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SHORT COMMUNICATION

The phytochrome B encoded by the *HLG* locus of *Nicotiana plumbaginifolia* is required for detection of photoperiod: *hlg* mutants show altered regulation of flowering and circadian movement

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Summary

The sensitivity of the *Nicotiana plumbaginifolia* wild-type and phytochrome B (*hlg*) mutant to photoperiod was investigated. Leaf number at bolting and rosette-leaf movement were measured under conditions of different light quality and photoperiod. Bolting in wild-type plants was slightly delayed under short days (SD), compared to plants grown under long days (LD). The *hlg* mutant bolted later than the wild-type under LD, but not under SD. An endogenous rhythm of rosette-leaf movement in the wild-type was present under SD, but suppressed under LD. The *hlg* mutant behaved indistinguishably from the wild-type under SD conditions, but the movements were not suppressed under LD in the mutants. The endogenous rhythm appeared to be entrained normally in the mutants under all the conditions investigated. It was concluded that the B-type phytochrome absent in the *hlg* mutant is required for the measurement of photoperiod duration in *N. plumbaginifolia*, but is not required for entrainment of the circadian clock.

Introduction

Most organisms amenable to investigation, including representatives of the plants, animals and prokaryotes, exhibit some form of endogenous rhythm. Since the day/night cycles of life on Earth are important in determining the priorities of existence for most organisms, their biological or circadian clocks have become adjusted to run with a period of roughly 24 h. However, the input of a photoreceptor is necessary in order to synchronise this clock with the environmental light / dark cycle (Dunlap, 1996). In plants, it has been known for some time that the red / far red reversible photoreceptor family, the phytochromes, are involved in this process of entrainment (Simon *et al.*, 1976).

The most obvious, and economically most important, aspect of endogenous rhythmicity in plants is the control of the time of flowering in response to photoperiod (Vince-Prue, 1975). The input of photoreceptors in this process must occur at at least two points. The endogenous oscillator must be entrained to run concurrently with the diurnal cycle, and the duration of the photoperiod and/or the dark period must be perceived by a photoreceptor, with reference to this oscillator. The phytochrome photoreceptor family is involved in the detection of photoperiod; in fact the perception of night-breaks and their effect on flowering time was one of the first phytochrome responses characterised (Borthwick *et al.*, 1952). The entrainment and floral induction responses are separate, and are both controlled by phytochrome(s) (Lumsden and Furuya, 1986; Lumsden, 1991).

The role of phytochromes in the perception of the length of a day has been studied in detail recently, thanks to the availability of mutant and transgenic plants with altered phytochrome levels. The Arabidopsis *phyA* mutant is deficient in day-extension perception, demonstrating that phytochrome A (phyA) plays a role in the perception of daylength in Arabidopsis, a long-day (LD) plant (Bagnall *et al.*, 1995; Johnson *et al.*, 1994). More recently, phyA deficient *fun* mutants of pea have been described (Weller *et al.*, 1997) which show an even greater deficiency in day-extension perception. The Arabidopsis *phyB* mutant is early flowering under all conditions, but this is not thought to be a photoperiodic effect, and is ascribed to loss of R:FR perception (Halliday *et al.*, 1994; Goto *et al.*, 1991), as the shade-avoidance response includes the acceleration of flowering (Smith, 1995). Current opinion is that multiple phytochromes control the perception of daylength and timing of flowering in Arabidopsis (Peeters and Koornneef, 1996).

Overexpression of a *PHYA* gene from *Avena* or a *PHYB* gene from Arabidopsis in day-neutral (DN) or shortday (SD) tobacco can cause increased sensitivity to night breaks (Halliday *et al.*, 1997). However, antisense ablation of *PHYB* transcript in *Solanum tuberosum* ssp. *andigena* (a species which requires SD for tuberisation) renders

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tuberisation constitutive (Jackson *et al.*, 1996). Consequently it may be that the native solanaceous phyB has a major role in the induction of responses dependent on SD, despite the fact that monocot phyA can also act in this manner when transgenically overexpressed (Halliday *et al.*, 1997).

The role of phytochromes in the entrainment of the circadian clock has also proved resistant to simple characterisation. Although entrainment has been shown to behave as a classic inductive phytochrome response (Simon *et al.*, 1976), it is also modulated by the very low fluence (VLFR) response mode of phytochrome action (Nagy *et al.*, 1993). The oscillation of *CAB* promoter activity and transcript levels has provided a means of studying the endogenous oscillator of plants from a molecular perspective (Millar and Kay, 1997). However, single phytochrome mutants appear to entrain this oscillation normally, and only the *hy1* mutant, which is deficient in phytochrome chromophore (Parks and Quail, 1991), displays the longer oscillation period expected from a mutant deficient in entrainment (Millar and Kay, 1997). Therefore, while previous work has demonstrated the importance of phytochromes in the entrainment of rhythms and the detection of photoperiod, multiple photoreceptors have been implicated in these processes. It appears that parallel responses can be mediated by multiple phytochromes, and that single photoreceptors rarely have sole responsibility for photoperiodic responses, as is the case in other aspects of higher-plant photomorphogenesis (Smith and Whitelam, 1997).

Possibly the easiest manifestation of the endogenous rhythm in plants to observe is the circadian movement of leaves. Despite being known for some time (see Sweeney, 1969), these movements are still important in tying together observations of oscillations at the molecular level with plant physiology (Carré, 1996). They are present in many species and represent a method of studying endogenous rhythms which is entirely non-invasive.

In addition to the rhythmic circadian movement of leaves, leaf movement also occurs in response to canopy shade. One of the many effects of the shade-avoidance syndrome (reviewed by Smith and Whitelam, 1997) is to cause an acute increase in the angle to the horizontal at which the leaves are held (Whitelam and Johnson, 1982). This has been observed in many species in our laboratory, including the model species *Arabidopsis thaliana* (H. Smith and G.C. Whitelam, unpublished results).

In *Nicotiana plumbaginifolia,* all the aerial parts of the rosette-stage plant display rhythmic morphological oscillations. The results presented here demonstrate that these movements display a free-running, endogenous rhythm, which is regulated in response to photoperiod.

Results

The hlg *mutants flower at a later developmental stage than the wild-type under LD, and show a reduced delay in flowering in response to SD*

The *hlg* mutants of *N. plumbaginifolia* have a mutation in one of what is probably a sub-family of two or more *PHYB*-type genes. They show no obvious phenotype at the vegetative rosette stage (Hudson *et al.*, 1997). However, observation of plants at the point of bolting suggested that *hlg* mutants bolt later than the wild-type, and develop more rosette leaves before doing so. This leads to the *hlg* mutants forming the abnormal, bushy rosettes seen in Figure 1(a), which also shows the retarded bolt development caused by the late-flowering phenotype. This phenotype includes greater leaf biomass and abnormal chlorophyll levels found in the mutants (Hudson, 1997).

Table 1 shows the mean leaf number at bolting of plants grown under 18 h light periods. The wild-type plants bolt with an average of 14 leaves (to 2 s.f.), while the *hlg* mutants bolt having developed an average of 16 leaves. This is inconsistent with the behaviour of the *phyB* mutant in Arabidopsis, which bolts with fewer rosette leaves than the wild type under LD (Bagnall *et al.*, 1995; Goto *et al.*, 1991).

Despite being for all practical purposes DN, wild-type *N. plumbaginifolia* shows a small delay in bolting in response to growth under SD conditions. The flowering response is delayed under SD by the developmental time equivalent of two rosette leaves (Table 1). This is equivalent to the extent to which flowering is delayed in the *hlg* mutants with respect to the wild-type under LD (Table 1). The response of the *hlg-1* mutant to SD in terms of flowering time is negligible (Table 1). It follows that the response of flowering time to photoperiod in *N. plumbaginifolia* appears to be eliminated in the absence of the phyB encoded by the *HLG* locus.

Leaf movement in N. plumbaginifolia *maintains a free-running rhythm. This movement displays an altered amplitude in the hlg-1 mutant after a 12 h light/12 h dark entrainment*

The movement of leaves in plants previously grown under entraining light/dark cycles was followed under continuous illumination by timed-interval photography. Rosette stage plants were assayed in this way for endogenous leaf-movement rhythms after entrainment for two weeks in 12 h fluorescent light periods in a 24 h cycle. The consequent oscillation in leaf angle can be seen in Figure 1(b). The mean-leaf-angle measurements are presented in Figure 2. Leaves of the wild-type plants display a small oscillation in their angle, which seems to be damped relatively rapidly

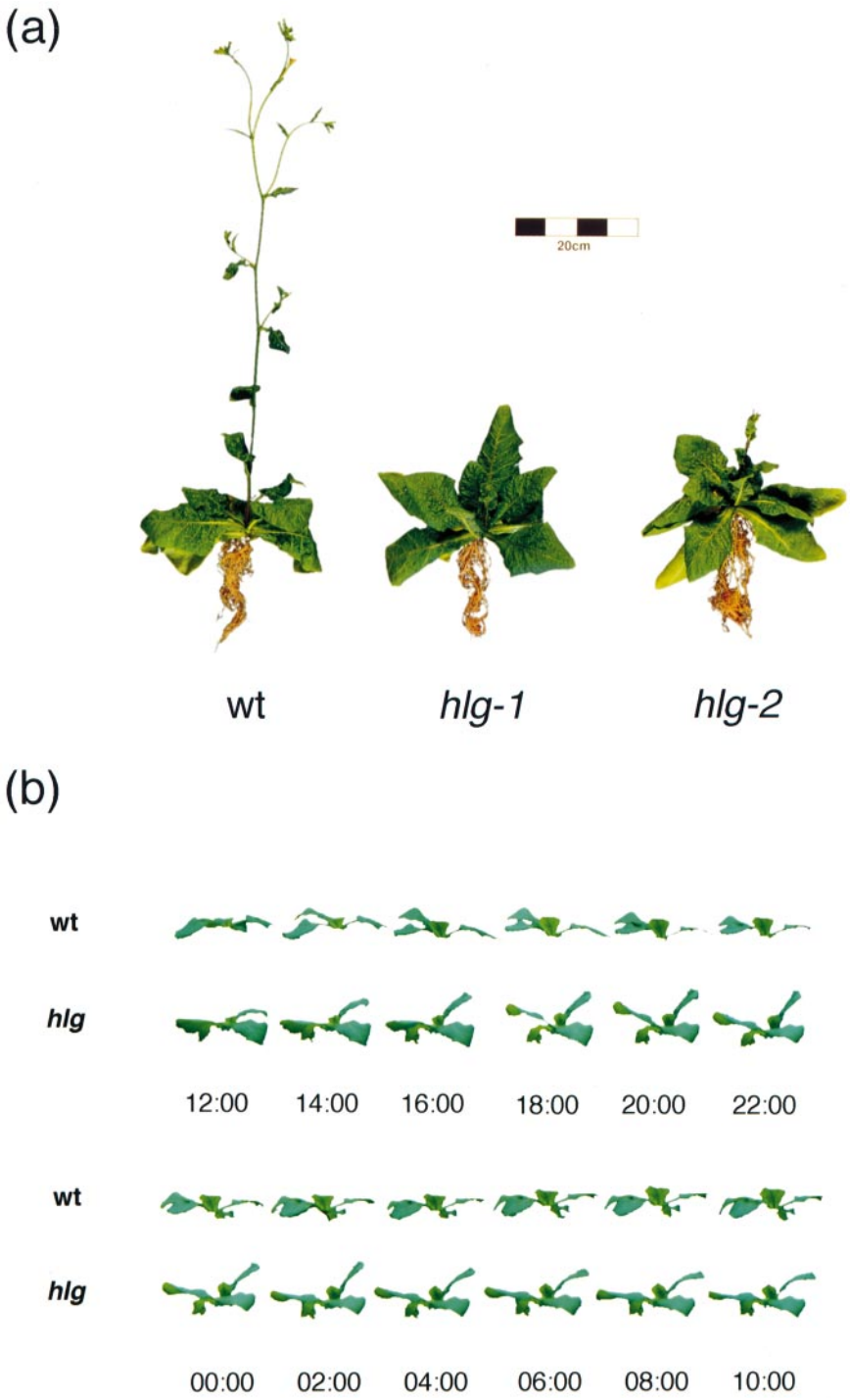

Figure 1. (a) Phenotype of *N. plumbaginifolia* wild-type and *hlg* mutants at 9 weeks after germination, grown in high R:FR W with an 18 h light period. Plants were typical of a large population, and show the increased leaf number and late bolting characteristic of the *hlg-1* and *hlg-2* mutants (centre and right) compared to the wild-type (left).
(b) Rhythmic movements of the leaves of *hlg*-mutant and wild-type plants over a 24 h period. Plants were entrained under a 12 h light / 12 h dark regime to an age of 5 weeks. The leaf movements were photographed under subsequent 24 h illumination in white light.

Table 1. The number of rosette leaves at bolting of *N. plumbaginifolia* wild-type and *hlg* mutants grown under different photoperiods of simulated daylight. Photoperiods were either 8 h of light followed by 16 h of darkness (SD) or 18 h of light followed by 6 h of darkness (LD)

	wt	*hlg*-1	*hlg*-2
Leaf no. at bolting, LD (± SE)	13.8 (±0.20), n=29	16.1 (±0.24), n=30	16.5 (±0.22), n=28
Leaf no. at bolting, SD (± SE)	15.8 (±0.47), n=20	16.9 (±0.19), n=18	not done

under continuous illumination. Leaves of the *hlg-1* mutant, however, display a strong rhythmic oscillation in angle, with a period of between 24 and 26 h.

Rhythmic leaf-angle movements are distinct from the acute alteration in leaf angle in response to R:FR

Leaf movement rhythms were also examined in wild-type and *hlg*-mutant plants entrained as described above, but under a 12 h light period of low R:FR. The shade-avoidance response (Smith, 1995; Smith and Whitelam, 1997) can induce leaf-angle alterations in response to lowered R:FR (Whitelam and Johnson, 1982). Since some *phyB* mutants display altered sensitivity to R:FR, and show some aspects of shade-avoidance under high R:FR conditions (Smith and Whitelam, 1997), it is feasible that the altered leaf angle phenotype observed in *hlg* is caused by shade-avoidance rather than effects on endogenous rhythms. However, when the leaf angles of plants entrained under low R:FR cycles are monitored, no rhythmic oscillation is displayed, and the mutants and the wild-type plants behave indistinguishably (Figure 2). The leaf-angle to the horizontal of plants entrained under W + FR is much higher than those entrained under W (Figure 2). It seems likely, therefore, that the shade-avoidance response of leaf angle overwhelms rhythmic movements, which are no longer seen in plants entrained under these conditions.

The free-running oscillation of leaf angle is induced in the wild-type by SD entrainment conditions

Wild-type plants entrained under fluorescent light periods of 8 h, with a subsequent 16 h dark period, show a marked free-running oscillation in leaf angle (Figure 2). This is in contrast to the minimal leaf movement observed when the wild-type plants are entrained under 12 h light/12 h dark conditions. It is therefore likely that the duration of the light period has an effect on the amplitude of leaf movements in the wild-type, as shade-avoidance does not cause rhythmic leaf movement. The phase and amplitude of the rhythm appear identical in the mutant and wild-type plants under 8 h light periods. The mean overall leaf angle is slightly higher in the mutants than in the wild type plants. In contrast to the situation in plants entrained in 12 h light/12 h dark conditions, the *hlg-1* mutant and wild-type plants display an identical amplitude and period of oscillation when entrained under an 8 h light period (Figure 2). There is consequently a response to light period duration in the wild-type plants which is absent in the *hlg-1* mutant. The effects of this response must be relatively slow, as the rhythm displayed by wild-type plants entrained in 8 h light periods is clearly maintained throughout the 72 h of continuous light at the end of the experiment.

Discussion

The wild-type *Nicotiana plumbaginifolia* used in these experiments is for practical purposes day-neutral. However, when examined sufficiently closely, many apparently day-neutral plants respond to photoperiod to some extent. While the flowering response may be unaffected by day-length in many species, responses such as cold acclimation, changes in bud dormancy and tuberisation may be induced by changes in photoperiod, particularly by SD (Vince-Prue, 1994). Also, slight alteration in the internode number or leaf number at bolting or flowering may be observed in plants that are outwardly day neutral (e.g. DN tobacco varieties as described by Halliday *et al.*, 1997).

Such sensitivity to photoperiod is apparent in the wild-type *N. plumbaginifolia.* The flowering time, in terms of rosette-leaf number at bolting, is slightly delayed under SD (Table 1). While no day-extension or night-break analysis was performed to confirm that this was not a photosynthetic effect, the W irradiance given to the plants under SD was sufficient to make this unlikely. The cabinets necessary for these growth conditions could not, unfortunately, deliver low-irradiance day extensions.

The *hlg* mutants bolt after forming more leaves than the wild-type plants under LD (Table 1, Figure 1). In other words, the *hlg* mutant is late flowering in LD. This is in direct contrast with the phenotype of the Arabidopsis *phyB* mutant, which is early flowering in LD (Bagnall *et al.*, 1995; Goto *et al.*, 1991). The bolting of the *hlg* mutant plants was not significantly delayed under SD conditions compared to LD conditions, unlike that of the wild-type plants. It is therefore concluded that the phyB absent in the *hlg* mutant is required for the sensitivity of bolting time to photoperiod.

The angle at which *N. plumbaginifolia* leaves are held is controlled by a free-running endogenous rhythm. The rhythm is obvious when wild-type plants are conditioned by entrainment under short (8 h) light periods, but is suppressed when entrainment conditions involve a 12 h

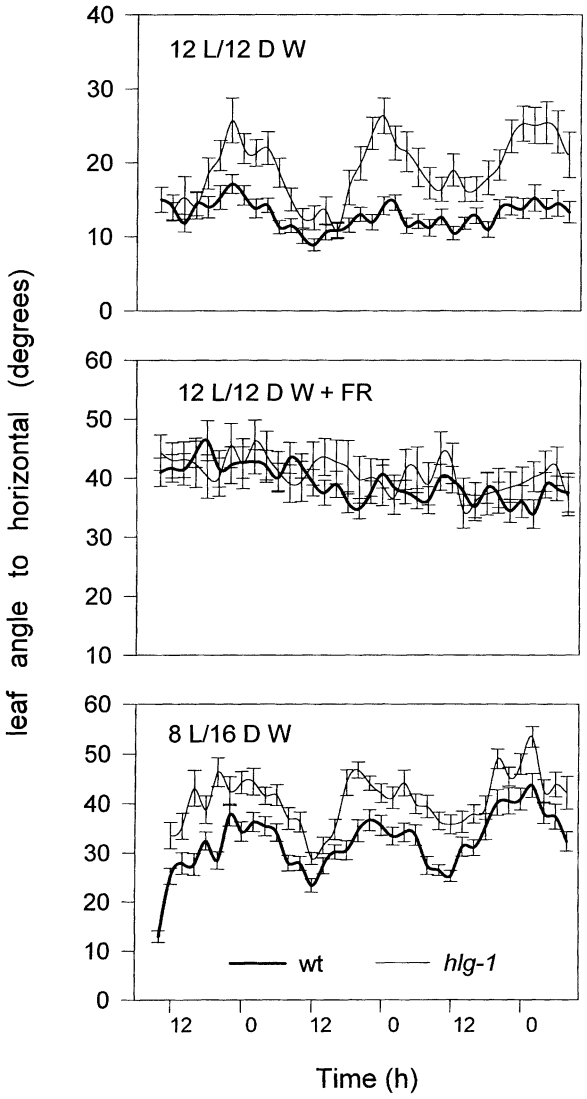

Figure 2. (Top) Changes in leaf angle to the horizontal in *N. plumbaginifolia* wild-type and the *hlg-1* mutant entrained in a 12 h light period in a 24 h cycle. (Centre) Changes in leaf angle to the horizontal in *N. plumbaginifolia* wild-type and the *hlg-1* mutant entrained in a 12 h light period of low R:FR. (Bottom) Changes in leaf angle to the horizontal in *N. plumbaginifolia* wild-type and the *hlg-1* mutant entrained in an 8 h light period.
Plants were 5–6 weeks-old and entrained for at least 2 weeks under fluorescent W/ dark cycles or W + FR / dark cycles of 24 h as described. Measurements were conducted under constant fluorescent illumination over a 72 h period. Each data point represents the mean of at least 20 leaf angles, from at least 10 individuals. Error bars represent the standard error of the mean. The 'time' axis represents 2 hourly measurements during continuous illumination, which were begun immediately after the plants were removed from entraining light/dark cycles. The 12:00 and 0:00 points represent the 'virtual' midday and midnight points which would be synchronous with the entraining zeitgeber had the treatment been continued.

light period. There is no evidence that the entrainment of the circadian oscillator itself is affected by alteration in photoperiod; rather, the coupling of this rhythm to the movement of the leaves occurs much more strongly in SD. It therefore appears that leaf movements of large amplitude are only seen in the wild-types under SD, in the same manner as that of the flowering response of a SD plant, or the SD-dependent tuberisation response of *Solanum andigena* (Jackson *et al.*, 1996).

The phytochrome-B deficient *hlg-1* mutant lacks sensitivity of the leaf movement rhythm to photoperiod, which is observed in the wild-type plants. The leaf oscillations of *hlg-1* under LD or SD are analogous to those that occur in the wild-type when it is entrained under SD. Hence the SD-dependent leaf movements of the wild-type are constitutively displayed in the mutant. The SD-dependent tuberisation response in *Solanum tuberosum* ssp. *andigena* also becomes constitutive when antisense RNA is used to reduce the level of native phyB (Jackson *et al.*, 1996). The most likely interpretation is that the condition of the wild-type plants under SD entrainment is the default pathway, and that the measurement of photoperiod is disrupted in the mutant. In the absence of the phyB mutated in *hlg*, the leaf movements of *N. plumbaginifolia* are synchronised with each other and with the light/dark cycle, but their amplitude is not suppressed under LD. Consequently, in the *hlg* mutant, the oscillator is normally entrained by the photoperiod. This implies that another photoreceptor is responsible for the entrainment of the leaf-movement rhythm, but that phyB is required in order for the endogenous oscillator to be used to determine daylength.

The acute increase in leaf angle to the horizontal in both the wild-type and mutant plants grown under simulated canopy-shade conditions must be mediated by one or more phytochrome(s) (see Smith, 1995; Smith and Whitelam, 1997). However, the absence of the *HLG* encoded phyB seems not to affect this response in *N. plumbaginifolia* (Figure 2). It seems likely that the phyB absent from the *hlg* mutant is not required for this response, as for other shade-avoidance responses in *N. plumbaginifolia* (Hudson *et al.*, 1997). However, the decrease in R:FR would be expected to affect phyB photoequilibrium as well as those of other phytochromes, reducing the phyB Pfr level and presumably triggering the rhythmic movements seen in wild-type plants grown under SD. The fact that no oscillation is seen in the plants grown in W+FR conditions (Figure 2) makes it likely that the rhythmic movement displayed under SD by the wild-type is suppressed under shade-avoidance conditions. This may be explained by the leaf-angle attained by shade-avoiding plants being a maximum, and no movement consequently being possible. For this to be the case, the shade-avoidance response must be in some way 'dominant' over the rhythmic leaf-movement response.

In conclusion, a functional copy of the *PHYB* gene described by Hudson *et al.* (1997) is required for *N. plumbaginifolia* to regulate both bolting time and leaf movement in response to daylength. With reference to the role ascribed to the homologous phyB in *Solanum tuberosum* ssp. *andigena*

(Jackson *et al.*, 1996), this finding provides evidence for a role for phyB in daylength perception, which may be conserved within the Solanaceae. As this paper underwent the review process, findings of a similar nature were reported by Finlayson *et al.* (1998), who found that the amplitude of ethylene production rhythms in *Sorghum* is greatly increased in *phyB* mutants, relative to the wild-types, under 12 h entrainment conditions. Although the flowering responses and the effect of FR-enriched light on the rhythm were different to those described here for *N. plumbaginifolia*, the results with *Sorghum* show that phyB acts to control the amplitude of circadian rhythms even in distantly related plant species.

Experimental procedures

Growth of adult plants in controlled environments and defined light conditions

Nicotiana plumbaginifolia seeds were germinated using gibberellic acid (GA_3, Sigma, Poole, Dorset, UK) to break dormancy. Seeds were incubated in 500 μg ml^{-1} GA_3 for 1 h before washing with three changes of deionised water. They were then surface dried on filter paper before being sown onto compost. Seedlings were germinated under identical conditions in white light (W) on compost in a greenhouse. Plants for bolting experiments were grown under SD (8 h) or LD (18 h) of simulated daylight of at least 250 μmol m^{-2} s^{-1}, provided by HQI metal halide bulbs. The number of rosette leaves when the bolt reached a height of 1 cm was counted. The cabinets capable of delivering W together with far-red light (FR) were described in detail by Keiller and Smith (1989). The W irradiance was kept at 150 μmol m^{-2} s^{-1} in both cabinets, which were maintained at a constant 22°C. The W only cabinet had a red:far red photon-fluence-rate ratio (R:FR) of 4.31 and the W + FR cabinet had a R:FR of 0.17.

Entrainment and analysis of endogenous rhythms

Seeds of *N. plumbaginifolia* were treated for 1 h in 500 μg cm^{-3} GA_3 to induce germination. They were then grown on compost under continuous fluorescent illumination (150 μmol m^{-2} s^{-1}) at 22°C. Once a rosette was formed, the seedlings were transferred to entrainment conditions of different photoperiods under a controlled temperature of 22°C. After 2–4 weeks of entrainment, the plants were placed under continuous (10 μmol m^{-2} s^{-1}) cool-white fluorescent illumination in a room kept at 22°C ± 2°C. The plants were arranged in front of a black background with the largest two healthy leaves (usually the second pair of true leaves) at 90° to the viewing axis. A Nikon F-801 35 mm SLR camera fitted with a 60 mm Micro-Nikkor lens, loaded with Fuji Provia colour transparency film and mounted on a tripod, was used to record the angle of the leaves to the horizontal every 2 h. The exposures were triggered by an MF-21 multi-control back (Nikon), which also recorded the exact time of each exposure on the film for future reference. The transparencies were then projected onto paper and the midribs of the leaves placed at 90° to the viewing axis traced, along with the lip of the pot (as a horizontal reference). The angles of two leaves from at least 10 different plants were then used to calculate the mean and standard error of the leaf angle to the horizontal.

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