Involvement of phytochrome in regulation of transpiration: red-/far red-induced responses in the chlorophyll-deficient mutant of pea


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Abstract. Transpiration rhythmicity and intensity were investigated in the chlorophyll-deficient mutant XL18 of Pisum sativum L. and in the phytochrome-deficient mutant aurea of Lycopersicon esculentum Mill. A custom-built psychrometer was used. In the XL18 mutant an acute transpiration response to monochromatic irradiation was observed such that red (R) light increased and far-red (FR) decreased transpiration rate, with equal rates of change. This result indicates that phytochrome is involved in regulation of transpiration. In wild type pea the chlorophyll-dependent component of transpiration was also shown to involve phytochrome. Monochromatic irradiation by red or far-red light induced an increase in transpiration with acceleration dependent on time of day. The response was irreversible by light of either wavelength. We conclude that both photoreceptors are involved in the acute response. Investigation of the daily course of transpiration revealed rhythmic changes in wild type pea and tomato under natural light conditions and in constant darkness. The rhythm was not apparent in the XL18 mutant in constant darkness, or in the aurea mutant under natural illumination. The latter results show that phytochrome, as a photoreceptor, is essential for maintaining the rhythm upon irradiation, while the photosynthetic component is crucial in darkness.

Keywords: chlorophyll deficiency, circadian rhythm, cryptochrome, Lycopersicon esculentum, mutant, pea, Pisum sativum, photoreceptors, phytochrome, stomata, tomato, transpiration, water relations.

Introduction

Transpiration is the process of water loss, which occurs constantly in plants and depends on water vapour pressure difference between the leaf interior and the external air. Changes in transpiration rate are attributed to guard cell movement, although the extent to which stomata control transpiration depends on the leaf water vapour boundary layer. (Weyers and Meidner 1990; Aphalo and Jarvis 1993; Meinzer et al. 1997).

Two photoreceptors directly promote the reduction of osmotic potential in guard cells, resulting in water influx and stomatal opening (Sharkey and Ogawa 1987). The first is a blue light (B) photoreceptor, which induces starch degradation at lower fluence rates and K⁺ uptake at higher fluences (Assman et al. 1985; Tallman and Zeiger 1988). The second is chlorophyll, which provides the photosynthetic production of sugars and depletion of CO₂ in internal leaf spaces. (Sharkey and Rashkey 1981; Shimazaki et al. 1982; Gotow et al. 1988).

Possible involvement of phytochrome, the third photoreceptor, in stomatal movement is discussed in several publications (Roth-Bejerano and Itai 1981, 1987; Nejidat et al. 1989). In these papers stomatal aperture of Commelina communis L. was measured upon monochromatic irradiation. Far-red light given after red light accelerated stomatal closure in darkness. Addition of far-red light to red light reduced the initial rate of opening and the final aperture. These authors also mentioned phytochrome-like effects on Mg, K–ATPase activity and K⁺ fluxes in guard cells of Commelina. Recently reduction of transpiration intensity upon red light irradiation was shown for the phytochrome-A-deficient mutant phyA-103 of Arabidopsis thaliana (Eckert and Kaldenhoff 2000). In contrast, guard cells of white leaves of the variegated mutant of Pelargonium hortorum L. or of Triticum aestivum L. bleached with the herbicide SAN 9789 did not show any red light response (Karlsson et al. 1983; Laffray et al. 1991). Red light did not
promote transpiration in the chlorophyll-deficient mutant Xan-U21 of Hordeum vulgare L., while blue light did. (Skaar and Johnsson 1980). Only one exception was found; red-/far-red-dependent stomatal movement was observed for the xantha mutant of Helianthus annuus L. (Habermann 1973), but the insensitivity of sunflower stomata (mutant and wild type) to individual red light treatments ruled this study out of consideration. The fact that neither transpiration nor stomata of ‘white’ leaves showed any red light-dependent response discounted the possible involvement of phytochrome in the process.

On the one hand two photoreceptors such as chlorophyll and the blue light photoreceptor are sufficient to promote simple diurnal opening and nocturnal closure of stomata (see above). On the other hand guard cell movement is known to be a clock-controlled circadian rhythm (Millar 1999). One might expect any of three events in C3 plants as preparation for further light or temperature alterations; stomata could open before dawn, close before sunset or remain open at night (Dewar and Meidner 1990; Yakushkina 1993). For higher plants different phytochromes and cryptochromes are involved in fine-tuning the circadian rhythm to a local photoperiod. But neither phytochrome, nor cryptochrome was found to regulate stomatal movement. Red-/far-red-dependent responses have never been observed in chlorophyll-deficient stomata (see above), and the blue light photoreceptor was found to be phototropin, rather than cryptochrome (Kinoshita et al. 2001).

This contradiction keeps us looking for a photoreceptor involved in tuning the internal biological clock in guard cells to a local photoperiod. In this paper we report red-/far-red-dependent transpiration responses in the chlorophyll-deficient XL-18 mutant of pea and discuss the possible role of phytochrome in regulation of transpiration intensity and rhythmicity.

Materials and methods

Plant material

Experiments were carried out with the chlorophyll-deficient mutant XL of pea (Pisum sativum L. cv. Early Green) and the phytochrome chromophore mutant au (auera) of tomato (Lycopersicon esculentum Mill. cv. Ailsa Craig).

XL (line xantha) was isolated by treatment of pea seeds with ethylmethanesulfonate (Ezhova and Gostimsky 1979). The mutation was lethal and the lifetime of seedlings was limited by the nourishing capacity of cotyledons to approximately three weeks. An outcome of the mutation was a disturbance of nuclear gene(s), controlling chloroplast membrane structure development; mesophyll plastids had enlarged lamellae, abolished grana and a large number of membrane vesicles. Chlorophyll absorption and fluorescence was not detected in acetone extracts (Gostimsky et al. 1982). Although production of mutant seeds from the heterozygote was 1:3 and seemed to be in sufficient amounts for experiments, these seeds were characterised by caterpillar damage and low and rapidly declining germination. Therefore, we had to use every germinating seed for experiments regardless of its weight, as well as each leaf regardless of its size. This brought about variation in the number of stomata per leaf from $10^5-10^7$, which we had to discount in calculations. Wild type and mutant plants (10–19 d old) were used for experiments. At this age a seedling had 2–4 expanded leaves. They were grown in tap water at 17–25°C under white fluorescent lamps (170 µmol m–2 s–1) for 14 h d–1.

The auera mutant of tomato was the well-known phytochrome chromophore mutant (Koornneef et al. 1985). At the seedling stage, no phytochromobilin synthesis and photoactive phytochrome was observed, while the mature plants accumulated approximately 70% of the holophytochrome of wild type (Lopez-Juez et al. 1990; Terry and Kendrick 1996; Sineshchekov et al. 1998). Tomato plants were grown in pots under natural photoperiod and illumination (11–17 h photoperiod) supplemented with fluorescent lamps (irradiance 130–280 µmol m–2 s–1). Plants that were 30–50 d old were used for experiments. At this age seedlings had 2–4 expanded leaves, that is, their ‘physiological’ age was comparable with the 20 d-old pea plants. Seeds were obtained from Prof. R. Kendrick, Wageningen Agricultural University, The Netherlands.

Equipment

Transpiration measurements

Measurements of transpiration were carried out with a custom-built differential psychrometer modified from that described by Mokronosov (1994). Briefly, an intact leaf from a plant was placed into a hermetically sealed cuvette, ventilated by room air. A thermocouple cell was used to sense the increase in humidity within the cuvette as the leaf transpired. The cell was based on a wet-and-dry pair of copper–constantan thermocouples connected in series (minus to minus). A difference in the voltage output from the two junctions was measured by a millivoltmeter (F136, Ministry of Precision and Ultrasensitive Instruments, Moscow, Russia), amplified by a millivoltmeter (LPU-01, Ministry of Precision and Ultrasensitive Instruments, Moscow, Russia) and permanently recorded by a plotter (KSP-4, Microsensor Technologies, Moscow, Russia). This equipment was used to investigate acute transpiration changes. This equipment could not be used for measurement of rhythmic transpiration changes over several consecutive days as it was excessively precise and susceptible to overheating during constant operation. Thus, in order to record daily transpiration changes, the voltage output signal was digitised by a 24-bit ADC (ZAO L-card, Moscow, Russia), connected to a PC with LabView software (National Instruments, Austin, TX). The set-up was calibrated with air mixtures of known humidity. In order to measure smaller changes of humidity (approximately 0.5%) against a high background humidity (40–70% comprising the sum of room air humidity plus an additional component arising from steady-state transpiration), a resistor compensation was added into the circuit scheme. Finally the signal of background humidity was subtracted from the whole signal, which allowed only changes of transpiration to be measured.

Monochromatic light quality and intensity

A slide projector was used as the irradiation instrument. Monochromatic light was obtained by combining cut-off and interference filters. Light intensity was measured with a thermopile (RTN 125, GMNC National Research Institute of Measurements in Optics and Physics, Moscow, Russia), connected to a microvoltmeter, and a PAR-radiometer (IL 1400 A, Nema Electronics, Amsterdam, The Netherlands). Fluxes of different intensities were obtained with neutral interference filters. Red light (R) = IF 670 ($\lambda_{max} = 670$ nm, $\lambda_{1/2} = 12$ nm) + KS-14 (> 630 nm), Flux intensities were 0.3 W m–2, 3.0 W m–2 and 10.1 W m–2. Far-red light = IF 726 ($\lambda_{max} = 726$ nm, $\lambda_{1/2} = 12$ nm) + FS-6 (> 700 nm). Flux intensities were 1.3 W m–2 (FR) or 0.3 W m–2 (FRabax). Blue light (B), 21.4 W m–2 = SS-8 (340–500 nm).
Spectral measurements

Spectral measurements were performed at a spectral resolution of 2 nm with a Hitachi 150-20 spectrophotometer (Hitachi, Tokyo, Japan) equipped with an integrating sphere. First to fourth leaves of wild type pea and the XL18 mutant were taken for this investigation. Reflectance and transmittance spectra, \( R(\lambda) \) and \( T(\lambda) \) of adaxial and abaxial surfaces of a leaf were recorded in the range of 400–800 nm against a barium sulfate standard. Spectra were measured on the background of either black velvet or a white reflective coating (2–3-mm-thick layer, Munsell Art. 6080, Edmund Industrial Optics, Barrington, NJ) applied from a glass plate. Since the integrating sphere did not provide complete collection of light transmitted by turbid plant tissues, \( T(\lambda) \) spectra were subjected to correction as described by Merzlyak et al. (2002), and the corresponding corrected spectra, \( T_c(\lambda) \), were calculated. Corresponding absorption spectra were calculated as follows:

\[
A(\lambda) = 100\% - R_{\text{black}}(\lambda) - T_c(\lambda).
\]

Procedure of transpiration measurement

In majority of experiments a leaf was placed in the cuvette such that its adaxial surface was exposed to light. This was done to simulate natural illumination, but it is essential to note that pea and tomato leaves are hypostomatous, that is, stomata are localised on the abaxial leaf surface. The procedure for measurements differed between two types of experiments described below.

Investigation on transpiration acute response upon monochromatic irradiation

Transpiration acceleration was investigated in the XL18 mutant and wild type pea upon R and FR irradiation. A plant was adapted in darkness for 12–16 h and then a leaf was irradiated by monochromatic light for 10 min, followed by a 15-min dark period, then by light of the alternate wavelength for 10 min, followed by a 15-min dark period. Additionally, the abaxial leaf surface only of wild type pea was exposed to far-red light of reduced intensity (FRabax).

In order have a ‘physiological control’, transpiration acceleration was examined under blue light, following the same scheme.

Investigation on transpiration rhythmicity

We found out that tender leaves of pea wilted in 24 h when exposed to strong wind (above 3 m s\(^{-1}\)), which had to be applied to remove the water vapour boundary layer. So we reduced the wind speed to 0.2 m s\(^{-1}\) and recorded of transpiration changes for just two days. Experiments were carried out on pea and tomato and their mutants under natural illumination (30–210 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and photoperiod (14 h light/10 h dark) and in constant darkness.

Calculations

Following the experimental procedure several parameters were calculated.

1. Transpiration rate, \( E \) (g H\(_2\)O stoma\(^{-1}\) s\(^{-1}\)). \( E \) was used to characterise daily transpiration changes.

\[
E = \frac{\Delta \times P \times v \times M}{100\% \times R \times T \times n \times S},
\]

where \( \Delta \) = difference in air humidity in the cuvette (%), calculated from a voltage signal, according to the calibration; \( P \) = water vapour pressure at the defined temperature (Pa); \( v \) = speed of air pumping, \( 3 \times 10^{-8} \text{ m}^2 \text{s}^{-1}; \) \( M \) = molar mass of water (18 g mol\(^{-1}\)); \( R \) = gas constant (8.31 J mol\(^{-1}\) K\(^{-1}\)); \( T \) = temperature in cuvette (K); \( n \) = number of stomata mm\(^{-2}\); \( S \) = leaf area (mm\(^2\)).

2. Transpiration acceleration, \( e \) (g H\(_2\)O stoma\(^{-1}\) s\(^{-2}\)). We used the parameter ‘transpiration acceleration’ to characterise acute changes in transpiration upon monochromatic irradiation. The value of \( e \) was negative or positive, depending on decrease or increase of transpiration rate, respectively. When no change in transpiration was observed, the parameter equalled zero. The parameter \( e \) has been used in previous studies under similar irradiation conditions (Mansfield and Heath 1963) and has been found to be more sensitive than \( E \) to endogenous rhythm.

\[
e = \frac{E}{\Delta t},
\]

where \( \Delta t \) = examined time period, s.

It is essential to notice that both parameters (\( E \) and \( e \)) were expressed per stoma, but not per unit area. This allowed us to take into consideration the whole evaporation surface especially in the case of the chlorophyll deficient mutant XL18. The number of stomata per leaf in the mutant varied up to 10-fold (see also Plant material).

3. \( \phi \), phase, (h) defined at a local time, when transpiration rate was maximal during the day.

4. \( \tau \), period, (h), calculated as a difference between the phases on first and second day.

5. \( \Delta \phi \), phase shift, calculated as a difference between the observed and predicted phases on the second day.

Between 4 and 20 independent experiments were carried out on different leaves of the XL18 and aurea mutants and their wild types; \( n = 4 \) for spectral measurements, \( n = 4–7 \) in experiments on transpiration rhythmicity, \( n = 10 \) in experiments on monochromatic irradiation of the XL18 of either wavelength, \( n = 6–20 \) in experiments on monochromatic irradiation of wild type pea. Results are presented as mean ± s.e. in tables or as mean curve and standard error bars in figures.

Results

Acute transpiration response upon monochromatic irradiation

Dark-adapted leaves of the chlorophyll deficient mutant XL18 irradiated by R or FR displayed various changes in transpiration rate (transpiration accelerations, \( e \)). When R was applied first, transpiration increased, and FR applied second decreased it with the same efficiency (Table 1, Fig. 1a) or at least stopped the increase. The response was reversed, when the first light applied was FR; this promoted a decline in transpiration rate, and subsequent exposure to R arrested the decline and increased transpiration (Table 1, Fig. 1a).

We checked the dependency of the response on fluence rate. As shown in Fig. 1b the response was not detected below 0.3 W m\(^{-2}\). Above this intensity the response increased proportionally to the logarithm of the fluence rate.

The irradiation of wild type by R and FR gave us more complicated data than of the mutant. When plants were irradiated by R first the transpiration acceleration was positive, as for the mutant, but the amplitude of the response was dependent on time of day. Acceleration was higher in the morning, from 1000–1200 h, and 2-fold lower in the evening, from 1600–2000 h. At midday the response was not detected (Table 1, Fig. 2a). Irradiation by R followed by FR induced a ‘chaotic’ reaction; increase, decrease, and reaction-stopping were observed. Therefore, we calculated a mean value that was close to zero and exhibited a high
standard error, indicating the complicated nature of the response to FR applied after R (Table 1).

When leaves of the wild type were first exposed to FR, simultaneous increase and decrease of transpiration rate (positive and negative accelerations, respectively) were observed on different leaves: (Table 1, Fig. 2b).

Examination of the FR-induced increase showed that it depended on time of day. The high positive acceleration was observed in the morning from 1100–1300 h, the lower acceleration occurred in the evening from 1430–2000 h. A period during which plants were unresponsive to light was found in the middle of the day (Table 1, Fig. 2b). Subsequent exposure to R (FR/R irradiation) promoted a further increase in transpiration rate (Table 1).

The FR-promoted decrease in transpiration was induced uniformly during the day and its acceleration magnitude did not depend on time of day (Table 1, Fig. 2b). FR/R irradiation stopped or reversed this response (Table 1).

In order to separate two types of response to FR (the time-dependent increase and time-independent decrease of transpiration rate) a flux of lower intensity was applied directly to stomata (FRabax). As shown in Fig. 2c this brought about disappearance of the time-dependent transpiration increase, while the decrease remained.

Blue light always induced an increase of transpiration intensity. The acceleration of the blue-light-promoted response was the highest among all responses (Table 1).

### Optical properties of pea leaves and estimation of light environment around stomata

Leaf absorption spectra of pea wild type and the XL18 mutant were calculated as described in Materials and methods (see also Merzlyak et al. 2002). The absorption spectrum of wild type leaves comprised two major bands in the blue (400–500 nm) and red (600–750 nm) regions of the spectrum, attributable to carotenoids and chlorophyll or to chlorophyll alone, respectively. The absorption spectrum of the XL18 mutant did not show the band in the red region, as a result of chlorophyll depletion (Fig. 3a).

Leaf reflectance and transmittance spectra (Fig. 3b) allowed us to estimate the light environment around stomata in hypostomatic pea leaves. In the case of the XL18 mutant, characterised by a negligible absorption in the range of 590–800 nm (Fig. 3a), both transmittance and reflectance were featureless and equalled 50% in this range (Fig. 3b). Therefore, the intensity of light reaching the abaxial epidermis of the leaf and hence the stomata, was equal to half of the flux intensity applied from above. In our experiments on fluence response we used R incident to the adaxial surface (Fig. 1b). The applied light intensities were 10.1, 3.0 and 0.3 W m$^{-2}$. At the lowest light intensity, transpiration changes were no longer detected, therefore this intensity has been considered close to the threshold. The light intensities

### Table 1. Transpiration acceleration upon monochromatic irradiation of wild type pea and its chlorophyll-deficient mutant XL18

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Transpiration acceleration, $e \times 10^{-15}$ (g H$_2$O stoma$^{-1}$ s$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>XL18</td>
<td>17.8 ± 4.3</td>
</tr>
<tr>
<td>Wild type</td>
<td>14.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>morning</td>
</tr>
<tr>
<td></td>
<td>evening</td>
</tr>
</tbody>
</table>

![Fig. 1](image_url) Effects of R and FR irradiation on transpiration acceleration in the XL18 mutant of pea. Plants were adapted in darkness for 12–16 h. The adaxial leaf surface was irradiated for 10 min by light of either wavelength. (A) Light was given in sequence: R (670 nm, 3 W m$^{-2}$), then FR (726 nm, 1.3 W m$^{-2}$), or vice versa. (B) R of different intensities was applied (670 nm, 0.3, 3 and 10.1 W m$^{-2}$). Data are the mean ± s.e. of 10 independent replicates.
reaching stomata, that is, the applied intensity corrected for transmittance, were 5.1, 1.5 and 0.2 W m$^{-2}$, respectively, and thus the ‘true’ threshold was considered to be $\geq$ 0.2 W m$^{-2}$.

In the case of wild type, values of reflectance and transmittance near 726 nm (Fig. 3b) were taken into account to estimate light intensity around stomata irradiated by FR or FR$_{abax}$ (Figs 2b, c). FR (1.3 W m$^{-2}$) was incident to the adaxial leaf surface and when corrected for transmittance near 726 nm (40%) gave the value of 0.5 w m$^{-2}$ around stomata. FR$_{abax}$ was incident to the abaxial leaf surface, that is, stomata were irradiated directly and light intensity (0.3 W m$^{-2}$) would not have required any correction if we had considered chlorophyll as photoreceptor. But we considered phytochrome to control transpiration changes upon FR$_{abax}$ (see Discussion), so correction was performed. Taking into account that phytochrome absorbance in the cell is negligible compared with chlorophyll (Sineshchekov 1995), the intensities of incident light and light reflected back from mesophyll layers were summarised to estimate photoreceptor irradiation in stomata. Addition of the abaxial leaf surface reflectance at 726 nm (46%) to the intensity of FR$_{abax}$ gave the value of 0.44 W m$^{-2}$.

Red light applied to wild type leaves was always incident to the adaxial surface, and its intensity (3 W m$^{-2}$) when

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Fig. 2. Effects of R and FR irradiation on transpiration acceleration in wild type pea. Distribution of the acceleration magnitude during the day is shown. Plants were adapted in darkness for 12–16 h and a leaf was irradiated for 10 min by light of either wavelength. (A) R (660 nm, 3 W m$^{-2}$) was incident to the adaxial surface. (B) FR (726 nm, 1.3 W m$^{-2}$) was incident to the adaxial surface. (C) FR$_{abax}$ (726 nm, 0.3 W m$^{-2}$) was incident to the abaxial surface. Data are the mean ± s.e. of 6–20 independent replicates.

Fig. 3. Optical properties of pea leaves. Individual spectra of a first developed leaf are shown. (A) Calculated absorption spectra of wild type and the XL18 mutant. (B) Trace 1, abaxial surface reflectance spectrum of wild type. Trace 2, corrected transmittance spectra in the direction of adaxial to abaxial surface of wild type. Trace 3, corrected transmittance spectra in the direction of adaxial to abaxial surface of the XL18 mutant.
corrected for transmittance at 670 nm (3% according to Fig. 3b) gave the value of 0.09 W m⁻².

Investigation of transpiration rhythmicity

Investigation of diurnal transpiration changes with natural photoperiod and illumination showed that amplitude (E) changed periodically in pea wild type and in the chlorophyll-deficient mutant (Figs 4a, b). On the first experimental day transpiration maxima occurred in the late evening at approximately 2100 h, and the maximum amplitude was approximately the same in the mutant and wild type (Table 2). On the second experimental day the amplitudes did not change significantly, and maxima again occurred in the late evening (Table 2, Figs 4a, b). Calculated periods of the rhythms were approximately 24 h.

Reaction to constant conditions (complete darkness for 2 d) differed for XL18 and wild type pea. The rhythm was not apparent in the chlorophyll-deficient mutant. Each plant examined showed an individual pattern of transpiration change, and it was impossible to determine a common period or phase. Two traces are given as an example in Fig. 4d, as well as a mean curve (note the large standard error bars in the figure). In wild type pea the rhythmicity persisted during 2 d of constant darkness, but the amplitude was smaller and the period shorter, compared with natural illumination conditions (Figs 4a, c).

Possible phytochrome involvement in regulation of transpiration rhythmicity was investigated using the phytochrome chromophore aurea mutant of tomato. In contrast with the XL18 mutant, the aurea mutant exhibited loss of rhythmicity upon natural illumination. Two individual traces are shown as an example in Fig. 5b. Plants transpired asynchronously and chaotically, and at the time when one showed a maximum rate of transpiration, others showed a minimum or a steady-state level. On the first experimental day it was possible to calculate a mean value for transpiration rate of approximately 1 × 10⁻¹¹ g stoma⁻¹ s⁻¹. On the second day E varied by one order of magnitude because of the chaotic course of curves (Fig. 5b).

When illumination was excluded and investigation of transpiration rhythmicity was carried out in constant darkness, a normal rhythm was shown for the aurea mutant (Fig. 5d). On both days of experiments transpiration maxima were observed in the late evening (Table 2, Fig. 5d), and the estimated period was approximately 24 h. The amplitude of these changes was the smallest one throughout all plants and

Fig. 4. Daily changes of transpiration in pea. (A, B) Natural illumination and photoperiod. (C, D) Constant darkness. (A, C) Wild type. (B, D) The XL18 mutant. Data are presented as the mean curve with several bars indicating s.e. In (D) two individual traces are included.
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conditions examined, and didn’t alter on the second day (Table 2). Wild type tomato plants showed periodic changes of transpiration rate in constant darkness and under natural illumination (Figs 5a, c). Upon natural illumination maximal transpiration was observed in the late evening on the first and second days. The amplitude was at the same value on both experimental days (Table 2, Fig. 5a). In constant darkness the period was extended, and the amplitude decreased on the second day, compared with the first day (Table 2, Fig. 5c).

We conclude that chlorophyll-deficiency affected rhythm maintenance in constant darkness. In contrast, phytochrome depletion caused loss of rhythmicity upon natural illumination.

Discussion

The first conclusion that can be drawn from our results is that we detected phytochrome involvement in transpiration regulation in ‘white’ plants. Leaves of the XL18 mutant do not show absorption in the red region, as a result of chlorophyll depletion. Therefore transpiration responses induced by R and FR should be attributed to phytochrome alone. We ascribe the response to the low-fluence response (LFR) type of phytochrome reactions, because, on the one hand, it is reversible; R increases and FR decreases transpiration with equal efficiency, which is typical for LFR. On the other hand, the response is dose-dependent above a threshold ($\geq 0.2 \text{ W m}^{-2} = 2 \mu\text{mol m}^{-2} \text{s}^{-1}$), which falls into the range of light intensities for this type of reaction, from $1\text{--}10^3 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Kendrick and Kronenberg 1993).

Responses of stomata to R and FR were observed by Habermann (1973) in the sunflower chlorophyll-deficient mutant, but differed from those reported here. Sunflower responses were more complicated; R was not effective in stomatal opening in either wild type or the mutant, but FR induced stomatal opening in the range from 0–4 $\text{W m}^{-2}$. Responses to double beam irradiation — R/FR or FR/R — were nevertheless not additive and depended on duration of the first light exposure. In our opinion the results indicated involvement of a complex, non-chlorophyll photoreceptor, absorbing in red region. The case probably should be re-examined based on contemporary knowledge of the types of phytochrome-induced physiological reactions: very low-fluence response (VLFR), low-fluence response (LFR) and high-irradiance response (HIR).

Another C3 plant examined for chlorophyll-independent sensitivity to R was the variegated mutant of Pelargonium hortorum L. (Laffray et al. 1991). In green leaves of the mutant plant the response induced by R was slightly above the noise level of the equipment. In white leaves the response to R was not detected, but if we take into account that white leaves were 3-fold less sensitive to B than green leaves, we might suggest that the response to R in white leaves was below the sensitivity of the equipment.

Chlorophyll-deficient mutants of barley (Skar and Johnsson 1980), as well as bleached wheat plants (Karlsson, et al. 1983), did not show any response to R. Both are grasses, which are commonly less sensitive to R than non-grasses (Johnsson et al. 1976). The grass-type stomata open precisely at sunrise and close at sunset, compared with stomata of non-grass plants, which open before dawn or stay open all day.

Table 2. Transpiration rhythm characteristics in the chlorophyll-deficient mutant XL18, the phytochrome-deficient mutant aurea, and their isogenic wild types

Changes in transpiration rate were recorded for 2 d under natural irradiation (130–210 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 14 h day/10 h night) or in complete darkness. Calculation of parameters characterising the rhythm is described in the text. The upper row in a cell with data is the value of a parameter on the first day, the lower row the value on the second day. Values are mean ± s.e. –, no rhythm apparent

<table>
<thead>
<tr>
<th>Irradiation conditions</th>
<th>Plant material</th>
<th>Time of maximum $\phi$ (h:min)</th>
<th>Rhythm characteristic</th>
<th>Period $\tau$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural photoperiod</td>
<td>Wild type pea</td>
<td>21:00 ± 0:10</td>
<td>$15.4 \pm 1.3 \times 10^{-11}$ (g stoma$^{-1}$ s$^{-1}$)</td>
<td>3:00</td>
</tr>
<tr>
<td></td>
<td>XL18</td>
<td>21:20 ± 0:30</td>
<td>$13.9 \pm 0.24$</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Wild type tomato</td>
<td>18:20 ± 1:10</td>
<td>$2.0 \pm 0.4$</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>aurea</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Complete darkness</td>
<td>Wild type pea</td>
<td>13:50 ± 2:00</td>
<td>$6.9 \pm 1.6$</td>
<td>5:30</td>
</tr>
<tr>
<td></td>
<td>XL18</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Wild type tomato</td>
<td>1:30 ± 1:30</td>
<td>$3.1 \pm 0.2$</td>
<td>3:00</td>
</tr>
<tr>
<td></td>
<td>aurea</td>
<td>22:10 ± 0:30</td>
<td>$0.4 \pm 0.09$</td>
<td>2:20</td>
</tr>
</tbody>
</table>


open after dusk (Yakushkina 1993). In other words, the composition of photoreceptors involved in regulation of transpiration and stomatal movement could be different for grasses and other C3 plants.

Based on these observations we conclude that phytochrome-dependent responses occur in C3 plants (at least in pea and sunflower) and possibly could be discovered in other species.

In wild type pea irradiated by R or FR three different types of responses are observed: (i) transpiration decrease upon illumination with FR, (ii) transpiration increase upon FR illumination, or (iii) transpiration increase upon illumination with R. The diversity of responses induced in ‘green’ plants has to be examined, taking into account possible involvement of both red light photoreceptors, chlorophyll and phytochrome. Both pigments absorb in the red and far-red regions; at 730 nm, which is the maximum absorbance of phytochrome in Pfr form, chlorophyll still makes a significant contribution to overall absorption (16%) (Fig. 3a). Hence both chlorophyll- and phytochrome-dependent changes of transpiration simultaneously occur in the wild type under FR and R. This observation indicates the necessity of separating the responses and establishing the role of each photoreceptor in regulation of transpiration.

The most complex pattern of reaction is observed in the response to FR. Irradiation from above, that is, through mesophyll layers containing bulk leaf chlorophyll, brings about a simultaneous increase and decrease in transpiration rate (Fig. 2b), clearly indicating involvement of more than one photoreceptor. In order to reveal the phytochrome component, stomata were irradiated directly from below by FRabax. The intensity of this radiation (0.3 W m–2) was below the threshold of photosynthetic response determined for Xanthium strumarium of 2 W m–2 (Sharkey and Rashkey 1981). If phytochrome is to be considered to control transpiration response under FRabax, its intensity must be corrected for reflectance from mesophyll layers. As shown in results, the corrected intensity of FRabax for the phytochrome-mediated response is 0.44 W m–2. This intensity is above the threshold that we estimated for the phytochrome-dependent response to R of ≥ 0.2 W m–2. Upon FRabax the transpiration decrease remains, whereas the transpiration increase disappears (Fig. 2c). Taking into account that, on the one hand, the intensity of FRabax was below the photosynthetic but above the phytochrome threshold, and on the other hand, that in the mutant phytochrome induced a decrease in transpiration upon FR illumination, we conclude that we have successfully separated the phytochrome component in the

Fig. 5. Daily changes of transpiration in tomato. (A, B) Natural illumination and photoperiod. (C, D) Constant darkness. (A, C) Wild type. (B, D) Aurea mutant. Data are presented as the mean curve with several bars indicating s.e. In (B) two individual traces are included.
wild type response. It controls transpiration decrease upon FR illumination. This decrease could be induced uniformly during the day (Fig. 2c).

Consequently, the FR-induced increase in transpiration should be chlorophyll-mediated. In fact it is generally known that photosynthetic production of sugars and consumption of CO₂ induces an increase in transpiration, regardless of wavelength (Sharkey and Rashkey 1981; Gotow et al. 1988). This statement is justified by our observations. On the one hand this response was not observed in the chlorophyll-deficient mutant. On the other hand in wild type the response was not induced until light intensity was above the photosynthetic threshold. It is essential to emphasise that the amplitude of this response, induced by chlorophyll, depends on the time of day, and that two photosensitive periods (in the morning and in the evening) are separated by a period of insensitivity to light in the middle of the day (Fig. 2b).

The third type of reaction induced in wild type plants by light is an increase in transpiration under R. In contrast to the chlorophyll mutant, in which the response upon R is uniform and reversible, in the wild type it is dependent on the time of day and irreversible. A diurnal pattern of the transpiration response under R was previously shown for Avena (Brogardh 1975; Brogardh and Johnsson 1975). The transpiration increase was maximal from 1000–1200 h, then regardless of wavelength (Sharkey and Rashkey 1981; Gotow et al. 1988). This statement is justified by our observations. On the one hand this response was not observed in the chlorophyll-deficient mutant. On the other hand in wild type the response was not induced until light intensity was above the photosynthetic threshold. It is essential to emphasise that the amplitude of this response, induced by chlorophyll, depends on the time of day, and that two photosensitive periods (in the morning and in the evening) are separated by a period of insensitivity to light in the middle of the day (Fig. 2b).

The third type of reaction induced in wild type plants by light is an increase in transpiration under R. In contrast to the chlorophyll mutant, in which the response upon R is uniform and reversible, in the wild type it is dependent on the time of day and irreversible. A diurnal pattern of the transpiration response under R was previously shown for Avena (Brogardh 1975; Brogardh and Johnsson 1975). The transpiration increase was maximal from 1000–1200 h, then it dropped to zero and was restored after 1800 h. In the absence of CO₂ the response was not detected, allowing the authors to identify its photosynthetic origin. The sensitivity of pea to R (Fig. 2a) is similar to that observed previously in oat. We suggest that this response is more likely controlled by chlorophyll, than phytochrome, according to its dependence on the time of day and similarity to the FR-dependent increase in transpiration. This speculation is supported by the fact that R intensity reaching stomata is just 0.09 W m⁻² due to it absorption in mesophyll, i.e. below the phytochrome threshold ≥ 0.2 W m⁻².

We conclude that both photoreceptors, chlorophyll and phytochrome, play an essential role in the specific regulation of the acute transpiration response. The photosynthetic component demonstrates the pattern of diurnal variation, which is probably controlled by a ‘gating’ mechanism, that is, the correct sequence of light-sensitive and light-insensitive phases. It was proposed that ‘gating’ is the result of the clock-controlled expression of phytochrome or a second shared messenger (Millar 1999). Our data show that the phytochrome-dependent component is not sensitive to time of day, that is, its involvement in ‘gating’ is not obvious. This fact indicates the need to reveal the role of phytochrome in the daily transpiration changes occurring in response to natural conditions.

Underlying the daily pattern of transpiration is the circadian rhythm of stomatal movement. It is a well-known endogenous rhythm, which persists under constant conditions and its phase can be reset by environmental factors (Weyers and Meidner 1990; Yakushkina 1993; Millar 1999). But the extent to which stomatal movement controls transpiration is a function of ratio of stomatal conductance to boundary layer conductance. The boundary layer is constituted by a laminar, slow-moving air layer parallel to the leaf surface, enriched in water vapour transpired by stomata. When the boundary layer is removed by wind at speeds above 3 m s⁻¹ stomatal control of water efflux is strong. When wind speed is less than 0.3 m s⁻¹ transpiration intensity depends on boundary layer conductance unless stomata are closed or narrow in width (Weyers and Meidner 1990; Meinzer et al. 1997). In other words, in the presence of weak wind the pattern of transpiration rhythm is shifted and delayed in comparison with the usual traces of stomatal movement (Yakushkina 1993). In our investigations we used a low wind speed because tender leaves of the XL18 mutant wilt in 24 h when exposed to strong wind. For the same reason we recorded transpiration changes just for two days, although usually plants are measured for three cycles to see if the rhythm persists.

Our data show that the 24-h rhythm is sustained for wild type pea and tomato upon natural photoperiod at least for two days. The curve of transpiration intensity has only one maximum at 1800–2100 h. This shift to the late evening is likely to be the result of low boundary layer conductance, compared with traces of stomatal movement, where maximum occurs at about midday (Sokolskaya et al. 2001). The rhythm persists for two days in constant darkness, showing its endogenous character.

Chlorophyll deficiency does not affect the transpiration rhythm upon natural illumination; the XL18 mutant possesses the same amplitude, phase and period as wild type. In constant darkness the rhythm is not apparent in the mutant. Each plant shows individual cycling of transpiration changes (see examples in Fig. 4d) with periods much shorter or longer than 24 h. Hence we cannot assert that the rhythmicity completely disappears, but evaluation of the mean period and estimation of the mean curve were impossible. The conclusion that could be drawn from these results is that a photosynthetic component is essential to maintain rhythmic changes of transpiration in darkness. We suggest that production of sugars is crucial in this process.

The most interesting fact is the loss of rhythm in the aurea mutant upon natural illumination, and its restoration with a period of approximately 24 h in constant darkness. It is essential to emphasise that the mutation in aurea is complex. On the one hand, aurea is known as a phytochrome-chromophore mutant in which phytochrome is not detected at the seedling stage (Terry and Kendrick 1996; Sineshchekov et al. 1998), but up to 70% of holo-phytochrome is re-established at the mature-fruit stage (Lopez-Juez et al. 1990). On the other hand, the mutant is yellow–green as a consequence of several defects in
chloroplast development (Koornneef et al. 1985). However at the age of one month the impact of the mutation becomes attenuated; chloroplast structure, net CO₂ uptake, photochemical and non-photochemical fluorescence quenching and transpiration rate are found to be similar to wild type (Biehler et al. 1997). We assume that at the age of one month, when we used aurea for the experiments, its photosynthetic activity levels were equivalent to those of wild type, but the phytocrome content is much lower than in the mature plant, therefore, we attribute all mutant responses to the shortage of phytocrome. This allows us to speculate that phytocrome is essential to maintain transpiration rhythmicity under natural illumination, probably by setting up a ‘gating’ mechanism for chlorophyll.

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References

Mokronosov AT (1994) (Ed.) ‘Practical training in plant physiology.’ (Moscow University Press, Moscow, Russia)


Yakushkina NI (1993) ‘Plant physiology.’ (Prosveshchenie: Moscow, Russia)

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