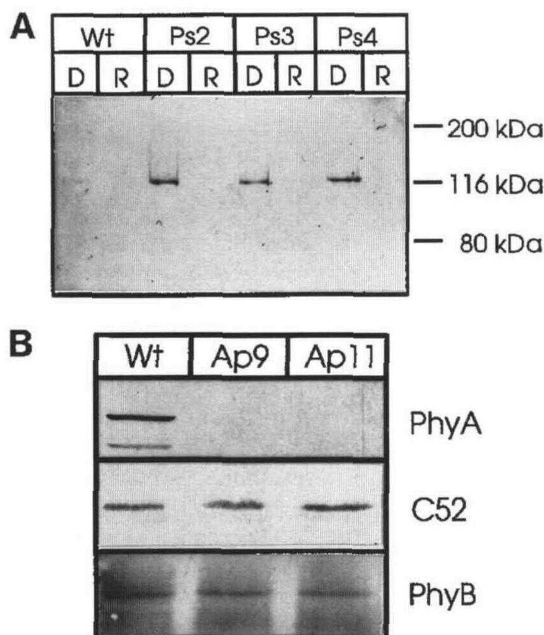


**Figure 4.** Immunoblot analysis of protein extracts from transgenic BINPS and BINAS plants. A, Analysis of overexpressors. Crude protein extracts were prepared from green tissue culture plants that were incubated in the dark for 4 d and either irradiated for 1 h with R followed by 2 h of darkness (R) or left in darkness (D) for the whole experiment. The immunoblot was probed with P25. B, Analysis of antisense plants. Top, Crude protein extracts were prepared from etiolated sprouts. The immunoblot was probed with a polyclonal potato phyA antiserum. Middle, Enriched protein extracts (see "Materials and Methods") from green leaves were probed with antiserum C52. Bottom, Enriched protein extracts were probed with a polyclonal potato phyB antiserum.

Preliminary screening of transgenic plants transformed with the antisense constructs was done by western blot analysis of protein extracts from 11 etiolated BINAP and 12 etiolated BINAS sprouts. Two BINAS plants (AS11 and AS14) and four BINAP plants (AP2, AP9, AP11, AP13) showed reduced phytochrome levels. These plants, as well as a wt control plant and two plants of each construct with unaffected phytochrome levels (AS3 and AP3), were subjected to northern blot analysis, using a sense-specific probe. Figure 3B shows that in BINAP plants, AP9 and AP11, *PHYA* mRNA levels were severely depleted. Reprobing the northern blot with a *PHYB* sequence (Heyer and Gatz, 1992b) indicated that *PHYB* mRNA levels were also affected, even though less severely. PhyA protein levels in AP9, AP11, and wt control plants were analyzed by western blot analysis of extracts of etiolated sprouts (Fig.

**Table I.** *Spectrophotometrically detectable phytochrome in etiolated sprouts of potato*

Values are means ± SE of at least three independent samples. $\Delta(\Delta A)_{660-730}$ is defined in "Materials and Methods."

| Measurement | wt | AP9 | AP11 | PS4 |
|---|---|---|---|---|
| $\Delta(\Delta A)_{660-730} \times 10^{-3}$ | 8.6 ± 1.8 | 1.2 ± 0.1 | 0.7 ± 0.1 | 15.7 ± 0.7 |

4B). PhyA levels were greatly diminished in the two selected BINAP plants. The amount of photostable phytochrome in green tissue of these plants was analyzed using two different antibodies: C22 is a rabbit antibody that was raised against a peptide containing sequences of *Avena PHYA*. It is interesting that in potato it recognizes a light-stable band, which is also recognized by the anti-phyB serum. Both sera show that the light-stable phytochrome pool is not significantly affected in the phyA antisense plants. Spectrophotometric analysis of extracts from etiolated sprouts of AP9 and AP11 revealed a 7- and 10-fold reduction in total spectral activity, respectively (Table I).

### Photoresponses of Etiolated Sprouts

Changing the level of phyA expression affected hook opening and leaf expansion when plants were grown in FR. Development was suppressed relative to the wt control in antisense plants, but in the overexpressors hook opening occurred earlier and leaf expansion was promoted both at the apex and the subapical nodes (Fig. 5).

The effect of R and FR on the growth of etiolated sprouts was tested in transgenic lines PS2, PS4, and AP9 (Fig. 6). Tubers were germinated in the dark and transferred to the different light treatments for 5 d. In the wt controls sprout growth was strongly inhibited by R, especially during the first 3 d of treatment. Continuous FR inhibited sprout growth to a lesser extent, indicating the operation of an FR-HIR. In contrast, FR was as effective as R in inhibiting growth of PS2 and PS4 sprouts. In the antisense line AP9, growth inhibition of both R and FR was reduced so that growth over 5 d was not significantly different for the light treatments.

The reduced sensitivity to R in AP9 concerning growth rates suggests that phyA contributes to the sensing of R in potato sprouts. We further tested this possibility by analyzing the effect of R on the expression of *RBCS* genes in etiolated BINAP and wt sprouts. Sprouts were given a 15-min R treatment and kept in darkness for 18 h before harvest. Figure 7 illustrates that *RBCS* RNA induction by an R pulse does not reach maximum levels in the AP9 and AP11 sprouts as compared to controls.

### Photoresponses of W-Grown Plants

Wt and transgenic plants were grown under fluorescent W (high R:FR) or fluorescent W supplemented with

**Figure 5.** Effect of continuous FR on apical development of wt and transgenic potato sprouts. Potato sprouts were germinated in darkness and transferred to continuous FR when the sprouts penetrated the soil. Photographs were taken in W after 8 d of FR treatment.

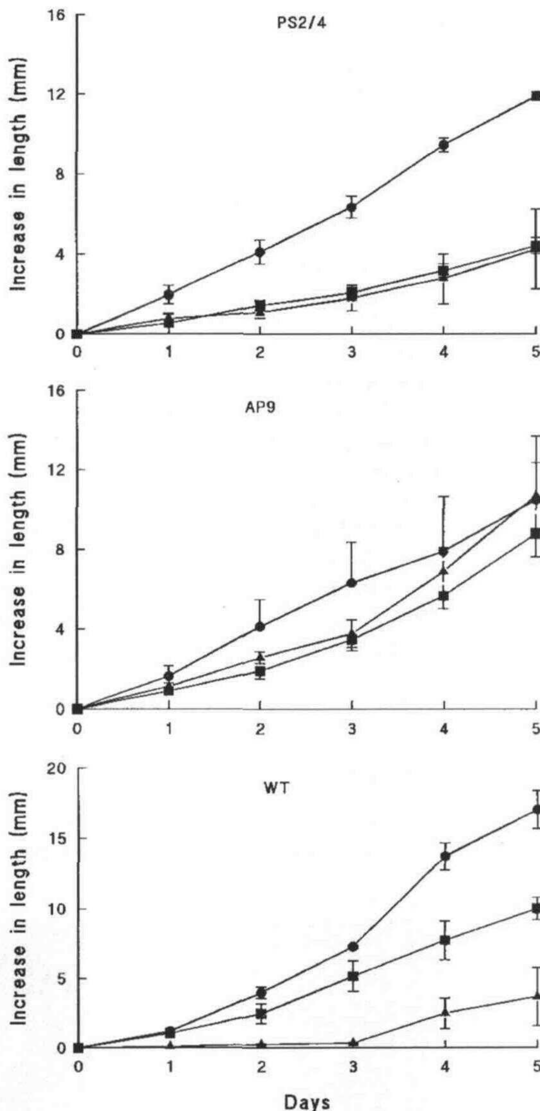


**Figure 6.** Effect of R and FR on stem extension in potato sprouts. Tubers were germinated in darkness and transferred to R (▲) or FR (■) or remained in darkness (●). Daily measurements were made under green safelight. Values are means ± SE of at least three independent samples. Values taken for overexpressors PS2 and PS4 were combined.

FR (medium and low R:FR). Wt plants exhibited a typical increase in stem extension rates in response to decreasing R:FR ratios. Stem extension slowed drastically after growth for 30 d in the high R:FR treatment, but continued in the low R/FR treatment. A comparison of overexpressor and antisense lines after 20 d from shoot emergence is shown in Table II. There was little difference between the shoot lengths of the transgenic and wt plants in either the high or medium R:FR treatment. However, under low R:FR ratios, the overexpressor lines were conspicuously shorter and the antisense line was taller than wt plants.

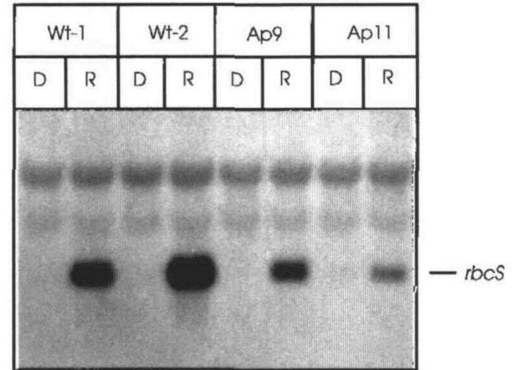


**Figure 7.** *RBCS* mRNA induction in etiolated wt and BINAS plants by a flash of R. Sprouts were grown in the dark (D), given 15 min of R, and then returned to the dark for 16 h until RNA was extracted.

## DISCUSSION

In this paper we describe the generation and analysis of transgenic potato plants with altered levels of phyA. These plants were generated by introducing a potato *PHYA* cDNA in sense orientation (for overexpression) or antisense orientation (for reduced expression) behind the CaMV 35S promoter. The overexpressor lines showed a 2-fold increase in spectral activity in dark-grown material. This is comparable to results from other laboratories, which expressed monocotyledonous *PHYA* cDNAs in transgenic plants: Keller et al. (1989) observed a 2.6-fold increase in spectral activity in dark-grown tobacco seedlings expressing oat phytochrome; Boylan and Quail (1991) report a 4-fold increase in spectral activity of etiolated Arabidopsis seedlings transformed with a chimeric oat *PHYA* cDNA clone. Considering that the CaMV 35S promoter is more than 4-fold stronger than the phytochrome promoters, unexpectedly low expression levels could be explained either by posttranscriptional control mechanisms (for example by limiting amounts of the chromophore) or by selection against highly expressing plants. Northern blot analysis of BINPS plants (Fig. 3A) also showed only a moderate excess of transgenic RNA as compared to the endogenous RNA, supporting the latter explanation. Only four phyA-overexpressing shoots could be regenerated with amounts of leaf discs normally yielding 20 to 30 transformants using the same vector. This observation also favors the theory of counterselection against strong overexpressors.

Expression of monocotyledonous phyA leads to a 16-fold (Keller et al., 1989) or 20-fold (Boylan and Quail, 1991) increase in spectral activity in light-grown plants. Based on

**Table II.** *Effect of different R:FR ratios on plant height*

Mean plant height (cm) ± SE of three independent samples after 20 d of growth in high, medium, or low R:FR ratios.

| Plant Type | High R:FR | Medium R:FR | Low R:FR |
|---|---|---|---|
| wt | 16.6 ± 1.3 | 23.1 ± 1.2 | 57.0 ± 6.1 |
| PS2 | 16.0 ± 2.3 | 22.9 ± 2.2 | 47.0 ± 4.5 |
| PS4 | 14.3 ± 0.7 | 29.6 ± 9.3 | 44.1 ± 10.9 |
| AP9 | 20.5 ± 1.4 | 34.8 ± 5.3 | 80.1 ± 5.9 |

western blot analysis we were unable to detect any increase of potato phyA in light-grown material, although reaccumulation in the dark was higher in the overexpressors compared to control plants (Fig. 4A). This difference in abundance in light-grown material must be due to the enhanced stability of monocotyledonous phyA in a dicotyledonous host (Boylan and Quail, 1989, 1991; Cherry et al., 1991; McCormac et al., 1992b), whereas the native phyA seems to be efficiently degraded in potato.

Antisense inhibition of phyA accumulation was more efficient using the entire coding region (BINAP plants) compared to a 780-bp internal fragment (BINAS plants) directly following the chromophore attachment region. Two of 11 BINAP plants tested showed a 7- and 10-fold decrease in the amount of spectrally active phytochrome (Table I). Because the *PHYA* and *PHYB* cDNAs are 61% identical, it was important to test for any effect of the *PHYA* antisense RNA on phyB expression. There was some evidence for slightly lower *PHYB* mRNA levels (Fig. 3B). Arabidopsis *phyB* mutants are partially dominant, which indicates that plants can be very sensitive to changes in phyB levels. Therefore, it was important to test whether phyB protein levels were significantly altered in AP9 and AP11. We used two different antibodies, one directed against the C terminus of phyB and the second one directed against a peptide of *Avena* phyA. Both antibodies reacted with the same light-stable band of the expected molecular size. Because western blots are not as quantitative as ELISAs, we cannot exclude a potential decrease of phyB levels up to approximately 20% (Fig. 4B). However, there was no effect on growth in W, which indicates that phyB levels were not modified to an extent that would influence photoresponses.

The effects of light quality on potato sprout development were first shown by Withrow (1941), who found that sprout extension was inhibited and that hook unfolding and leaf expansion were stimulated by FR. In our experiment, phyA levels had a striking effect on hook opening and early leaf development in continuous FR, which was suppressed in the antisense plants and accelerated in the overexpressors (Fig. 5). These data are consistent with the sensing of FR being primarily the function of phyA (McCormac et al., 1991, 1992a, 1992b, 1993; Whitelam et al., 1992).

Inhibition of stem extension in sprouts of phyA antisense plants was reduced in response to both R and FR. Thus, phyA is involved in sensing R, which indicates that even under continuous R-sufficient Pfr molecules remain available to mediate the response. The relative insensitivity of phyA antisense sprouts to R contrasts with results obtained with phyA-deficient Arabidopsis and tomato plants (Nagatani et al., 1993; Reed et al., 1994; van Tuinen et al., 1995). After 5 d of continuous growth in R, the length of wt potato sprouts was reduced to 20% of the level of sprouts grown in the dark, whereas the sprout length of antisense plants reached 80% of the value of dark-grown controls. As discussed above, *PHYB* mRNA levels or phyB protein levels were at most only slightly reduced, indicating that the unexpected low sensitivity of AP9 and AP11 sprouts to R was due to decreased phyA levels. Under continuous R, hypocotyls of wt Arabidopsis seedlings reached 37% of the length of the dark-grown controls, whereas seedlings of a *phyA* mutant reached 54% (Reed et al., 1994). R-mediated inhibition of hypocotyl elongation of wt tomato seedlings led to seedlings reaching 23% of the size of dark-grown seedlings, whereas phyA-deficient seedlings were slightly less inhibited (32%; van Tuinen et al., 1995). Taken together, these data reveal that in the etiolated stage potato phyA mediates a considerably larger component of the response to R than is observed in Arabidopsis and tomato. This difference might be due to either less efficient destruction of Pfr under R or a higher sensitivity to the existing Pfr levels in potato sprouts. In the overexpressing lines, sensitivity to R was not increased. This could mean either that the response was already saturated by the endogenous phytochrome levels or that under R the Pfr pool was not increased in the transgenics. The response to FR was more pronounced compared to wt plants.

The finding that potato phyA is an important component of the R-mediated de-etiolation process is supported by studies of the induction of *RBCS* transcripts by R in etiolated sprouts (Fig. 7). Induction of *RBCS* transcription in the potato phyA antisense plants was reduced compared to wt plants, suggesting that the lack of phyA cannot be fully compensated by phyB. In Arabidopsis, full induction of expression of the CAB gene by R can be mediated through either phyA or phyB (Reed et al., 1994). These results reveal that the roles of phyA and phyB in the de-etiolation process as deduced from studies of Arabidopsis seedlings do not entirely predict the photoresponses of potato sprouts.

De-etiolated transgenic potato plants with reduced levels of phyA did not show any obvious phenotypic alterations when grown in W. This corresponds with the phenotype of the Arabidopsis *phyA* mutants in W (Whitelam et al., 1993). We did not observe the increased tendency to wilt that has been described for phyA-deficient mutants in tomato (van Tuinen et al., 1995). Increased levels of phyA also did not affect the phenotype of potato plants grown in W. This result contrasts with the observation that overexpression of monocotyledonous phyA in a dicotyledonous background leads to an altered phenotype (Keller et al., 1989; Boylan and Quail, 1989, 1991; Nagatani et al., 1991b). This discrepancy can be explained by the inability of native phyA to accumulate because of its light lability in its homologous background. Whereas Keller et al. (1989) and Boylan and Quail (1991) observed a 16- to 20-fold higher spectral activity in plants grown in W, we could not detect any increase in our transgenic plants when these were grown in W. Because even different cultivars of tobacco react differently to increased monocotyledonous phyA levels (Nagatani et al., 1991b), it could also be that our potato plants are generally less sensitive to altered phyA levels in W, irrespective of whether it originates from a monocotyledonous or a dicotyledonous species. It remains an open question whether phyA can interact with partners of the signal transduction chain that are normally addressed by phyB. The experiments with the monocotyledonous phyA overexpressors are not conclusive, because the monocotyledon-

ous phyA might not be specifically recognized as type A because of its heterologous structure. The ideal experiment would be to overexpress a native phyA that cannot be recognized by the host degradation machinery.

When wt potato plants were grown at constant photosynthetically active radiation but varying R:FR ratios, plant height varied dramatically in a pattern typical for the shade-avoidance syndrome (Smith, 1992). At low R:FR ratios, plants were taller with longer internodes, and axillary branching was suppressed. Both the overexpressors and antisense plants showed a similar overall shade-avoidance response to these treatments (Table II). Based on the observation that Arabidopsis *phyB* mutants are saturated for the shade-avoidance response (Robson et al., 1993), this photoresponse has been assigned to the action of phyB: lack of functional phyB (either by mutation or by keeping most of it in the Pr form under low R:FR conditions) allows promotion of stem extension. In the potato system, phyA antisense plants were taller than the controls and phyA overexpressors were shorter, indicating a regulatory role of phyA in controlling stem extension under low R:FR ratios. This is supported by results obtained with Arabidopsis *phyA*/*phyB* double mutants. The hypocotyls of the *phyA*/*hy3* double mutant are significantly longer under low R:FR than those of the *hy3* mutant (Johnson et al., 1994), which suggests that phyA is also involved in reducing stem extension. Obviously, this response can be elicited only under low R:FR ratios where phyA can accumulate. McCormac et al. (1992b) have shown that endogenous dicotyledonous phyA levels increase when W is supplemented with FR. In the potato system, we would thus predict that phyA levels in overexpressors increase over the level reached in wt. Consequently, we observe a reduction in plant height compared to control plants. In the potato antisense plants that show a decreased abundance of phyA, plant height is increased under shade conditions. Thus, there seems to be an overlap in the physiological function of phyA and phyB with regard to reducing stem extension. Differences in their responsiveness to different light conditions seem to arise from differences in stability and expression levels.

## CONCLUDING REMARKS

By analyzing transgenic potato plants with altered phyA levels we were able to show that phyA elicits or modulates photoresponses under conditions that allow its accumulation (dark-grown plants, plants grown under low R:FR ratios). These data confirm the general picture that the function of phyA is to sense the difference between darkness and shade conditions. In this respect it was surprising that phyA is also needed for sensing R to initiate inhibition of sprout elongation and to elicit maximal *RBCS* expression levels.

## ACKNOWLEDGMENTS

We would like to thank Natalie Wright for technical assistance in the potato growth experiments and Michael Herold for anti-phyB serum.

Received February 23, 1995; accepted May 22, 1995.
Copyright Clearance Center: 0032-0889/95/109/0053/09.

## LITERATURE CITED

**Boylan MT, Quail PH** (1989) Oat phytochrome is biologically active in transgenic tomatoes. Plant Cell **1:** 765–773

**Boylan MT, Quail PH** (1991) Phytochrome A overexpression inhibits hypocotyl elongation in transgenic *Arabidopsis*. Proc Natl Acad Sci USA **88:** 10806–10810

**Cherry JR, Hershey HP, Vierstra RD** (1991) Characterization of tobacco expressing functional oat phytochrome. Plant Physiol **96:** 775–785

**Clack T, Mathews S, Sharrock RA** (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: the sequences and expression of PHYD and PHYE. Plant Mol Biol **25:** 387–400

**Cordonnier MM, Greppin H, Pratt LH** (1986) Identification of a highly conserved domain on phytochrome from angiosperms to algae. Plant Physiol **80:** 982–987

**Deblaere R, Bytebier B, De Greve H, Debroeck F, Schell J, van Montagu M, Leemans J** (1985) Efficient octopine Ti plasmid derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic Acids Res **13:** 4777–4788

**Dehesh K, Franci C, Parks BM, Seeley KA, Short TW, Tepperman JM, Quail PH** (1993) *Arabidopsis HY8* locus encodes phytochrome A. Plant Cell **5:** 1081–1088

**Devi KRG, Chan Y-L, Wool IG** (1989) The primary structure of rat ribosomal protein S4. Biochim Biophys Acta **1008:** 258–262

**Eckes P, Schell J, Willmitzer L** (1986) Isolation and characterization of a light inducible, organ-specific gene from potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. Mol Gen Genet **205:** 14–22

**Flavell RB** (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci USA **91:** 3490–3496

**Furuya M** (1993) Phytochromes: their molecular species, gene families, and functions. Annu Rev Plant Physiol Plant Mol Biol **44:** 617–645

**Heyer A, Gatz C** (1992a) Isolation and characterization of a cDNA clone coding for potato typeA phytochrome. Plant Mol Biol **18:** 535–543

**Heyer A, Gatz C** (1992b) Isolation and characterization of a cDNA clone coding for potato typeB phytochrome. Plant Mol Biol **20:** 589–600

**Höfgen R, Willmitzer L** (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res **16:** 9877

**Höfgen R, Willmitzer L** (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). Plant Sci **66:** 221–230

**Johnson E, Bradley M, Harberd NP, Whitelam GC** (1994) Photoresponses of light grown *phyA* mutants of *Arabidopsis*. Phytochrome A is required for the perception of daylength extensions. Plant Physiol **105:** 141–149

**Kay SA, Nagatani A, Keith B, Deak M, Furuya M, Chua N-H** (1989) Rice phytochrome is biologically active in transgenic tobacco. Plant Cell **1:** 775–782

**Keller JM, Shanklin J, Vierstra RD, Hershey HP** (1989) Expression of a functional monocotyledonous phytochrome in transgenic tobacco. EMBO J **8:** 1005–1012

**Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

**Logemann J, Schell J, Willmitzer L** (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

**Mancinelli AL** (1980) The photoreceptors of the high irradiance responses of plant photomorphogenesis. Photochem Photobiol **32:** 853–857

**Mancinelli AL** (1988) Some thoughts about the use of predicted values of the state of phytochrome in plant photomorphogenesis research. Plant Cell Environ **11:** 429–439

**McCormac AC, Cherry JR, Hershey HP, Vierstra RD, Smith H** (1991) Photoresponses of transgenic tobacco plants expressing an oat phytochrome gene. Planta **185:** 162–170
**McCormac AC, Wagner D, Boylan MT, Quail PH, Smith H, Withelam GC** (1993) Photoresponses of transgenic *Arabidopsis* seedlings expressing introduced phytochrome-B-encoding cDNAs—evidence that phytochrome-A and phytochrome-B have distinct photoregulatory functions. Plant J **4:** 19–27
**McCormac AC, Whithelam GC, Boylan MT, Quail PH, Smith H** (1992a) Contrasting responses of etiolated and light-adapted seedlings to red:far-red ratios: a comparison of wild-type, mutant and transgenic plants has revealed differential functions of members of the phytochrome family. J Plant Physiol **140:** 707–714
**McCormac AC, Whitelam GC, Smith H** (1992b) Light-grown plants of transgenic tobacco expressing an introduced oat phytochrome A gene under the control of a constitutive viral promoter exhibit persistent growth inhibition by far-red light. Planta **188:** 173–181
**Muleo R, Rugini E, Thomas B** (1994) Differential exhibition of photoreversible phytochrome in callus from two woody species, *Actinidia deliciosa* and *Olea europea*. Plant Sci **98:** 81–85
**Nagatani A, Chory J, Furuya M** (1991a) Phytochrome B is not detectable in the *hy3* mutant of *Arabidopsis*, which is deficient in responding to end-of-day far-red light treatments. Plant Cell Physiol **32:** 1119–1122
**Nagatani A, Kay SA, Deak M, Chua N-H, Furuya M** (1991b) Rice type I phytochrome regulates hypocotyl elongation in transgenic tobacco seedlings. Proc Natl Acad Sci USA **88:** 5207–5211
**Nagatani A, Reed JW, Chory J** (1993) Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol **102:** 269–277
**Parks BM, Quail PH** (1993) *hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. Plant Cell **5:** 39–48
**Pratt LH** (1983) Assay of photomorphogenic photoreceptors. *In* W Shropshire, Jr, H Mohr, eds, Encyclopedia of Plant Physiology, New Series, Vol 16A: Photomorphogenesis. Springer-Verlag, Berlin, pp 152–177
**Quail PH** (1991) Phytochrome: a light activated molecular switch that regulates plant gene expression. Annu Rev Genet **25:** 389–409
**Robson PRH, Whitelam GC, Smith H** (1993) Selected components of the shade avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiol **102:** 1179–1184
**Rocha-Sosa M, Sonnewald U, Frommer W, Stratmann M, Schell J, Willmitzer L** (1989) Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J **8:** 23–29
**Reed JW, Nagatani A, Elich TD, Fagan M, Chory J** (1994) Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol **104:** 1139–1149
**Reed JW, Nagpal P, Poole DS, Furuya M, Chory J** (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell **5:** 147–157
**Salisbury FB, Ross CW** (1985) Plant Physiology. Wadsworth, Belmont, CA
**Sharrock RA, Parks BM, Koorneef M, Quail PH** (1988) Molecular analysis of the phytochrome deficiency in an *aurea* mutant of tomato. Mol Gen Genet **213:** 9–14
**Sharrock RA, Quail PH** (1989) Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution and differential expression of a plant regulatory photoreceptor family. Genes Dev **3:** 1745–1757
**Smith H** (1992) The ecological functions of the phytochrome family. Clues to a transgenic programme of crop improvement. Photochem Photobiol **56:** 815–822
**Smith H, Whitelam GC** (1990) Phytochrome, a family of photoreceptors with multiple physiological roles. Plant Cell Environ **13:** 695–707
**Somers DE, Sharrock RA, Tepperman JM, Quail PH** (1991) The *hy3* long hypocotyl mutant of *Arabidopsis* is deficient in phytochrome B. Plant Cell **3:** 1263–1274
**van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE, Koorneef M** (1995) Far-red light-insensitive phytochrome A-deficient mutants of tomato. Mol Gen Genet **246:** 111–141
**Whitelam GC, Harberd NP** (1994) Action and function of phytochrome family members revealed through the study of mutant and transgenic plants. Plant Cell Environ **17:** 615–625
**Whitelam GC, Johnson E, Peng J, Carol P, Anderson ML, Cowl JS, Harberd NP** (1993) Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. Plant Cell **5:** 757–768
**Whitelam GC, McCormac AM, Boylan MT, Quail PH** (1992) Photoresponses of *Arabidopsis* seedlings expressing an induced oat phyA cDNA persistence of etiolated plant type responses in light grown plants. Photochem Photobiol **56:** 61–62
**Withrow RB** (1941) Response of seedlings to various wavebands of low intensity irradiations. Plant Physiol **16:** 241–256