THE LEVEL OF phyA IN *PHARBITIS NIL* CHOIS DURING THE PHOTOPERIODIC FLOWER INDUCTION

ADRIANA SZMIDT-JAWORSKA, KRZYSZTOF JAWORSKI, JAN KOPCEWICZ

Nicholas Copernicus University, Institute of General and Molecular Biology
Department of Plant Physiology and Morphogenesis
Gagarina 9, 87-100 Toruń, Poland; tel. (056) 61 14 456; fax: + 48-56-61-14-478

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ABSTRACT

The aim of this work was to determine if there is any relationship between an endogenous phyA level and photoperiodic flower induction. The level of phyA was characterised with polyclonal antibodies directed to phyA from pea. At first it was detected that phyA level is predominant in cotyledons, whereas in roots and stems the concentration of labile phytochrome is rather low. So cotyledons were used for later experiments. In these cotyledons exposed to light illumination a rapid destruction of phyA has been observed. The loss of extractable phyA chromoprotein occurs already after 60 min of irradiation. *Pharbitis nil* is a short-day plant and a single 16-hours–long dark period is fully inductive. We assessed that phyA level is extremely low during a long inductive night and an immunodetectable phytochrome appears only after 24 hours of darkness. The obtained results suggest that labile phytochrome is not taking part in the direct control of the photoperiodic flower induction.

KEY WORDS: *Pharbitis nil*, labile phytochrome, phytochrome degradation and reaccumulation, photoperiodic flower induction.

INTRODUCTION

Pharbitis nil is a very sensitive short-day plant that develops flower buds when 4-5 day old seedlings are exposed to a single short-light / long-dark cycle. A single 16-hour-long dark period is fully inductive (6-7 flower buds with a terminal one per plant) for such young plants. The mechanism of flower induction in *Pharbitis nil*, such as in other short–day plants, remains vague. Plants sense the quality, quantity, direction and duration of the incident light and use it as a signal to optimise their growth and development. The light is perceived by a complex system of different photoreceptors (Batschauer 1998). They include the phytochromes which are responsible for the detection of red and far-red light and the receptors for blue and UV light (Chory et al. 1996). Phytochromes are also involved in photoperiodic timekeeping. Recently genetic, molecular and cell biology studies have led to the characterisation of the different phytochrome populations (labile-phyA and stabile-phyB-E), and their cellular localization, but the role of these phytochromes in various growth and development processes is still under investigation. Phytochromes are present in different organs of mono- and dicotyledons and also in lower plants. However, the investigations on the phytochrome in *Pharbitis nil*, which is the model plant in most photoperiodic studies, are very scarce.

Recently, we employed newly produced polyclonal antibodies (PAbs) raised against *Pisum* phyA to cross react with a labile phytochrome from *Pharbitis nil* (A. Szmidt-Jaworska et al. 2000). Thus, the aim of the present study was to determine if the level of phyA in different organs of *Pharbitis nil* seedlings are similar to that observed in other dicots and if there is any relationship between an endogenous phyA level and photoperiodic flower induction.

Abbreviations:
PAbs – polyclonal antibodies, phyA – labile phytochrome, P_r – red absorbing form of phytochrome, P_{fr} – far-red absorbing form of phytochrome, SDS – sodium dodecyl – sulfate

MATERIALS AND METHODS

Plant material

Seeds of *Pharbitis nil* Chois cv. Violet (Japan) were scarified with a scalpel and left for swelling for 24 h at 26°C in darkness.

Seeds for phyA localization were grown in darkness for 5 days. Then different organs like cotyledons, roots, hypocotyls were harvested and frozen in liquid nitrogen.

Seeds for phyA reaccumulation measurements were sown in moist vermiculit and grown in light (130 μmol $m^{-2}s^{-1}$, cool white fluorescent tubes Polam, Warsaw, Poland) at 25°C for 5

days. After that seedlings were moved into the inductive darkness. At different times of the dark treatment cotyledons were harvested and frozen in liquid nitrogen.

Seedlings used for phyA degradation measurements were grown in darkness for 5 days and then moved to the light. Cotyledons were harvested at the different times of the light treatment and frozen in liquid nitrogen.

Phytochrome extraction

To prepare total cotyledon proteins from etiolated *Pharbitis nil* rapidly for SDS-PAGE, previously frozen tissue was ground to a fine powder with a mortar and pestle. After adding insoluble PVP, proteins were extracted with ice-cold 50 mM Tris buffer pH 8.5 containing 50% ethylene glycol, 140 mM ammonium sulphate, 56 mM 2-mercaptoethanol, 10 mM EDTA and protease inhibitors: aprotinin (5 μg/ml), leupeptin (2 μg/ml), 4 mM jodoacetamide at the ratio of 62 mg powder to 1 ml buffer. The extract was clarified by centrifugation at 20000 × g for 15 min. The supernatant was carefully collected and polyethylenimine was added to the final concentration of 0.1%. The extract was vortex and after the centrifugation for 10 min at 12000 × g saturated ammonium sulphate solution (0.725:1; v/v) was added to the supernatant and the extract was gently stirred for 30 min. The ammonium sulphate precipitate was collected by centrifugation at 12000 × g for 15 min, directly resuspended into 25 μl SDS-sample buffer (100 mM Tris H-Cl, pH 6.8, 4% SDS, 20% glicerin, 10% β-mercaptoethanol, 0.1% bromphenolblue). A sample was kept at 100°C for 5 min, cooled rapidly and clarified by centrifugation. Supernatants were used immediately or stored at -70°C for later use.

SDS-PAGE

SDS-polyacrylamide gel electrophoresis and subsequent staining of the gels with Coomassie brilliant blue were performed by the procedure of Leammli (1970) using a 7.5% acrylamide running gel and a 4% acrylamide stacking gel.

Molecular weight standards were obtained from Sigma (mixture SDS-7). The intensity of the bands on the gel was measured with a reflectance scanning densitometer (Image Master®, Pharmacia Biotech.), which was on line with a computer.

Antibodies

PAbs against purified labile phytochrome from pea were raised in rabbits and purified as described before (Szmidt-Jaworska et al., 2000).

Immunobloting

After separation by SDS-PAGE, polypeptides were electroblotted onto a nitrocellulose (Sigma, 2 μm) in 100 mM Tris-HCl, 192 mM glycine and 25% (v/v) methanol with a Transblot cell (BioRad Labolatories) at 150 mA constant current for 8 hours (Burnett 1981). The membrane was blocked for 1 hour at 25°C in a TBS buffer containing 3% (w/v) fat free milk powder. The membrane was incubated with the primary antibody (PAbs against phyA, 1:1000) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1% (w/v) fat free milk powder for 2 h at 25°C. After washing three times in 20 mM Tris-HCl (pH 7.5), 0.05% Tween 20 and 150 mM NaCl, the membrane was incubated for 1-2 h with alkaline-phosphatase-conjugated goat antibodies (1:20000) to rabbit immunoglobulin G (IgG), washed and stained for alkaline phosphatase according to the manufacturer's instructions (Protoblot kit; Sigma). The reaction was terminated by rinsing the blots in water.

Protein assay

The concentration of proteins in the sample was determined by dye-binding assay (Bradford, 1976), using bovine gamma globulin as a standard.

RESULTS

Both the extraction of phytochrome and selection of assayed conditions were based on previous experience with etiolated tissue (A. Szmidt-Jaworska et al. 2000). The level of phyA used for SDS-PAGE was very low and we were not able to see any high range bands on the gels (data not shown). Fortunately technics using poly- or monoclonal antibodies are more sensitive, so Western-bloting assay has been adapted for quantitation of phytochrome.

It was detected that a labile phytochrome (phyA) is predominant molecular species in cotyledons, whereas in roots and stems the concentration of this phytochrome population is rather low (Fig. 1). To the next experiments only cotyledons were taken.

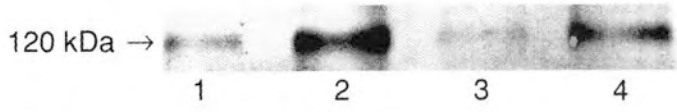

Fig. 1. Immunoblot analysis of 120-kDa phytochrome from *Pharbitis nil* whole seedlings, roots, hypocotyls and cotyledons. Equal protein amount (10 μg) was separated on 7.5% SDS-PAGE, electroblotted onto nitrocelulose and immunostained by PAbs raised against pea phyA.
Lane 1 – phyA level in whole etiolated plants,
Lane 2 – phyA level in cotyledons,
Lane 3 – phyA level in roots,
Lane 4 – phyA level in hypocotyls.

Phytochrome content during continuous irradiation was followed by Western-blotting assay of crude extract from cotyledons as a function of time after the transfer of the latter to light. After the synthesis as P_r, a phytochrome is remarkably stable (half-life > 100 h) allowing it to accumulate to high level in etiolated seedlings (Fig. 2, lane 2). The kinetic of de-

Fig. 2. Phytochrome degradation in etiolated *Pharbitis nil* cotyledons. Seedlings were grown in darkness for 5 days and then transferred into the continuous light. At various times, cotyledon tissue was assayed for phytochrome content. The nitrocelulose shows the kinetics of phyA degradation, the migration position during SDS-PAGE of the 120-kDa *Pharbitis nil* labile phytochrome.
Lane 1 – marker 116-kDa,
Lane 2 – phyA level in etiolated cotyledons,
Lane 3 – 10 – phyA level after moving plants into white light (10, 20, 30, 45, 60, 90, 240 min of white light).

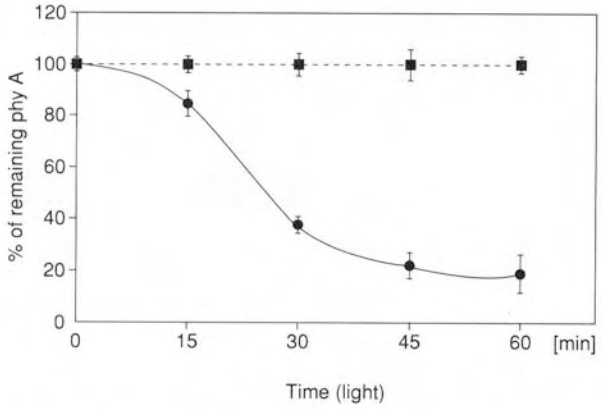

Fig. 3. Percentage of immunologically detectable phytochrome remaining in *Pharbitis nil* cotyledons following the exposure to white light at 26°C for the time indicated. The amounts present at etiolated cotyledons were set to 100%.

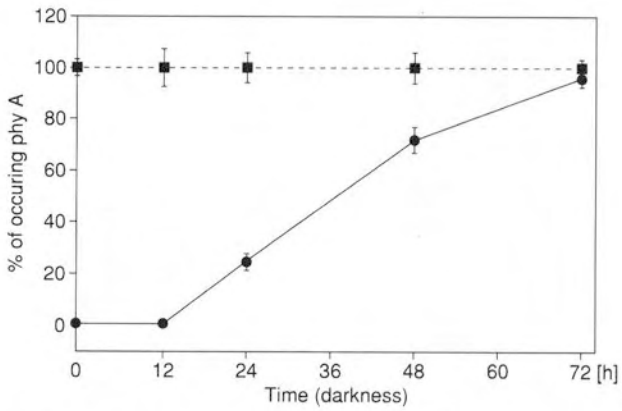

Fig. 4. Percentage of immunologically detectable phyA occurring in *Pharbitis nil* cotyledons which were put into darkness for the time indicated. The amounts present at etiolated cotyledons were set to 100%.

gradation of the 120-kDa phytochrome polypeptide was determined immunologically, and the half life of a phytochrome in vivo was approximately 30 min at 25°C (Fig. 2). The quantity of a phytochrome in vivo was determined by the measurement of the intensities of bands on blot by reflectance scanning densitometry, and the results are shown in Fig. 3. The amount of phyA detected in crude extract after 60 min of white light treatment was reduced to 20% of the controle extract from dark-grown cotyledons.

An experiment with phyA degradation was accomplished by following the reaccumulation of a phytochrome after transferring light-grown seedlings into darkness. The kinetic of reaccumulation during a long night was also determined immunologically, and an immunodetectable phytochrome appeared after 24 h of darkness (Fig. 5). The amount of a phytochrome during reaccumulation was estimated by the quantitation of the intensities of bands on blot by reflectance scanning densitometry. After first 24 hours only 20% of total phyA level was immunoprecipitated and in 72 h the level was 80%. Percentage of immunologically detectable phyA is shown in Fig. 4.

DISCUSSION

It is well known that a phytochrome controls many physiological processes during plant growth and development. After the earlier discovery that higher plants contain several different types of a phytochrome (Quail 1991), the question concerning the role of the various phytochromes in the photoperiodic induction of flowering arises. There is evidence that the phytochrome A is an essential component of the daylength-sensing mechanism in long-day plants (Johnson et al. 1994). Contrary there is so far little direct evidence for the role of phyA in short-day-plants (SDP) (Jackson and Thomas 1997).

P. nil is a short-day model plant, but the knowledge concerning the role of a labile phytochrome and even the quantitation of phyA level in this plant is very limited. For other species it has been assumed, using immunoassay, that a labile phytochrome is a predominant molecular species (about 98-99% of total phytochrome) in etiolated tissue (Abe et al. 1989; Somers et al. 1991). The low level of a phytochrome was determined in roots and stems, whereas meristematic tissue, hook (in pea) and leaves (Nagatani 1997) contain a high level of phyA. This pattern of distribution has been also confirmed for *P. nil* using antibodies to phytochrome apoprotein. The highest level was obtained for cotyledons, and the lowest for roots (Fig. 1).

It is not surprising that plants developed exquisite mechanisms to regulate the synthesis and stability of the chromoproteins. This regulation is especially obvious for phyA, which is controlled at the levels of *PHYA* gene transcription, mRNA stability, protein availability and protein turnover (Higgs and Colbert 1994; Quail et al. 1995). Of these, the most dynamic and influential is the rapid proteolysis of the chromoprotein following the photoconversion of P_r to P_{fr}. Pratt et al (1974) first showed that P_{fr} degradation involves the loss of both spectrometrically and immunologically detectable phyA. In etiolated seedlings the transformation of P_r to P_{fr} increases the degradation rate over 100-fold (Quail et al. 1973). However, the kinetic of *P. nil* labile phytochrome degradation was more rapid ($t_{1/2}$ = 30 min) than that observed by radioimmunoassay for other dicots, with half-lives of 2.9 and 3.5 h (Shimazaki et al. 1983).

Fig. 5. Dark reaccumulation of labile phytochrome in *Pharbitis nil* cotyledons. Seedlings were grown for 5 days under continuous light and then transferred into darkness. At various times, cotyledon tissue was frozen, homogenized and the phytochrome assayed by immunoblot analysis with anti-pea phytochrome antibodies. The nitrocelulose shows the migration position during SDS-PAGE of *Pharbitis nil* 120-kDa labile phytochrome.
Lane 1 – marker 116-kDa,
Lane 2 – phyA level in etiolated cotyledons,
Lane 3 – phyA level in green cotyledons,
Lane 4-8 – phyA level in green cotyledons moved into the darkness (6, 12, 24, 48, 72 hr of darkness).

For many plants both high expression levels of *PHYA* in the dark and phyA stability as P_r allow these molecules to accumulate to a high level in dark-grown plants. However, it has been seen in the previous experiments with *P. nil*, that *PHYA* mRNA is heavily down-regulated by light and the expression level is extremely low (Carter et al. 2000). In our experiments we determined the kinetics of phyA level reaccumulation during a long night and an immunodetectable phytochrome appeared only after 24 h of darkness.

The present experiment on both chromoprotein degradation and reaccumulation was aimed at evaluating the function of phyA in the photoperiodic induction. The observation that phyA is rapidly degraded after light treatment and the chromoprotein and mRNA reaccumulation during the induction night is very slow, longer than 16-hour-inductive night, could suggest that phyA is not an influential factor in the control of photoperiodic flower induction in short-day plants. However, more informative experiments, using transgenic qualitative *P. nil* seedlings with low phyA level, are needed before the role of phyA in this plant becomes definitely clear.

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POZIOM FITOCHROMU LABILNEGO PODCZAS FOTOPERIODYCZNEJ INDUKCJI KWITNIENIA U *PHARBITIS NIL.*

STRESZCZENIE

Celem pracy było określenie czy istnieje zależność pomiędzy poziomem fitochromu labilnego a fotoperiodyczną indukcją kwitnienia. Poziom phyA był badany z wykorzystaniem przeciwciał poliklonalnych skierowanych na fitochrom labilny z grochu. Stwierdzono, że fitochrom ten występuje we wszystkich badanych organach, jednakże jego poziom jest najwyższy w liścieniach. Z tego powodu materiałem do dalszych analiz były liścienie.

W wyniku przeprowadzonych badań zaobserwowano gwałtowną destrukcję fitochromu labilnego w liścieniach *Pharbitis nil* pod wpływem światła. Całkowity rozpad fitochromu występuje już po 60 minutach naświetlenia. Stwierdzono jednocześnie, że poziom phyA w 5 dniowych siewkach poddanych długiej indukcyjnej nocy jest bardzo niski i w większych ilościach pojawia się dopiero w 24 godzinie ciemności. Wyniki badań sugerują więc, że labilny fitochrom nie bierze bezpośredniego udziału w kontroli fotoperiodycznej indukcji kwitnienia.

SŁOWA KLUCZOWE: *Pharbitis nil*, fitochrom labilny, degradacja i reakumulacja fitochromu, fotoperiodyczna indukcja kwitnienia.