Phytochrome A resets the circadian clock and delays tuber formation under long days in potato

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Received 28 March 2000; accepted 6 April 2000.
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Summary

Transgenic potatoes (Solanum tuberosum) with either increased (sense transformants) or reduced (antisense transformants) phytochrome A (phyA) levels were used, in combination with specific light treatments, to investigate the involvement of phyA in the perception of signals that entrain the circadian clock. Far-red or far-red plus red light treatments given during the night reset the circadian rhythm of leaf movements in wild-type plants and phyA over-expressors, but had little effect in phyA under-expressors. Far-red light was also able to reset the rhythm of leaf movement in wild-type Arabidopsis thaliana but was not effective in mutants without phyA. Blue light was necessary to reset the rhythm in phyA-deficient potato plants. Resetting of the rhythm by far-red plus red light was only slightly affected in transgenic plants with reduced levels of phytochrome B. The production of tubers was delayed by day extensions with far-red plus red light, but this effect was reduced in transgenic lines deficient in phyA. We conclude that phyA is involved in resetting the circadian clock controlling leaf movements and in photoperiod sensing in light-grown potato plants.

Keywords: circadian rhythms, entrainment, photoperiod, phytochrome, potato, tuberisation.

Introduction

Many biological activities in eukaryotes and prokaryotes are organized into precise daily cycles. These rhythms persist under constant environmental conditions with a period of approximately 24 h, indicating the involvement of an internal circadian oscillator (Dunlap, 1999). Endogenous clocks allow organisms to anticipate the periodic changes in their environment, and to co-ordinate different physiological functions accordingly. In order to keep their anticipatory function throughout the year, circadian clocks are synchronized daily by environmental signals such as light and temperature changes (Pittendrigh, 1993).

In plants, photobiological evidence pointed to the control of clock entrainment by phytochrome and blue-light photoreceptors (Halaban, 1969; Hillman, 1971; Nagy et al., 1993; Satter et al., 1981; Simon et al., 1976). Somers et al. (1998) have recently found that the period of cab2::luciferase expression is affected by phytochrome A (phyA), phytochrome B (phyB) and cryptochrome 1 (cry1) under particular fluence rates of red light (R) and blue light. These observations indicate that cry1, phyA and phyB are components of the input pathway to the clock, components of the central oscillator, or both. Although based on the functions of phyA, phyB and cry1 in photomorphogenesis, a role in the input pathway would appear to be the most favourable hypothesis; the effects of phyA and cry1 on the period of cab2::luciferase expression are maximum at the lowest fluence rates tested (Somers et al., 1998) and cannot be accurately measured in darkness (Millar et al., 1995). If the effects of phyA and cry1 were present in darkness, a role as components of the central oscillator would appear more likely than participation in the input to
the clock. In favour of the latter view, phytochromes contain a bipartite PAS domain (Lagaris et al., 1995) typical of several clock proteins (Kay, 1997), and the expression of PHYB shows circadian rhythmicity, also typical of clock component proteins (Kozma Bognar et al., 1999). There are examples in other systems, where photoreceptors appear to be part of the clock rather than part of the input to the clock. In mice, cry1 and cry2 were formerly proposed to be in the input to the clock (Miyamoto and Sancar, 1998), but current evidence is more easily accounted for by considering a role for these photoreceptors as central components of the clock itself (Griffin et al., 1999; Kume et al., 1999; Lucas and Foster, 1999; Okamura et al., 1999; Thresher et al., 1998; van der Horst et al., 1999; Vitaterna et al., 1999).

In this paper we specifically address the role of phyA in the light input pathway to the clock. For this purpose, we have analysed the response of potato plants over- or under-expressing phyA, as well as the response of Arabidopsis phyA mutants, to light treatments that normally advance the phase of circadian rhythms compared to dark controls.

In addition to its effects on circadian rhythmicity, phyA is also involved in the perception of daylength in long-day plants (Johnston et al., 1994; Weller et al., 1997). Circadian clocks are part of the photoperiodic time-measuring mechanism (Thomas and Vinc Prue, 1997), and therefore the role of phyA in daylength perception could be associated with its effects in clock entrainment. Here we have analysed whether tuberization in potato, a process that requires short days, is also affected by phyA when the length of the day is extended using a light of similar composition to that used to reset the clock.

Results

Far-red light fails to reset the circadian clock in potato and Arabidopsis plants deficient in phyA

Control plants cultivated under natural photoperiods were transferred to constant (free-running) conditions of light (continuous dim white light) and temperature (25°C). Treated plants were exposed to a 5 h light treatment immediately prior to transfer to free-running conditions (i.e. at the end of the last night) (Figure 1). Similar protocols have successfully been used to investigate the effect of different light qualities in resetting the circadian clock in Chlamydomonas (Kondo et al., 1991) and Gonyaulax (Ronneberg and Deng, 1997). Effective light treatments late in the night are expected to produce advances in the phase of the rhythm (Pittendrigh, 1981).

In a first set of experiments, the plants were exposed to 5 h of far-red light (FR) because phyA is the only photoreceptor known to retain biologically significant amounts of its active form under FR (Heyer et al., 1995; Nagatani et al., 1993; Parks and Quail, 1993; van Tuinen et al., 1995; Whiteham et al., 1993). Compared to control plants, exposure to FR during the final hours of the night caused an almost immediate and persistent phase advance in the free-running rhythm of leaf movement in WT and phyA over-expressors, but not in phyA antisense transgenics (Figure 2a,c). The period of the rhythm under continuous dim white light was not significantly affected by the FR treatment or by the genotype (Figure 2b), but a direct relationship was observed between phyA levels and the phase shift produced by the FR treatment (Figure 2c). The phase shift caused by FR was significantly larger (P<0.05) in the phyA over-expressor (approximately 3 h) than in the WT (approximately 2 h), and significantly smaller (P<0.05) in the phyA under-expressor (less than 1 h) than in the WT. The phase shift produced by continuous FR was not a consequence of reduction in the Pfr levels of stable phytochromes. A single FR pulse of 5 min given 5 h before the plants were transferred to continuous light, although predicted to reduce the level of Pfr of stable phytochromes, had no effect on the rhythm (data not shown).

To investigate whether the ability of phyA to reset the circadian rhythm is common to other species, we used seedlings of Arabidopsis thaliana grown under photoperiods of fluorescent white light. Exposure to 5 h of FR at the end of the last night caused a strong shift (5 h) in the phase of the rhythm of leaf movement in WT seedlings but had no significant effect in mutant plants lacking phyA (Figure 3a,c). The maximum phase shift caused by the FR treatment was not reached immediately (it was reached immediately in potato), and this resulted in a small but significant shortening of the period in the WT (Figure 3b).

Roles of phyA and phyB in resetting the circadian clock in potato

High fluence rates of pure FR are useful as a photobiological tool but are not frequent under natural radiation conditions. A FR+R mixture providing a R/FR ratio similar to sunlight (i.e. 1.1) was used to simulate light conditions experienced by plants grown in open areas. Control plants were transferred to free-running conditions at dawn, whereas treated plants were exposed to 5 h of FR+R before being transferred to free-running conditions simultaneously with control plants. Compared to the controls, the FR + R treatments caused a persistent shift (more than 4 h) of the circadian rhythm of leaf movement in WT and phyA over-expressors (Figure 4a,c). This shift was small (approximately 1.5 h) in transgenic seedlings with the PHYA antisense construct (Figure 4a,c). The period of the rhythm was not significantly affected (Figure 4b).
To investigate whether other phytochromes could be involved in resetting the rhythm under FR+R, transgenic lines over-expressing the antisense gene of the apoprotein of phyB were compared to the WT. The phase advance was 3.1 h compared to 4.5 h in the WT (Figure 5a,c). Thus, the difference caused by reduced phyB (1.4 h) was smaller than that caused by reduced phyA (approximately 2.5 h) despite similarly severe reductions of phyB (Jackson et al., 1996) and phyA (Heyer et al., 1995) levels in the respective antisense transgenics. The period of the rhythm was not significantly affected (Figure 5b).

**Blue light resets the circadian clock in phyA-deficient plants**

Although resetting the circadian rhythm using FR+R with a R/FR ratio similar to sunlight was severely impaired in phyA-deficient plants, WT plants, phyA over-expressors and phyA under-expressers showed no differences in leaf movement under natural radiation in the glasshouse (Figure 6). Probably, changes in blue light and/or temperature were able to compensate for the lack of phyA.

To test the effect of blue light, potato plants were exposed to FR+R+ blue light before transfer to free-running conditions. In WT and phyA over-expressers, FR+R or FR+R+ blue light caused a similar phase shift compared to control plants transferred to free-running conditions without the 5 h treatment (phase shift (h): WT, FR+R = 3.6 ± 0.5, FR+R+ blue = 4.7 ± 0.6; phyA over-expressor, FR+R = 4.3 ± 0.6, FR+R+ blue = 4.8 ± 0.6; means ± SE of 15 replicate plants). This indicates that in WT and phyA over-expressor plants, phytochromes were able to cause maximum resetting of the rhythm. In the antisense PHYA transgenics, blue light added to a background of FR+R induced a phase advance of the rhythm that was not observed with FR+R alone (Figure 7a,c). The period of the rhythm was not significantly affected (Figure 7b). Blue light was added to a background of phytochrome-absorbable radiation to minimize the effects of blue light via phytochrome (Thomas and Dickinson, 1979). The calcu-
lated phytochrome photoequilibrium (i.e. the proportion of Pfr) established by the FR + R + blue light source used here was similar to that established by FR + R alone (i.e. 69%), and the blue light added to the background of FR + R increased the rate of phytochrome photocconversion by only 15%. In addition, in plants with reduced phyA levels, increasing phytochrome photocconversion by the addition of more FR + R to the background did not mimic the effect of blue light (data not shown). Thus, the effect was specific for blue light.

**phyA delays tuber production in potato**

To investigate whether light of the spectral composition effective to shift the phase of a circadian rhythm via phyA is also able to affect a photoperiodic response, potato plants were grown in a glasshouse (photoperiod 10–12 h) with or without a 6 h extension of the day with FR + R. Photoperiod extension caused a delay in tuber production. This effect was significantly less intense in transgenic plants deficient in phyA (Figure 8). The last date shown for each light-condition indicates the final number of tubers. Similar results were obtained with independent sense and antisense transgenic lines (data not shown).

**Discussion**

phyA is one of the photoreceptors involved in resetting the circadian clock controlling leaf movements in light-grown plants. Exposure to FR during the final 5 h of the night (immediately before dawn) advanced the phase of the circadian movement of the leaves in light-grown plants of potato (Figure 2) and Arabidopsis (Figure 3) but was not effective in antisense transgenic potato plants with reduced levels of phyA or phyA mutants of Arabidopsis. Artefacts caused by the antisense technology in potato (somaclonal variation or effects on other members of the phytochrome family) may be ruled out because overexpression of phyA increased the phase shift in response to FR (Figure 2), independent antisense transformants showed a similar behaviour in response to FR + R (Figure 4), phyB levels are unaffected in antisense transgenics (Heyer et al., 1995), and antisense expression of PHYB had reduced effects compared to antisense expression of PHYA (Figure 5).

Somers et al. (1998) showed that phyA lengthened the period of the circadian rhythms under very low fluences of R or blue light. In principle, these effects could be accounted for by a role of phyA in the input to the clock.
and/or a role of phyA as component of the central oscillator. The results presented here indicate that phyA is involved in the input to the clock. Although we do not rule out a role of phyA as part of the circadian oscillator, the difference between WT and phyA-deficient plants was only observed under conditions where light treatments absorbed by phyA reset the rhythm compared to controls that remain in darkness.

When the light input simultaneously activates several photoreceptors, the output depends on the net of interactions (both positive and negative) among these photoreceptors (Casal, 2000). Direct molecular interaction between phyA and cry1 has also been observed (Ahmad et al., 1998). A given photoreceptor could affect the input to the clock either directly or by modifying the activity of other photoreceptors. In mice, for instance, the cry2 mutant is more, not less, sensitive to light treatments that cause phase delays (Thresher et al., 1998), suggesting that CRY2 would negatively regulate the action of the photoreceptor(s) causing phase delays. The input effect of phyA is direct because FR is predicted to activate only phyA. At least in potato, a single pulse of FR given at the onset of the 5 h treatment period was not effective in inducing a phase shift in WT plants, indicating that FR did not operate mainly via reductions in the levels of stable phytochrome(s). In addition, since the period of a circadian rhythm under

continuous light depends on the balance between phase advances and phase delays (Millar and Kay, 1997), a given photoreceptor mutant could increase the length of the period by either reducing phase advances or increasing phase delays. The protocol used here allows us to conclude that phyA deficiencies reduce phase advances.

The phase shift induced by 5 h of FR was significantly longer in Arabidopsis (up to 8 h, 3 days after the light treatment) than in potato (3 h, in the phyA over-expressor). While the direction of the phase shift induced by a particular light treatment is totally conserved across kingdoms, large species-specific differences are observed for the magnitude of the phase shift. For instance, a 0.25 h light pulse can induce a phase shift of 12 h in Drosophila pseudoobscura and only 3 h in Drosophila melanogaster (Pittendrigh, 1981). Following the criterion of Winfree (1970) based on the magnitude of the shift, the response to light is type 1 (small phase shifts) in potato and type 0 (large phase shifts) in Arabidopsis.

A mixture of FR + R with a R/FR ratio similar to that of sunlight in open places was also able to reset the circadian rhythm of leaf movement in WT and phyA over-expressors, but had little effect in phyA under-expressors. However, the antisense transgenics did not show a general failure of rhythm entrainment because addition of blue light to the FR + R mixture was enough to cause the phase shift (Figure 7). When blue light is compared to darkness, the effect is at least partially mediated by phytochromes (Casal and Mazzella, 1998; Neff and Chory, 1998). In the present experiments, blue light was added to a background of phytochrome-absorbable radiation, and this condition...
blue light photoreceptor (i.e. not by phytochrome). In *Arabidopsis*, cry1 affects the circadian rhythm of cab2::luciferase expression (Somers *et al.*, 1998). In potato, the effect of blue light could at least partially account for the normal rhythm of leaf movement under sunlight.

Some lines of *Solanum tuberosum* ssp. *Andigena* will not tuberize when the day exceeds a critical threshold. This inhibition is completely lost in PHYB antisense plants (Jackson *et al.*, 1996). A similar picture is found for flowering in the short-day plant *Sorghum bicolor* (Childs *et al.*, 1997). Interestingly, phyB mutants of *Arabidopsis* (Reed *et al.*, 1993) and peas (Weller *et al.*, 1995), two long-day plants, also flower earlier than their corresponding WT. Thus, phyB could be involved in making tuberization and flowering responses sensitive to photoperiod rather than in sensing the duration of the photoperiod. The situation is different for phyA because deficiencies in this photoreceptor delay long-day responses such as flowering in *Arabidopsis* (Johnson *et al.*, 1994) and peas (Weller *et al.*, 1997), but accelerate short-day responses such as tuber formation in potato (Figure 8). Photoperiodic responses depend on the interaction between light and an endogenous circadian rhythm of sensitivity towards light. In this scenario, light has a two-fold effect: first, a direct action inhibiting (short-day responses) or promoting (long-day responses) a given process (e.g. tuber formation, flowering), and second, resetting the circadian rhythm of sensitivity. In *Arabidopsis*, cry2 has a strong effect on flowering (Guo *et al.*, 1998) but a minor effect on at least some circadian rhythms (Somers *et al.*, 1998). Thus, cry2 appears to be involved mainly in the direct

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**Figure 7.** Blue light added to a background of FR + R resets the rhythm of leaf movement in potato plants under-expressing phyA (AP11). (a) Leaf angle, (b) period of the rhythm and (c) phase shift caused by FR + R and FR + R + blue light. Glasshouse-grown plants were exposed to 5 h FR + R or FR + R + blue light at the end of the last night, immediately before transfer to free-running conditions. Vertical lines indicate the times of maximum (solid lines) and minimum (dashed lines) leaf angles in control plants. Data are means ± SE of 10 plant replicates.

**Figure 8.** phyA delays tuber production in response to photoperiods extended with FR + R. Plants were grown in the glasshouse (photoperiod 10-12h) with or without extensions of the photoperiod (6h) with FR + R. The under-expressor line is AP11 and the over-expressor line is PS4. Data are means ± SE of at least 10 plant replicates.
effect of light on flowering. By contrast, the role of phyA in photoperiodic responses could involve resetting of circadian clocks.

phyA is involved (a) in de-etiolation and seedling survival, particularly under very dense canopies (Yanovsky et al., 1995); (b) in the perception of irradiance levels (Yanovsky et al., 1998); (c) in the modulation of responses to R/FR ratio perceived by phyB (Casal, 1996; Yanovsky et al., 1995; Yanovsky et al., 1998); (d) in resetting the circadian rhythm of leaf movement (Figures 2–4); and (e) in the perception of daylength controlling flowering time in long-day plants (Johnson et al., 1994; Weller et al., 1997), and tuberization in potato (Figure 8).

Experimental procedures

Plant material and growth conditions

Potato plants (Solanum tuberosum), either of the WT (cultivar Désirée) or transformed with the PHYA gene in sense (lines PS4 and PS2) or antisense (lines AP11 and AP9) orientation, were as described by Heyer et al. (1995). Transgenic plants of Solanum tuberosum ssp. andigena transformed with the antisense PHYB gene (lines α-PHYB4 and α-PHYB10, Jackson et al., 1996) were included in some experiments. Plants were propagated from small tubers and grown in 650 cm² pots containing a soil/sand mixture in a heated greenhouse. Plants were used approximately 3 weeks after the sprouts had emerged. For experiments on tuber production, the plants were grown in the greenhouse in a clear substrate (Quemisoil, Quemi International, Inc., Ecosistema Biotico SA, Buenos Aires, Argentina) wrapped in black plastic film. Plants of Arabidopsis thaliana of the ecotype Landsberg erecta or the phyA-1 mutant (Whitealam et al., 1993) were grown under fluorescent white light (80 μmol m⁻² s⁻¹), photoperiod 12 h) in 5 cm³ pots containing a soil/sand medium.

Leaf position measurements and data analysis

In potato, the three youngest leaves > 1 cm long were marked in each plant. The angle between the average position of the petiole of these leaves and an imaginary line normal to the soil was measured at regular intervals during the experiments. The angle of the three leaves per plant was averaged for statistical analysis. Each experiment was conducted on at least three occasions, and data from different experiments were pooled for presentation. In Arabidopsis, the position of the first pair of leaves was recorded every 2 h with a digital video camera (Quick Cam, Connectix Corporation, San Mateo, CA, USA) and the angle between the petioles was measured using the Scion Image analysis program (Scion Corporation, Frederick, MD, USA). Data were fitted, according to Millar et al. (1995), to the equation: L(t) = c₀ + c₁t + (a₁ − a₀)t sin [2π(t − t₀)], where L is leaf angle, c₀ is the estimated value of the leaf angle at t = 0, c₁ is an estimate of the linear rate of change in leaf angle, a₀ is the estimated value of the amplitude at t = 0, a₁ is an estimate of the linear change in cycling amplitude, t is time, T is the period estimate, and φ is the estimate of the phase at t = 0. The first 6 h of measurements were not included among the data used to estimate the parameters of the above equation to avoid effects produced by shifting the plants from coloured to dim white light. Phase shifts were calculated as the difference between the phases of light-treated and control plants. The phases estimated by the above equation were used for the analysis when the periods were not significantly affected by the light treatment. In Arabidopsis (Figure 3b), a small but significant change in the period was produced by the light treatment in WT seedlings and therefore the phases were calculated by linear regression through the peaks in the days following the light pulse, extrapolated back to the day of the pulse (Kondo et al., 1991; Roenneberg and Deng, 1997).

Light treatments

For leaf angle experiments, glasshouse-grown potato plants were exposed to FR, FR + R or FR + R + blue light treatments starting 5 h before dawn and subsequently transferred to free-running conditions, i.e. continuous white light at 25°C. The FR treatment (100 μmol m⁻² s⁻¹) was provided by incandescent lamps in combination with a water filter, six blue acrylic filters (Paolini 2031) and two red acetate filters (La Casa del Acetato, Buenos Aires, Argentina) (see Casal and Boccalandro, 1995 for spectrum). The same source was used for experiments with Arabidopsis. Incandescent lamps in combination with a water filter, two red acetate filters (La Casa del Acetato, Buenos Aires, Argentina) and a copper sulphate filter (1.5% water solution, 5 mm thick) were used to provide FR + R (30 μmol m⁻² s⁻¹) at an R/FR ratio similar to that of daytime sunlight. To obtain FR + R + blue light, a source of blue light (22 μmol m⁻² s⁻¹), consisting of a bank of fluorescent tubes (Philips, TLF 40 W/54) and a blue acetate filter, was used in combination with the FR + R source. Continuous white light (50 μmol m⁻² s⁻¹) with peaks in the orange and blue regions and a secondary peak in the near infra-red region (see Casal and Sánchez, 1994 for spectrum) was provided by high-pressure sodium lamps (Son Osram, Buenos Aires, Argentina) in combination with distilled water filters. For tuber production experiments, the plants were grown immediately after planting under natural photoperiods with or without a 6 h extension with FR + R (30 μmol m⁻² s⁻¹) provided at the end of each day.

Acknowledgements

We are grateful for financial support from FONCYT (PICT 08-00115-02088), Universidad de Buenos Aires (TG59), CONICET (PID 0888), and Fundación Antorchas (A-13622/1-40).

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