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ISOLATION AND CHARACTERIZATION OF
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WITH
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ZHANGLING REN

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ISOLATION AND CHARACTERIZATION OF ARABIDOPSIS THALIANA MUTANTS WITH ALTERED PHOTOTROPISM

By

Zhangling Ren

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF
ARABIDOPSIS THALIANA MUTANTS
WITH
ALTERED PHOTOTROPISM

By

Zhangling Ren

A screening procedure has been devised using multiple pulses of unilateral blue light to identify mutants in Arabidopsis thaliana with altered phototropism. Thirty five mutants have been isolated with two distinctly different phototropism phenotypes and with geotropic responses which are normal or impaired.

A fluence-response curve has been measured for ZR-8, which has altered phototropism and normal geotropism. The amplitude of the phototropic response to a single light pulse and multiple pulses by ZR-8 are lower than the analogous response by the wild-type. The thresholds for ZR-8 are shifted to higher fluence compared with the wild-type. The maximum of the first positive response of ZR-8 and the wild-type are at the same fluence, but the maximum response of ZR-8 to multiple pulses is at a higher fluence than that of the wild-type. It is suggested that such mutants should be useful in gaining an eventual understanding of phototropism in plants.

ACKNOWLEDGEMENT

I want sincerely to thank my major professor, Dr. Ken Poff for his guidance and advice in science and also in English. I also wish to thank Dr. Beni Steinitz for introducing Arabidopsis to me and guiding me into the world of phototropism. Thanks also to the members of my committee, Dr Barbara Sears and Dr. Hans Kende.

I am very appreciative of the technical assistance from Therese Best. I will always remember the good times with Carol, Brian and Mary. Thanks to all of you who have patiently explained and helped me to understand American culture and custom.

Finally, thanks go to my mother, father and grandmother, whose love is always with me.

TABLE OF CONTENTS

LIST OF FIGURES

Page

INTRODUCTION

The phenomenon of phototropism in plants was observed and described as early as 1880 by Darwin. He described phototropic movement and suggested that phototropic curvature was due to a modification of ordinary circumnutation. Since that time, the phototropism of stems has received a great deal of attention. The work on phototropism has been in three general areas: the nature of the photoreceptor pigment; the mechanism for detecting light direction; and the control of the growth response.

The nature of the photoreceptor pigment has been studied mainly through fluence response curves and action spectroscopy, and also through the use of inhibitors. Fluence response curves for plant phototropism are quite complex with the so-called first positive phototropism at lower fluence, an indifferent zone or negative phototropism at intermediate fluence, and the so-called second positive phototropism at higher fluence. This is further complicated by the fact that only first positive phototropism follows the Bunsen-Roscoe law of reciprocity (Dennison, 1979). The invalidity of reciprocity for second positive phototropism means that the response is not simply a function of the number of quanta absorbed, but depends mostly on the time of irradiation and only slightly on the fluence rate used

(Pickard, et al., 1969). It is of interest that essentially the same complex fluence response curve is found in both monocotyledonous (Galston, 1959; Briggs, 1963; Zimmerman and Briggs, 1963a,b; Curry, 1969), and dicotyledonous plants (Steyer, 1967; Steinitz and Poff, 1985). In addition, the action spectra are all similar to each other and to other "blue light responses", with peaks at about 370 nm, 440 nm, 460 nm, and 485 nm (Dennison, 1979; Shropshire, 1980). Based on the action spectra, carotenoids and flavins have been suggested as the photoreceptor pigment (Schmidt, 1980). Although the controversy will likely continue until the photoreceptor pigment has been isolated and identified, it is generally thought that the photoreceptor pigment is a flavoprotein (Dennison, 1979). The flavoprotein hypothesis is consistent with the data from action spectroscopy and with work using inhibitors specific to phototropism. Many such inhibitors have been described but these share one attribute - - they all in some way combine with flavins or interfere with flavin physiology or photochemistry.

Although the measurement of light direction could use either a refraction mechanism or a screening mechanism, the weight of evidence supports the former in the fungus Phycomyces blakesleeanus and the latter in flowering plants. Light is thought to be refracted by the Phycomyces sporangiophore growing in air such that the organ acts as a convergent lens, focusing unilateral light onto the distal

side which is thereby caused to grow faster than the proximal side (Shropshire, 1962; Bergman, et al., 1969; Castle, 1933). The shoot of the flowering plant is considerably more dense than a Phycomyces sporangiophore, and any focusing advantage is overcome by the screening of light through absorption and scatter of the plant tissue. As a result of this screening, more light is absorbed on the proximal side than on the distal side of the plant shoot, permitting the measurement of light direction (Thimann and Curry, 1961; Seyfried and Fukshansky, 1983; Parsons, et al., 1984; Piening, 1984)

The most carefully studied aspect of phototropism has been the mechanism whereby unequal light reception is translated into unequal growth and thereby into curvature of the shoot. For example, Blaauw (1915) suggested that the phototropic response might be caused by a relative inhibition of cell elongation. However, Went and Thimann (1937) proposed that the phototropic response is caused by the redistribution of some plant hormone (Cholodny-Went theory of tropism). This point continues to be controversial, but the Blaauw hypothesis seems most favored recently (Franssen, et al., 1981; Meijer, 1968; Black and Shuttleworth, 1974; Jose and Vince-Prue, 1977; Gaba and Black, 1979; Thomas et al., 1980; Cosgrove, 1981).

In most cases, the phototropism of monocotyledonous plants and fungi has been studied, but not much attention has been directed to the study of phototropism in dicotyledonous plants. In 1967, Steyer suggested that the two types of phototropic responses, first and second positive phototropism, existed in both monocotyledonous and dicotyledonous plants. Further studies on phototropic responses of dicotyledonous plants have been performed by Bruinsma et al. (1975), Shuttleworth and Black (1977) and Hart and MacDonald (1981). Recently, Steinitz and Poff (1985) characterized the phototropic response in hypocotyls of a dicotyledenous plant, Arabidopsis thaliana. They showed that the patterns of fluence-response relation (first and second positive responses) are the same as those of monocotyledous plants (Avena), and that sequential multiple pulses can induce a greater phototropic response than can a single pulse.

Phototropism and geotropism are the two major sensory systems responsible for regulating the orientation of aerial organs of plants (Briggs, 1963; Juniper, 1976; Wilkins, 1977; Dennison, 1979; Hart and MacDonald, 1981). In most cases, phototropism and geotropism have been considered and studied separately and independently (Briggs, 1963; Kang and Burg, 1972, 1974; Mohr and Pichler, 1960; MacArthur and Briggs, 1979). However, under natural conditions, the potential for both systems exists within one organ, and the

tropism of plant organs is determined by the balance of phototropism and geotropism. Moreover, the two sensitivities may interact. Hart and MacDonald (1980) reported that blue light, which induces the phototropic response, inhibited geotropic bending of Lepidium and Sinapis hypocotyls. Hart and MacDonald (1981) later described the degree of interaction or competition between phototropism and geotropism within a single organ of a dicotyledous plant, Lepidium sativum L. At best, the interaction of phototropic and geotropic systems is not clear.

A collection of phototropism mutants would be a valuable tool both for the study of phototropism itself and also for the study of the interaction between phototropism and other sensory responses. Since no such higher plant phototropism mutants were available, this work was undertaken to isolate such mutants and to begin their characterization. A screening technique has been devised and used for identifying phototropism mutants in Arabidopsis thaliana. In addition, phototropism and geotropism have been characterized for some of the mutants isolated.

MATERIALS AND METHODS

Plant Material: The Estland variety of Arabidopsis thaliana (L.) Heynh, a self-fertilizing crucifer, was used throughout this study. The seed stock was generously provided by Dr. C. Somerville.

Mutagenesis: The seeds of A. thaliana were mutagenized by Drs. C. Somerville and B. Steinitz. The seeds were placed in an erlenmeyer flask and soaked for 12 hr in an aqueous solution of 0.2% (w/v) ethylmethanesulfonate (EMS; Sigma Chemical Co., St. Louis, MO.). Following this treatment, the solution was discarded, and the seeds rinsed with running distilled water for 8 hr.

m_2 Seed Production: This m_1 generation of seed was sown in large flats containing a bottom layer of perlite, overlain with a pH-balanced peat mixture, sprinkled with a layer of fine vermiculite, and moistened with distilled water. After sowing, the flats were covered with clear cellophane to prevent desiccation, and held at 4° for 2 days to enhance germination. The seed were then incubated at $21\text{-}23^{\circ}$ under 16 hrs white light per day from above (10^4 mW/m^2 from General Electric Delux Cool White fluorescent tubes). The

self-pollinating m_1 plants were permitted to set seed, and about 8 weeks after sowing, the second generation mutant (m_2) seeds were harvested by cutting the plants at ground level and allowing them to dry for about 1 week in paper bags. The material was then threshed using a wire screen, and the m_2 seeds collected and pooled from all of the m_1 plants.

Growth of Seedlings: To produce material for phototropism, geotropism or fluence-response relationship tests, m_2 seed were sown on 0.8% (W/V) agar (Difco Bacto Agar, Difco Laboratories, Detroit, MI.) containing 1 mM KNO3 in 0.3ml microstrip wells (Lab Systems Inc., Morton Grove, Il) at one seed per well. Rows of the microstrip wells were set in a transparent box (3.5 X 17 X 21 cm) which was then wrapped with black cloth and incubated in darkness at 4^{o} for 4 days to potentiate germination (Shropshire, et al., 1961). The black cloth was then removed, and the seeds exposed at 25^{o} for 30 hr to white light (125 $mE/m^2/sec$ from General Electric Delux Cool White fluorescent tubes) also to potentiate germination. The box of seedlings was moved to a dark room at 25^{o} and 100% relative humidity for 42 hrs, after which they were used in phototropism or geotropism tests.

Phototropic Induction with Multiple Pulses: Seedlings grown in darkness for 42 hr following the potentiation of

germination, were stimulated unilaterally with nine 2.5 sec pulses of 450 nm light at 37.6 pE/cm^2/sec for a total fluence of 94 pE/cm^2 for each pulse. The pulses were given at 15 min intervals. Thus the length of time between the first and ninth pulse was 120 min. After an additional 30 min in darkness, the experiment was terminated and the curvature of the stimulated seedlings measured.

Geotropic Induction: Seedlings grown in darkness for 42 hr following the potentiation of germination were exposed to red light (500 ergs/cm^2/sec from red fluorescent tubes in combination with a 3-cm thick filter of 5% (w/v) aqueous $CuSO_4$ (giving a maximum output at 660nm) for one hour. The microstrips containing the seedlings were then turned within the plastic box such that the seedlings were in the horizontal position, and incubated for a period of time in darkness. The curvature of 100-200 seedlings was measured 2, 8, and 12 hrs after the onset of geotropic stimulus.

Curvature Measurements: Seedlings were gently affixed to sticky transparent tape in a way that the curvature angles were parallel to the tape surface. The tape was mounted in the slide holder of an enlarger, and an enlarged image of the seedlings projected onto paper on which two straight lines were drawn for each seedling, one representing the shoot, and one the final growth direction of the shoot tip. The angle of curvature was measured from these lines using a

protractor. In most cases, 100-200 seedlings were measured for each condition. The experiments were repeated 3-6 times, and the data point is the mean of the pooled data from these repetitions.

Light Sources: Light for phototropic stimulation was provided by a Bell and Howell projector equipped with a 300 W tungsten halogen lamp with the light beam filtered through a 450 nm interference filter (PTR Optics, Waltham, MA.; half-band width, 8.5 nm) and a 3 cm pathlength of 5% (w/v) aqueous $CuSO_4$ solution.

All phototropism experiments were performed in complete darkness, because green light had been previously shown not to be phototropically "safe" (Appendix I). Some of the geotropism experiments were conducted in darkness while others were carried out using a dim green light which had no apparrent effect on geotropism.

Light Measurements: Light intensities were measured with a model 68 Kettering Radiometer (Laboratory Data Control, Riviera Beach, FL.).

RESULTS

To identify mutants with altered phototropism, it is necessary that the phototropic response of the wild-type parent be significantly different from the phototropic response of the mutant. Assuming that the minimum response would be equivalent to that of the wild-type in darkness (e.g., zero response), then it is necessary that the phototropic response of the wild-type be significantly different from the curvature expressed in darkness. For this reason, the curvature of the wild-type was measured with and without a phototropic stimulus. The curvature of seedlings grown in complete darkness was measured, and as expected, is approximately zero (Fig. 1). In addition, the curvature of more than 60% of the seedlings was between $+5^{o}$ and -5^{o}. The phototropic curvature of a similar population of seedlings treated similarly but exposed to a single pulse of 846 pE/cm^2, was 8.9^{o} +/- 1.1^{o} (Fig. 2). In contrast, the curvature of a similar population of seedlings, treated similarly but exposed to nine 2.5 sec pulses of 450 nm blue light at 94 pE/cm^2 per pulse (for a total of 846 pE/cm^2) was 80^{o} (Fig. 3). Moreover, only 0.3% of the seedlings showed a curvature less than 30^{o} and none less than 20^{o}. Thus, the phototropic curvature significantly increased with the

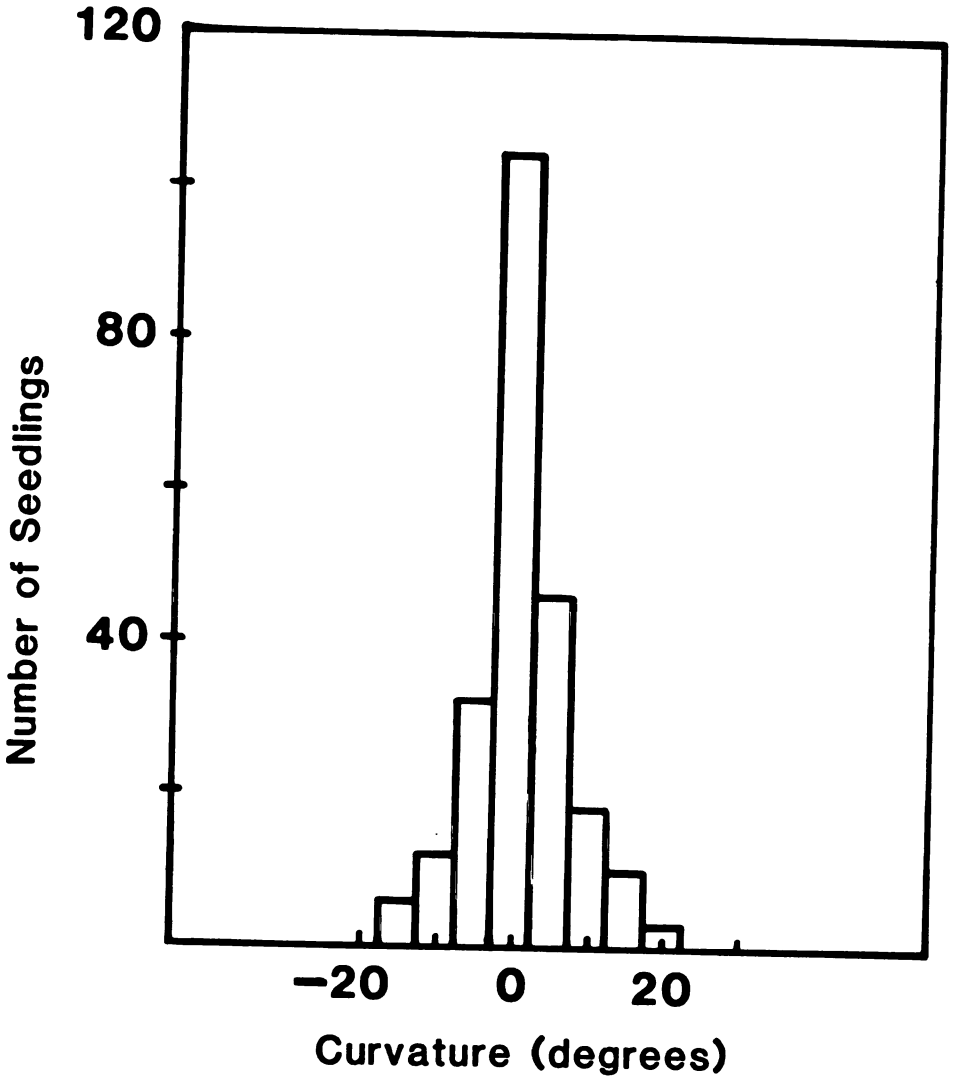


Figure 1. Frequency distribution for the curvature of the shoots of 251 wild-type seedlings prepared for phototropic stimulation but then left in darkness in the vertical position for 120 min. The mean of the population is 0.54° with a standard error of 0.36°.

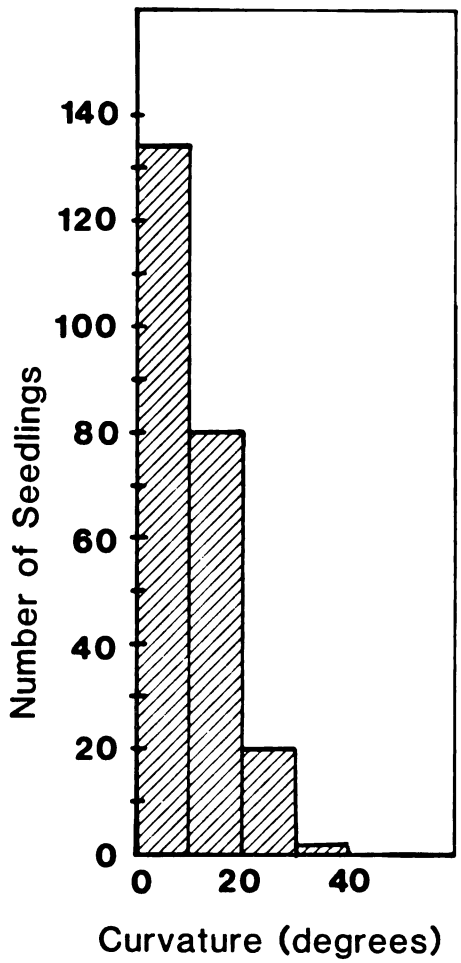


Figure 2. Frequency distribution for the curvature of the shoots of 236 wild-type seedlings prepared for phototropic stimulation, exposed to a single 22.5 sec pulse of unilateral light at 37.6 pE/cm^2/sec, and left in darkness for 120 min before the curvatures were measured. The mean curvature was 8.9° with a standard error of 1.1°.

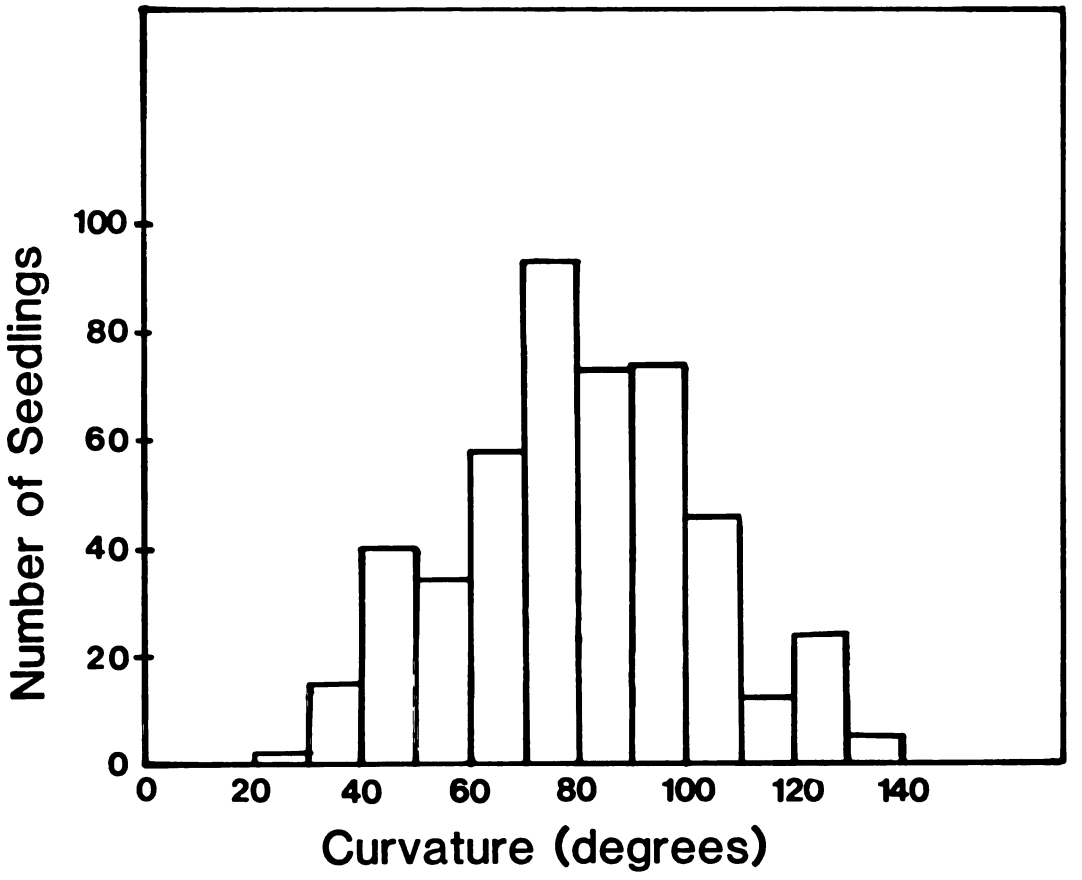


Figure 3. Frequency distribution for the curvature of the shoots of 576 wild-type seedlings prepared for phototropic stimulation, exposed to nine 2.5 sec pulses of unilateral light at 37.6 pE/cm^2/sec given at 15 min intervals, and left in darkness for 30 min before the curvatures were measured 150 min after the first pulse. The mean curvature was 79.6^o with a standard error of 1.8^o.

multiple pulse regime such that there was little overlap between the curvatures of a population of seedlings in darkness, and another similar population exposed to the nine pulses of light.

The (m_2) seeds of Arabidopsis thaliana were sown as described above for phototropic induction with multiple pulses. The seedlings were stimulated with nine 2.5 sec pulses of 450 nm blue light at 15 min intervals. Thirty min following the last pulse (150 min after the first pulse), those seedlings which appeared to have less than 20 curvature degrees were designated as ZR-1, ZR-2, etc. in sequence as they were found. The plants were allowed to green under white lights for 2 days and were then transplanted to clay pots containing moistened peat mixture. The plants were grown to adults in a growth chamber at 25° under 16 hr daylength. The m_3 seed from each plant were harvested for further testing.

Out of a population of 379,000 m_2 seedlings which were screened, 555 seedlings were identified with a phototropic curvature less than 20 degrees. Many plants were lost due to death or inadequate seed set. An adequate amount of seed were collected from 245 out of the 555 putative mutants. The m_3 seed from each of these 245 strains were sown, the seedlings exposed to nine pulses of light as described above, and the curvature of each seedling measured. Based

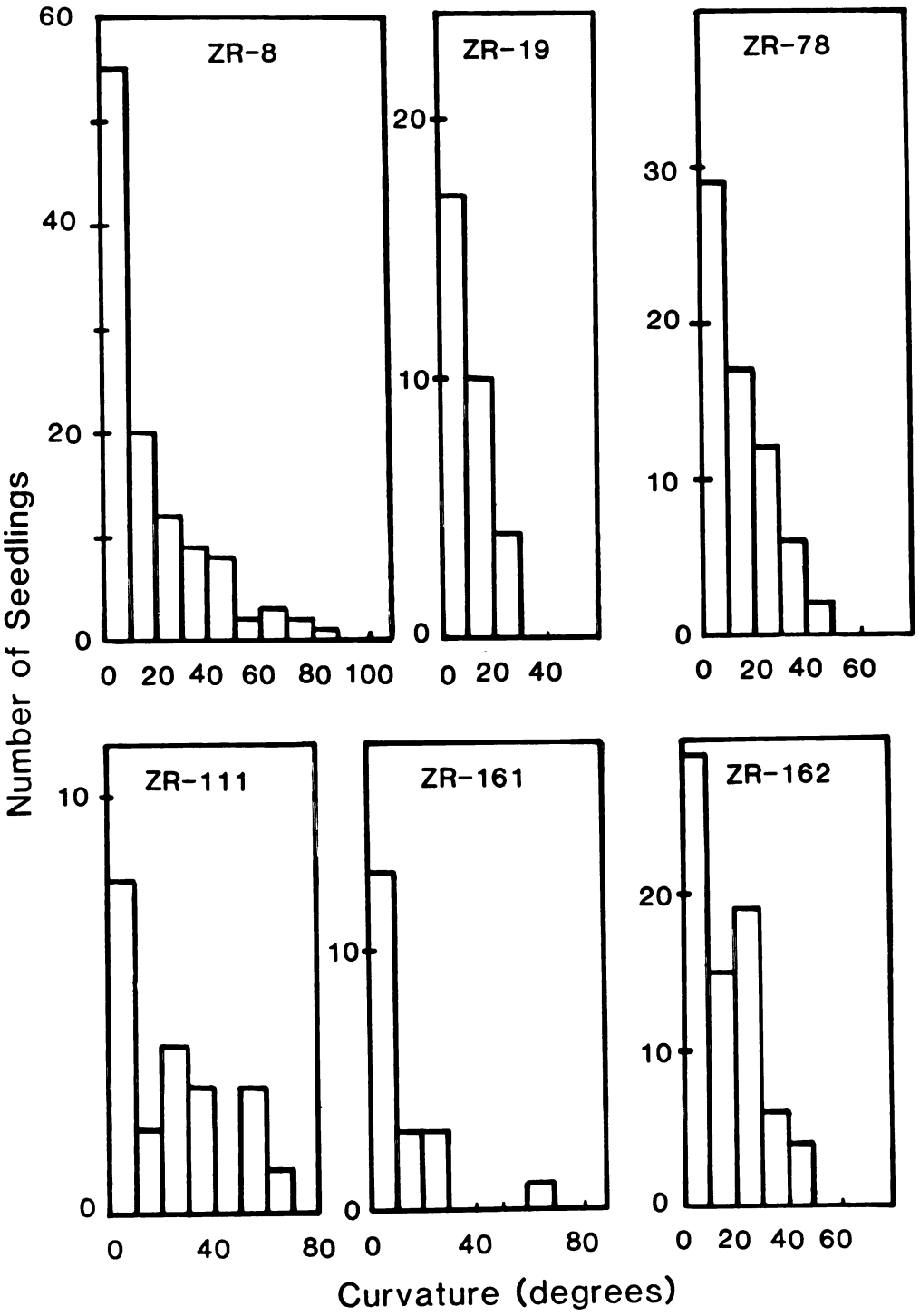
on the frequency distributions for these data, 35 strains were identified with a curvature significantly different from that of the wild-type parent (Fig. 4). At least two types of distributions are identifiable: 1). there were 21 mutants for which the peak of the frequency distribution was close to 0^{o} (Fig. 4a); 2). there were 14 mutants for which this peak was significantly greater than 0^{o} (Fig. 4b). Further work was carried out on mutants in the former category.

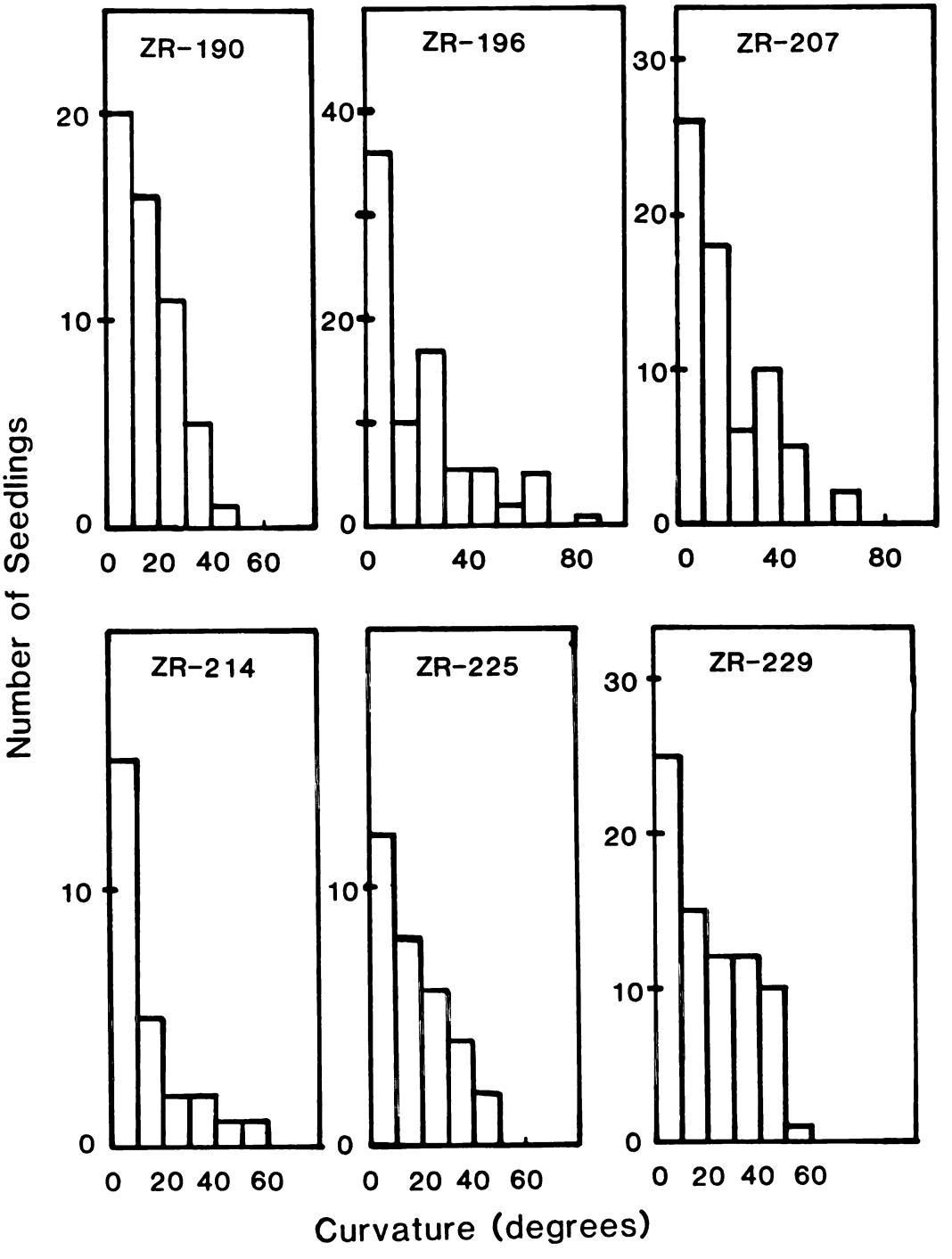
Eight of the mutants showing essentially zero phototropism also were tested for geotropism. The geotropic responses of ZR-8, ZR-362, ZR-469 and ZR-555 were essentially the same as the geotropic response of the wild-type parent (Fig. 5a). In contrast, the geotropic responses of ZR-19, ZR-162, ZR-196 and ZR-240 were less than that of the wild-type parent (Fig. 5b).

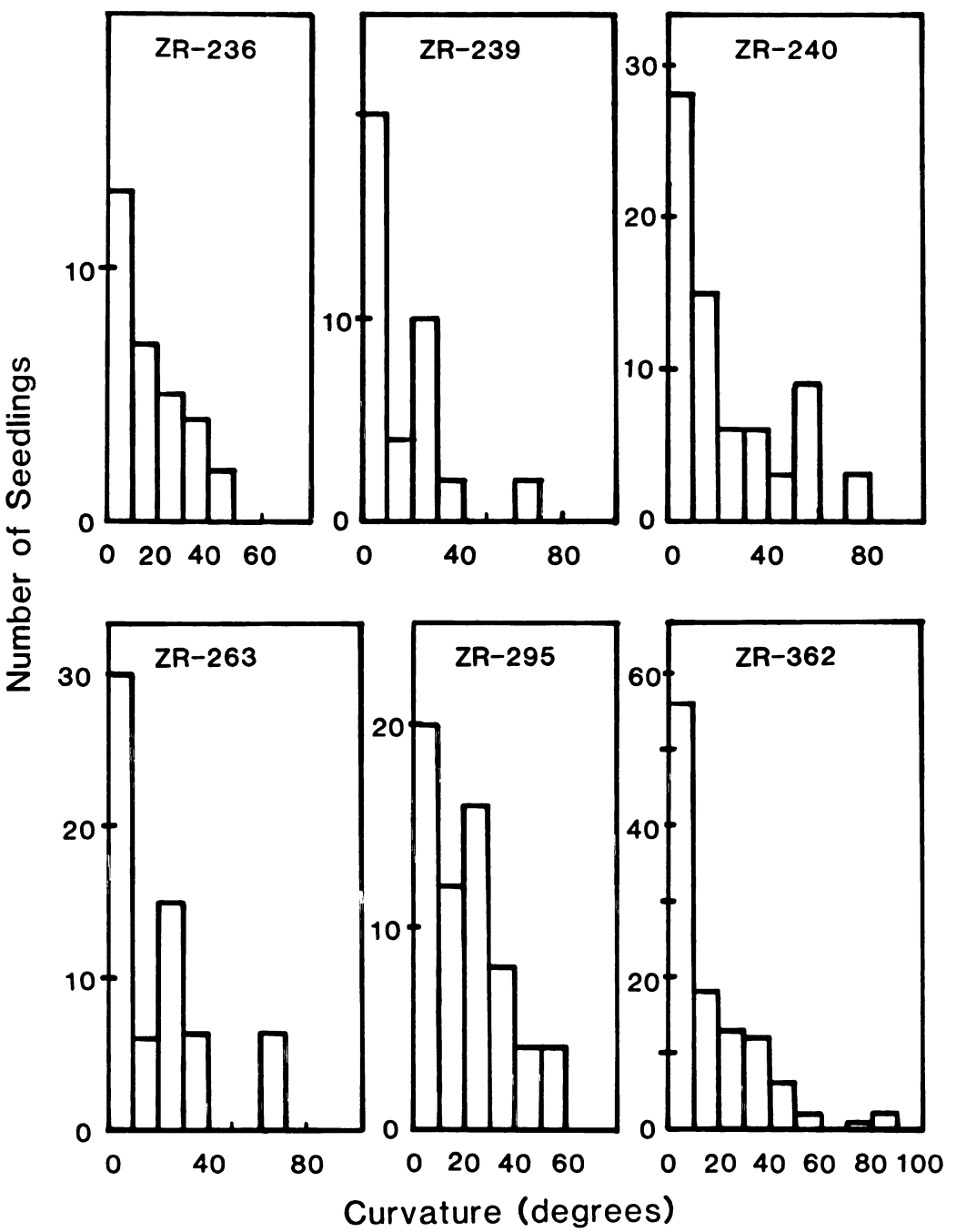
Assuming that geotropism and phototropism share a portion of their transduction chains, only those phototropism mutants which are normal in geotropism, are potential candidates for photoreceptor mutants. Therefore, a fluence response curve was measured for one of the photo-minus and geo-plus mutants, ZR-8. Seed (m_4) of ZR-8 were sown as before, the seedlings exposed for different times to a single pulse of light at each of three different fluence rates, 3.76 pE/cm^2/sec, 37.6 pE/cm^2/sec, and 376

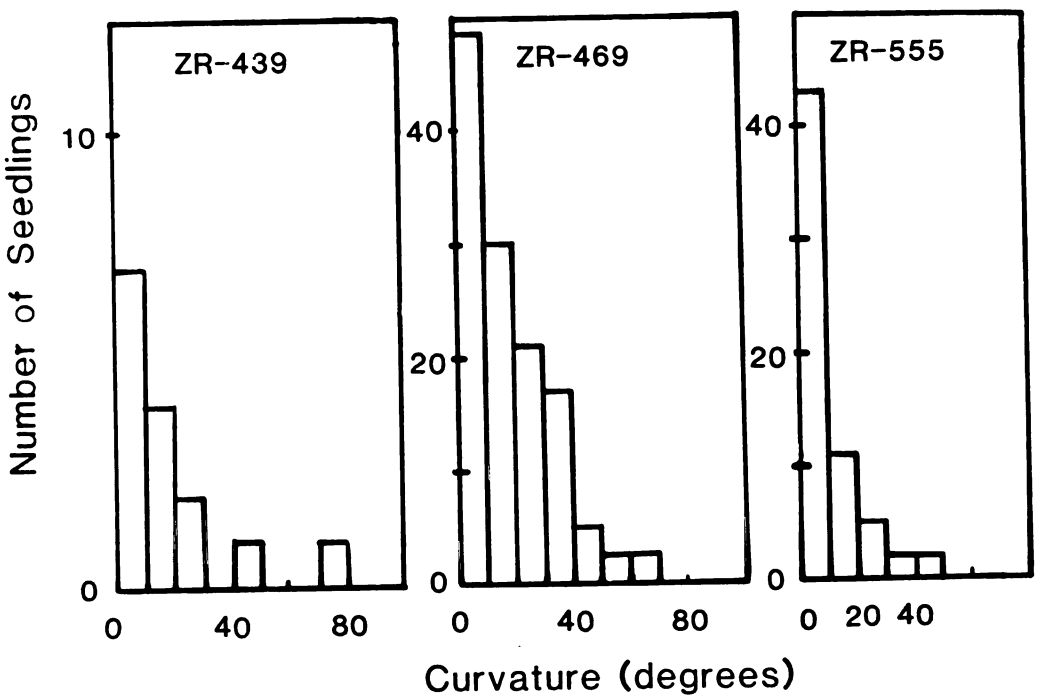
Figure 4. Frequency distribution for the curvature of the shoots of Arabidopsis seedlings with a phototropism phenotype different from that of the wild-type. The seedlings were prepared for phototropic stimulation, exposed to nine 2.5 sec pulses of unilateral light at 37.6 $pE/cm^2/sec$ given at 15 min intervals, and left in darkness for 30 min before the curvatures were measured 150 min after the first pulse. Vertical bars represent +/- one standard error. a. Strains ZR-8, ZR-19, ZR-78, ZR-111, ZR-161, ZR-162, ZR-190, ZR-196, ZR-207, ZR-214, ZR-225, ZR-229, ZR-236, ZR-239, ZR-240, ZR-263, ZR-295, ZR-362, ZR-439, ZR-469, and ZR-555; b. Strains ZR-95, ZR-237, ZR-288, ZR-293, ZR-301, ZR-328, ZR-337, ZR-338, ZR-422, ZR-436, ZR-438, ZR-443, ZR-296 and ZR-440.

a.

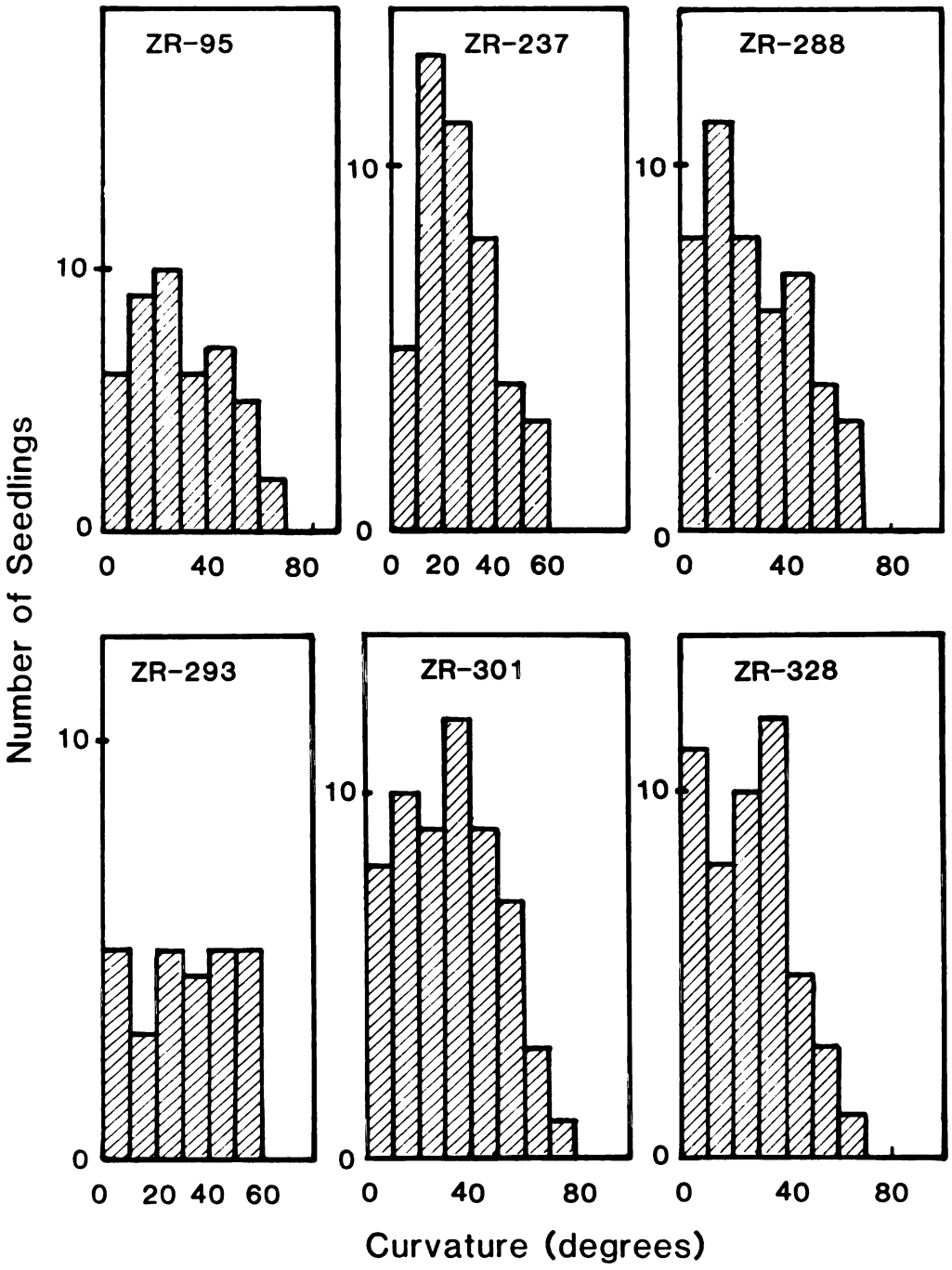

ZR-8
ZR-19
ZR-78
ZR-111
ZR-161
ZR-162
Number of Seedlings
Curvature (degrees)
0 20 40 60 80 100
0 20 40
0 20 40 60
0 40 80
0 40 80
0 20 40 60
60
40
20
0
20
10
0
30
20
10
0
10
0
10
0
20
10
0

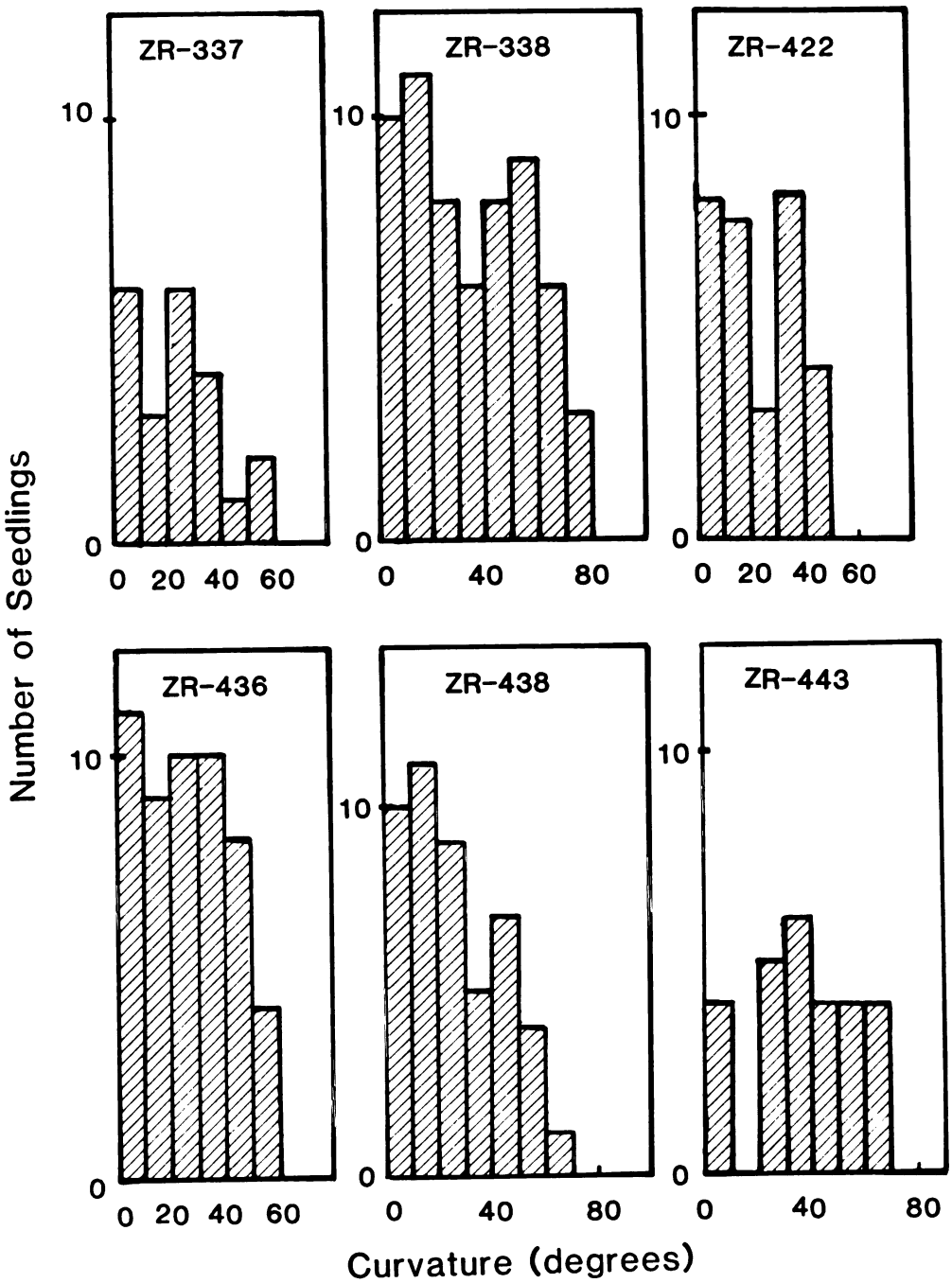

ZR-190
ZR-196
ZR-207
ZR-214
ZR-225
ZR-229
Number of Seedlings
Curvature (degrees)
0
10
20
30
40
0 20 40 60
0 40 80


ZR-236
ZR-239
ZR-240
ZR-263
ZR-295
ZR-362
Number of Seedlings
Curvature (degrees)


ZR-439
ZR-469
ZR-555
Number of Seedlings
10
0
40
20
0
0 40 80
0 40 80
0 20 40
Curvature (degrees)

b.


ZR-95
ZR-237
ZR-288
ZR-293
ZR-301
ZR-328
Number of Seedlings
Curvature (degrees)
0
10
0 40 80
0 20 40 60


ZR-337
ZR-338
ZR-422
ZR-436
ZR-438
ZR-443
10
0
0 20 40 60
0 40 80
Number of Seedlings
Curvature (degrees)

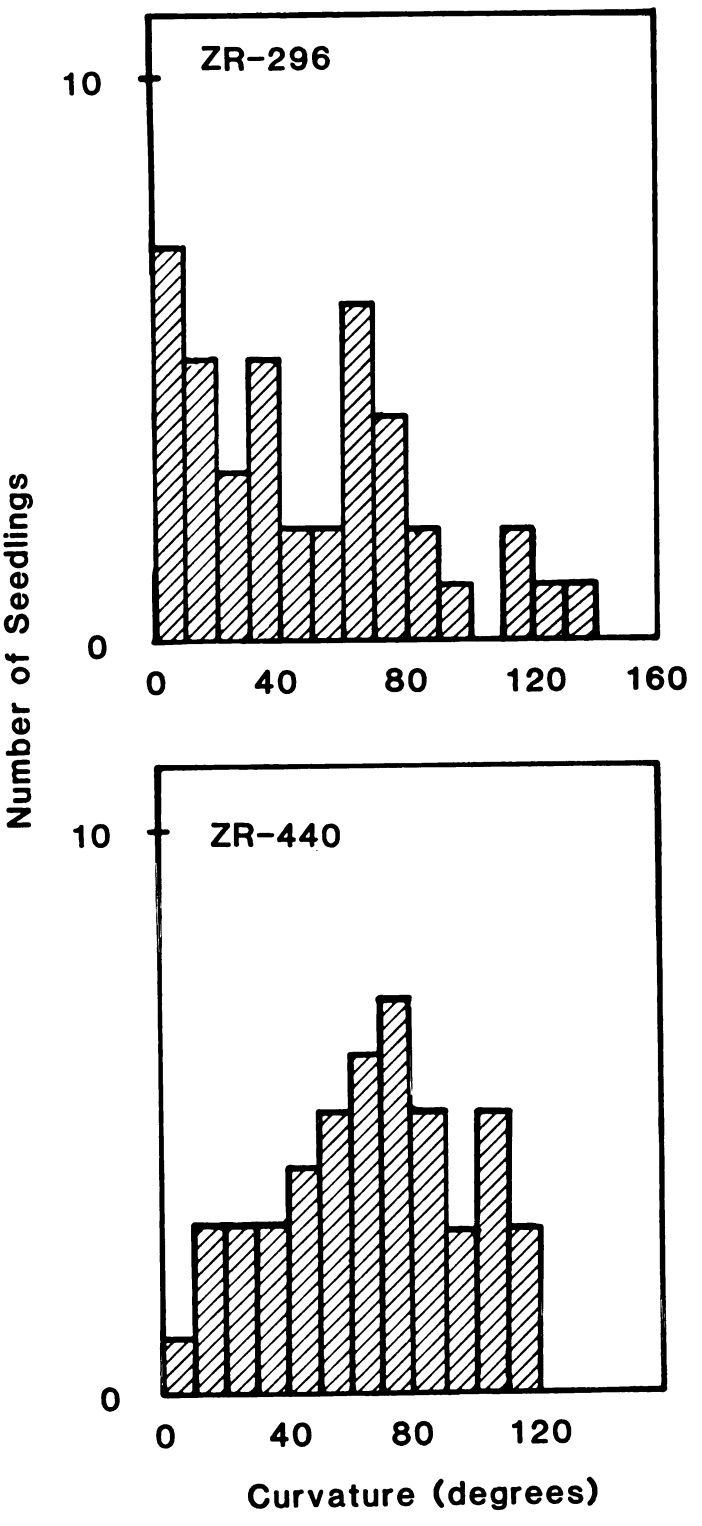
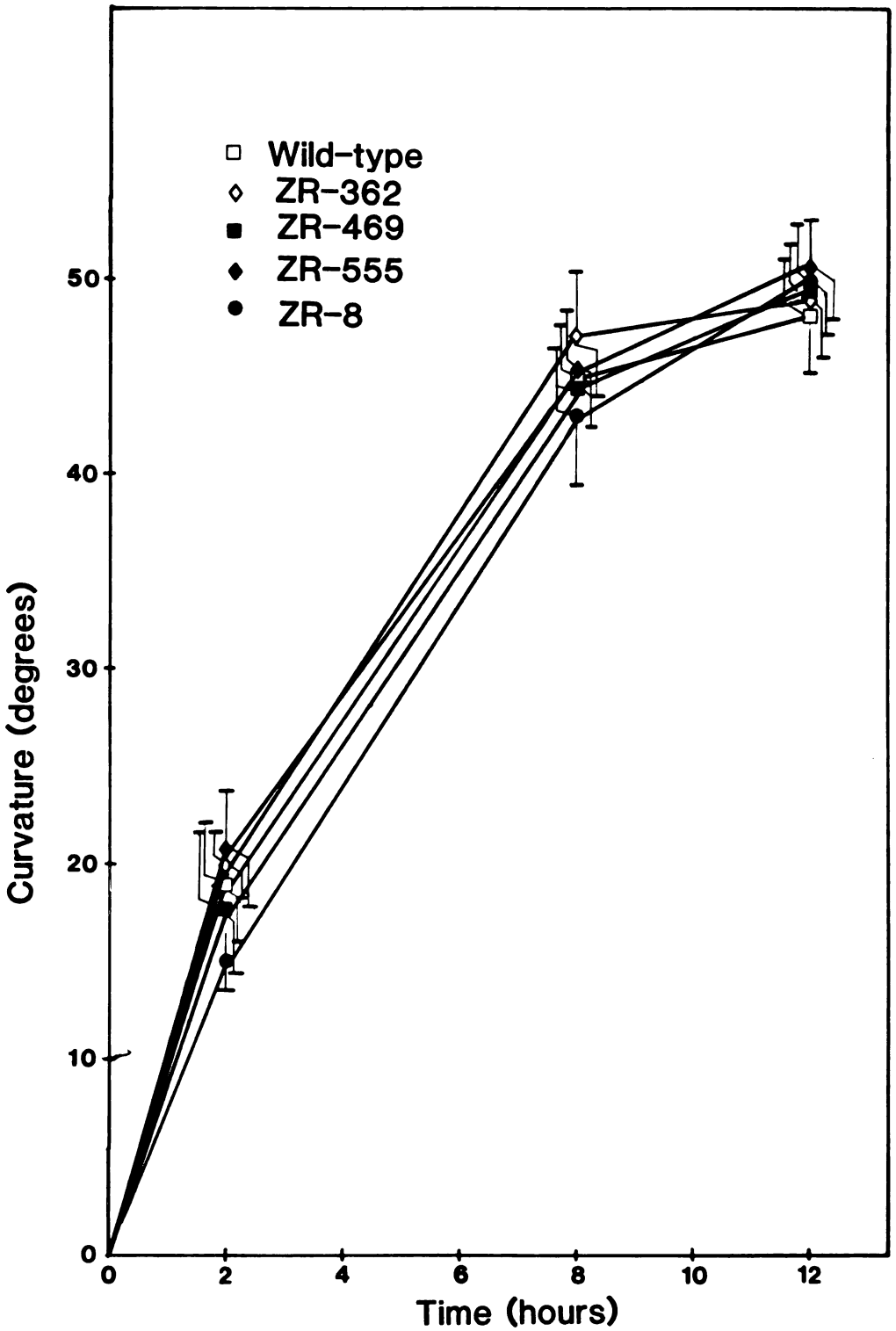
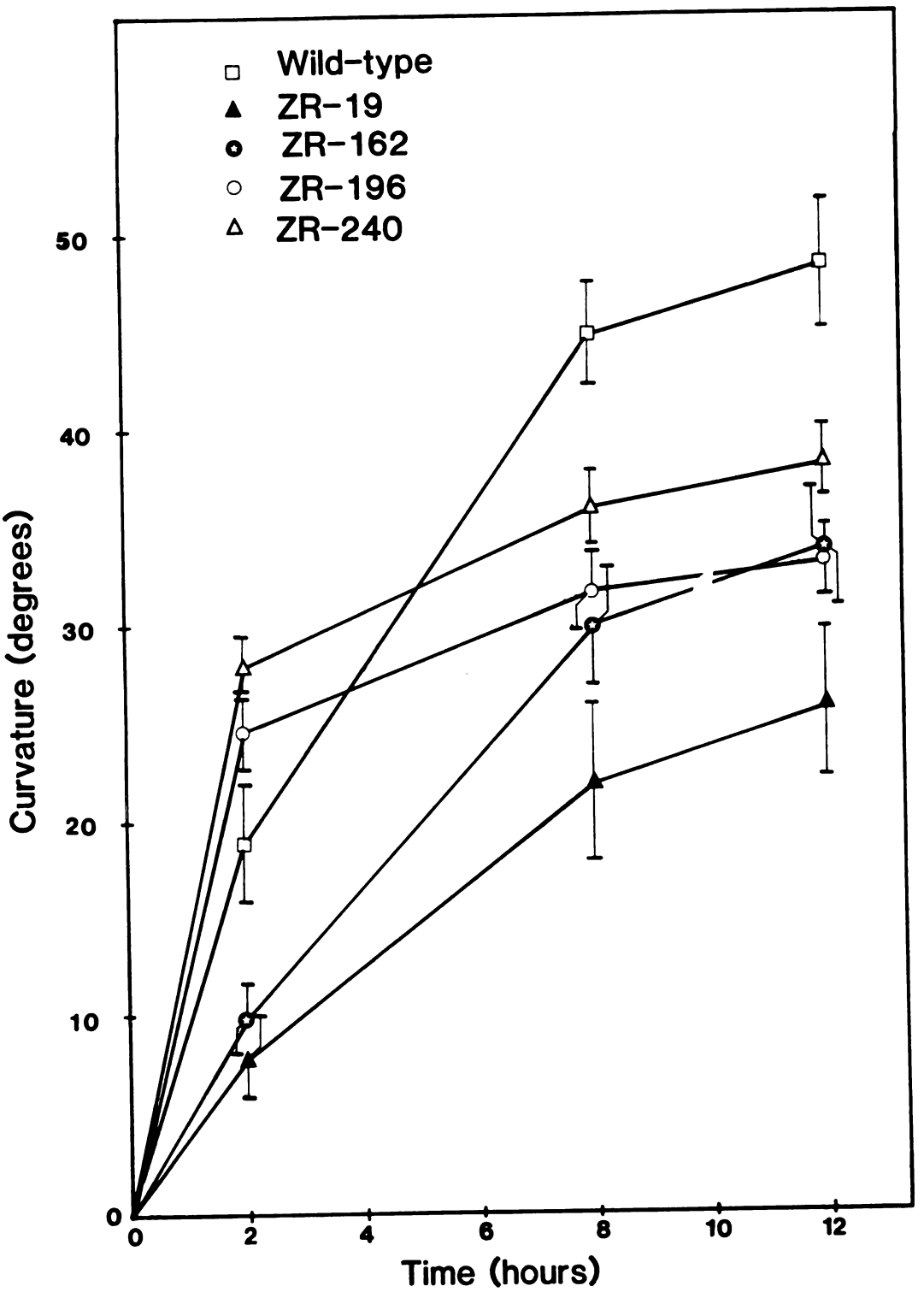

ZR-296
10
0
0
40
80
120
160
Number of Seedlings
ZR-440
10
0
0
40
80
120
Curvature (degrees)

Figure 5. Kinetics for geotropic curvature in Arabidopsis seedling shoots. Curvature was measured 2, 8, and 12 hours after the onset of the geotropic stimulus. Vertical bars represent +/- one standard error. a. Strains ZR-8, ZR-362, ZR-469, and Zr-555; b. Strains ZR-19, ZR-162, ZR-196, and ZR-240.

a.


Wild-type
ZR-362
ZR-469
ZR-555
ZR-8
Curvature (degrees)
0
10
20
30
40
50
0
2
4
6
8
10
12
Time (hours)

b.


Wild-type
ZR-19
ZR-162
ZR-196
ZR-240
Curvature (degrees)
0
10
20
30
40
50
0
2
4
6
8
10
12
Time (hours)

pE/cm^2/sec, and the resultant curvature measured. These data are plotted as fluence response curves (Fig. 6) which show that the amplitude of the phototropic response was decreased, but that the maximum first positive response is at the same fluence as in the wild-type (c.f., Fig. 6 and Fig. 7). In addition, the threshold of the first positive response may be higher in ZR-8 (about 5 pE/cm^2) than in the wild-type parent (about 0.3 pE/cm^2). It is also evident that the amplitude of the second positive response is decreased in ZR-8 relative to the wild-type parent.

A fluence response curve was also measured for ZR-8 with multiple pulses. Seed (m_4) of ZR-8 were sown as before, the seedlings exposed for different times to five pulses of light at 37.6 pE/cm^2/sec, the resultant curvature measured, and the data plotted in the form of a fluence response curve. The results (Fig. 8) show that the amplitude of the response was increased and that fluence required for the maximum response was shifted toward higher fluence compared with the results obtained using a single pulse (c.f., Fig. 6 and Fig. 8). The maximum response obtained using five pulses of light is less for ZR-8 than for the wild-type under comparable conditions, and the fluence required for the maximum response using five pulses is shifted for ZR-8 to higher fluence compared with the wild-type under comparable conditions (c.f., Fig. 8 and Fig. 9).

Figure 6. Fluence response curve for phototropism of the seedling shoots of Strain ZR-8 in response to a single pulse of light at 3.76, 37.6, 376 pE/cm^2/sec. Curvatures were measured 120 min after the onset of stimulation. Vertical bars represent +/- one standard error.

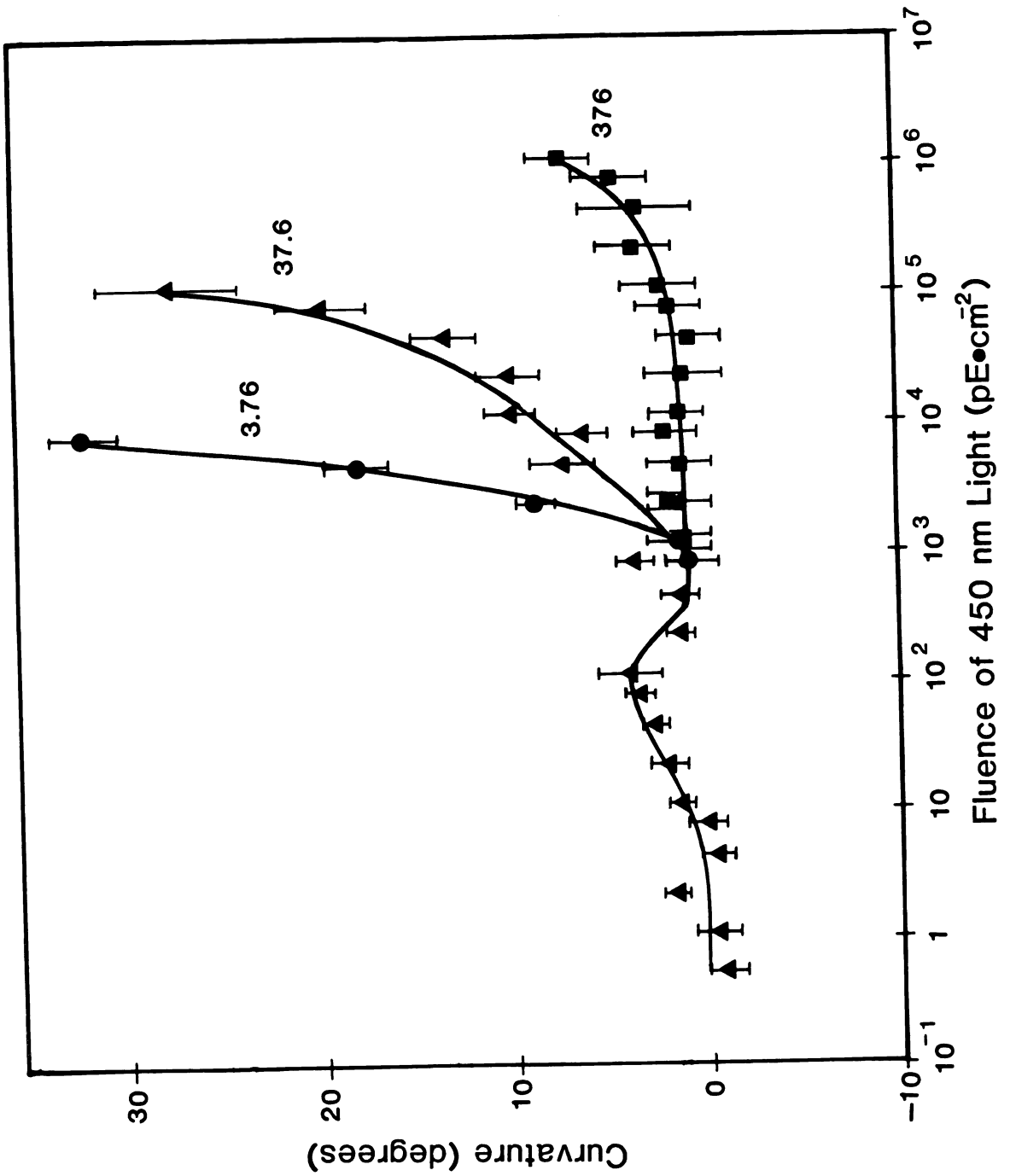
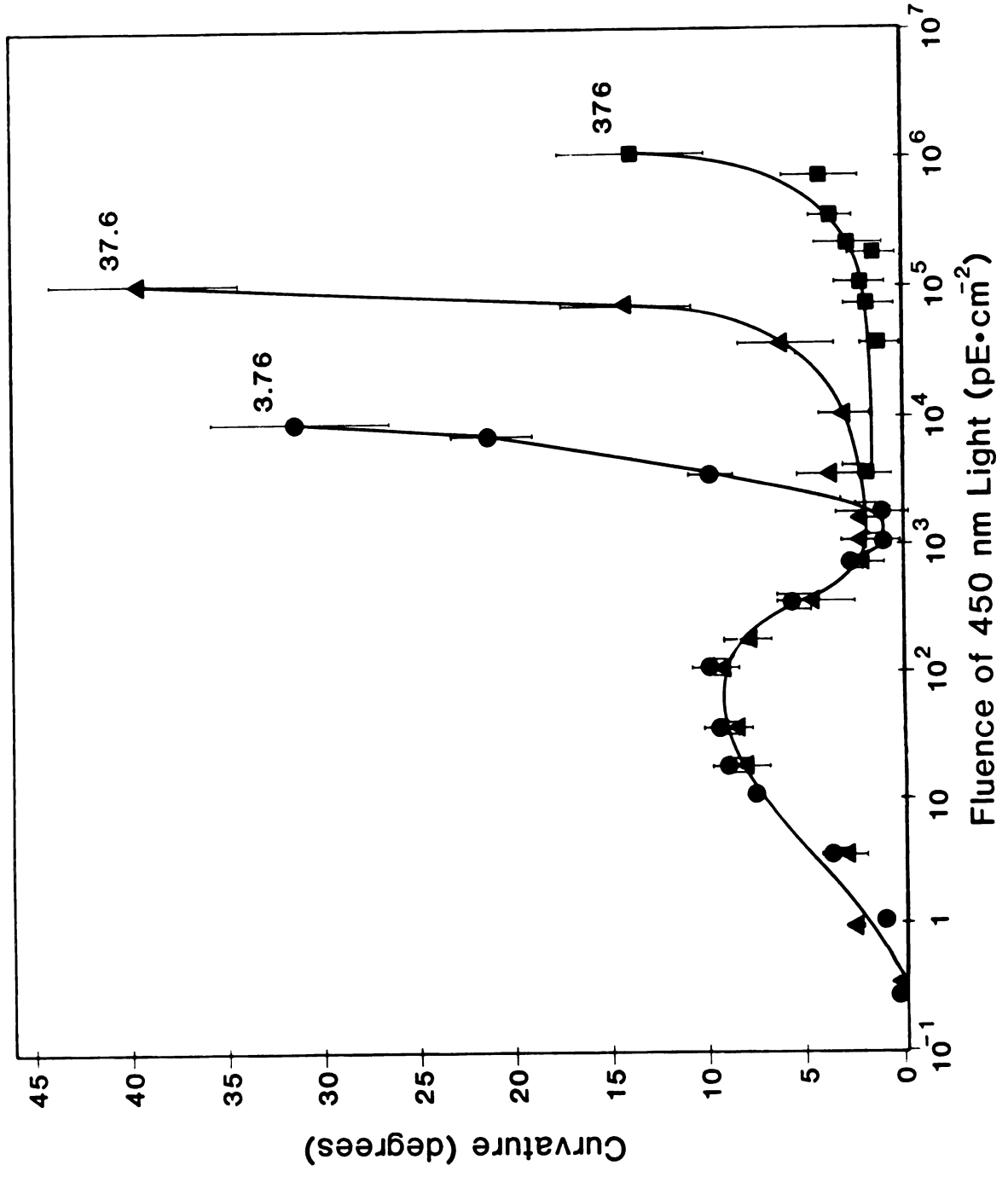

Curvature (degrees)
30
20
10
0
−10
10^{-1}
1
10
10^2
10^3
10^4
10^5
10^6
10^7
Fluence of 450 nm Light (pE•cm^{-2})
3.76
37.6
376

Figure 7. Fluence response curve for phototropism of the seedling shoots of wild-type in response to a single pulse of light at 3.76, 37.6 and 376 pE/cm^2/sec. Curvatures were measured 120 min after the onset of stimulation. Vertical bars represent +/- one standard error (From Steinitz and Poff, 1985).


Curvature (degrees)
45
40
35
30
25
20
15
10
5
0
10^{-1}
1
10
10^2
10^3
10^4
10^5
10^6
10^7
Fluence of 450 nm Light (pE•cm^{-2})
376
37.6
3.76

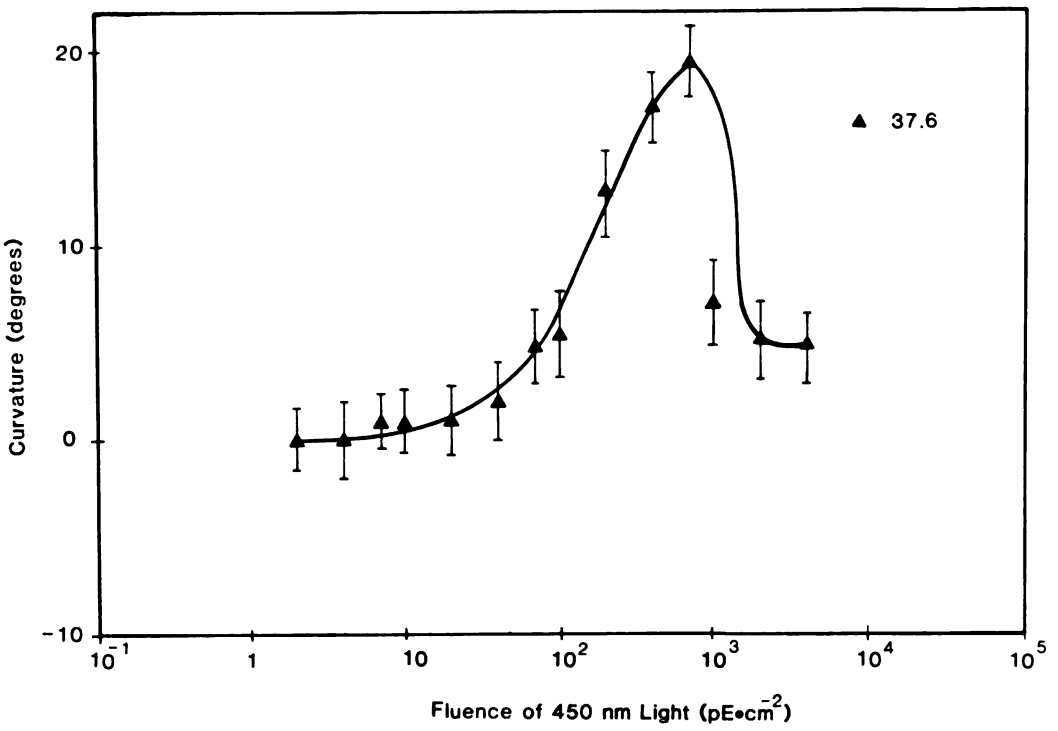


Figure 8. Fluence response curve for phototropism of the seedling shoots of Strain ZR-8 in response to five pulses of light at 37.6 pE/cm^2/sec. Curvatures were measured 120 min after the onset of stimulation. Vertical bars represent +/- one standard error.

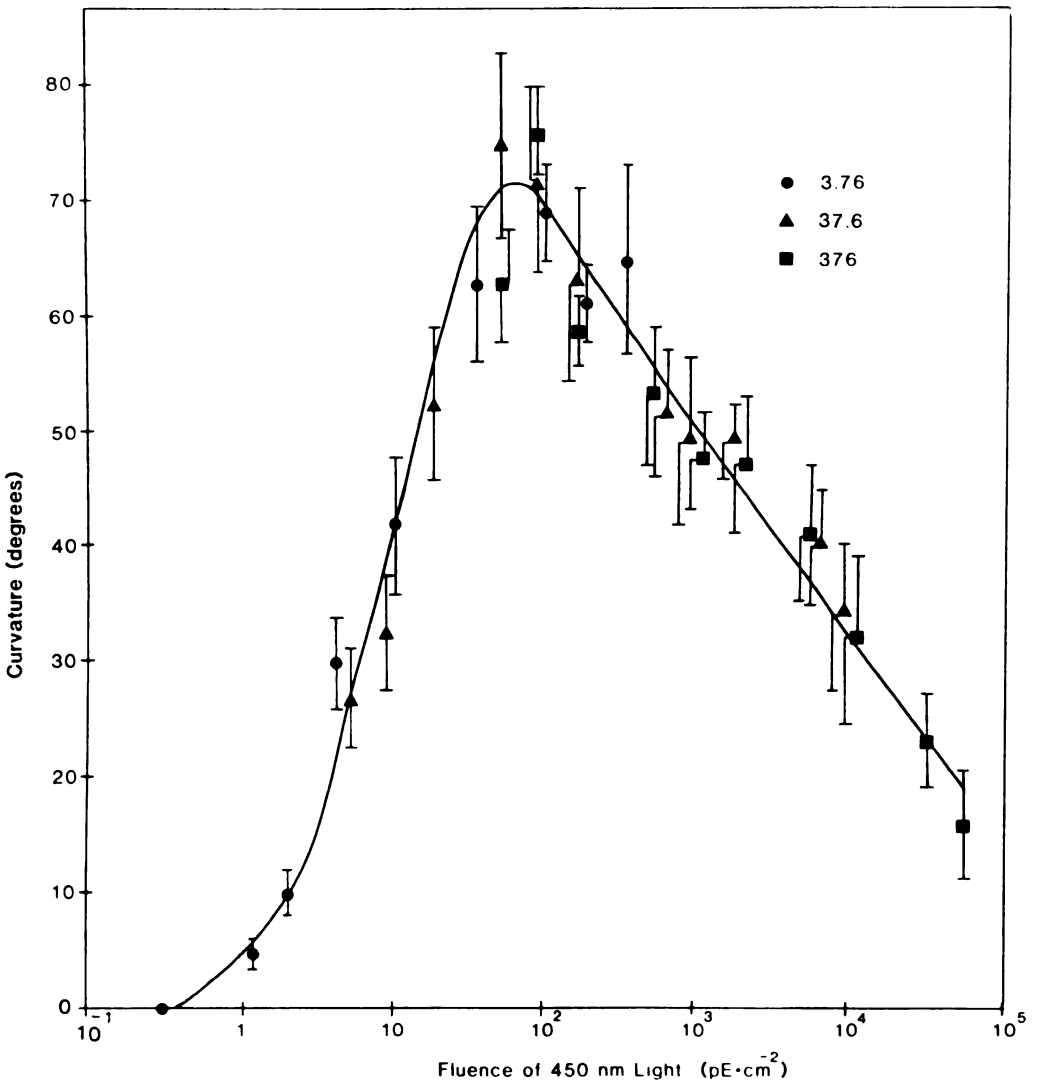


Figure 9. Fluence response curve for phototropism of the seedling shoots of wild-type in response to five pulses of light at 3.76, 37.6 and 376 $pE/cm^2/sec$. Curvature were measured 120 min after the onset of stimulation. Vertical bars represent +/- one standard error (From Steinitz and Poff, 1985).

The additivity of phototropic curvature with multiple pulses was measured for ZR-8. Seed (m_4) of ZR-8 were sown as before, the seedlings exposed to different numbers of pulses of light at 37.6 pE/cm^2/sec, and the resultant curvature measured. The results (Fig. 10) show that curvature increased about 4.5^o for each additional pulse for ZR-8 in contrast with an approximately 10^o increase per pulse reported for the wild-type (Steinitz & Poff, 1985).

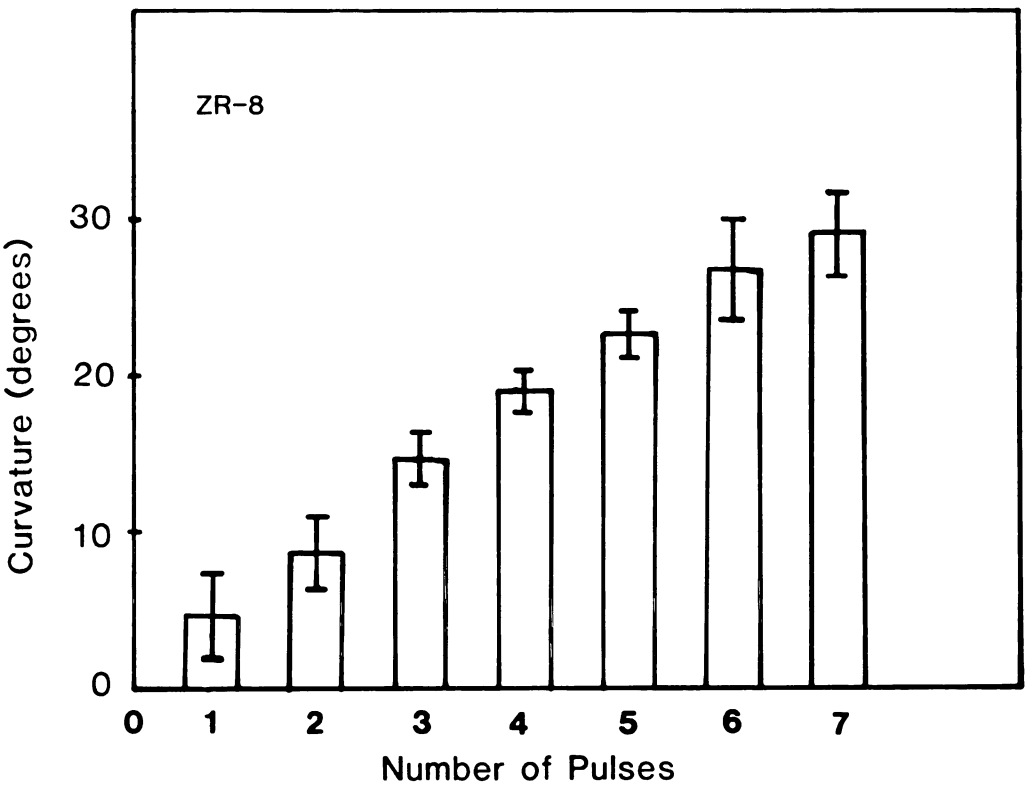


Figure 10. Induction of phototropic curvature in seedling shoots of Strain ZR-8 with sequential 2.5 sec pulses of light at 37.6 pE/cm^2/sec. The interval between sequential pulses was 15 min with curvature measured 120 min after the onset of stimulation. Vertical bars represent +/- one standard error.

DISCUSSION

It would be difficult at best to attempt any screening of phototropism mutants based on the differences measured here between the maximum phototropic curvature obtained in first positive phototropism (Fig. 2), and the curvature which might be expected by plants not exposed to any unilateral light (Fig. 1). Clearly, greater differences must be found to provide the basis for a screening procedure. Although a far greater amplitude of phototropic curvature can be obtained in second positive phototropism, it must be remembered that reciprocity is not valid for this response for which the amplitude of the response is primarily time dependent and only slightly dependent on the fluence rate. Thus it is doubtful whether or not one could find, using such a screen, mutants with a decreased amount of photoreceptor pigment. To locate such mutants, one must work under conditions in which the organism is a quantum counter - - conditions in which reciprocity is valid.

A second possible approach to the problem of how to screen for phototropism mutants was presented by the report of Steinitz and Poff (1985) that large angles of curvature could be induced under conditions where reciprocity is valid, if the organism is exposed to multiple pulses of

light at 15-20 min intervals. The value of this approach was supported by the frequency distribution obtained for seedlings exposed to nine pulses of light at 15 min intervals (Fig. 3). Since there is little overlap between this distribution and one for a similar population held in darkness, it appeared feasible to proceed with a screen. The validity of this decision was demonstrated by the mutants obtained.

The usefulness of mutants in gaining an understanding of phototropism requires ideally that they be stable, that every step in the transduction pathway be represented by a mutant, and that the mutants be characterized both with respect to genetics and with respect to biophysics. The first point has been partially accomplished by the fact that each of the 35 strains described have been carried at least to the m_4 generation, and some to the m_6 generation. It is difficult to respond to the second point without considerably more information. Once it is known from the genetic characterization how many genes are involved in the existing collection of mutants, it may be possible to guess whether or not all of the steps are represented. If only a few loci are found again and again, it is likely that most of the possibilities have been located. On the other hand, if few loci are represented more than once, it is likely that the pathway has not been saturated. In addition, this screening procedure has been designed for identifying

non-lethal mutants. If some specific step is critical for the plant, then the loss of that step may be lethal, and that step would not be represented in this collection of phototropism mutants. The ultimate way around this limitation would be the isolation of temperature-sensitive conditional mutants. Since the genetic characterization of these mutants will require a considerable amount of time, the remainder of this work was devoted to beginning the biophysical characterization.

It is well known that both phototropism and geotropism control the aerial orientation of plant organs. Given the fact that the expression of both responses requires a modification of the growth of the two sides of the shoot, it is likely that the two transduction pathways share common elements. Although this has not been proven, it has been sufficiently accepted to have been used as the basis for tests of the specificity of various potential inhibitors of phototropism. Given a collection of phototropism mutants, it was therefore desirable to test each strain for geotropism. Two types of geotropic responses were detected, one with the wild-type or "normal" response (Fig. 5a), and a second with impaired geotropism (Fig. 5b). This identifies at least two phenotypes, the phototropism minus, geotropism plus; and phototropism minus, geotropism minus. In addition, if the latter phenotype can be shown to result from single gene mutation, this supports the contention that

there are common elements in the phototropism and geotropism transduction pathways.

A single strain, ZR-8 mutant, which is phototropism minus and geotropism plus, was further characterized through the measurement of a fluence-response curve. For a simple photochemical sequence, where the response is regulated by a single photoreceptor pigment and proportional to the number of quanta absorbed by that pigment, it is clear that decreasing the "capture cross section" of the pigment should shift the fluence-response curve toward higher fluence. In contrast, for that simple case, altering the efficiency at some point "downstream" of the photoreceptor pigment should cause a decrease in the amplitude of the fluence response curve, but no shift along the fluence axis. The results obtained for ZR-8 do not easily fit into either category. It is clear that the amplitude of the response is decreased and that the response maximum for first positive phototropism is not shifted from that of the wild-type parent, and this is consistent with a modification "downstream" of the photoreceptor pigment. However, it appears from the fluence response measured using a single pulse that the threshold fluence for first positive phototropism is shifted toward higher fluence in comparison with that of the wild-type. It is possible that this apparent shift is actually statistical variation resulting from the extremely low curvatures measured in ZR-8. The

fluence-response curve measured using multiple pulses shows a decreased amplitude of response, a shift in the maximum response toward higher fluence, and an increase in the threshold fluence.

It is evident that these results are not easily reconciled with those expected for the simple hypothetical photoresponse system discussed above. One possible explanation for the threshold shift is the loss of one photoreceptor pigment out of a system containing two or more pigments. It is not clear what effect this should have on the fluence-response curve measured using multiple pulses, and this points to a need for much more work in this area. Any model which is developed to explain phototropism in response to multiple pulses of light must also include provision for the various changes in fluence response curve seen in ZR-8.

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APPENDIX I

Plant Physiol. (1985) 77, 248-251
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Short Communication

Blue and Green Light-Induced Phototropism in *Arabidopsis thaliana* and *Lactuca sativa* L. Seedlings[1]

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ABSTRACT

Exposure time-response curves for blue and green light-induced phototropic bending in hypocotyls of *Arabidopsis thaliana* (L.) Heynh. and *Lactuca sativa* L. seedlings are presented. These seedlings show significant phototropic sensitivity up to 540 to 550 nanometers. Since wavelengths longer than 560 nanometers do not induce phototropic bending, it is suggested that the response to 510 to 550 nanometers light is mediated by the specific blue light photoreceptor of phototropism. We advise care in the use of green 'safelights' for studies of phototropism.

There are several action spectra for the phototropic response in *Avena* coleoptiles, and all show that the activity of visible light is limited to the 400 to 500 nm region, with the major peak of activity being near 450 nm. Some of the action spectra end at about 500 to 520 nm (1, 7, 17, 19) making it difficult to assess whether these are indeed the longest wavelengths for phototropic activity. However, Everett and Thimann (7) and Elliot and Shen-Miller (6) state that there is no activity above 510 nm. Action spectra for phototropism of other higher plants are not availble. Recently Iino *et al.* (11) showed that phytochrome mediates red light-induced phototropism in corn, indicating that wavelengths longer than 510 nm induce phototropic bending.

While initiating a detailed study on phototropism in dicotyledonous plants, we encountered a high level of irregularity in our results, and tested the possibility that a green safelight might induce phototropic response. We report here results showing that green light induces phototropism in two dicotyledonous plants, and advise care in the use of green 'safelights' for studies of phototropism.

MATERIALS AND METHODS

Plant Growth. Seeds of *Arabidopsis thaliana* (L.) Heynh. were sown on 0.8% (w/v) agar (Difco Bacto-Agar) supplemented with 1.0 mM KNO_3 in rows of 0.3-ml wells prepared from Falcon 3911 microtest III flexible assay plates. Sowing was done at a density of one seed per well making it possible to expose individual rows of seedlings and to isolate single seedlings for measurement of curvature. The plant assay rows were placed in transparent plastic boxes (21 × 16 × 3 cm) lined with moistened paper to maintain high humidity. These incubation boxes were kept in the dark for 4 d at 3° ± 1°C to increase germination (16) and then transferred to a Sherer Gro Lab (Sherer-Gilbert, Marshall, MI) at 25°C and continuous white light for 30 h. This preirradiation was required to induce phytochrome-mediated germination (16). By the end of the preirradiation, only the radicle emerged from the seed coat. At this stage, seedlings were moved and kept in the dark at 25° ± 0.5° C, until the end of the experiment.

Lactuca sativa seeds (6058 Grand Rapids—Burpee's Greenhard brand; W. Atlee Burpee Co., Warminster, PA) were sown on Whatman 1 filter paper moistened with distilled water and incubated for 48 h in transparent plastic boxes at 25°C in dark. The germinated seedlings, while remaining in the boxes, were then transferred to 25°C and continuous white light for 8 h. This preirradiation was shown in preliminary experiments to increase the response to the subsequent phototropic stimulus (*cf.* 9). At the end of the preirradiation period, seedlings were selected for uniformity, transplanted into vials containing moistened vermiculite (one plant per vial), replaced in boxes, and kept in darkness.

The onset of the stimulus was at 72 h after transfer of the imbibed *Arabidopsis* seeds from 3° to 25°C, or at 96 h after sowing of lettuce seeds. For stimulation, *Arabidopsis* seedling rows or lettuce seedlings in vials were taken out from their incubation box, and the phototropic stimulus applied in a room with a controlled RH of more than 90%. Following stimulation, seedlings were again placed in the box and incubated in darkness for development of curvature. Curvature was measured 120 min after onset of the stimulus (Figs. 1 and 2).

Light Sources. White light during preirradiation, at 125 $mE \cdot m^{-2} \cdot s^{-1}$, was provided by General Electric Delux Cool White fluorescent tubes (F48T12/CWX/HO, 800 ma) and measured with a Li-Cor LI-185A radiometer. Phototropism was induced by a unilateral light stimulus. The source consisted of a projector equipped with a General Electric 300 w ELH multi-mirror quartzline bulb. The light was passed through 3 cm of 5% (w/v) cupric sulfate solution and through an interference filter. The wavelength maxima of the interference filters used were at 450, 500, 510, 521, 531, 540, 550, and 560 nm, (PTR Optics, Waltham, MA) with a half band width of 8.5, 8.2, 11.6, 9.6, 10.0, 12.0, 10.2, and 9.6 nm, respectively. To increase the ability to distinguish between 450 nm light and all other wavelengths used, and to avoid blue light contamination due to stray light, all the interference filters used for wavelengths of 500 nm and above were used in combination with a Corning 3-70 sharp cut filter (Corning Glass Works), which has greater than 30% transmission from 500 to 700 nm and less than 0.1% transmission at 480 nm.

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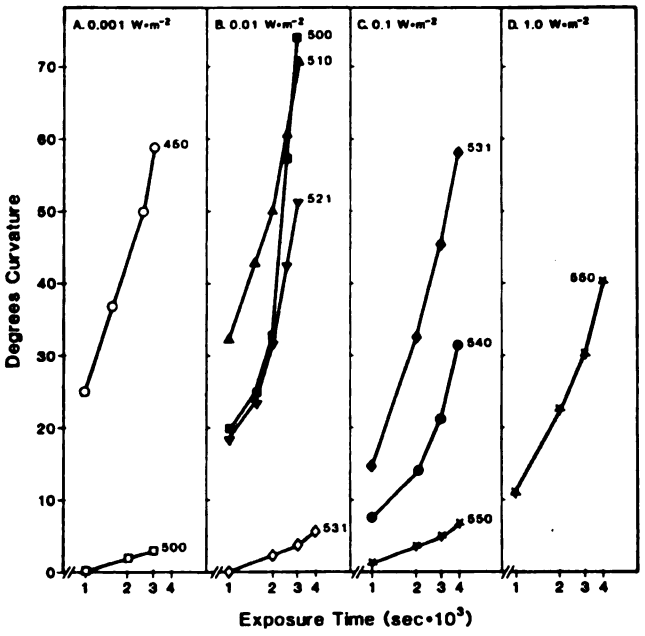

FIG. 1. Exposure time-response curves for phototropism by *A. thaliana* seedling hypocotyls. Seedlings were exposed to unilateral light with a fluence rate of 0.001 $w \cdot m^{-2}$ (A), 0.01 $w \cdot m^{-2}$ (B), 0.1 $w \cdot m^{-2}$ (C), and 1.0 $w \cdot m^{-2}$ (D). Curvature was measured 120 min after onset of exposure. SE ranged from 2 to 9% of the value. Numbers adjacent to curves indicate the actinic wavelength in nm.

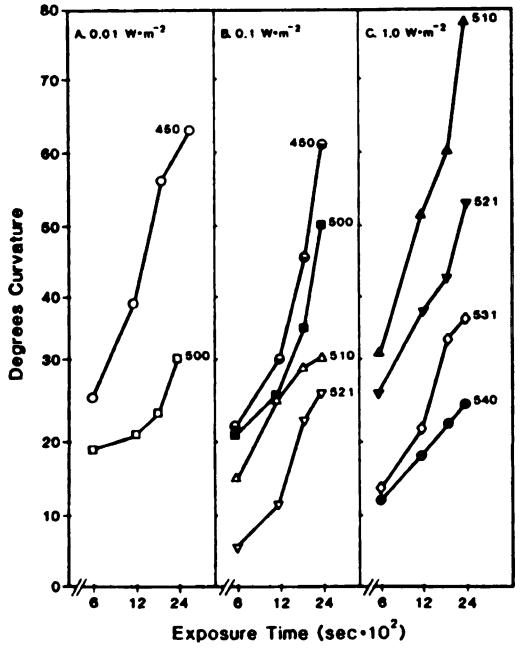

FIG. 2. Exposure time-response curves for phototropism in *L. sativa* seedling hypocotyls. Seedlings were exposed to unilateral light with a fluence rate of 0.01 $w \cdot m^{-2}$ (A), 0.1 $w \cdot m^{2}$ (B), and 1.0 $w \cdot m^{-2}$ (C). Curvature was measured 120 min after onset of exposure. SE ranged from 1 to 7% of the value. Numbers adjacent to curves indicate the actinic wavelength in nm.

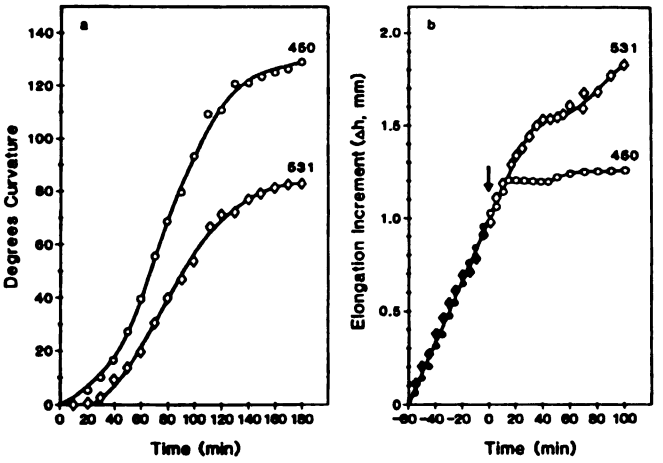

FIG. 3. a, Time course for bending by *Lactuca* seedling hypocotyls exposed to unilateral light of either 0.1 $w \cdot m^{-2}$ 450 nm light (O) or 1.0 $w \cdot m^{-2}$ 531 nm light (◇). Onset of exposure was at time zero. SE ranged from 0.3 to 10% of the value. b, Time course for growth increment below the tip of *Lactuca* hypocotyls. Onset of experiment in the dark at time −60 min. Arrow at time zero indicates onset of vertical irradiation. Seedlings were irradiated with either 0.1 $w \cdot m^{-2}$ 450 nm light (O) or with 10 $w \cdot m^{-2}$ 531 nm light (◇). (●, ◆), Growth in the dark. SE ranged from 0.1 to 7.6% of the value.

To test phototropic sensitivity above 560 nm, seedlings were exposed to light filtered through two Corning 2-62 sharp cut filters, which have greater than 50% transmission from about 600 nm above and less than 0.2% transmission from 400 to 580 nm. The fluence rate was varied by changing the distance between the plants and the light source and by using neutral density filters. Measurements of the monochromatic light were made using a model 68 Kettering radiometer (Laboratory Data Control, Riviera Beach, FL).

Measurement of Curvature and Growth Increment. At 120 min after onset of stimulus, *Arabidopsis* and *Lactuca* seedlings were gently adhered to a sticky transparent tape, with the direction of bending parallel to the tape surface. The tape with *Arabidopsis* seedlings was inserted into an enlarger and the curvature traced and measured from the enlarged image of the seedling. The tape with lettuce seedlings was photocopied and the curvature traced and measured from the copy. In preliminary experiments we found that the elongation of the lettuce hypocotyl between 56 h (end of the white light preirradiation) and 100 h after sowing was confined to a zone which, at 56 h after sowing, is within the first mm below the seedling's apical shoot meristem. At the end of the preirradiation period, a black Indian Ink mark was placed 1 mm below the tip. The time course of the growth increment of this region was followed by time lapse photography (Fig. 3b). Time lapse photography was also used to follow the development of curvature (Fig. 3a). Photographs were taken using a Minolta X-700 camera with Kodak IR film. IR light was provided by a Minolta auto 280 PX flash lamp equipped with an IR 87 C Kodak Wratten filter. Curvature and growth measurements were made on the enlarged negative images.

Twenty four *Arabidopsis* seedlings, sown in two rows, were used for each exposure. The rows were positioned to prevent shading and curvature was measured for seedlings with hyocotyls emerging vertically from the agar. A mean curvature value was calculated from measurements made on 15 to 20 seedlings at each exposure. Experiments were repeated four to six times. Thus, for each exposure, at least four mean values were obtained. The data in the figures represented a final mean value of these means. In experiments with lettuce, only two seedlings could be

used at a time because of the use of a macro lens for the time lapse photography of elongation (Fig. 3b). All experiments with lettuce seedlings were done in triplicate.

RESULTS AND DISCUSSION

The only appropriate way to evaluate the relative effectiveness of different wavelengths of light in the induction of a response is to compare fluence response curves for each wavelength. However, this can be done only when one can establish reciprocity for that response. For phototropism, reciprocity holds only over the range of fluences to which the plant exhibits what is traditionally referred to as the 'first positive' response (5). Unfortunately, the peak of the first positive response is only 9 to 10° curvature for *Arabidopsis* seedlings which does not make the desired quantitative approach feasible. A larger curvature can be induced with relatively longer unilateral exposures to the stimulating light (a condition in which reciprocity is not valid) and the magnitude of the resulting response depends on the fluence-rate used, as is known to occur in the 'second positive' response (5). Moreover, the dependency on the fluence rate is complex (see Fig. 5 in Ref. 7). Given these factors, comparison of fluence rate-response curves would be misleading. We have therefore chosen to present results in the form of exposure time-response curves. (Figs. 1 and 2).

Arabidopsis seedlings were exposed to durations of 10 to 40 min of unilateral light of different wavelengths and subsequently returned to darkness. Curvature was measured 120 min after onset of the stimulus. A low fluence rate of 1 $mw \cdot m^{-2}$ at 450 nm was sufficient to induce a very strong bending response (Fig. 1). Using the same fluence rate at 500 nm induced a minute response, yet the strong curvature was readily induced at 500, 510, and 521 nm given the fluence rate of 10 $mw \cdot m^{-2}$. Similarly, this latter fluence rate at 531 nm induced a significantly smaller bending response, but an increase of the fluence rate to 100 $mw \cdot m^{-2}$ led to a response comparable to that induced by 1 $mw \cdot m^{-2}$ at 450 nm. The degree of curvature was consistently lower for 540 and 550 nm light although a considerable response could be induced with 1 $w \cdot m^{-2}$ at 550 nm. At wavelengths of 560 nm and above, no bending was observed at any fluence rate from 10^{-3} to 10^{3} $mw \cdot m^{-2}$.

The response of lettuce seedlings was significantly less than that of *Arabidopsis* seedlings in the green light region (Fig. 2). At all wavelengths above 500 nm, a fluence rate 1 order of magnitude higher was required in order to detect the phototropic response. When the yellow-orange Corning cut-off filters were used (see "Material and Methods"), no curvature was observed in either *Arabidopsis* and *Lactuca* seedlings. Thus, 560 nm probably represents the upper limit of phototropic sensitivity in the visible region for both species.

Given the report of Elliot and Shen-Miller (6) that the fluence-response curves and action spectra are similar for growth inhibition and phototropism in *Avena* coleoptiles, and given the report of Hartmann (10) that 531 nm light induced no growth inhibition in lettuce hypocotyls, we were surprised to find green light-induced phototropism in lettuce (Fig. 2). To investigate further, we followed the time course for the blue light- and green light-induced growth inhibition and curvature development in the hypocotyls of lettuce seedlings. The first measurable blue light-induced growth inhibition and curvature development appeared about 10 min after onset of light. Green light-induced curvature development and inhibition of elongation could be detected 30 to 40 min after onset of light (Fig. 3, a and b). Hence, as in *Avena* (6), *Lactuca* spectral sensitivity for phototropism and photomorphogenesis is similar in the blue-green region.

This reported responsiveness of lettuce to 531 nm does not necessarily stand in disagreement with Hartmann's observations (10). The fact that he could not find an inhibitory growth response to 531 nm light might have resulted from a difference in experimental approach. It has been previously observed that the response by lettuce hypocotyls to red light is qualitatively different in different regions of the hypocotyl. This results in a zero net effect when a red light-induced growth response is measured on the level of the entire organ (8). Our elongation measurements were confined to that region of the hypocotyl in which the curvature response to unilateral light developed. In contrast, in the work where no inhibition of elongation by 531 nm light was reported, growth of the entire hypocotyl was measured (10).

Phytochrome is thought to be the photoreceptor mediating the photomorphogenic influence of green light (3, 4, 15). In some cases, a specific reversible green-orange-red (13) or far-red-green light photoreceptor has been suggested (18). In phototropism, we see no reason to postulate a photoreceptor pigment for the green region different from the blue light photoreceptor of phototropism. The sharp drop in the 480 to 510 nm region as seen in the action spectra of *Avena* coleoptile phototropism (19) may simply be red-shifted into the 520 to 550 nm region in *Arabidopsis* and *Lactuca*. This significant extension of phototropic activity into the green region resembles a similar extension of phototropic activity into the green region in *Celosia cristata* (2). These results definitely corroborate the variation in spectral phototropic sensitivity among different higher plant species (reported by Atkins [2]). A precise comparison of the spectral sensitivites of *Arabidopsis*, *Lactuca*, and *Avena* must await the development of a reasonable method for measuring an action spectrum in *Arabidopsis* and *Lactuca*, overcoming the limited first positive phototropic response.

The present work demonstrates that 500 to 550 nm light can serve as a vectorial signal for inducing phototropic responses. Green light has also been shown to have a scalar effect on subsequent gravitropic response (13, 14), and to have a potential photomorphogenic effect via reception by photochrome. Therefore, we suggest caution in the use of green light as safelight not only in studies on photomorphogenesis (12) but also in work on phototropism. The 'safety' of any light should be established for any given species.

Acknowledgments—We thank T. R. Best for excellent technical assistance.

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