Hormonal influence on photocontrol of the protandry in the genus *Helianthus*

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Abstract

Under natural photoperiodic conditions protandry in hermaphrodite disc flowers of sunflower (*Helianthus annuus* L.) is determined by the different elongation rates of the style and filaments. The elongation of the filament and style starts simultaneously after the daily dark period, but the style growth rate is slower. When plants close to anthesis are exposed to continuous white light (WL) a loss of protandry occurs: the filaments do not grow far enough to extrude the anthers from the corolla. The histological analyses show that the number of filament epidermal cells remains unaltered after organ elongation and that cells respond to photoperiod only by cell expansion. Emasculation does not substantially inhibit filament cell expansion, whereas isolation of the filament or stamen from the corolla suggests that this organ could be the perception site of the filament growth stimulus. In vitro treatments with auxin (indole-3-acetic acid, IAA or α-naphthaleneacetic acid, NAA) reverses the inhibition of cell expansion caused by continuous WL, whereas gibberellic acid (GA₃) at high concentrations reproduces the effect of continuous WL. Experiments carried out on various *Helianthus* spp. show that all these plants have evolved the same photo- and hormonal-control of the protandry. In experiments in which the light treatments were continued for 24 h, the auxins drastically reduced the inhibiting effect of red light (R) and dichromatic treatments FR (far red) + R, whereas GA₃ repressed filament extension regardless of light quality. As far as auxins are concerned, the response of sunflower filaments does not appear to be connected with the polar transport of the hormone. Moreover, the promoting effect of darkness is not mediated by an increase of endogenous free IAA in disc flowers. However, sunflower filaments manifested a similar temporal pattern of response to the light/dark cycle and to auxin.

Key words: Anthesis, auxins, gibberellic acid, filament, protandry.

Introduction

Mechanisms that prevent selfing and its harmful effects (Jarne and Charlesworth, 1993) and promote more proficient pollen dispersal, have shaped much of floral evolution (Harder and Barrett, 1996). The allogamy in the genus *Helianthus* is favoured by the particular mechanism of anthesis in which anthers shed pollen before the stigma of the same flower is ready to receive it. In *Helianthus* spp. anthesis in the disc flowers starts in the early morning, when the anther tube is extruded by the elongation of the filaments. The stigma appears at the top of the anther tube in the late afternoon of the same day and by the following morning it has fully emerged with the receptive surface exposed (Knowles, 1978).

Light may be an important environmental factor for the growth and development of the flower as well as for its inception (Batschauer, 1998; Thomas, 1999). Plants monitor the intensity, quality, direction, and duration of light, and modulate their development to maximize the acquisition of energy for photosynthesis and to synchronize reproductive development (Chory, 1997; Batschauer, 1998). The light-dependent responses of plants require the combined action of several photoreceptors as phyto-

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chrones (Quail et al., 1995), cryptochromes and UV-B receptors (Briggs and Huala, 1999). Among these, the photoperiodic induction of flowering has dramatic importance for plants and the involvement of photochromes and cryptochromes has been elucidated (Thomas, 1999). In sunflower, it has been demonstrated that inhibition of filament elongation under continuous white light (WL) is controlled by the phytochrome system (Baroncelli et al., 1990).

Many of the developmental processes that occur as a result of light signals are dependent, at least in part, on the action of the phytohormones (Behringer and Davies, 1992; Barker-Bridgers et al., 1998; Jensen et al., 1998). The suggestion that plant hormones might serve as a secondary messenger in the signal response cascade is supported by the fact that many light-regulated plant growth responses involve changes in hormone level or can be mimicked by hormone application (Kraepiel and Miginiac, 1997; Chory and Li, 1997; Hedden, 1999). Light-dependent alterations in auxin physiology, including changes in auxin levels (Iino, 1982; Jones et al., 1991; Behringer and Davies, 1992), transport (Shinkle et al., 1998) and in the amount of auxin-binding proteins (Walton and Ray, 1981; Jones et al., 1991) have been reported. Moreover, mutants in the light signal transduction role, on tms2, in which the gene encodes the indoleacetic amide hydrolase that catalyses the conversion of biologically inactive auxin amides into active auxins (Karlin-Neumann et al., 1995), have been reported here. A histological analysis has been conducted on epidermal cells of filaments which demonstrate that many light-regulated plant growth responses involve changes in hormone level or can be mimicked by hormone application (Kraepiel and Miginiac, 1997; Chory and Li, 1997; Hedden, 1999). Light-dependent alterations in auxin physiology, including changes in auxin levels (Iino, 1982; Jones et al., 1991; Behringer and Davies, 1992), transport (Shinkle et al., 1998) and in the amount of auxin-binding proteins (Walton and Ray, 1981; Jones et al., 1991) have been reported. Moreover, mutants in the light signal transduction pathway have been selected using the tms2 transgenic Arabidopsis, in which the tms2 gene encodes the indoleacetic amide hydrolase that catalyses the conversion of biologically inactive auxin amides into active auxins (Karlin-Neumann et al., 1995). In addition, an examination of Nicotiana plumbaginifolia phytochrome mutants showed that they have higher levels of free indole-3-acetic acid (IAA) than the wild type (Kraepiel et al., 1995), while phytochrome-deficient mutants of Arabidopsis thaliana require gibberellins (GAs) to express the elongated phenotype of these plants (Peng and Harberd, 1997). There is also evidence that the activity of GA3_3-β-hydroxylase in the le mutant of pea (Campbell and Bonner, 1986), cowpea (García-Martínez et al., 1987) and in seed of Grand Rapid lettuce (Toyomatsu et al., 1998) is regulated by phytochrome.

In several genera elongation of filament and style appears to be regulated by growth substances (Greyson and Tepfer, 1966, 1967; Koevenig, 1973; Koning, 1983a, b; Koning and Raab, 1987; Kiss and Koning, 1990; Fei and Sawhney, 1999). In Gaillardia (Compositae) the role of auxins in controlling filament elongation involves stimulation of ethylene biosynthesis and acid growth (Koning, 1983a). In Arabidopsis the rapid filament growth, which occurs during the anthesis, appears to be genetically controlled by the gene MS33 whose product either regulates temporal biosynthesis of growth substances (IAA and GAs) or makes the filament tissue sensitive to hormones, thereby enhancing the signalling pathway in cell elongation (Fei and Sawhney, 1999).

Although hormonal regulation of floral development and anthesis has been much studied (Parish and King, 1985; Levy and Dean, 1998), knowledge concerning the interactions between growth regulators and light signals on the growth of filament and style is scarce. A study on the anthesis of disc flowers in the genus Helianthus which revealed that photoperiod regulates protandry by inducing a different elongation speed of style and filaments is reported here. A histological analysis has been conducted on epidermal cells of filaments which demonstrate that the number of cells remains unaltered during organ elongation and that filaments respond to photoperiod only by cellular expansion. Since in some species it has been reported that sepals, petals and anthers influence filament growth (Koevenig, 1973; Raab and Koning, 1988; Fei and Sawhney, 1999), the effect of disc flower organs on filament elongation has been analyzed. In experiments conducted in vitro, it is also shown that the WL- and R-induced inhibition of filament elongation was reversed by exogenous treatments with auxins, while gibberellic acid (GA3) inhibited the anthesis regardless of all light conditions. In addition, it is shown that the endogenous levels of free IAA remained unchanged during the daily light/dark cycle. Finally, the fundamental role, on filament elongation, of a temporal pattern of competence that cannot be shifted by light or hormone treatments is discussed.

Materials and methods

Plant material and growing conditions

Two pure lines (EF and CM) of sunflower (Helianthus annuus L.) provided by the Agricultural Department of Plant Biology, University of Pisa and several wild species of the genus Helianthus (H. tuberosus L., H. neglectus Heiser, H. petiolaris Nuttal ssp. fallax Heiser, H. occidentalis Riddell, H. praecox Engleman and Gray ssp. hirtus Heiser, H. praecox Engleman and Gray ssp. ranunculoides Heiser, H. simulans Watson, H. debilis Nuttal ssp. debilis Nuttal, H. argophyllus Torrey and Gray) provided by the North Central Regional Plant Introduction Station, Ames, IA (USA), were used as starting material. Achenes were germinated in Petri dishes on distilled water and after 2 d were transferred to 20 cm diameter pots containing Mannaflor S compost (Manna Laives, Bolzano, Italy) plus an initial dose of complete fertilizer (Osmocote 14–14–14, Sierra Ltd, England). Plants were grown in a growth chamber with a light schedule of 16 h of WL at 22 ± 1 °C. In several experiments, 2–3 d before anthesis of the first whorl of disc flowers, the plants were transferred to continuous WL. The WL was obtained from Philips TLM 40 W/33 RS fluorescent tubes (Philips, Eindhoven, The Netherlands) with a photon fluence rate of 100 μmol m⁻² s⁻¹.

In vivo filament growth

In sunflower, the fixed number of whorls of disc flowers that daily open on the head, permits ready discrimination between flowers in anthesis after one dark period (Stage II) and disc flowers of more internal whorls, neighbouring to whorls of Stage II, that will open after two light/dark cycles (Stage I).
To determine the time-course of the filament and style elongation, 10 disc flowers (Stage II) were harvested at 30 min intervals, starting 12 h before the onset of anthesis, from plants grown in continuous WL (WL24) and from plants grown under 16 h white light/8 h dark (D) cycles (WL16/D8). Filament and style length was measured each time. In each flower the stamens were removed with the aid of microscapelips and were placed in a drop of water on a microscope slide. The anthers were removed and then a cover slip was placed over the filament. Slight pressure was applied to the cover slip to remove trapped air and to make the epidermal cells more visible. Filament lengths were measured with an eyepiece micrometer in a Wild Makroscop M420 (Wild Heerbrugg Ltd, Heerbrugg, Switzerland) inverted microscope. Lengths of epidermal cells in several rows were measured separately with an eyepiece micrometer Periplan 10 × MESS (Leitz, Germany) in a Leica DM-RB microscope. To assess the uniformity of elongation, the length of cells was plotted as a function of position along the length of the filament as described by Koning (1983a). To assay the influence of the anther on in vivo filament elongation, closed disc flowers (Stage II) were gently opened and emasculated before the dark period. The percentage of seed set in plants grown in WL16/D8 cycles or in continuous WL during the anthesis, was calculated as the ratio between seeds and the total number of disc flowers. In all experiments conducted in vivo filament elongation was measured 24 h after the beginning of the experimental treatments, each plant being randomly assigned to the different treatments. Experiments were repeated three times and the results are presented as means ± standard deviation (SD).

In vitro filament growth

To evaluate the influence of the flower’s organs on filament elongation complete disc flowers, disc flowers without ovary, disc flowers without ovary and anthers, anther plus filament, corollas plus filaments, and isolated filaments were excised from flowers in Stage II and cultured in Petri dishes (60 × 15 mm) containing solidified (8 g l−1 Bactoagar, Oxoid Ltd, Basingstoke, UK) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without hormones. Complete disc flowers, disc flowers without ovary, disc flowers without ovary and anthers and corolla plus filaments were placed in a vertical position on the MS medium, while anthers plus filaments and isolated filaments were placed horizontally on the same medium. In some experiments more internally positioned disc flowers (Stage I) were also used. In all experiments conducted to study the interaction between growth regulators and light signals, disc flowers deprived of the ovary were used. From 15–25 disc flowers in each Petri dish (60 × 15 mm) were placed in a vertical position on the MS medium supplemented with different concentrations of auxin (IAA and NAA), GA3, or inhibitors of auxin polar transport (TIBA, 2,3,5-triodo-2-benzoic acid and NPA; N-(napth-1-yl) phthalamic acid). The Petri dishes were not covered and were transferred to a growth chamber at 22 ± 1 °C, under different light regimes, as reported in the tables and figures.

In vitro light treatments

White light was obtained from Philips TLM 40 W/33 RS fluorescent tubes as above. Standard irradiation fluence rate was about 100 μmol m−2 s−1 (phytochrome photoequilibrium (φ) = 0.70; Baroncelli et al., 1990). Red light was produced by filtering the light from Philips TL 40 W/15 fluorescent tubes through a 3 mm PG 501 filter (IMPLA S.p.A.); the fluence rate was 34 μmol m−2 s−1 (φ = 0.80). Far-red light (FR) was produced by using Philips (Philips) 120 W 230–240 incandescent tubes filtered through PG 501/3 mm (IMPLA S.p.A.) and KG 3/2 mm (Schott & Gen, Mainz, Germany) filters, the photon fluence rate being 19.5 μmol m−2 s−1 (φ = 0.03). A dichromatic treatment was performed by adding R at a photon fluence rate of 34 μmol m−2 s−1 to standard FR 19.5 μmol m−2 s−1, thus establishing a φ higher than 60%. The spectral emission curves and the spectral fluence rate were measured by a Li-Cor 1800 spectroradiometer. Measurements of filament length were made after 12 h or 24 h of growth and presented as means (± SD) of three independent experiments with 4–5 replications (Petri dishes).

IAA analysis

Disc flowers at Stage II of the inbred line EF of sunflower were collected at different times in a light/dark cycle (WL16/D8) or continuous white light (WL24) as shown in Fig. 7. The equivalent 0.5 g fresh weight of freeze-dried disc flowers were extracted in 65% isopropanol (v/v) with 0.02 M imidazole buffer at pH 7 to which [1H]-IAA as a radiotracer and [13C6]-IAA (JD Cohen, USDA-ARS, Beltsville, Maryland, USA), as an internal standard for quantitative mass-spectral analysis, were added. After overnight isotope equilibration, the analysis of free IAA was performed according to Chen et al. (Chen et al., 1988). IAA was purified using a Beckman System Gold HPLC with UV detector (Varian UV 50) equipped with a C18 Partisphere column (Whatman, 110 × 5 mm i.d.) and the samples were eluted at 1 ml min−1. Far-red light (FR) was produced by a Philips TL UV 30 W/5 fluorescent tube filtered through a 3 mm PG 501 filter. The spectral fluence rate was measured by a Li-Cor 1800 spectroradiometer. Measurements of filament length were made after 12 h or 24 h of growth and presented as means (± SD) of three independent experiments with 4–5 replications (Petri dishes).

Statistical methods

The data were treated using analysis of variance procedures and means were separated using Tukey’s test (P = 0.05). Homogeneity of variances was evaluated by Bartlett’s test (P = 0.05). Differences between means of Tables 1 and 5 were tested using the Student t-test (P = 0.05); for data of Table 1 statistical analysis was performed after arcsin transformation of the percentages of seed set. In addition, regression analysis was used for experiments shown in Figs 4 and 6.

Results

In vivo filament elongation

The inflorescence of the genus Helianthus is a capitulum or head, characteristic of the Compositae family. The flowers of the outer whorl of the head (ray or ligulate flowers) generally have five elongated petals united to form straplike structures and are restricted to the periphery of the radiate head. The disc flowers, arrayed in arcs radiating from the centre of the head into distinct left and right turning spiral rows, are monocious with

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both male (staminate) and female (pistillate) organs. Each disc flower is surrounded by a sharp-pointed, chaffy bract and consists of a basal inferior ovary, two pappus scales, and a tubular corolla. Five introrse anthers are united to form a cylinder around the style while the separate filaments are attached to the base of the corolla tubes. Inside the anther tube is the style which terminates in a divided stigma whose receptive surfaces are in close contact during the bud stage.

When sunflower plants are grown in a growth chamber under WL16/D8 cycles, a whorl of one to four rows of disc flowers opens daily, as under natural conditions. The time-course of filament and style elongation is shown in Figs 1 and 2. The filament elongation begins immediately after the dark period and lasts 6 h. About 80% of the distension is completed after 2–3 h from the beginning of the light period (Fig. 1). Immediately afterwards, pollen is discharged inside the anther tube. The style elongation starts at the same moment (Fig. 1), but with a slower elongation rate and longer duration (16–20 h): thus the two hairy stigma branches are able to remove the pollen grains from the anther tube (Fig. 2). In plants placed under continuous WL the filament elongation is drastically inhibited, while the growth of the style is only delayed (Fig. 1). In such a way, the style pushes the stigma through the anther tube but the two hairy stigma branches run along only the apical end of the anther tube (Fig. 2). Since much of the pollen remains inside the anther tube it is not available for pollination. In fact, as shown in Table 1 the percentage of seed set in sunflower plants grown under continuous WL, is much reduced (11.40%) when compared with those of plants grown under photoperiodic condition (58.26%).

The histological analysis indicated that it is only the distension of the basal and central epidermal cells that determines the final length of the filament (Fig. 3). Indeed the 15 cells at the tip of the filament did not undergo elongation (Fig. 3). Moreover, when the filament growth was completed (FL), the number (65 ± 2) of epidermal cells remained constant in each longitudinal row.

The inhibition of filament elongation induced by continuous WL is not limited to sunflower. In fact, in several Helianthus spp., in plants grown under continuous WL, a significant reduction of filament growth, with respect to plants grown in light/dark cycle (WL16/D8) was observed (Table 2).

**Influence of disc floral organs on filament elongation**

To evaluate the influence of the anther on filament elongation, the in vivo filament growth of emasculated
Fig. 3. The length of epidermal cells as a function of their position along the sunflower filaments before (IL: Initial Length) and after (FL: Final Length) filament elongation. The cells were counted from the tip to the base of the filament to designate the cell position. Ten filaments with 10–12 epidermal rows each were counted for determination of IL and FL. The data were pooled but to simplify the figure the standard deviations (SD) are not reported.

Table 2. Effect of different light treatments (WL16/D8 or WL24) on in vivo filament length (mm) in several species of the genus Helianthus

All plants were grown in photoperiod (WL16/D8) until 2–4 d before anthesis. The data are means ± SD of three independent experiments with 50–60 replicates (filaments) each. Statistical evaluation was done separately for each species; values within lines followed by the same letter are not significantly different at the 0.05 probability level according to Tukey’s test.

<table>
<thead>
<tr>
<th>Species</th>
<th>IL*</th>
<th>FL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WL16/D8</td>
<td>WL24</td>
</tr>
<tr>
<td>H. tuberosus</td>
<td>1.50±0.06 b</td>
<td>4.58±0.12 a</td>
</tr>
<tr>
<td>H. neglectus</td>
<td>1.80±0.06 c</td>
<td>5.66±0.10 a</td>
</tr>
<tr>
<td>H. petiolaris ssp. fallax</td>
<td>1.08±0.16 b</td>
<td>3.44±0.25 a</td>
</tr>
<tr>
<td>H. occidentalis</td>
<td>1.14±0.05 b</td>
<td>3.62±0.08 a</td>
</tr>
<tr>
<td>H. praeox ssp. hirtus</td>
<td>1.16±0.05 b</td>
<td>3.62±0.07 a</td>
</tr>
<tr>
<td>H. praeox ssp. runyonii</td>
<td>1.30±0.06 c</td>
<td>4.46±0.08 a</td>
</tr>
<tr>
<td>H. simulans</td>
<td>1.58±0.07 b</td>
<td>4.54±0.05 a</td>
</tr>
<tr>
<td>H. debilis ssp. debilis</td>
<td>1.02±0.07 b</td>
<td>3.01±0.06 a</td>
</tr>
<tr>
<td>H. argophyllus</td>
<td>1.32±0.04 c</td>
<td>4.18±0.08 a</td>
</tr>
</tbody>
</table>

*IL (Initial Length), length of the filament (mm) before the treatment; FL (Final Length), length of the filament (mm) after the treatment.

Table 3. Effect of the emasculation on in vivo filament length (mm) of sunflower (Helianthus annuus L.)

The plants were grown in photoperiodic (WL16/D8) conditions. The data are means ± SD of three independent experiments with 50 replicates (filaments). Different letters indicate statistically different values (P = 0.05) according to Tukey’s test.

<table>
<thead>
<tr>
<th>IL*</th>
<th>FL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc flowers with anthers</td>
<td>Disc flowers without anthers</td>
</tr>
<tr>
<td>1.39±0.09 c</td>
<td>3.86±0.17 a</td>
</tr>
<tr>
<td>3.29±0.22 b</td>
<td></td>
</tr>
</tbody>
</table>

*IL (Initial Length), length of the filament (mm) before the treatment; FL (Final Length), length of the filament (mm) after the treatment.

The effect of growth regulators on filament elongation was studied in vitro, under light/dark cycle (WL16/D8) or in continuous white light (WL24) using disc flowers at Stage II, deprived of the ovary.

The inhibitory effect of the continuous white light was reversed by the auxins IAA and NAA (Fig. 4A) in a concentration-dependent fashion (regressions were: y = 2.5099–0.3035x + 0.1598x² for IAA, R² = 0.991 and y = 2.3745 + 0.58932x – 0.0400x² for NAA, R² = 0.81). However, in contrast to IAA, NAA at 10⁻³ M showed a significantly minor promotive effect compared to lower concentration (e.g. 10⁻⁴ M; Fig. 4A). In continuous white light (WL24) the filament elongation is not influenced by exogenous treatments with GA₃ (Fig. 4A).

Under light/dark cycle, low concentrations of GA₃ (<10⁻⁶ M) did not significantly affect filament elongation (Fig. 4B); by contrast a strong reduction of filament growth was observed at concentrations higher than 10⁻⁶ M (regression was y = 4.857–0.1105x – 0.099x², R² = 0.92). The two auxins (IAA and NAA) showed only a marginal effect at the highest concentrations. The response to GA₃ was reverted if IAA was added to the culture medium (Table 5).

In all Helianthus spp. analysed, IAA, under continuous WL that per se hinders filament elongation, showed a promoting effect as observed in sunflower (Table 6).

Exposure to WL (photostationary state: (φ = 0.70), and R (φ = 0.80)), inhibited expansion of the filament cells (Fig. 5), whereas in disc flowers transferred to FR (φ = 0.03) or darkness (D) for 24 h the filaments elongated normally (Fig. 5). Dichromatic light R + FR (φ = 0.60) prevented the filament from lengthening similarly to R (φ = 0.80). Both the auxins (IAA and NAA) drastically reduced the inhibitory effect of R or of dichromatic
The experiments were conducted in vitro on MS basal medium. The data are means ± SD of three independent experiments with 40–50 replicates (filaments); values within lines followed by the same letter are not significantly different at the 0.05 probability level according to Tukey’s test.

<table>
<thead>
<tr>
<th>Light treatments</th>
<th>Explicts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O+C+S+A+F*</td>
</tr>
<tr>
<td>WL24</td>
<td>1.92 ± 0.10 b</td>
</tr>
<tr>
<td>WL16/D8</td>
<td>4.78 ± 0.21 a</td>
</tr>
</tbody>
</table>

*O, ovary; C, corolla; S, style; A, anther; F, filament.

In continuous WL, filaments of disc flowers treated in vitro with auxins (Fig. 6) showed an elongation trend analogous to filaments of plants grown in vivo under photoperiodic condition (Figs 1, 2). In fact, the filaments treated with IAA and NAA, after a lag period of about 4 d before anthesis, the data are means ± SD of three independent experiments with 50 replicates (filaments); values within lines followed by the same letter are not significantly different at the 0.05 probability level according to Tukey’s test.

### Table 6. Effect of IAA (10⁻³ M) on filament length (mm) in several species of the genus Helianthus grown in continuous white light during the anthesis

All plants were grown in photoperiod (WL16/D8) until 2–4 d before anthesis. The data are means ± SD of three independent experiments with 50 replicates (filaments); values within lines followed by the same letter are not significantly different at the 0.05 probability level according to Tukey’s test.

<table>
<thead>
<tr>
<th>Species</th>
<th>Filament length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS (Control)</td>
</tr>
<tr>
<td>H. tuberosus</td>
<td>1.80 ± 0.22 b</td>
</tr>
<tr>
<td>H. neglectus</td>
<td>1.80 ± 0.25 b</td>
</tr>
<tr>
<td>H. petiolaris ssp. fallax</td>
<td>1.31 ± 0.16 b</td>
</tr>
<tr>
<td>H. occidentalis</td>
<td>1.43 ± 0.18 b</td>
</tr>
<tr>
<td>H. praepax ssp. hirtus</td>
<td>1.27 ± 0.15 b</td>
</tr>
<tr>
<td>H. praepax ssp. runyonii</td>
<td>1.40 ± 0.14 b</td>
</tr>
<tr>
<td>H. simulans</td>
<td>2.43 ± 0.49 b</td>
</tr>
<tr>
<td>H. debilis ssp. debilis</td>
<td>1.15 ± 0.21 b</td>
</tr>
<tr>
<td>H. argophyllus</td>
<td>2.03 ± 0.20 b</td>
</tr>
</tbody>
</table>
To investigate whether IAA analysis by an increase in endogenous auxin, the levels of free IAA in disc flowers (Stage II) were analysed during the various stages of anthesis in a light/dark cycle (WL16/D8) and in continuous white light (WL24). Modest changes were observed in the endogenous level of the hormone and only two slight rises of IAA concentration were observed under photoperiodic conditions, prior to the dark period and when the filaments were almost completely elongated (Fig. 7).

**IAA analysis**

To investigate whether filament elongation was induced by an increase in endogenous auxin, the levels of free IAA in disc flowers (Stage II) were analysed during the various stages of anthesis in a light/dark cycle (WL16/D8) and in continuous white light (WL24). Modest changes were observed in the endogenous level of the hormone and only two slight rises of IAA concentration were observed under photoperiodic conditions, prior to the dark period and when the filaments were almost completely elongated (Fig. 7).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Filament length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS (Control)</td>
<td>4.48 ± 0.37 ab</td>
</tr>
<tr>
<td>TIBA 10⁻⁴ M</td>
<td>4.25 ± 0.60 ab</td>
</tr>
<tr>
<td>3 × 10⁻⁴ M</td>
<td>3.41 ± 0.45 b</td>
</tr>
<tr>
<td>NPA 10⁻³ M</td>
<td>4.67 ± 0.31 a</td>
</tr>
<tr>
<td>3 × 10⁻⁴ M</td>
<td>4.88 ± 0.26 a</td>
</tr>
</tbody>
</table>

**Table 7. Effect of auxin transport inhibitors (TIBA and NPA) on filament length (mm) of sunflower (*Helianthus annuus* L.)**

The experiment was conducted in photoperiodic conditions (WL16/D8). The concentrations are expressed in molarity. The data are means ± SD of three independent experiments with 50 replicates (filaments); values followed by the same letter are not significantly different at the 0.05 probability level according to Tukey’s test.

**Discussion**

**In vivo filament elongation**

In sunflower (*Helianthus annuus* L.) light, through the action of the photoreceptor phytochrome (Baroncelli et al., 1990), regulates one of the mechanisms that favours cross-pollination: the different lengthening speed of style and filament in the disc flowers. In natural photoperiodic conditions flowering of disc flowers starts at dawn, with

**Table 8. Effect of the disc flower stage on filament length (mm) of sunflower (*Helianthus annuus* L.), under photoperiodic (WL16/D8) or continuous white light (WL24) conditions**

The experiments were conducted in media without growth regulators (MS) or supplemented with IAA (10⁻³ M). The data are means ± SD of three independent experiments with 50 replicates (filaments); values within lines followed by the same letter are not significantly different at the 0.05 probability level according to Tukey’s test.

<table>
<thead>
<tr>
<th>Light treatments</th>
<th>Disc flowers: Stage I</th>
<th>Disc flowers: Stage II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS        IAA</td>
<td>MS        IAA</td>
</tr>
<tr>
<td>WL24</td>
<td>1.73 ± 0.06 c 2.06 ± 0.07 b</td>
<td>1.97 ± 0.07 b 3.98 ± 0.20 a</td>
</tr>
<tr>
<td>WL16/D8</td>
<td>1.77 ± 0.06 c 2.20 ± 0.20 b</td>
<td>3.81 ± 0.22 a 4.04 ± 0.21 a</td>
</tr>
</tbody>
</table>

**Temporal patterns of response of the filament to light or hormonal signals**

In contrast to the behaviour of disc flowers at Stage II, in which the filaments elongate on MS medium under inductive photoperiod (WL16/D8) or when IAA was present under continuous white light (WL24), the filaments of the disc flowers at Stage I did not elongate under a light/dark cycle, even in the presence of high auxin concentration (10⁻³ M) in the medium (Table 8). The same result was obtained with NAA (data not shown).
the filaments rapidly lengthening and protruding from the corolla after 3–5 h (Figs 1, 2). As observed in other plants (Greyson and Tepfer, 1966; Koevenig, 1973; Koning, 1983a; Fei and Sawhney, 1999), histological analyses showed that the number of filament epidermal cells remains unaltered during organ elongation and cells respond to photoperiod only by cell expansion.

As shown in Cleome hassleriana (Koevenig, 1973) and Arabidopsis (Fei and Sawhney, 1999), the removal of sunflower anthers inhibits filament cell expansion. Greyson and Tepfer reported that anther removal from Nigella hispanica flower buds reduced filament elongation to 60% of normal length whereas emasculated filaments of sunflower manifested only a 15% reduction in growth compared to the intact control disc flowers (Table 3) (Greyson and Tepfer, 1967). On the contrary, a strong inhibition of elongation in filaments or stamens in disc flowers deprived of the corolla was observed (Table 4), suggesting that this organ could be the perception site of the filament growth stimulus. Nevertheless, it cannot be excluded that reduced growth caused by emasculation or removal of the corolla could be due to wound-induced ethylene production. In Ipomoea nil filament, growth was restored by suppressing ethylene production from the cut corolla tissues (Kiss and Koning, 1990).

The crucial role of the light signal in the regulation of protandry is evident when plants of sunflower, near to anthesis, are exposed to continuous WL. In fact under this light condition, it was observed that filaments did not grow long enough to extrude the anthers from the corolla; as a consequence the stigma, pushed through the anther tube by the style, only collects a small amount of pollen, thus inhibiting pollination and reducing the seed production (Table 1). Experiments carried out on various species of Helianthus show that the same kind of response to light is present in all plants analysed. This behaviour presumably reflects a evolutionary adaptation of Helianthus progenitor(s) to favour allogamy and to escape the deleterious effect of inbreeding.

**Interaction between hormones and light signals**

In many species, filament growth appears to be regulated by growth substances (reviewed in Greyson, 1994). These results showed that in Helianthus spp. auxins (IAA and NAA) stimulated filament elongation by reverting the anther removal from disc flowers. Moreover, GA3 also had an inhibitory effect on style growth irrespective of light conditions (data not shown), showing a more general influence on the whole process of anthesis in Helianthus. In Sorghum bicolor, the concentration of GAs shows diurnal rhythms with a peak concentration near midday and minimum concentration at night (Foster and Morgan, 1995). The need for a dark period for filament elongation (Fig. 1) and the inhibition of filament extension due to GA3 (Figs 4B, 5) or constant WL or R (Figs 1, 5) could suggest that different light conditions influence filament growth by altering GAs metabolism.

Following the pioneer experiment by Miller (1958), it has long been speculated that light is one of the environmental factors that act on plant physiology affecting its hormonal status (Kraepiel and Miginiac, 1997; Chory and Li, 1997). However, although current knowledge of the photoreceptor signalling pathway has recently improved (Frohnmeyer, 1999), the intermediate steps between light reception and hormone action are still unclear. These results obtained in vitro confirm the importance of the phytochrome system in filament growth and demonstrate that auxins can reduce the inhibiting effect of R or dichromatic treatments FR + R on cell expansion of the filament (Fig. 5). These results are consistent with the hypothesis of an IAA involvement in the light regulation of elongation. Iino demonstrated a strong inhibitory effect of R on IAA biosynthesis in maize coleoptile associated with a decrease in mesocotyl elongation rate (Iino, 1982). Moreover, it is known that light induces the biosynthesis of several plant growth inhibitors (Muir and Zhu, 1983). In particular, it has been shown that light-grown sunflower seedlings contain a higher level of 8-epixanthatin than the dark control (Yokotani-Tomita et al., 1997). 8-Epixanthatin plays an important role in the phototropic response and in the light-induced growth of sunflower hypocotyls by suppressing auxin action (Yokotani-Tomita et al., 1997, 1999).

In this study’s experiments exogenous auxins (IAA and NAA) induced, even under continuous WL, the same distension kinetic shown in vivo under photoperiodic condition (Figs 1, 6). This finding, like those for Cleome (Koevenig, 1973), Citrus (Goldschmidt and Huberman, 1974), Lilium (Hess and Morrè, 1978), and Gaillardia (Koning, 1983a), could indicate that auxin is required for filament growth in vivo. In addition, in several species, it has been shown that the content of natural auxin changes in parallel with flower opening stages (Ilahi, 1979; Koning, 1983a). For example, the level of free auxin in Gaillardia disc flowers increased 10-fold just before anthesis and filament elongation (Koning, 1983a). In sunflower, however, although throughout anthesis some differences in the endogenous levels of free IAA were observed, the results obtained do not indicate that they are linked to the daily light/dark cycle and therefore rule out the hypothesis that the promoting effect of darkness is mediated by an increase in free IAA.

The light-induced changes in polar transport of endogenous or applied auxins are consistent with the rapid changes in organ elongation (Jones et al., 1991; Behringer...
It is noteworthy that sunflower filaments manifest the same temporal pattern of response either to light dark/cycle and to auxin (Table 8). Numerous photomorphogenic responses have been described that have light-sensitive and -insensitive phases. For example, leaf movements are photoregulated, but the response depends upon the time during a diurnal cycle at which the light stimulus is given (Satter and Galston, 1981). The observation that filaments of flowers at Stage I did not elongate either under the inductive light/dark cycle, or in the presence of high auxin concentration in the medium (Table 8), indicates that the starting point is determined endogenously and cannot be shifted by light signals or auxins and suggests that both dark/light cycle and auxin supply require the same critical phase of the flower’s sensitivity to express their effect on filament elongation.

In conclusion, evidence is provided here that protandry, in hermaphrodite flowers of Helianthus, is established by the different lengthening speed of style and filaments in response to photoperiodic treatments. In particular, it has been shown that filament elongation was determined only by cellular extension and that the growth stimulus required by this process appears to be perceived by the corolla. Furthermore, it has been demonstrated that auxins and gibberellins altered the photocontrol of protandry exerting an opposite effect on filament extension. However, since the promoting effect of darkness is not mediated by an increase of endogenous free IAA in disc flowers, the general question on how the light signals interact with hormonal metabolism remains unanswered. The ability of IAA to revert the inhibitory effect of GA₃ (Table 5) could suggest the involvement of a critical balance between growth inhibitors and promoters. To evaluate this hypothesis the analysis, in disc flowers of sunflower, of the endogenous levels of bioactive GAs and inhibitors of auxin activity (e.g. 8-epi-yanthathin) will be necessary.

References


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