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Daily Changes in the Competence for Photo- and Gravitropic Response by Potato Plantlets

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Abstract Competence for phototropic (PT) and gravitropic (GT) bending by potato plantlets grown in vitro manifests regular daily changes indicating possible involvement of circadian regulation. Unilateral stimulation of plantlets with blue light at dawn resulted in moderate PT response regarding both attained curvature and long lag phase. The PT response was the strongest between 8:00 and 12:00 h. Throughout the afternoon and in the evening, bending rate and maximal PT curvature declined significantly until 23:00 h. The GT response was fastest and strongest for plantlets stimulated early in the morning and late in the evening. During the rest of the day, GT competence did not change much apart from a minimum at 15:00. In conditions comprising either prolonged day or prolonged night, plantlets appeared to maintain rhythmicity of competence for PT and GT at least in the short-term. Introduction of a dark period prior to the tropic stimulation at 11:00 h when both PT and GT responses were strong resulted in the opposite effect: PT was depressed, and GT was enhanced. There was a time threshold of 60 min for the duration of the dark period so the plants can sense interruption in the daylight. Levels of relative expression of a *PHOT2* gene indicate rhythmic daily changes. The *PHOT2* gene was present at high levels during morning hours and late in the evening. As the mid-day and the afternoon hours approached, *PHOT2* expression decreased and reached a daily minimum at 17:00 h. We believe that our data offer strong support for the conclusion that there is an involvement of circadian rhythms in control of both PT and GT.

Keywords Potato · Phototropism · Gravitropism · Circadian rhythm

Introduction

Although considered as sessile, higher plants are well equipped to respond to environmental changes by performing movements known as tropic responses. Phototropism, as the response to unidirectional light stimulation, and gravitropism, the response to gravity stimulation, are considered main factors regulating the spatial position of the plant body. They enable plants to quickly rearrange their position to optimally utilize the incoming light.

Photo- and gravitropism are complex physiological responses occurring both in shoots and roots as a consequence of differential cell elongation. These are synchronized responses of plant organs and not of individual cells or cell groups. Tropisms are traditionally studied in dark grown, etiolated seedlings with only a limited number of studies of shoot and root responses reported for green, light-grown plants.

Recently, we described tropic responses of potato using plantlets produced in vitro from single node explants (SNE; Vinterhalter and others 2012). Under conditions of a long day 16/8 LD photoperiod, light-grown plantlets manifested vigorous movements after 2 h of tropic stimulation. During our initial studies, we noticed that competence for tropic

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response of SNE plantlets significantly varied through the day indicating possible involvement of circadian regulation. Circadian rhythms are internally driven plant responses that help plants synchronize their daily activities. They enable plants to anticipate and correctly respond to the regular daily shifts of night and day compensating seasonal changes. They also prevent plants from responding to unexpected light and temperature stimuli. The complex and sophisticated gene expression machinery underlying circadian rhythms (Más 2005) presents a significant adaptational advantage (Johnson 2001).

A major breakthrough in circadian rhythm studies in plants was made in the 1990s with development of a method enabling bioluminescence variation measurements in transgenic plants carrying a construct containing a CAB2 promoter fused to a functional firefly luciferase *Luc* gene (Millar and others 1992). This method provided a simple and accurate non-invasive technique demonstrating cycling of daily levels of the CAB2::LUC fusion protein. The method was later widely accepted in various approaches including those enabling the isolation of individual components of the circadian clock. Studies done in *Arabidopsis* pigment mutants showed a major role for cryptochrome, phytochrome (Somers and others 1998), and to a lesser extent, zeitlupe genes (Somers and others 2000) in the entrainment of the circadian clock. None of these or other studies showed direct involvement of phototropin in the clock entrainment (Millar 2003; Webb 2003) and as a consequence phototropism was not considered as a process with a possible circadian regulation. However, it should be noted that a co-action between phototropins and cryptochromes in phototropism has been reported by Whippo and Hangarter (2003).

Circadian rhythms have been studied mostly in the model plant *Arabidopsis thaliana*. Covington and others in 2008 showed that the true number of genes manifesting daily changes in mRNA expression was approximately 36 % of the total genome, which is higher than previously reported (Harmer and others 2000).From their results, it became obvious that many plant processes and responses are probably affected by circadian rhythms. According to McClung (2006), *Arabidopsis* exhibits myriad of rhythmic outputs of the clock including (1) rhythmic cotyledon and leaf movement, (2) elongation rate of abaxial and adaxial cells of the cotyledonele and leaf petiole, (3) elongation of the hypocotyl, and (4) elongation of inflorescence. Circadian regulation was also found to affect or regulate other processes like mineral nutrition and solute transport (Haydon and others 2011) or stomatal conductance and CO_2 assimilation (Dodd and others 2004). Unfortunately, the experimental approaches for many processes affected by circadian regulation like those presented here are still cumbersome and time consuming.

In trying to elucidate possible involvement of circadian regulation in the phototropic bending of potato plantlets, we focused our attention on kinetics of the bending process at various times of day and under the free running conditions comprising prolonged day or prolonged night. As supporting evidence, we also studied the relative expression of the *PHOT2* gene throughout the day looking for signs of its daily cycling shown to exist for other blue light (BL) receptors (Fankhauser and Staiger 2002).

A limitation of our initial study of potato plantlet tropisms (Vinterhalter and others 2012) was that it showed the response in a single time point, recording the data only at the end of 2 h of tropic stimulation. In the present study, we opted to investigate the kinetics of the bending process utilizing time-lapse digital photography, recording the tropic movements of individual plantlets throughout the whole period of tropic stimulation. It enabled us to get a clear insight into different phases of the bending process. Apart from measuring the maximum and bending angle after 120 min, we also calculated the lag phase of bending as time required by shoots to reach 10° of bending curvature. Thus, the main goals in this study were to document daily changes in the kinetics of the photo- and gravitropic responses of potato plantlets and investigate how the changes in light regime and free running conditions affect the observed diurnal changes of tropic competence.

Materials and Methods

Plantlet Growth and Tropic Stimulation

Shoot cultures of potato (*Solanum tuberosum* L.) cv. Desiree, confirmed by ELISA tests to be virus-free were obtained from the Agricultural Combine Belgrade (PKB). They were grown on plant growth regulator-free MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose and 0.7 % agar according to the continuous propagation procedure suggested by Hussey and Stacey (1984). Single node explants were excised from shoots avoiding the 2–3 basal and 1–2 apical nodes. Groups of six SNE explants arranged in a circle were cultured in 270 ml volume glass jars (Φ 60 × 120 mm) with 50 ml of medium and translucent polypropylene closures. Sub-culturing was done at 3–4 weeks intervals always prior to the activation of axillary buds. SNEs required 9–14 days in the growth chamber to reach the height suitable for tropic treatments. At this stage, explants had well-developed adventitious roots and were therefore referred to as plantlets.

The growth chamber from which flasks with cultures were sampled for treatments was adjusted to maintain temperature at 24.5 ± 0.5 °C and a long-day photoperiod (16 h light/8 h darkness). Light was produced by fluorescent lamps (Philips

TLD 18w) providing a fluence rate of 74 μmol/m^2 s as measured by a LiCor 1400 spectrophotometer with a Quantum sensor. The beginning of the day (dawn) in the growth chamber was fixed at 7:00 h and the end of the day (dusk) at 23:00 h. For the 13 h light/10 h darkness photoperiod, the end of the day was fixed at 20.00 h. For constant light conditions, lights were continuously turned on. Experiments were performed in 60 × 80 × 30 cm (H × L × W) black-walled cabinets (black boxes) situated in a dark room adjusted to the same temperature conditions as the growth chamber. The light-isolated growth chamber with cultures was situated in the same dark room. There was no other light (safe light) in the dark room during treatments apart from the light sources providing unilateral BL for phototropism or brief orange light used in gravitropic studies. Commercial narrow-beam and 1.2 W spot LED lamps produced by Phillips (for the emission spectral characteristics see our previous paper Vinterhalter and others 2012), equipped with a GU10 socket, were used as a sources of unilateral BL. These blue lamps provided a fluence rate of 24 μmol/m^2 s at a distance of 40–42 cm. Yellow Orange LED lamps provided less than 2 μmol/m^2 s, which was sufficient to take photographs during gravitropic stimulation. The peak of the emission spectrum was at about 580 nm for yellow LED lamps as measured by an Ocean Optics HR2000-CR UV-NIR spectrometer (data not shown).

During unilateral BL stimulation, each culture jar containing six plantlets was continuously illuminated by a single, BL LED lamp. For gravitropic stimulation, jars with SNE plantlets were turned on the side and placed horizontally (at 90°) in darkness in shallow grooves preventing jars from rolling. Jars were briefly illuminated with yellow LED lamps (6–10 s) to enable photographs to be taken.

Data Collection and Analyses

Treatments were applied to 4 culture flasks each containing 6 plantlets and were replicated 2–3 times. For both phototropism and gravitropism experiments, flasks were photographed at 3 or 4 min intervals. Close up, 3.5 Mpix large photographs were made with a Panasonic Lumix DMC-FZ28 digital camera. Unilateral BL illumination used for PT treatments was continuous whereas in the GT studies, yellow light from a mobile light source positioned lateral to cultures was briefly turned on for every photograph. Continuous illumination of cultures with yellow light used in GT studies did not induce a visible PT response. Quantitative measurements of curvature angles were done from stored digital images with the UTHSCSA Image tool for Windows 3.0 or Linux Gimp.

Graphic presentations of tropic curvatures were drawn for each shoot in the treatment. They were all aligned to zero angle at start, and their curvatures at 10 min increments, if missing, were extrapolated from graphs. Average curvatures calculated for 10 min increments were used to create the average curvature plots for each treatment. To prevent misinterpretation of data due to variability of responses at different times of day, we arbitrarily assigned the angle of 10° to be a threshold for both PT and GT. Therefore, duration of lag phase was determined as the time needed for plantlets to reach 10° of curvature. Under tropic competence or potential we consider the ability of plantlets to perform tropic bending in time. Therefore, high competence denotes treatments in which plants exhibit vigorous tropic curvatures in short periods of time. Graphs were drawn and statistics calculated using the Qtiplot for Linux software.

Quantitative Real Time PCR

Total RNA was isolated from samples using a GeneJET RNA Purification kit (Thermo Fisher Scientific, Pittsburgh, PA), according to manufacturer's instructions. Samples consisting of the upper approximately 20 mm of shoots were prepared from 10 plantlets each in order to minimize the individual plant variation in gene expression. The quantity as well as the purity of total RNA was determined by measuring optical density at 260 nm and the A260/A280 absorption ratio using NanoVue spectrophotometer (GE Healthcare, Sweden). Only the RNA samples with an A260/A280 ratio between 1.9 and 2.1 and A_{260}/A_{230} greater than 2.0 were used in the analysis. To avoid any genomic DNA (gDNA) contamination, total RNA was treated with DNase I (RNase- free) (AM2222-Ambion, Life Technologies, Carlsbad, CA). First strand cDNA synthesis (starting from 1 μg of RNA) was primed with an oligo hexamer primer using RevertAid reverse transcriptase (Thermo Fisher Scientific, Pittsburgh, PA) according to manufacturer's instructions. Primers for *PHOT2* (Table 1) were designed using Primer3 software according to tomato *PHOT2* gene (Acc. No. EU021291.1). As a reference gene *EF1α* (Acc. No. AB061263) was used (Nicot and others 2005). Polymerase chain reactions were performed in a 96-well plate with ABI Prism 7500 (Applied Biosystems, Life Technologies, Carlsbad, CA) thermal cycler, using SYBR Green to monitor dsDNA synthesis. Reactions contained 12.5 μl 2× SYBR Green Solution (Thermo Fisher Scientific, Pittsburgh, PA), 10 pmol of each primer, and 1 μl of 100-fold diluted cDNA (1.5 ng). The following standard thermal profile was used for all PCR reactions: polymerase activation (95 °C for 10 min), amplification, and quantification cycles repeated 40 times (95 °C for 1 min, 60 °C for 1 min). The efficiency of primers was determined using the standard curve method (User bulletin #2, Applied Biosystems). The specificity of the amplicons was checked by

Table 1 Primers used in quantitative real time PCR

	F primer sequence 5′–3′	R primer sequence 5′–3′
PHOT2	AGTGGGGATTGACTGTGAGG	CCTCGGATGTCCTTGTTGAT
EF1α	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA

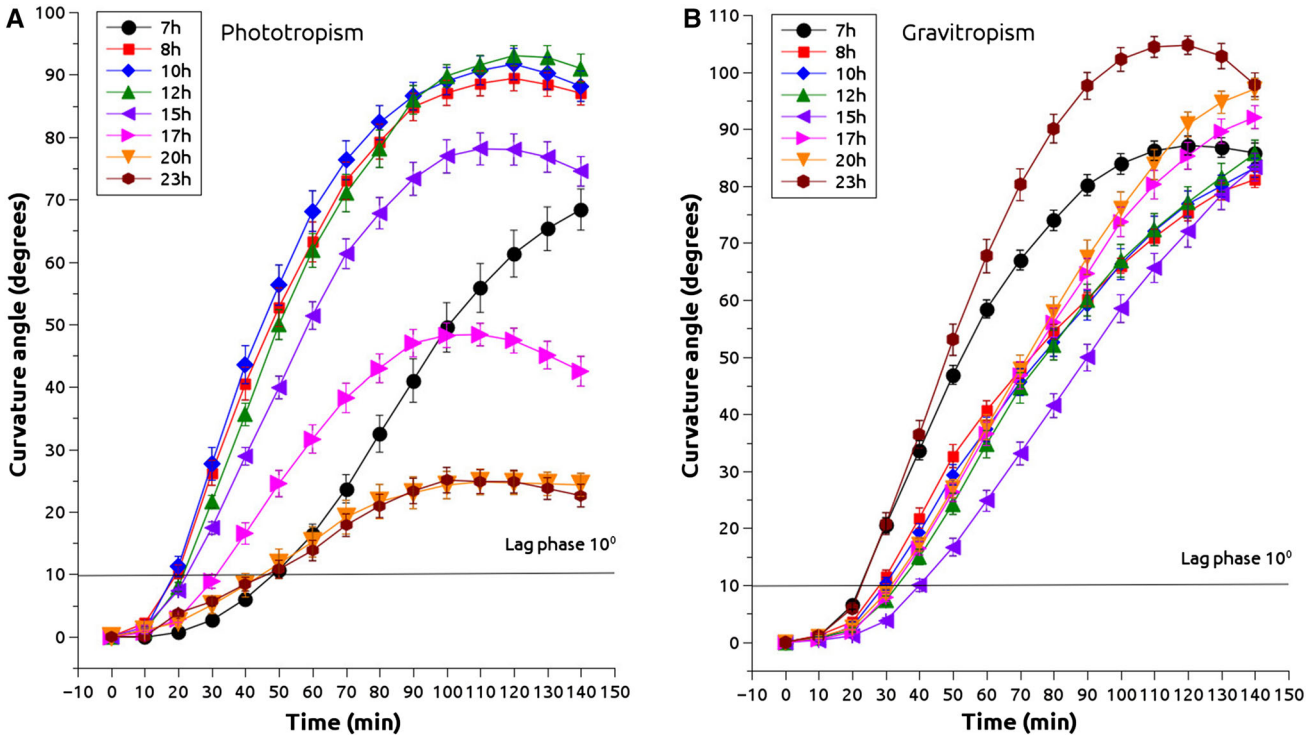

Fig. 1 Tropic curvatures of potato plantlets grown in 16 WL/8 D photoperiod at different times of day. Culture flasks each containing 6 plantlets were transferred from a growth chamber and: **a** placed in the beam of a single blue-light emitting LED lamp for PT; **b** overturned at 90° and placed in darkness for GT. The flasks were briefly (5–6 s) illuminated with yellow LED lamps to take photographs

electrophoresis in 2 % (w/v) agarose gel and a melting-curve analysis performed by the PCR machine after 40 amplification cycles (60–95 °C with one fluorescence read every 0.6 °C). All investigated qPCR products showed only single peaks and no primer-dimer peaks or artifacts. Three biological repetitions were used for the measurement, and three technical replicates were analyzed for each biological repetition. Relative expression of the *PHOT2* gene was calculated using the ddCt-comparative method (Livak and Schmittgen 2001), using etiolated plants as calibrator.

Results

Kinetics of Phototropic Response

Flasks with potato plantlets growing under 16/8 h light to darkness photoperiod were sampled at different times of day and unilaterally illuminated with BL in a dark chamber to induce a phototropic response. Kinetics for the obtained phototropic curvature at certain time points are presented in Fig. 1a. The PT response at different times of the day showed changes in the lag phase duration and slope of the curves representing the rate of PT bending. The response of plantlets at dawn (7:00 h, just before the lights were turned on) took more than 50 min to start and was moderate but much higher than at dusk (23:00 h) at the beginning of night. In the first hour of the day (7:00–8:00), the lag phase duration rapidly decreased and the magnitude of the PT curvature increased. From 8:00 to 12:00 h, the PT response was fast and plantlets reached about a 90° angle of curvature during 120 min of stimulation. Plantlets stimulated at these times had a lag phase between 18-and-22 min long. This significant morning increase was followed by an afternoon decline in the rate and magnitude of the PT response all the way until the end of day (23:00 h). The drop in PT competence was obvious already at 15:00 h

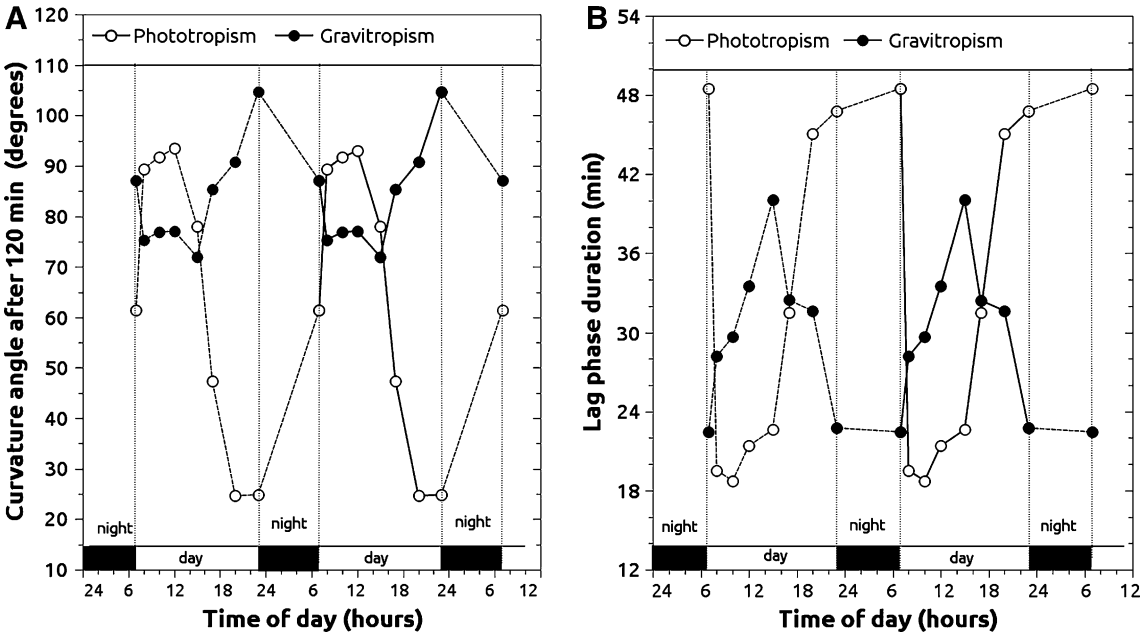

Fig. 2 Daily changes of parameters defining the PT and GT response. **a** Magnitude of tropic curvature after 120 min stimulation. **b** Duration of lag phase calculated as time to reach 10° curvature. Plants grown under 16 WL/8 D photoperiod were used in these experiments

because the plantlets stimulated at this time had a slower rate of curvature, and the maximal attained angle was 78°. From Fig. 1a, it is evident that there was a prominent change in PT competence through the day. Both the highest curvature angles and lag phase durations plotted on a graph against corresponding times of days (Fig. 2a) appeared to change in a distinct daily rhythm.

Kinetics of Gravitropic Response

For the study of GT bending kinetics, plantlets were sampled at different times of day and stimulated in darkness (Fig. 1b). The strongest GT response was recorded in plants at dusk (end of day at 23:00 h). The maximum angle attained was higher than 90° (around 105°), and the lag phase was short (22 min). Plantlets stimulated at dawn responded in a similar manner except that the maximal angle they reached was closer to a right angle (87°). Turning the light on at dawn induced a small but visible decline in the GT competence. For the plantlets stimulated at 8:00, 10:00, 12:00, and 17:00 h, duration of the lag phase was around 30 min and the maximal angle of bending between 77° and 85°. The longest lag phase of 40 min was recorded for plantlets stimulated gravitropically at 17:00 h. These plantlets reached a maximum angle of curvature of 72° (Fig. 1b). In general, the GT response was more consistent throughout the day than the PT response. When plotted on a graph against corresponding times of day, the highest curvature angles and lag phase durations for the GT responses (Fig. 2b) also appeared to follow distinct daily rhythms.

Effect of Interruption of Day with the Short Period of Darkness

Because both the PT and GT response were strongly affected at dawn when the light was turned on, we investigated how a dark pretreatment applied in the midst of the morning PT maximum (4 5 h after the beginning of day) would affect the response of potato plantlets. A 60-min-long period of darkness applied at the time of day when plants exhibit a vigorous PT response induced a drop in competence represented by a loss of sensitivity to subsequently applied light (Fig. 3a). The PT response was delayed for at least 20 min but the magnitude of curvature was unchanged after 140 min of stimulation (Fig. 3a). The introduction of a 60-min-long period of darkness at 11:00 h prior to GT stimulation produced the opposite effect to the one induced in plantlets responding to unilateral BL. The lag phase of the GT response was not prolonged, and the maximum angle of curvature was higher when compared to the response of plantlets that stayed in WL all the time (Fig. 3b). Placing plantlets into darkness for 20 min did not induce any change in either the PT or GT response.

Therefore, a 60-min long dark pretreatment delayed the PT response by prolonging the lag phase duration, but at the same time, it improved the GT response increasing the maximum curvature angle while the lag phase duration remained the same.

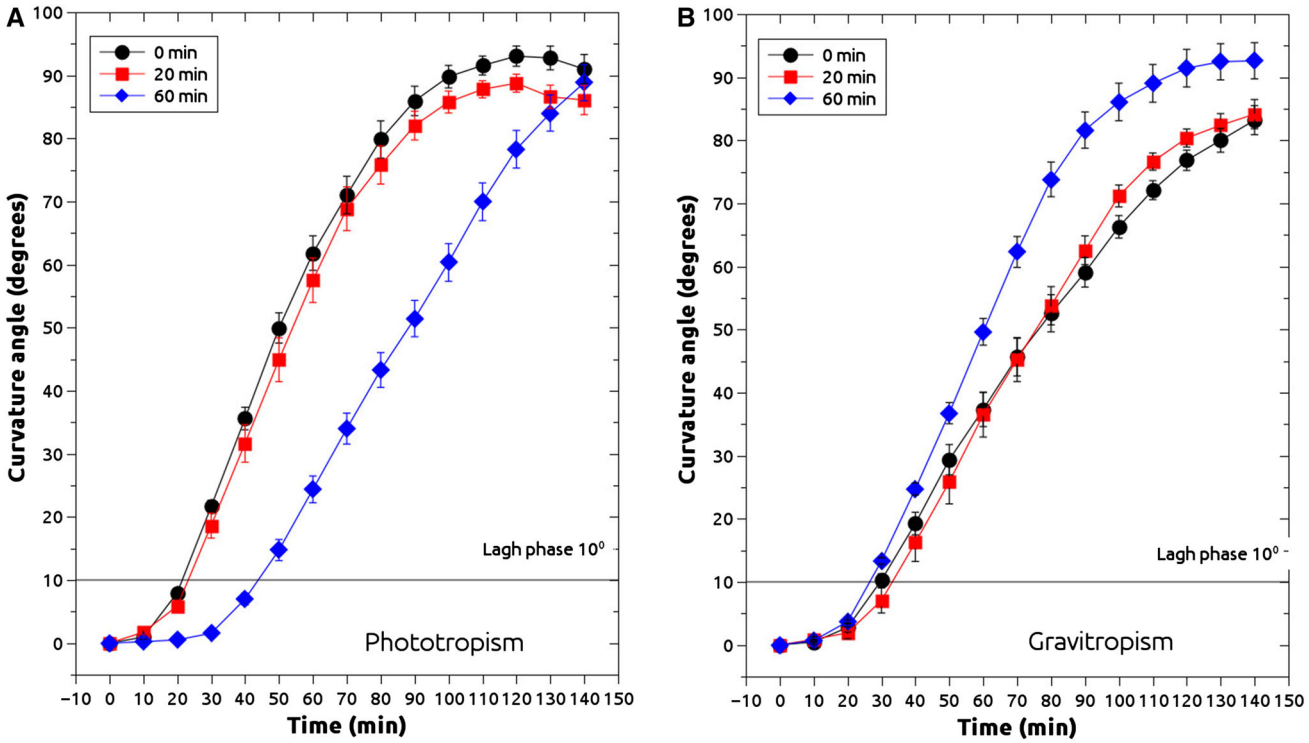

Fig. 3 Effect of 20 and 60-min-long dark pretreatments on tropic responses of plants grown under 16 WL/8 D photoperiod. **a** phototropic response. **b** Gravitropic response. Plants were placed in the dark for different period of time at 11:00 h

Prolonged Day and Night Experiments

To evaluate the possibility that daily changes in the potato tropic capacities are under circadian regulation, it would be necessary to place cultures under free-running conditions meaning continuous night and/or continuous day. Although such conditions can be easily established for material grown in vitro, they seriously affect the PT response of potato plantlets making this approach unsuitable. In continuous darkness, the PT response is absent as a fast response in etiolated plantlets (Vinterhalter and others 2012). In continuous light, the PT response maintained the magnitude similar to the one recorded in the afternoon (between 15:00 and 17:00 h) for the plants grown in 16/8 light to darkness photoperiod (Figs. 1a, 4a).

Our approach for getting a better insight into how potato plantlets grown under "free running conditions" respond to PT and GT stimulation was to prolong the length of the last day or night the plantlets were grown in the 16/8 light to darkness photoperiod prior to tropic stimulation. Like this we were able to record response trends that clearly indicated that potato plantlets anticipate the next night-to-day or day-to-night change of photoperiod.

When the duration of the last day (before treatment)was extended from 16 to 24 h, the maximum curvature of the PT response did not change much (26° vs. 30°; Fig. 4a) whereas the lag phase duration somewhat decreased. When the "day" was prolonged to 30 h, the bending rate improved and the maximum was achieved already after 70 min whereas the lag phase did not change significantly (Fig. 4a). Under conditions of 34-h-long day, the PT bending response dropped to a very low magnitude and the lag phase duration was extended to 55 min (Fig. 3a).

SNE potato explants kept continuously in light from the time of subculturing onward developed into plantlets manifesting no detectable rhythms in either PT or GT response. Their PT response (Fig. 4a) was constant regardless of the time of day, reaching a maximum curvature angle of 56° after 70 min of unilateral BL stimulation following a 22-min long lag phase. The GT response of these plantlets was constantly at the maximum (data not shown).

Plotting the highest curvature angles and lag phase durations in relation to the time of day reveals that under prolonged day plantlets anticipated the change of light regime although it actually did not occur. Thus after 30 h of continuous day at the time that corresponded to subjective morning (after the missing night) maximum curvature angle started to increase as an expected "start of new day" response whereas the lag phase duration started to decrease (Fig. 5a, c).

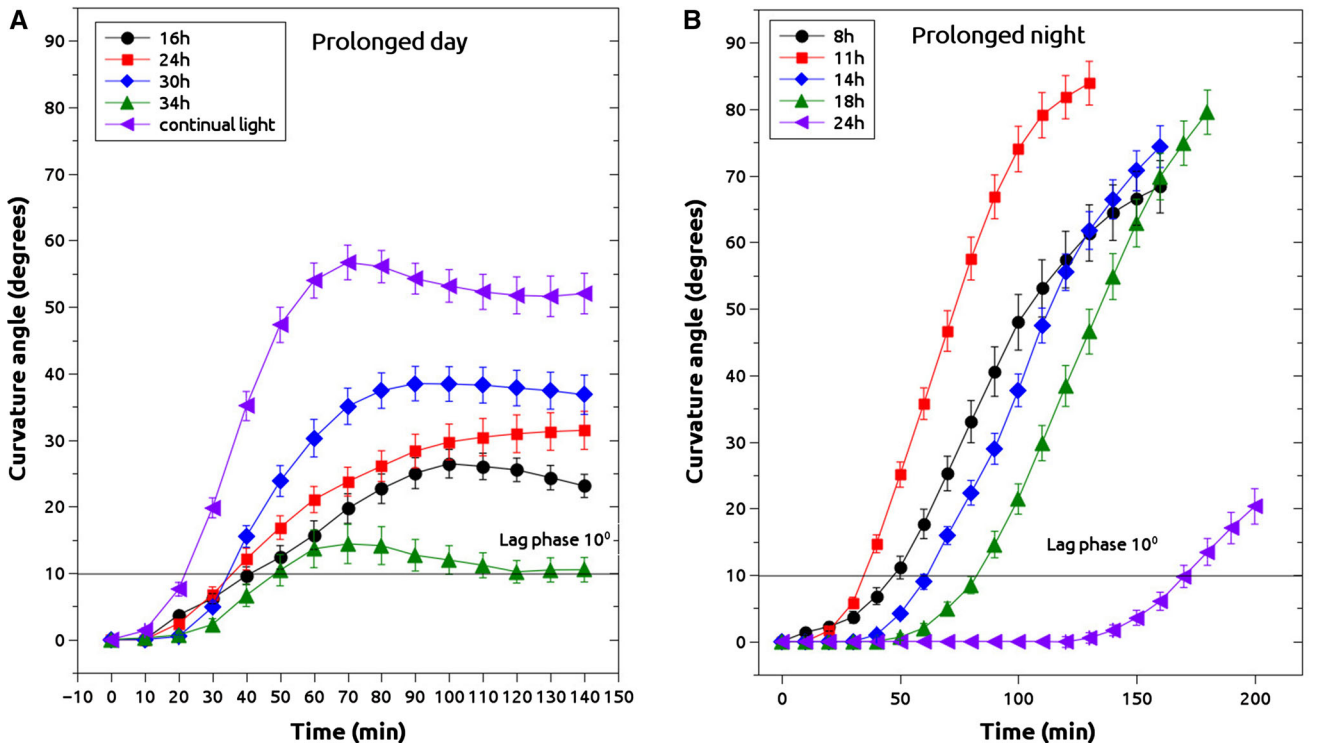

Fig. 4 Tropic response of plants in conditions of prolonged (extended) last day or night prior to tropic stimulation. Plants grown under 16 WL/8 D photoperiod were used and to create prolonged day treatments (**a**), light was not turned off at 23:00 h at the end of the last day which was extended to last 24, 30, and 34 h. The end of these light periods corresponded to 7:00, 13:00, and 17:00 h of the next day's local time, respectively. The exceptions were plants from the continuous light treatment that were grown in continuous light through the whole duration of subculture. Plants grown under 16 WL/8 D photoperiod were also used to create prolonged night treatments (**b**), by not turning the light on at 7:00 at the end of the last night which was therefore extended to last 11, 14, 18, or 24 h. The end of these dark periods corresponded to 10:00, 13.00, 17:00 and 23:00 h of the next day's local time, respectively

Prolonging the duration of night from 8 to 11 h resulted in promotion of the PT response from both aspects, providing a shorter lag phase and a higher bending rate (Fig. 4b).

The maximum PT response was still high for plantlets that experienced a night lasting 14 and 18 h but then it rapidly deteriorated. After a night lasting 24 h, plantlets reached the average maximum curvature angle of only 18°, and the lag phase duration increased to nearly 3 h.

Under prolonged night, plantlets also anticipated the change of light regime. After 11–18 h of continuous night at the time that corresponded to subjective morning, the maximum curvature angles remained high (Fig 5b). The plantlets that were incubated in 14 h of darkness, exhibited a prominent but transient decrease in PT lag phase duration (Fig 5d).

Daily Changes in PHOT2 Relative Expression Levels

Because both, significant daily changes in the PT bending capacity and the absence of this rhythm in etiolated plantlets may results from changes in levels of pigments involved in BL perception, we investigated the abundance of *PHOT2* mRNA throughout 1 day. Using the levels present in etiolated plantlets as a standard, we showed that the relative abundance of *PHOT2* mRNA changed significantly throughout the day (Fig. 6). At dawn and in the early morning hours, the level of *PHOT2* expression levels was four times higher than those recorded in etiolated plantlets. Later in the morning, the abundance of *PHOT2* mRNA decreased and reached minimal values in the afternoon hours between 13:00 and 17:00 h. Relative expression of *PHOT2* increased again later in the afternoon and continued to rise until the end of the day at 23:00 h. The *PHOT2* mRNA level in etiolated plantlets was low and was used as a reference value equaling 1.0. Even after 16–18 h of unilateral irradiation that induced a 90° curvature angle (Vinterhalter and others 2012), the estimated relative abundance of *PHOT2* mRNA was only ×1.1. The highest *PHOT2* mRNA expression levels (up to ×32 times higher than in etiolated material) were detected in plantlets under prolonged night conditions.

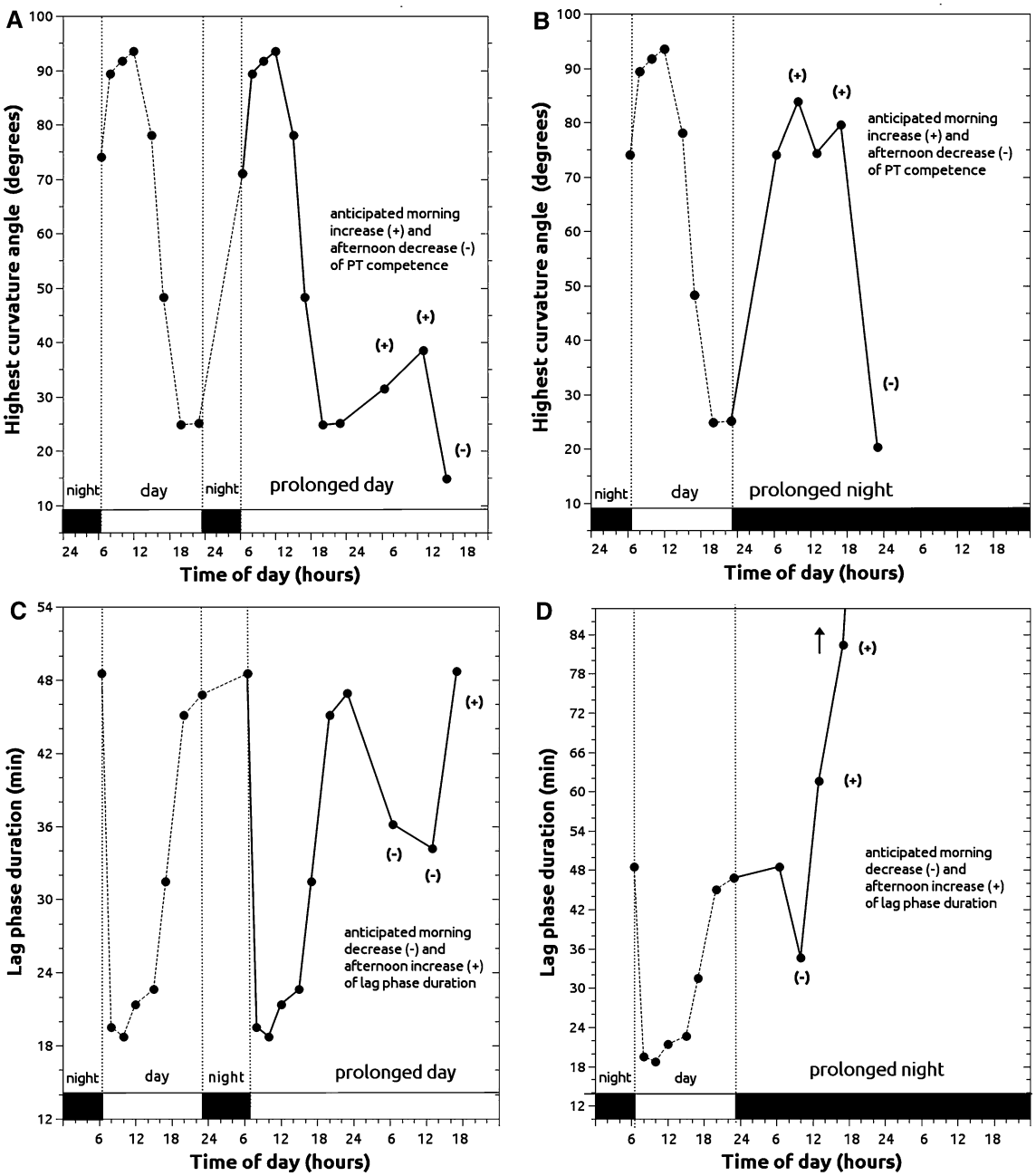

Fig. 5 Changes in maximum curvature angles (**a**, **b**) and in lag phase duration (**c**, **d**) induced by extended duration of the last day or night. These data were derived from Fig. 4. In (**d**) the value for duration of lag phase in the night extended to last 24 h was far out of the range (over 180 min) and could not be plotted

Discussion

Our results strongly support the idea that the competence of potato plantlets to perform phototropism is under the control of circadian rhythms. Major parameters that define the PT response of potato plantlets exhibited regular daily variations which persisted through conditions of prolonged day and night consistent with other plant responses that are under circadian regulation. As a result of changes in the duration of the lag phase (Figs. 1a, 2b) and the rate of bending, the maximum PT response was recorded in the period that spans over morning and early afternoon hours, and the minimum was recorded late in the evening (Figs. 1a, 2a).

On the other side, daily variability in gravitropism was far less apparent. Lag phase duration changed throughout the day to a lesser degree for plants exhibiting GT than for those responding to BL (Figs. 1b, 2b). The magnitude of

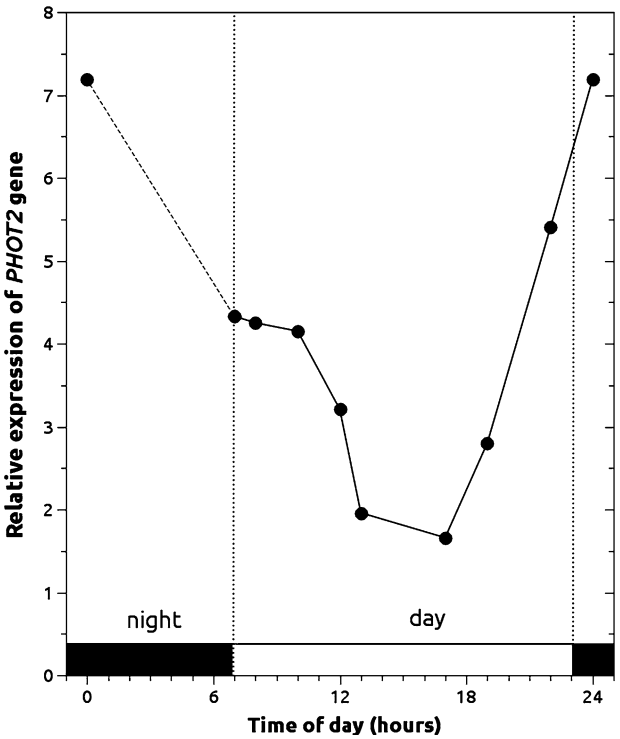

Fig. 6 Diurnal changes of *PHOT2* mRNA relative expression levels in plants grown under 16 WL/8 D photoperiod

the GT response also varied although not as much as it did for the PT response (Figs. 1b, 2a). Plots describing the duration of the lag phase and maximum curvature for PT are almost mirror images of those recorded for GT but with smaller amplitudes (Fig. 2). The absence of a large magnitude does not negate the rhythmic nature of GT which suggests that the GT response may also be under the control of circadian rhythm.

A careful look into our results describing the PT response of potato plantlets throughout the day (Fig. 1a) can lead to a suggestion that plants undergo effector adaptation on a daily basis. This type of adaptation is characterized by initial desensitization followed by recovery of sensitivity and enhancement of response (Poff and others 1994). At dawn, potato plantlets could have initially been desensitized by incoming BL. Because they needed time to regain their sensitivity, these plants exhibited a longer lag phase. Transfer of plantlets to WL at 7:00 h might have represented the beginning of period of irradiation that can induce enhancement of PT and shortening of the lag phase. When etiolated seedlings of tobacco and *Arabidopsis* were pre-irradiated with red light 2 h prior to unilateral stimulation with BL, the time threshold for a second positive phototropism was decreased four times (Janoudi and Poff 1992). Putting plantlets into darkness for 60 min at 11:00 h could have hypothetically brought them back into a highly sensitized state similar to the one they were in at dawn (Fig. 1a). After being exposed to unilateral BL at 12:00 h, plantlets went through the same steps of adaptation including desensitization, recovery of sensitivity, and enhancement of response. The most obvious outcome of this would be a much longer lag phase before onset of curvature recorded for this group of plantlets compared to those that spent no time or only 20 min in darkness. And indeed, such a response was recorded (Fig. 3a). However, this theory still fails to explain the loss of bending competence in the hours of late evening and night (Fig. 5). At those times, plantlets should not be highly sensitive to BL but would have already received an excessive amount of WL to induce the enhancement of the PT response. Instead of exhibiting vigorous PT curvature, plantlets were slow to initiate bending, and the attained angles of curvature were much lower than in preceding hours. It appears as though plantlets in the evening can be desensitized by long-term irradiation but lose the ability to induce enhancement of the PT response. This may be the consequence of changing levels of phytochrome which is known to be a major factor mediating PT enhancement (Janoudi and Poff 1992).

One of the most characteristic features of circadian rhythms distinguishing them from diurnal changes in general is that they continue under free running conditions, comprising continuous day or continuous night (Harmer and others 2000; Nozue and others 2007). For that reason, we provided conditions of prolonged continuous light or prolonged continuous darkness to cultures previously maintained for some 10–11 days in a (16 h WL/8 h dark) photoperiod and examined the PT response. Extension of "day" (light period) from 16 to 24 h and further to 30 h led to a modest increase of bending capacity in plantlets (Figs. 4a, 5a). In the 34-h-long "day," bending capacity was greatly diminished which corresponded well with results recorded for plantlets that were still under 16 h WL/8 h dark photoperiod as that time point represents 17:00 h of the "next day" (Figs. 4a, 5a, c). The increase in the PT response and shortening of the lag phase in the 24- and 30-h-long "day" (Fig. 5c) can be explained as an anticipation of the coming day after the missed night and the ability of potato plantlets to continue to exhibit entrained changes under "free-running" conditions. The PT response did not change throughout the day for the plantlets grown under continuous WL (Fig. 4a). The lag phase duration, bending rate, and the magnitude of the response were similar regardless of when the plantlets were exposed to BL. Because there was no "entrainment" period, factors mediating the PT response were not under the control of the circadian clock, and as a result bending proceeded in a similar manner at any given time, unilateral stimulation

was delivered. Interestingly, the magnitude of the PT response of plantlets grown under continuous WL was lower than in plantlets entrained by the photoperiod.

In our experiments, plantlets grown under conditions of 24-h-long night exhibited a weak PT response (Fig. 4b). Such a low response is a reaction of plants to disruption of the circadian rhythm. When plants are grown in dark for extended periods of time they become etiolated. Conditions of continuous darkness are mostly experienced by plants when they are just-germinated seedlings. Whether plants that never experienced light, or those kept in darkness for a long time, can exhibit responses that are rhythmic in nature is doubtful. It is known that etiolated hypocotyls of *Arabidopsis* seedlings entrained with as little as two (Dowson-Day and Millar 1999) or seven (Covington and Harmer 2007) L/D cycles exhibited circadian rhythmicity in elongation growth and expression of many genes, respectively. In our previous study, we have reported on the poor ability of etiolated potato plantlets to exhibit vigorous PT (Vinterhalter and others 2012). All these data suggest that PT evolved as a response of plants experiencing diurnal cycles and is strongest under such conditions regardless of the light developmental program plants may presently be in.

Incubation of plantlets in the "night" longer than 11 h resulted in incremental loss of bending competence of plantlets mostly exemplified by the progressive increase in the lag phase duration. As would be expected for a process controlled by the circadian rhythm, there was a sign of entrained changes of the lag phase that became shorter for plantlets that experienced 11-h long "night" (Figs. 4b, 5d). When the "night" was extended to be over 18-h long, the lag phase duration exceeded 120 min resulting in a collapse of the PT response. Results from these experiments further discredit the possibility that plantlets are undergoing effector adaptation on a daily basis. If this was the case, plantlets experiencing prolonged night should have been (over)sensitized and responded to BL stimulation at least as much as plantlets that were in the dark for 8 h. Maximum PT curvature of plantlets grown in prolonged night was never higher than the curvature of plants grown under a 16 h WL/8 h dark photoperiod (Figs. 4b, 5b). Also, under conditions of extended "day," the lag phase should have continued to become longer and the maximum curvature lower than in plantlets grown under a 16-h WL/8 h dark photoperiod. The opposite results were recorded in these experiments (Figs. 4a, 5c). Light is necessary to maintain periodicity of hypocotyl elongation in *Arabidopsis* seedlings (Dowson-Day and Millar 1999; Nozue and others 2007). In the case of potato plantlets, both lag phase duration and maximum PT response were changed in a way that led to the loss of periodicity even in the presence of light during the prolonged "day" experiments (Fig. 5a, c). It took only about 4–5 h longer than the duration of "regular" night for the periodicity of the lag phase for PT to be lost (Fig. 5a, c). The maximum PT response stayed at the level that would suggest maintenance of periodicity within the length of the night period that was tested. On the contrary, during free-running dark conditions, hypocotyls of *Arabidopsis* seedlings exhibited completely arrhythmic and faster than usual growth (Nozue and others 2007). Despite the variable nature of the recorded data, we believe that the results from our experiments under free-running conditions represent a strong argument for the circadian nature of daily changes in the PT competence of potato plants. Tropisms are complex processes dependent upon proper perception of external signals and redistribution of growth (Poff and others 1994). Auxins that have been implicated as major contributors to regulation of uneven growth on two sides (Estelle 1996; Liscum and Stowe-Evans 2000) of plant organs exhibiting tropic responses are also heavily controlled by circadian rhythms (Covington and Harmer 2007). During the "entrainment" period, all the systems that were involved in control of the PT response were "in phase" and as a result potato plantlets bent according to the direction of the incoming BL (Fig. 1a). However, when the duration of the "day" changed, the systems involved in mediation of bending could have fallen "out of sync" and due to differences in their periodicity and amplitude the kinetics of PT could have changed, too (Fig. 5). Because perception of gravity should not be heavily influenced by light in light-grown plantlets, the GT response depended mostly on systems controlling redistribution of growth under the "regular" (16 h WL/8 h dark) photoperiod resulting in smaller daily variability (Figs. 1b, 2).

This discrepancy in the control of different tropisms became especially obvious in the experiments where we interrupted the "day" with the period of darkness. Upon the insertion of a 60-min-long period of dark into the "day" phase, the GT response of potato plantlets barely changed, and the bending rate actually increased in comparison to the other two treatments (Fig. 3b). On the other hand, the capacity of plantlets to respond to unilateral BL was significantly altered and the lag phase duration doubled (Fig. 3a). Once plantlets regained their ability to perceive the light signal, they continued to bend at a similar rate as plantlets in the other two batches. Our results suggest that there is a time threshold for disruption of the circadian rhythm in both types of tropism, and it is between 20 and 60 min. And there is also a threshold for duration of phases within the cycle. When 4 h WL/4 h dark cycles were exerted upon *Arabidopsis* seedlings, they disregarded two out of three cycles and produced a 24 h instead of an 8 h rhythm (Nozue and others 2007). Organization of phases within the circadian rhythm of systems controlling

tropic responses of potato plantlets could be investigated further in a different study.

Our data suggest that some factors mediating PT are being synthesized (activated) and depleted (de-activated) in accordance with the circadian clock. Considering that phot2 is generally accepted as the photoreceptor responsible for the absorption of light, inducing second positive phototropism (Jarillo and others 1998) and functions in the range of fluence rates, we used herein (Sakai and others 2001), we decided to examine if its levels changed throughout the day. We are well aware that the level of transcription of *PHOT2* gene does not necessarily offer much information about the level and activity of phot2. Still, this type of data can serve as an indication of the role phot2 plays in control of the PT response. The relative abundance of *PHOT2* changed significantly during the day by slowly decreasing in the morning and declining faster in the afternoon (Fig. 6). In the evening, the level of *PHOT2* went up again. Despite the increase in transcription of *PHOT2* that took place between 17:00 and 23:00 h, the PT response did not recover. There are at least two possibilities for these results. One is that a component of the transduction chain other than the photoreceptor became more important in mediation of the PT response. The second possibility is that there was a post-transcriptional regulation of *PHOT2* expression that led to a reduced PT response. Regardless of these two possibilities, our results do point to diurnal periodicity in the change of *PHOT2* levels during the day, and circadian clocks were shown to control expression of *PHOT1* levels as well (Harmer and others 2000).

Because the GT stimulation is constant and unavoidable, it is difficult to imagine there would be some need for plants to have the GT response under circadian regulation. However, there is a clear advantage for plants to have their capacity for bending toward incoming light very high during the day and low during the night. By being able to direct their growth toward incoming light, plants can maximize their photosynthesis and further promote overall growth (Dodd and others 2005). For that reason, PT and GT responses of potato plantlets seem to be well coordinated in time so that they complement each other throughout the day (Fig. 2). Based on the long duration of the lag phase and low maximum response, it seems that by having a weak gravitropism, plants favor phototropism during the "day" when it is important to "see" where the light is coming from and grow toward it. On the contrary, during the night, the strength of the PT response fades and the GT response gets to be the prevalent tropic movement.

In conclusion, tropic responses of in vitro-produced potato plantlets appear to be under the control of circadian rhythms. Phototropism is favored by the plantlets during the day and gravitropism during the night. The time threshold for the disruption of the circadian rhythm during the "day" phase is longer than 20 min and equal to (or shorter than) 60 min.

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