Evidence for Two Photoreactions and Possible Involvement of Phytochrome in Light-dependent Stomatal Opening

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ABSTRACT

Leaves of the xantha mutant of Helianthus annuus have a higher rate of transpiration and a lower diffusive resistance in the light than in the dark. Stomates of this nonphotosynthetic mutant open in the light and close in the dark.

Comparative studies of tobacco, xantha mutant, and wild-type sunflower stomatal opening over a range of light intensities in isolated portions of the spectrum reveal two patterns of response: (a) a low intensity opening in the green and far red characterized by partial opening, absence of a threshold, and saturation of the response at low light intensities; (b) a high intensity response in the blue characterized by a threshold (intensities greater than 100 microwatts per square centimeter needed for opening) and a linear opening response at higher incident light intensities. In xantha mutant stomates only the low intensity system appears to be operational, while both low and high intensity systems are present in the wild-type sunflower and tobacco.

Red light has an inhibiting effect on stomatal opening in both mutant and wild-type sunflowers. They require prior exposure to far red for opening to occur in red light. This red-far red antagonism suggests the involvement of phytochrome.

The stomatal apparatus controls not only transpirational water loss but also the exchange of gases between the interior and exterior of leaves. Stomatal opening or closing is a consequence of turgor changes in the guard cells that are influenced by a number of environmental factors including CO₂ and O₂ tension, water supply, temperature, and light. Of these controlling factors, the influence of light is perhaps the least understood.

Intensity, quality, and periodicity of light influence stomatal opening (9, 10, 13). Most hypotheses concerning the mechanism of light regulation of stomates postulate the involvement of the photosynthetic apparatus, an implication of the almost universal presence of chloroplasts in the guard cells and their absence in other epidermal cells. This circumstantial evidence based on the pattern of chloroplast distribution is supported by reports of the unresponsiveness to light of stomates in nonphotosynthetic mutants of higher plants and in etiolated leaves (19–21). However, some aspects of the photoreponse of stomates are inconsistent with, or even contradic-

MATERIALS AND METHODS

Plant Materials. The xantha and albina mutants of Helianthus annuus were propagated by grafting seedlings onto wild-type stocks according to procedures previously described (6, 22). Grafted mutant and control sunflowers were grown in pebbles irrigated with Hoagland’s solution supplied with an automatic nutrient cycling system.

Because large amounts of leaf material were needed, the xantha rather than the albina mutant was utilized for most of the experiments to be described. Xanthas can be grown in our greenhouse in any season, but the albina mutant must be grown

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under filters (4) and therefore can be propagated only during winter months.

Tobacco plants (Nicotiana tabacum L., variety Broadleaf John Williams) were grown in pots containing a soil, sand, and peat mixture. Some experiments with two additional varieties of tobacco (Florida Green and Florida Gold) indicated that there are no detectable varietal differences in stomatal response.

The guard cells of all experimental materials have been examined by phase contrast and fluorescence microscopy of freshly prepared epidermal strips from lower leaf surfaces. The presence or absence of chlorophyll fluorescence and structural characteristics were noted. Observations were documented by photomicrographs in which the same fields were photographed first under phase contrast and then under fluorescence optics.

Leaves were harvested late in the afternoon on the day before use. They were transferred (with petioles in water) to a constant temperature (22 C) dark room. The next morning leaf discs (1.5 cm diameter) were cut with a No. 11 cork borer. There is no true “safe light” for this operation. A green filtered lamp was placed at least 1 m away from the work surface and discs were floated on water in 50-ml beakers and transferred to a light-tight box immediately after cutting.

Measurements. Transpiration rates were estimated as weight loss per unit time of single excised leaves mounted in vials of water. Leaves were equilibrated in the dark for 45 to 90 min before weighing was begun. The glass doors of a Mettler analytical balance were covered with several layers of black greenhouse cloth during the dark periods. Daylight was supplemented with a single 150-w incandescent bulb aligned with the level of the leaf but placed 1.5 m away. Weights were recorded at 5-min intervals. Between readings in the light, the glass doors of the balance were opened. There were no measurable changes in temperature within the balance chamber during the light period. At the end of the sequence of readings which included an initial dark, a light, and final dark periods, the leaf blade was severed at the level of the stopper holding it in the vial. It was weighed, and its area was determined with a planimeter. Transpiration rates could thus be expressed on a weight or an area basis.

Diffusive resistance was measured with a Lambda diffusive resistance meter. Measurements with either the tubular or horizontal sensors generally agreed. Readings were taken on leaves of intact plants under the following conditions: in the greenhouse (full sunlight) and in a constant temperature dark-room (22 C) after dark equilibration and under a bank of Sylvania Gro-Lux lamps (approximately 500 µW/cm² incident light energy).

Stomatal aperture was estimated by means of the silicone impression technique (15). In all experiments in which apertures were measured, leaf discs were floated on a 1-cm layer of water. Samples exposed to the light (as well as dark controls) were maintained in constant temperature rooms (22–23 C). The standard duration of illumination was 2 hr. Stomatal aperture was measured under oil immersion with a calibrated ocular micrometer. Samples examined were cellulose acetate replicas of silicone impressions. For estimates of per cent open stomates, stomates with apertures of 2 µ or more were scored as open, those with apertures of less than 2 µ as closed.

Control of Light Quality and Intensity. Light intensity was varied by controlling the distance between samples and the source of illumination. The range of distances available was approximately 10 to 100 cm. Light quality was controlled by selecting appropriate lamp and filter combinations. Spectral distribution of incident light intensity was measured for every experimental condition with an ISCO model SR spectroradiometer. Representative curves for each of the lamp and filter combinations used to control light quality are shown in Figure 1.

RESULTS

Guard Cell Structure and Chlorophyll Content. The xantha and albina mutants of Helianthus annuus L. are nonphotosynthetic (22). The complete absence of detectable chlorophyll and carotenoids in the albina and the presence of only trace levels of chlorophyll in the xantha (4, 22) are consistent with the absence of light-dependent changes in O₂ uptake and CO₂ evolution. But are the trace amounts of chlorophyll in the xantha mutant distributed through all of the normally photosynthetic cells and tissues of the leaf or localized within a single cell type such as the guard cells? Fluorescence microscopy, which provides a sensitive test for the presence of chlorophyll, and observations of the same fields under phase contrast optics were utilized to answer this critical question and to make general comparisons of cell structure in the epidermal layers of all experimental materials used in these studies.

The guard cell chloroplasts of both wild-type sunflower and tobacco leaf epidermal strips exhibit the bright red fluorescence characteristic of chlorophyll. Under fluorescence optics, albina guard cells exhibit no traces of red fluorescence. In the xantha, there is a barely perceptible pink fluorescence associated with the poorly developed plastids in some preparations and an absence of detectable fluorescence in others. Thus, fluorescence microscopy is consistent with chemical analyses of the pigment content of mutant leaves and provides no evidence that the persistent traces of chlorophyll in the xantha mutant are localized within the guard cells.

Structural peculiarities were evident only in the epidermal cells of the albina mutant. In this complete albino, there is wide variation in size of the stomatal apparatus with unusually small to exceptionally large pairs of guard cells. Xantha mutant and wild-type stomata are structurally almost identical, the most distinguishing feature being the absence or presence of strong chlorophyll fluorescence in guard cell plastids.

Transpiration Rates and Diffusive Resistance of Mutant and Wild-type Sunflowers. Transpiration rates in light and dark measured as weight loss from single excised leaves are summarized in Table I. In both xantha and albina mutants there is a measurable response to illumination. Although individual leaves exhibit wide variations in rate, there is clearly a more rapid weight loss in the light than in the dark. Mutant leaves
tend to have more rapid rates of transpiration in the dark than do wild-type leaves because their stomates tend to remain partially open in the dark.

Transpiration rates have been expressed on a weight and on an area basis. There are differences between mutant and wild-type leaves in weight per unit area that are related to differences in leaf thickness. The albina has an abnormal leaf development with little differentiation of the mesophyll and inhibited lamina expansion (4), while the xantha more nearly approximates the wild type in leaf development (22). When transpiration rates are expressed as weight loss per cm² leaf area, the albina has the highest rate of transpiration in both the light and dark with a smaller difference in rate between light and dark than exhibited by the xantha and wild type.

As would be anticipated, there are measurable differences in values for diffusive resistance measured in the light and the dark (Table I). Only data for the xantha and wild type sunflower are available because leaves of the albina mutant are too small and irregular to fit in the sensors of the diffusive resistance meter.

The comparative data summarized in Table I indicate that the guard cells of mutant leaves do respond to light and that a phototoxic stomatal opening can occur in the absence of photosynthesis.

Responses to Light Intensity. Preliminary studies with leaf discs exposed to low intensity monochromatic light obtained by means of interference filters presented a very confusing picture of stomatal response to light as a function of wavelength. With xantha mutant and wild-type sunflower leaf discs, stomatal opening was observed not only at 460 and 600 but also at 720 nm. Parallel runs with tobacco leaf discs revealed negligible opening at 460 and 600 nm with only partial opening at 720 nm. It seemed possible that the light intensities used were too low for significant opening to be observed and therefore a series of light intensity-response curves were run beginning with "white" light from Sylvania Gro-Lux lamps.

White Light. The spectral output of Gro-Lux lamps is predominantly in the blue and red, regions of the spectrum that are most effective for photosynthesis. In "white" light obtained from these lamps without filters, two patterns of stomatal response were evident (Fig. 2). With xantha mutant leaf discs, maximal stomatal aperture and the highest per cent of open stomates were found in leaf discs exposed to 300 µW/cm². At higher intensities, stomates closed. Wild-type sunflower and tobacco stomates responded to low light intensities, as did the xantha mutant. In the range of 200 to 300 µW/cm² both exhibited a plateau in their response with greater opening at higher intensities. Without knowledge of the pattern of response in the xantha mutant, the nonlinearity in wild-type sunflower and tobacco light intensity-response curves might have been attributed to experimental error. However, a similar pattern of response has been described by Liebig (10) in which opening occurred at very low intensities, stomates closed partially at higher intensities, but at still higher intensities opened further. With comparative data for the nonphotosynthetic mutant available, a plausible hypothesis for this strange behavior is that in wild-type stomates two light-dependent reactions are involved in opening. Only one of these reactions (a low intensity response) is operational in the mutant. By next running light intensity-response curves in isolated portions of the spectrum, it was possible to demonstrate that one or the other of the two postulated photoreactions could be activated selectively by utilizing the appropriate light quality.

Blue Light. The intensity-response curves of wild-type sunflower and tobacco in blue light exhibit a pattern that will be referred to here as the "high intensity" photoreaction of stomates, a response that is not seen in the xantha mutant (Fig. 3). There is a distinct threshold with a minimal light intensity of approximately 100 µW/cm² needed for opening. At higher intensities there is a fairly linear increase in stomatal aperture.
and per cent open stomates. This pattern of response has been reported by Kuiper (9). The maximal light intensity that was available under the conditions of blue light used was less than 4 times the threshold level. The stomatal response to higher light intensities can be estimated by extrapolation of the relatively linear curves shown in Figure 3. The estimated light intensity needed for 100% open stomates is approximately 450 μW/cm² for tobacco and 700 μW/cm² for wild-type sunflower. The estimated stomatal apertures at these light intensities are 5 μ for tobacco and 7 μ for wild-type sunflower. The term “high intensity” response has been used for this pattern of stomatal opening because the estimated levels of light energy needed for maximal response are of the order of 5 to 20 times those needed for saturation of the “low intensity” response exhibited by stomates in green and far red light. The unresponsiveness of xantha stomates to blue light suggests that the high intensity system is inoperative in the mutant and that the photoreceptor for the low intensity system does not absorb significantly in the blue region of the spectrum.

Green Light. In green light, xantha mutant and tobacco stomates exhibit practically identical patterns of response (Fig. 4). The wild-type sunflower also follows this pattern in which there is no threshold, a partial opening, and saturation of the opening response at relatively low light intensities. The maximal aperture in green light is about 2 μ, which is the dividing line between the arbitrary definitions of open versus closed stomates. Thus, the apparent difference in response of the wild-type sunflower stomates is a consequence of the way that open and closed have been defined.

From these data it appears that the low intensity response is operational in the stomates of all three kinds of leaf material used and that the photoreceptor for this response absorbs in the green portion of the spectrum. It is difficult to imagine that this stomatal response can be linked in any way to the photosynthetic apparatus.

Red Light. It has been recognized for some time that red light is less effective than blue light in promoting stomatal opening (9, 12). Nevertheless, the patterns of response observed in the red were not anticipated. Tobacco stomates exhibited the expected high intensity response with its characteristic threshold, but both xantha and wild-type sunflower stomates remained closed (Fig. 5). The unresponsiveness of the xantha mutant can be explained by its lack of the high intensity photosystem. There are two possible explanations for the inactivity of the wild-type. If it is assumed that the low intensity response must precede the high intensity response in sunflower stomates, then opening does not occur either because the low intensity response is not activated in red light or because the low intensity response is inhibited by red light.

Far Red Light. All three kinds of experimental material responded identically to far red light (Fig. 6) with a pattern characteristic of low intensity stomatal opening. From estimates of the initial slopes of the light intensity-response curves, far red light appears to be approximately three times as effective as green light in promoting low intensity opening.

Hypotheses Based on Light Intensity Response Curves. The light intensity response patterns of stomates seen in isolated portions of the spectrum support the hypothesis that two photoreactions are involved in stomatal opening. The response to white light by stomates with both systems operational is precisely what one would anticipate from the additive effects of the two systems working in sequence. The active response of the low intensity system to far red light and the strange behavior of stomates in the red suggest that phytochrome may be involved in the low intensity response.

Red-Far Red Antagonism. As a test for the involvement of phytochrome, the effects on stomatal opening of red and far
red light in sequence were investigated. The experiments summarized in Table II were done with xantha mutant and wild-type sunflower leaf discs. The protocol involved exposure to red or far red light for a variable time (0-120 min) followed by the standard 2-hr period in light of the other color. The conditions of illumination used (204 μW/cm² red and 140 μW/cm² far red) were saturating for the low intensity opening in far red but only twice the threshold level of the high intensity response to red light exhibited by tobacco stomates. The results of these experiments can be summarized briefly as follows. (a) Red preceding far red inhibits opening. The greatest inhibition is achieved by relatively short exposures to red (5-10 min) preceding 2 hr in far red. Longer preillumination (which significantly lengthens the total light period) is less inhibitory but maximal opening is never significantly greater than in far red alone. (b) Far red preceding red promotes opening. The extent of opening after the final 2 hr of red light is proportional to the length of prior exposure to far red. The greatest opening response occurred in 2 hr far red plus 2 hr red.

**DISCUSSION**

The results of these experiments clearly demonstrate a red-far red antagonism in the light-dependent opening of both xantha and wild-type sunflower stomates. They suggest the involvement of phytochrome and also imply that there may be a single mechanism for turgor movements in plants. The nyctinastic movements of leaves were first shown to be under phytochrome control when Fondeville et al. (3) demonstrated that a period of red prior to darkness hastens the closing movement of *Mimosa pudica* leaves while far red prior to darkness delays this movement. Satter et al. (16, 17) have shown that in the nyctinastic movements of *Albizia* leaflets opening is accompanied by a flux of potassium ions from dorsal to ventral pulvinule cells while in closing there is a movement of potassium ions in the opposite direction.

Both the nyctinastic movements of leaves and stomatal opening and closing are complicated by the fact that they exhibit endogenous rhythms which are synchronized to diurnal cycles of light and dark but can persist through periods of experimentally imposed constant light or constant dark conditions. It was from studies of such rhythmic stomatal behavior that Mansfield (11) first demonstrated a low intensity light effect which caused a phase shift in the rhythm of stomatal opening ability. The most effective wavelength for induction of such phase shifts was found to be 703 nm, while comparable effects with red light could be obtained only by continuous low intensity or intermittent high intensity interruption of the dark period. Mansfield considered the involvement of phytochrome in the low intensity phase shift phenomenon, but he rejected this hypothesis because of the absence of a reversal of red effects by far red and the requirement for frequent interruptions of the dark period for a phase shift response to be elicited.

The interactions of red and far red on the low intensity response are complex. There is no simple way to explain why a long exposure to red preceding far red should be less inhibitory than shorter exposures. However, sufficient interaction has been demonstrated to support the proposed involvement of phytochrome.

The experiments reported here provide little information about the photoreceptor for the high intensity photoreponse of stomates. Yet they do provide an explanation for the generally poor response to red as compared to blue light. If the function of the low intensity response is, as Mansfield (11) has suggested, one of effecting a “readiness to open” or, as we prefer to view it, to serve as an “unlocking mechanism,” and if this response is inhibited by red light, then the generally greater response of stomates to blue light is understandable.

### Table 11. Effects of Red and Far Red Light in Sequence on Stomatal Opening in Xantha and Wild-type Sunflowers

<table>
<thead>
<tr>
<th>Length of First Light Period (min)</th>
<th>Open stomates</th>
<th>Aperture</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Xantha</td>
<td>Wild type</td>
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<tr>
<td>0</td>
<td>39.8 ± 3.3</td>
<td>33.8 ± 2.3</td>
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<tr>
<td>5</td>
<td>14.8 ± 4.7</td>
<td>14.8 ± 1.3</td>
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<td>10</td>
<td>18.2 ± 5.9</td>
<td>6.2 ± 2.8</td>
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<tr>
<td>15</td>
<td>22.2 ± 7.6</td>
<td>10.8 ± 6.3</td>
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<tr>
<td>30</td>
<td>30.5 ± 12.6</td>
<td>11.0 ± 6.0</td>
</tr>
<tr>
<td>60</td>
<td>23.8 ± 10.8</td>
<td>14.2 ± 9.4</td>
</tr>
<tr>
<td>90</td>
<td>36.8 ± 2.6</td>
<td>38.5 ± 13.5</td>
</tr>
<tr>
<td>120</td>
<td>52.0 ± 8.5</td>
<td>34.5 ± 14.9</td>
</tr>
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</table>

A. Red preceding 2 hr far red

<table>
<thead>
<tr>
<th>Length of Read preceding 2 hr far red (min)</th>
<th>Open stomates</th>
<th>Aperture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.8 ± 8.0</td>
<td>5.5 ± 3.4</td>
</tr>
<tr>
<td>5</td>
<td>16.0 ± 6.3</td>
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<td>10</td>
<td>21.8 ± 9.5</td>
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<td>15</td>
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<td>18.2 ± 9.1</td>
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<td>90</td>
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<td>34.8 ± 9.4</td>
</tr>
<tr>
<td>120</td>
<td>76.5 ± 5.2</td>
<td>51.2 ± 15.7</td>
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C. Dark controls

<table>
<thead>
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<th>Mean ± sd for four runs</th>
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<tbody>
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</tr>
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</table>

1. Mean ± sd for four runs.
2. Intensity of illumination: red, 209 μW/cm²; far red, 140 μW/cm².

**LITERATURE CITED**