

# Mutants of *Arabidopsis thaliana* with Decreased Amplitude in Their Phototropic Response<sup>1</sup>

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## ABSTRACT

Two mutants of *Arabidopsis thaliana* have been identified with decreased phototropism to 450-nanometer light. Fluence-response relationships for these strains (ZR8 and ZR19) to single and multiple flashes of light show thresholds, curve shapes, and fluence for maximum curvature in 'first positive' phototropism which are the same as those of the wild type. Similarly, there is no alteration from the wild type in the kinetics of curvature or in the optimum dark period separating sequential flashes in a multiple flash regimen. In addition, in both strains, gravitropism is decreased compared to the wild type by an amount which is comparable to the decrease in phototropism. Based on reciprocal backcrosses, it appears that the alteration is due to a recessive nuclear mutation. It is suggested that ZR8 and ZR19 represent alterations in some step analogous to an amplifier, downstream of the photoreceptor pigment, and common to both phototropism and gravitropism.

Although considerable effort has been invested over the past century in the study of phototropism in plants, we still do not have an understanding at the molecular level of any step in the sensory transduction pathway leading from light reception to curvature. Phototropism has been a particularly difficult process to study because the only step which can be directly observed is the final curvature. Other changes have been suggested to participate in phototropism, but their involvement is, in the final analysis, correlative. For example, it has been suggested that the spectrophotometrically observable, blue light-induced absorbance changes are mediated by the photoreceptor pigment for phototropism, and can therefore be used to assay for that pigment (1, 9, 12). However, the evidence to support this suggestion is that the spectral

sensitivity for the blue light-induced absorbance change is similar to the spectral sensitivity for phototropism. It has been clear for some time that additional evidence is needed to establish a causal relationship between parameters such as the light-induced absorbance changes and the physiological process of phototropism.

One technique to establish such a causal relationship and dissect a complex physiological pathway is the use of mutants. The genetic approach has been particularly valuable for the study of chemotaxis in bacteria (8, 13), but its value has also been evident in eukaryotic sensory systems. In *Phycomyces*, a tentative transduction sequence or pathway has been suggested for phototropism and carotenogenesis based on a set of mutants (10). In addition, evidence has now been accumulated that phototropism by *Phycomyces* involves multiple photoreceptor pigments (4–6). In 'higher' plants, mutants have been described with alterations in phytochrome (7, 11), but the genetic approach has not been exploited to the extent that it has in 'lower' plants.

This work was undertaken as part of an effort to systematically develop a genetic system for the study of phototropism and gravitropism in plants. A number of *Arabidopsis thaliana* mutants with alterations in phototropism have been identified. Two of these mutants exhibit a decreased amplitude in their phototropic response. In this paper, we describe the isolation and initial characterization of these mutants.

## MATERIALS AND METHODS

### Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh., var Estland, were used throughout this study. For seed multiplication, plants were grown in clay pots containing equal parts of perlite, sphagnum, and vermiculite, and saturated with a mineral nutrient solution (3). The plants were grown under a photoperiodic schedule of 18 h light and 6 h darkness.

### Screening for Mutants

For mutagenesis, seeds of *A. thaliana* were treated with 3% ethyl methane sulfonate for 16 h, and then washed for 4 h with distilled water (3). Following treatment with the ethyl methane sulfonate, these M1 seeds were sown in clay pots, as described above, and kept in darkness at  $4 \pm 1^\circ\text{C}$  for 3 d. They were then transferred to  $22 \pm 1^\circ\text{C}$  under white light as above. The M2 seeds were harvested at maturity, 6 to 8 weeks later, and allowed to dehydrate for 3 to 4 weeks before use.

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Etiolated seedlings raised from the M2 seed were analyzed for their phototropic response to nine flashes of 450-nm light ( $1 \mu\text{mol m}^{-2}/\text{flash}$ ) at 15-min intervals using the multiple flash technique previously described (14). The seedlings that curved less than  $20^\circ$  in response to this stimulus were identified as putative mutants, assigned a strain number, and allowed to green under white light. These seedlings were then transferred to plastic pots and allowed to mature and set seed under the standard growth conditions. The M3 seed was collected from each of the M2 strains, and the phototropic response of about 50 seedlings of each strain was tested and compared to the response of the wild-type parent. Those strains showing phototropic curvature significantly different from that of the wild type were identified as mutants. Two of these strains, ZR8 and ZR19, have been selected for more intensive characterization, for which M4 to M6 seed was used.

### Assay for Phototropic Response

Seeds were sown on 0.8% (w/v) Difco-bacto agar, containing  $1.0 \text{ mM KNO}_3$  in 0.3 mL wells in strips prepared from Falcon 3911 microtest III flexible assay plates (Becton Dickinson Labware, Oxnard, CA). One seed was planted in each well and the strips were placed in transparent plastic incubation boxes ( $14 \times 13 \times 3.5 \text{ cm}$ ) lined with moistened paper on the bottom, and the lid was sealed with parafilm to maintain high humidity. Germination was potentiated by chilling in darkness for 3 to 4 d at  $4 \pm 1^\circ\text{C}$  and then exposing to white light at  $24 \pm 1^\circ\text{C}$ . The radicle usually emerges from the seed coat following 24 to 30 h of preirradiation with white light for the wild type and ZR19, but following 20 h preirradiation for ZR8. At the stage of radicle emergence, seedlings were moved to darkness at  $24 \pm 1^\circ\text{C}$ , and 90% relative humidity.

After 42 h in darkness, the seedlings were exposed to the phototropic stimulus. The microassay strips with seedlings were removed from the incubation boxes and arranged on a 'staircase' stand, designed to prevent shading. The seedlings were irradiated with unilateral blue light, either as a single flash or a series of flashes, separated by a dark interval. After irradiation, the seedlings were moved back into the incubation boxes and curvature allowed to develop in darkness. All manipulations were carried out in darkness since green light is known to induce curvature in *A. thaliana* (15).

### Assay for Gravitropic Response

Seedlings were grown as in the assay for phototropism but, after the 42-h period in darkness, the seedlings were exposed to red light at  $2.4 \mu\text{mol m}^{-2} \text{ s}^{-1}$  for 1 h, and then turned onto their sides for 10 h for gravistimulation. Following stimulation, curvature of each hypocotyl was measured.

### Measurement of Curvature

The experiments with phototropism were terminated 2 h after the beginning of the stimulus. The seedlings were gently laid onto sticky transparent tape, with the direction of bending in the plane of the tape surface. The tape with seedlings attached was inserted into a photographic enlarger. The enlarged image of each seedling was traced and the curvature

was measured from the traced image. For each exposure (with a defined fluence), 60 seedlings planted in five rows of microassay wells were used. Curvature was measured only of the seedlings that emerged upright from the agar surface; usually 45 to 55 seedlings from a set of 60 were averaged to obtain mean values. Frequency distribution histograms for curvature were generated as previously described (2) using VP-planner (Stephenson Software, Inc).

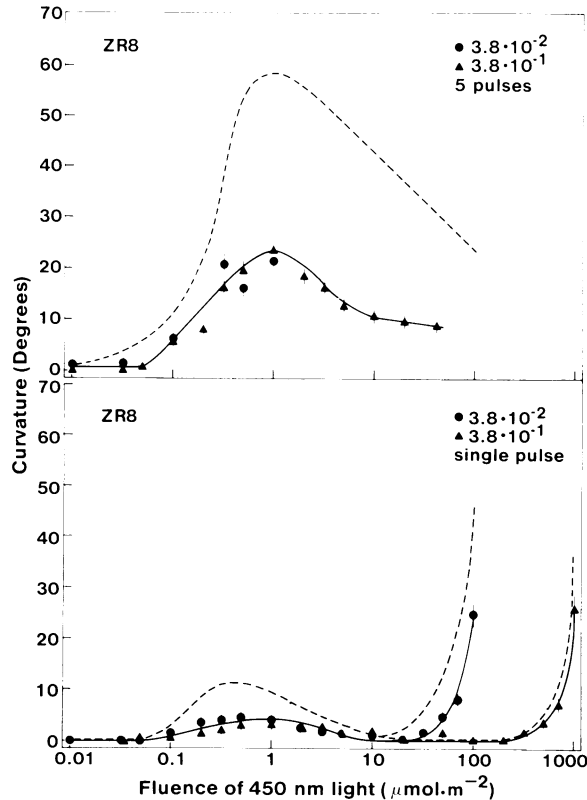
### Light Sources

For preirradiation, white light of  $125 \text{ mmol m}^{-2} \text{ s}^{-1}$  was provided by General Electric (Cleveland, OH) DeLux Cool-white fluorescent tubes and measured with a model LI-185A radiometer (Li-Cor, Lincoln, NE). The light source for phototropic experiments consisted of a projector equipped with a Sylvania 300 W ELH tungsten halogen lamp used in combination with a 12 cm layer of aqueous cupric sulfate solution (1.25%, w/v) and 450 nm interference filter with a half-band width of 10 nm (PTR Optics, Waltham, MA). The fluence rate was varied by changing the distance between the plants and the light source and by using neutral density filters. The energy measurements of the monochromatic light were made by using a model IL 700A Research Radiometer (International Light, Newburyport, MA). The duration of actinic irradiation was controlled with a Uniblitz shutter (Vincent Associates, Rochester, NY).

## RESULTS

Fluence-response relationships have been measured for phototropism of strains ZR8 and ZR19 to single flashes of light and five flashes of light. Each has been measured at two different fluence rates ( $0.038$  and  $0.38 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ). The general shapes of the fluence-response relationships to a single flash of light for ZR8 (Fig. 1) and ZR19 (Fig. 2) are quite similar to the shape of the relationship for the wild type (dashed curves in Figs. 1 and 2) which also was described previously (14). Both strains exhibit 'first positive' and 'second positive' curvature. First positive curvature is approximately the same for the mutants as for the wild type at both fluence rates, while second positive curvature for the mutants may exhibit a slightly higher threshold than that of the wild type when measured at the lower fluence rate. The major difference between the fluence-response relationships for the mutant strains and that for the wild type is the decreased amplitude of the response for the mutants. The maximum response expected in first positive curvature for the wild type is about  $10$  to  $12^\circ$ , while that found for ZR8 is about  $4$  to  $5^\circ$ , and that for ZR19 is about  $3^\circ$ . Because of this decreased amplitude it is difficult to make any statement about the threshold for first positive curvature for the mutants. Moreover, the slight change in threshold for second positive curvature may only be a consequence of this decreased amplitude of the response.

The fluence-response relationships for ZR8 and ZR19 to five flashes of light at 15 min intervals (Figs. 1 and 2) exhibit a shape similar to that of the wild type (dashed curves in Figs. 1 and 2, and also previously described [14]). However, the maximum curvature of ZR8 is about  $23^\circ$ , and the maximum curvature of ZR19 is about  $30^\circ$ , while that of the wild type is

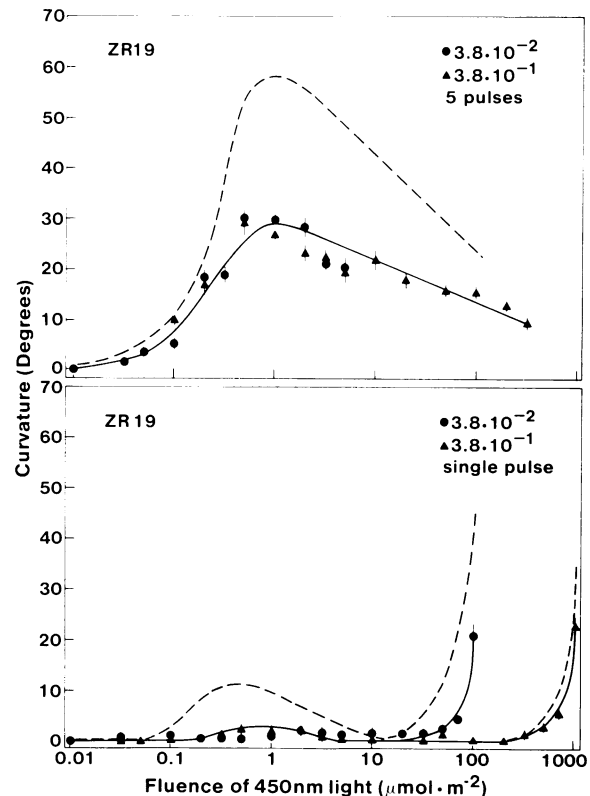


**Figure 1.** Fluence-response relationship for phototropism by ZR8 to a single flash or to five flashes of 450-nm light. Upper panel, five pulses of light at 15 min intervals; lower panel, single pulse of light. (●),  $0.038 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; (▲),  $0.38 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Vertical bars represent  $\pm 1$  SE. Dashed lines represent the fluence-response relationships for wild type.

almost  $60^\circ$ . It can be seen from the fluence-response relationships to five flashes of 450-nm light that the thresholds and fluence for maximum response of ZR8 and ZR19 are not substantially different from that of the wild type. The threshold of ZR8 could be shifted by as much as 5 times to a higher fluence, but, given the error bars, and given the fact that the fluence for maximum curvature is not shifted, the possible change in threshold is not regarded as significant.

Steinitz and Poff (14) described the dependence of phototropic curvature of *A. thaliana* to multiple flashes of blue light on the dark interval between flashes. This has been interpreted to indicate a kinetic limitation in the transduction sequence. A similar dependence has been measured for ZR8 and ZR19, by varying the time in darkness between light flashes, and measuring the final curvature to five flashes of light at  $0.2 \mu\text{mol m}^{-2}/\text{flash}$ . Curvature increases with time in darkness between flashes for ZR8, ZR19, and the wild type to a maximum at about 20 min (Fig. 3).

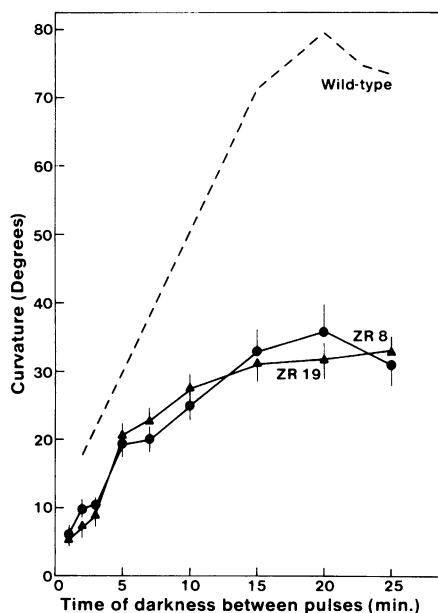
The time required for curvature to completely develop in wild-type *A. thaliana* is 100 to 120 min following the first exposure of the seedlings to light (14), depending on the irradiation sequence. Development of curvature was measured in ZR8 and ZR19 as a function of time following the last of the five flashes of light at 15 min intervals. In both strains, most of the curvature had developed within 30 min



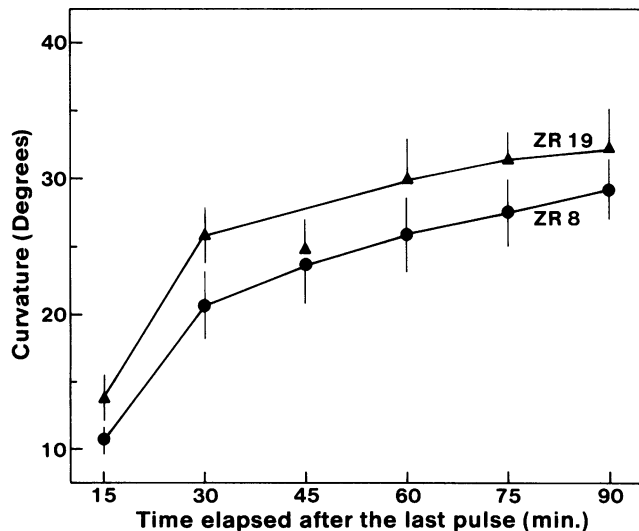
**Figure 2.** Fluence-response relationship for phototropism by ZR19 to a single flash or to five flashes of 450-nm light. Upper panel, five pulses of light at 15 min intervals; lower panel, single pulse of light. (●),  $0.038 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; (▲),  $0.38 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Vertical bars represent  $\pm 1$  SE. Dashed lines represent the fluence-response relationship for wild type.

after the last pulse of light, with only a slight increase in curvature beyond 60 min after the last light flash (Fig. 4). Since five flashes at 15-min intervals takes 60 min, the development of curvature was essentially complete within 120 min after the first exposure of the seedlings to light. Therefore, the reduced curvature of ZR8 and ZR19 was not a consequence of an alteration in the kinetics with which curvature was developed, and choosing to measure curvature 120 min after the onset of irradiation (60 min after the last flash) was reasonable.

The capacity of an *A. thaliana* hypocotyl to curve decreases in very long hypocotyls. The length of wild-type, ZR8, and ZR19 hypocotyls was measured as a function of time after transferring seedlings to darkness. The hypocotyl length of ZR19 is slightly less than that of ZR8, and the hypocotyl length of both is somewhat less than that of the wild type beyond about 33 h (Fig. 5). In other experiments (data not shown), the hypocotyl length of seedlings from different seed batches of wild-type *A. thaliana* was closer to that of ZR8 and ZR19. Thus, the decreased phototropic curvature of ZR8 and ZR19 cannot be attributed to the use of seedlings with extremely long hypocotyls. Moreover, the alteration in hypocotyl growth beyond 33 h is about 20% whereas the alteration in phototropic curvature is about 50%. Thus, although phototropic curvature has been measured 42 h following transfer



**Figure 3.** Dependence of phototropic curvature to five flashes of 450-nm light (1 s/flash) at  $0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$  on the time interval in darkness between the flashes. (●), ZR8; (▲), ZR19. Dashed line represents the dependence of phototropic curvature on the time interval in darkness between flashes for the wild type (data taken from Steinitz and Poff, 1986). Vertical bars represent  $\pm 1$  SE.

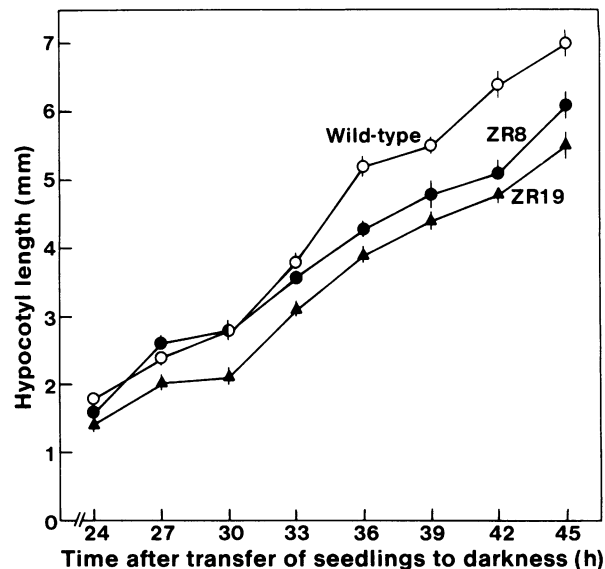


**Figure 4.** Kinetics for the development of phototropic curvature by ZR8 and ZR19 to five flashes of 450-nm light (1 s/flash) at  $0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ . (●), ZR8; (▲), ZR19. Vertical bars represent  $\pm 1$  SE.

of the seedlings to darkness, it seems unlikely that any change in growth has resulted in the decreased phototropic curvature of ZR8 and ZR19.

Gravitropic curvature of the hypocotyls of both ZR8 and ZR19 has been measured following 10-h gravistimulation with the seedlings turned onto their sides. Gravitropism of ZR8 and ZR19 is reduced about 50% compared to that of the wild type (Table I).

Reciprocal backcrosses have been made of ZR8 and ZR19



**Figure 5.** Kinetics for hypocotyl growth of the wild type (○), ZR8 (●), and ZR19 (▲). Vertical bars represent  $\pm 1$  SE.

**Table I.** Gravitropic Response of the Hypocotyls of Mutants with Altered Phototropism

Strain	Gravitropic Curvature <sup>a</sup> (°) $\pm$ SE
Wild type	$44.8 \pm 2.7$ ( $n = 80$ )
ZR8	$27.3 \pm 2.5$ ( $n = 67$ )
ZR19	$23.4 \pm 2.1$ ( $n = 66$ )

<sup>a</sup> Measured following 10 h of gravistimulation.

**Table II.** Phototropic Curvature of Wild-Type, ZR8, ZR19, and F<sub>1</sub> Progeny of Backcrosses of ZR8 and ZR19 to the Wild Type

Cross	Phototropic Curvature (° $\pm$ SE) of the Progeny
Male $\times$ Female	
Wild type $\times$ wild type <sup>a</sup>	$57.9 \pm 2.6$
ZR8 $\times$ ZR8 <sup>a</sup>	$23.7 \pm 2.1$
ZR19 $\times$ ZR19 <sup>a</sup>	$27.1 \pm 2.2$
ZR8 $\times$ wild type	$56.9 \pm 4.2$
Wild type $\times$ ZR8	$60.5 \pm 3.3$
ZR19 $\times$ wild type	$58.6 \pm 3.5$
Wild type $\times$ ZR19	$61.1 \pm 2.9$

<sup>a</sup> Self-fertilized.

with the wild type. The F<sub>1</sub> progeny of these crosses exhibit curvature to five flashes of 450-nm light ( $0.2 \mu\text{mol m}^{-2}$ /flash) at 15-min intervals which is the same as that of the wild type (Table II). Thus, the decreased curvature of ZR8 and ZR19 appears to be due to a recessive nuclear mutation.

## DISCUSSION

It can be assumed that the sensory pathway for phototropism is relatively complex, and the existence of several elements can be argued on solely physiological grounds. These elements include the photoreceptor pigment, at least one element providing a kinetic limitation downstream of the photoreceptor

pigment (14), some mechanism for establishing a gradient of at least one plant growth regulator across the hypocotyl, and dependence of cell elongation on that regulator. Because of this complexity, a genetic system affords a unique opportunity to dissect the pathway and to expand the basis for the identification of other elements in the sensory pathway.

Perhaps the most interesting mutant which could be identified at present would be one with an alteration in the photoreceptor pigment. Such a mutant might permit the isolation and identification of that pigment. However, a mutant with an altered photoreceptor pigment should exhibit a shift in the threshold for the response in a fluence-response relationship. Since neither ZR8 nor ZR19 shows a significant shift in threshold (Figs. 1 and 2), it can be concluded that they do not represent alterations in the photoreceptor pigment. Since the optimum dark period between flashes is not altered (Fig. 3), it can also be concluded that ZR8 and ZR19 do not represent alterations in the kinetic limitation described earlier (14).

Based on the growth of the hypocotyls of ZR8 and ZR19 (Fig. 5), it can be argued that the decreased phototropic curvature does not result either from a general impairment of vigor or from a change in hypocotyl length at the time of the phototropic stimulus and response. However, the impairment in the gravitropic response of both ZR8 and ZR19 is approximately the same compared to the wild type (Table I), as is the impairment in the phototropic response. Thus, it appears that the alteration is in an element common to phototropism and gravitropism.

Based on the reciprocal backcrosses, we conclude that ZR8 and ZR19 represent recessive nuclear mutations. Preliminary data indicate that ZR8 and ZR19 do not complement, *i.e.* the F<sub>1</sub> progeny of a cross between ZR8 and ZR19 exhibit the same reduced phototropic curvature which is exhibited by ZR8 and ZR19 alone. Thus, if further work proves this lack of complementation to be correct, then ZR8 and ZR19 may be considered to be alterations at the same genetic locus.

Combining all these data, we conclude that ZR8 and ZR19 represent alterations in some step analogous to an amplifier, downstream of the photoreceptor pigment, and common to phototropism and gravitropism. This class of mutants may represent our first indication of the existence of this step. Without an alteration in some biophysical parameter (*e.g.*

threshold fluence), further study of these mutants may be difficult. However, they may later be valuable as material with which one may identify the altered transduction step.

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