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DEVELOPMENT OF A GENETIC SYSTEM FOR THE STUDY OF GRAVITROPISM IN *ARABIDOPSIS THALIANA*

By

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ABSTRACT

DEVELOPMENT OF A GENETIC SYSTEM FOR THE STUDY OF GRAVITROPISM IN *ARABIDOPSIS THALIANA*

By

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Genetic systems have been successfully utilized in the dissection of several biochemical and sensory pathways. However, this approach has not been exploited in the study of gravitropism due to the lack of a comprehensive collection of gravitropism mutants within a single species. To establish such a genetic system in *Arabidopsis thaliana*, gravitropism mutants were identified using a direct screening procedure. Five mutants from this screen were chosen for further study on the basis of their starch content and gravitropism phenotypes.

F2 segregation ratios from reciprocal backcrosses, complementation analyses, and dihybrid analyses comprised the subsequent genetic study of the five mutant lines. Genetic analyses suggested that each of the five mutants carried single recessive nuclear mutations in two loci affecting gravitropism and one locus affecting starch content. The affected starch locus, phosphoglucomutase (*pgm*), was shown to be genetically separable from the gravitropism lesion. These data suggest that previous conclusions based on the gravitropic response of starchless and starch-deficient mutants should be re-examined.

Physiological and biophysical analyses of the mutant lines included measurements of elongation growth rates, phototropism, and stimulus-response curves for gravitropism. The absence of any correlation between elongation growth and gravitropism phenotype, and the capability of each line to respond phototropically, suggested that the observed gravitropism phenotypes were not due to alterations in either elongation or differential growth. Light-grown seedlings exhibited a similar light-induced enhancement of gravitropism in mutant and wild-type seedlings, regardless of the starch content phenotype. These data support the hypothesis that starch content is not involved in the light-enhancement of gravitropism.

The work described in this dissertation demonstrates the utility of a genetic system for the study of gravitropism in higher plants. The importance of controlling additional parameters in future screening procedures is also discussed.

To my parents,
Fred E. Bullen and Bertha Lasley Bullen,
who through their quiet love and steadfast support
gave me the courage to question, and
the freedom to seek my own answers.

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INTRODUCTION

Plants, animals, bacteria, and fungi all perceive and respond to certain features of their environment in ways which maximize survival under the perceived conditions. These sensory systems may result in the acquisition of nutrients (for examples, see Ordal & Gibson, 1977; Ordal, *et al.* 1977; Clark & Koshland, 1979), the avoidance of toxic situations (for a discussion, see Macnab, 1984), the regulation of growth and development (for example, see Briggs, *et al.*, 1984), the optimization of light or temperature conditions (for examples, see Feinleib, 1980; Hertel, 1980; Nultsch, 1980; Poff, 1984; Fortin & Poff, 1991), and the maintenance of contact with a substrate for anchorage and/or access to water and minerals (Jaffe & Galston, 1968; Sack, 1991). This last category includes gravitropism, the directional growth of plant organs with respect to the gravity vector.

In spite of over 100 years of investigation, the sequence of events which constitutes sensory transduction in gravitropism remains unknown. Conflicting data regarding the role of starch in gravitropism has continued to confound any conclusion regarding the amyloplast hypothesis of gravity perception. Although calcium and auxin are likely involved in transmission, virtually nothing is known about how they interact with the gravity receptor or with each other. Identification of the mechanism(s) through which phytochrome mediates the gravitropic response, and an understanding of the relationship(s) between the transduction pathways for gravitropism and phototropism, could provide additional avenues for dissection of the gravitropism pathway. Genetic approaches have proven successful in the dissection of biochemical and sensory pathways in the past. However, no comprehensive collection of gravitropism mutants existed within a single species to facilitate such dissection of the gravitropism transduction pathway.

The goal of this work was to develop a genetic system in *Arabidopsis thaliana* for the study of gravitropism, and to use that system to study the roles of starch and light in the transduction of the gravity signal.

REVIEW OF LITERATURE

To respond to a stimulus, an organism must first detect the stimulus signal, and then translate that signal into the physiological steps which culminate in the response. Thus, sensory systems are often conceptually subdivided into three phases: 1) perception of the stimulus, 2) transmission or transduction of the perceived signal, and 3) response (Koshland, 1980). Difficulties in defining boundaries between these phases in some systems has led to the suggestion that transduction be defined as inclusive of perception and response (Haupt, 1984). This is the sense in which these terms will be used here.

Models for sensory transduction in gravitropism suggest that physical interactions between the plant and gravity are distinct from the actual perception of the gravity signal. Thus, the transduction of gravitropism has been subdivided into four conceptual phases: 1) susception, 2) perception, 3) transmission, and 4) response (Hensel, 1986; Björkman, 1988). Susception results from the initial physical influence of gravity on the susceptor, presumably some mass or component in or on the cell which is especially susceptible to changes in the gravity vector. Perception is the conversion of this physical signal into a physiological signal, and requires interaction between the susceptor and a receptor (Sack, 1991). Transmission includes all of the physiological events through which the signal moves from perception to response. The final phase, response, refers to those events which affect the direction of organ growth. Although response typically refers to the differential growth resulting in curvature, the plant is responding to gravity even when maintaining a vertical orientation and straight growth pattern.

It is evident from the numerous reviews of gravitropism that considerable physiological, biophysical, and genetic evidence has accumulated which both support and refute the numerous transduction models proposed. Reviews which discuss elements of all four phases of transduction include Larsen (1962), Johnsson (1971), Audus (1975), Juniper (1976), Jackson & Barlow (1981), Wilkins (1984), Pickard (1985), Moore & Evans (1986), Timmell (1986), and Hart (1990). Reviews which focus on the susception and perception phases of gravitropism include Audus (1962), Juniper (1977), Audus (1979), Volkmann & Sievers (1979), Björkman (1988), Sievers & Hensel (1990), and Sack (1991). A thorough review of the use of mutants in studies of gravitropism has also been published (Roberts, 1987).

To simplify the discussion here, the following review is divided into three major sections: 1) sensory transduction in gravitropism, 2) interactions between light and gravity, and 3) genetic systems in sensory physiology.

SENSORY TRANSDUCTION IN GRAVITROPISM

Several models for the transduction of the gravity signal have been proposed in the 100 years since Charles Darwin (1896) first defined the problem. Since gravitropism can be envisioned as progressing in phases, the models will be discussed here according to the four phases of transduction. Although some overlap will undoubtedly be necessary, it is hoped that this approach will simplify the discussion since the elimination of one phase in a model would not necessarily render other phases of that model invalid.

Susception

Gravity susceptors have been proposed based on the presumed relationship between gravity and the mass of the susceptor. Proposed susceptors fall into two broad categories: 1) intracellular particles or organelles called statoliths, and 2) the total mass of the protoplast acting as a statolith.

Intracellular Statoliths

The "statolith theory," first proposed by in 1886 by Berthold (after Rawitscher, 1932), suggests that gravity susception occurs through the sedimentation of high-density intracellular particles. A particle functioning as a statolith must move with respect to the gravitational vector, and be of sufficient mass to be more susceptible to gravity than to Brownian motion (Johnsson & Pickard, 1979; Björkman, 1988). A system consistent with this theory has been described for the alga *Chara* (for a review, see Sievers and Volkmann, 1979). In *Chara*, the settling of barium sulfate crystals toward the lower side of the rhizoid results in growth inhibition on that side of the rhizoid. This local inhibition of growth results in downward curvature of the rhizoid.

The likelihood of organelles such as mitochondria, dictyosomes, nuclei, spherosomes, vacuoles, or amyloplasts functioning as the gravitropism statolith in higher plants has been examined both theoretically and empirically. Based on assumptions about cytoplasmic viscosity, statolith shape and statolith behavior, sedimentation has been predicted for each organelle using the physical equations which describe the movement of a body through a liquid (Audus, 1962; Shen-Miller, 1970; Pollard, 1971). However, deviations from the assumptions, and the possible effects of cytoplasmic streaming and intracellular electrostatic forces, render these calculations of limited value in identifying the statolith (Volkmann & Sievers, 1979). Empirical evidence consists largely of cytological studies of organelle distributions in vertically-oriented organs, and/or redistributions in horizontally-oriented organs (for examples, see Griffiths & Audus, 1964; Moore, 1983; Moore & Pasieniuk, 1984; Moore, 1985a,b; Moore,1986; Sack & Kiss, 1989). Of all the organelles examined, only the amyloplast fulfills the theoretical requirements for the gravitropism statolith, and consistently exhibits sedimentation in all species examined (for a review, see Sack, 1991).

First proposed independently by Haberlandt (1900) and Nemec (1900), the starch-statolith or amyloplast hypothesis is supported by correlative evidence. Amyloplasts are generally abundant in the presumed sites of gravity perception, and exhibit a marked redistribution upon horizontal placement of the plant organ (for reviews, see Poff & Martin, 1989; Sack, 1991). Several early investigators also observed positive correlations between loss of starch and loss of gravity response (Haberlandt, 1902; Nemec, 1902; Darwin, 1903). Chemical destarching revealed similar correlations in later works (Iversen, 1969; Zagorski & Lewak, 1980). Similarly, in the *amylomaize* mutants of corn, a reduced gravitropic response was positively correlated with the reduced size of the amyloplasts (Hertel, *et al.*, 1969; Filner, *et al.*, 1970).

Evidence contrary to the amyloplast theory has also been reported in experiments very similar to those described above. Chemical removal of starch did not always result in loss of gravitropism response (Went & Pekelharing, 1909; Syre, 1938; von Bismarch, 1959; Pickard and Thimann, 1966), although the completeness of chemical destarching in these experiments has been questioned (Filner, *et al.*, 1970). More recently, a starchless mutant of *Arabidopsis thaliana* with abnormally shaped, nearly starchless amyloplasts has been described to exhibit gravitropic curvature to within 70% of that observed in the wild-type parent (Caspar & Pickard, 1989). However, an observed decrease in this mutant's sensitivity to gravity, as well as the intermediate gravitropic response of a *Nicotiana* mutant with intermediate levels of starch, led to the suggestion that starch-depleted plastids may still function as the gravity susceptor, and that starch may be necessary for full sensitivity to gravity (Kiss & Sack, 1989; Kiss, *et al.*, 1989). Whether or not these abnormal plastids exhibit significant sedimentation under the influence of gravity has not been resolved (Caspar & Pickard, 1989; Kiss, *et al.*, 1989).

Space-flight experiments have yielded other data which conflict with the amyloplast hypothesis. Under microgravity conditions, root cap amyloplasts became randomly distributed and starch was depleted (Merkys, *et al.*, 1981), implying that the amyloplasts were susceptible to gravity. However, gravity was required for root cap development, indicating that the root cap is not the sole site of gravity perception in roots (Moore, *et al.*, 1987). These data are consistent with arguments based on a review of early earth-based experiments (Poff & Martin, 1989). However, since elements of plant growth and development are variously retarded or accelerated in microgravity environments, the possible influence of parameters unrelated to the gravitropism mechanism must also be considered (for a review, see Halstead & Dutcher, 1987).

Thus, although gravitropism can occur in the absence of amyloplast starch, unequivocal evidence that the plastid is not the gravity susceptor has not been reported.

Protoplast

Several models for gravitropism in which the mass of the protoplast functions as the gravity susceptor vary only in the proposed means of perception (Czapek, 1898; Pickard & Thimann, 1966; Edwards & Pickard, 1987; Wayne, *et al.*, 1990). Evidence which fails to support the amyloplast hypothesis has often been cited as indirect evidence for non-plastid based susception. More convincing, the absence of any obvious statolith in *Phycomyces* suggests that a non-plastid system in plants may exist (Shropshire & Lafay, 1987). More direct evidence in support of these hypotheses has not been reported.

The theoretical feasibility of the protoplast as susceptor depends on the perception mechanism proposed. If the weight of the protoplast was perceived as a pressure differential across the plasmalemma or the cell wall (Pickard & Thimann, 1966), a very large gravity signal amplification of 10^6 would be necessary for the signal to exceed background noise (Björkman, 1988). If gravity induced a tension differential across the membrane (Edwards & Pickard, 1987), the resulting energy would be less than the activation energy required for opening most stretch-activated ion channels (Björkman, 1988). However, a potential energy sufficient for membrane channel activation would result if the increased pressure of the falling protoplast on one side of the cell and the decreased pressure of the protoplast on the other side of the cell resulted in a differential force across the cell (Wayne, *et al.*, 1990). Under this model of perception, amyloplasts could contribute to susception by either increasing the total mass of the protoplast (Wayne, *et al.*, 1990), or by amplifying the gravity signal through a localized increase in mass (Sack, 1991).

Summary

The identity of the gravity susceptor remains undetermined. Although evidence favors the plastid-based model, it is possible that the weight of the protoplast contributes to gravity susception. In some species, such as *Phycomyces*, protoplast weight may constitute the only means of gravity susception.

Perception

Perception of the gravity signal requires that the susceptor and the receptor interact. This interaction may involve changes in the physical contacts between the susceptor and the receptor, or perturbations in electrostatic potentials or cytosolic pressures within the cell. The endoplasmic reticulum, the plasmalemma, and the cytoskeleton have all been proposed as possible gravity receptors.

Endoplasmic reticulum

The endoplasmic reticulum (ER) has been proposed as the gravity receptor based on its polar distribution in columella cells, and on the asymmetrical distribution of calcium which develops in gravistimulated organs. This model assumes that sedimenting statoliths press against the ER inducing the release of calcium into the cytoplasm (for a review, see Evans, *et al.*, 1986). Redistribution of ER in gravistimulated *Chara* rhizoids has also been cited as evidence for the involvement of the ER in the curvature response (Bartnik & Sievers, 1988).

The coincident polar distribution of amyloplasts and ER has been cited as evidence for the involvement of the ER in gravitropism. Enrichment of ER in the distal portion of columella cells has been observed in some species (for examples, see Volkmann, 1974; Olsen & Iversen, 1980; Sack & Kiss, 1989), but is apparently lacking in others (for examples, see Philips & Torrey, 1974; Ransom & Moore, 1983; Barlow, *et al.*, 1984). Disruption of the distal ER in *Lepidium* roots reduced the gravitropic response, but did not eliminate it (Wendt, *et al.*, 1987). However, since a few ER cisternae remained in the distal portion of the treated cells, these results did not eliminate the ER as the possible gravity receptor. Rather, the polarity of ER distribution found in some species could represent a means of signal amplification (for a discussion, see Sack, 1991).

It has also been suggested that actual physical contact may not be necessary for amyloplasts to interact with the ER. Physical contact has been observed in *Arabidopsis* (Sack & Kiss, 1989); however, such contacts have not been demonstrated unequivocally in all species examined (for examples, see Volkmann, 1974; Stephenson & Hawes, 1986). Alternatives to actual contact include localized cytosolic pressure induced through displacement of the amyloplasts, and electrostatic interactions between the plastid and ER membranes (for discussions, see Volkmann & Sievers, 1974; Björkman, 1988).

Plasmalemma

Current models proposing the plasmalemma as the gravity receptor assume that the mass of the protoplast is the gravity susceptor. Earlier models in which sedimenting statoliths either interacted directly with the plasmalemma (Haberlandt, 1914; Iversen & Larsen, 1973) or physically controlled symplastic continuity by blocking the plasmodesmata (Arisz, 1969; Barlow, 1974a,b) have been discounted. Extensive cytological studies of amyloplast sedimentation failed to demonstrate any contact with the plasmalemma (for reviews, see Juniper, 1976; Perbal, 1978), and further suggested that ER situated between the amyloplasts and the plasmalemma prevented direct contact (Audus, 1975). Experiments demonstrating apoplastic transmission of the gravitropism signal suggested that plasmodesmatal gating was not involved (Moore, *et al.*, 1990).

Ion channels within the plasmalemma could open or close in response to changes in membrane pressure or tension, resulting in changes in ion transport (Pickard & Thimann, 1966; Edwards & Pickard, 1987). Such channels have been described in animals and fungi (Guharay & Sachs, 1984; Zhou, *et al.*, 1991), and plasma membrane ion channels have been documented in plants (for an example, see Bush, *et al.*, 1988). However, estimates of the activation energy required to open such channels are significantly greater than calculations of the energy supplied to the cell by gravity, even if that energy were focused on the channels by proposed linkages between the plasmalemma and the cell wall (Edwards & Pickard, 1987; Björkman, 1988).

Cytoskeleton

As a gravity receptor, the cytoskeletal complex of microtubules and microfilaments could activate ion channels in the amyloplast envelope (Björkman, 1988), or focus the mechanical energy of susception on ion channels in the plasmalemma (Edwards & Pickard, 1987; Hensel, *et al.*, 1989). Cytological evidence for these models is lacking due to difficulties in visualizing the cytoskeleton (White & Sack, 1990). Interactions between the cytoskeleton and amyloplasts were suggested by the increased rate of amyloplast sedimentation observed when the cytoskeleton was disrupted by cytochalasin (Sievers, *et al.*, 1989).

However, microtubules and microfilaments also appear to influence the intracellular positioning of the ER (Hensel, 1984; Hensel, 1987; Hensel, 1989b). Thus, direct relationships between the cytoskeleton and gravitropism may be difficult to demonstrate.

Summary

The identity of the gravity receptor remains undetermined. Although evidence favors the ER as the gravity receptor, this model relies on the plastid-based model of gravity susception and the assumption that calcium distribution is the first event of signal transmission. Since other means of susception may contribute to gravitropism, other means of gravity perception may also be involved.

Transmission

Transmission refers to the series of events which link perception and response. The intercellular communication through which a signal travels from the site of perception to the site of response can be accomplished either chemically or electrically (for a review, see Björkman, 1988). Both modes of intercellular communication have been examined with respect to gravitropism.

Chemical

Possible components of chemical transmission in gravitropism include calcium ions, calmodulin, phosphoinositides, and auxin. Accumulation of apoplastic calcium on the concave side of gravistimulated organs has been observed within a few minutes of gravistimulation, and before the onset of curvature (Goswami & Audus, 1976; Lee, *et al.*, 1983; Slocum & Roux, 1983). A relationship between calcium redistribution and gravitropism was suggested by the inhibition of both responses by the calcium chelator EGTA (Daye, *et al.*, 1984; Lee, *et al.*, 1984). Similarly, high concentrations of calmodulin in columella cells and shoot apices (Lin, *et al.*, 1986), the inhibition of gravitropism by calmodulin inhibitors (Björkman & Leopold, 1987; Stinemetz & Evans, 1987), and an apparent increase in calmodulin mRNAs in response to touch (Braam & Davis, 1990) suggest that calmodulin may be involved in signal transmission. Phosphoinositides could also contribute to signal transmission by enhancing the release of calcium from the ER (for a discussion, see Björkman, 1988). Evidence for the phosphoinositide pathway in plants has been reviewed (Poovaiah, *et al.*, 1987a,b), and its involvement in red-light mediation of orthogravitropism has been suggested in maize (Roux and Serlin, 1987; Perdue, *et al.*, 1988). Another event of signal transmission may be the redistribution of growth regulators, evidenced by the redistribution of auxin (for a review, see Evans, *et al.*, 1986) and small-auxin-up RNAs or SAURs (McClure & Guilfoyle, 1989) prior to the onset of curvature. Inhibition of calcium redistribution by auxin transport inhibitors has also suggested a link between calcium and auxin transport (Lee, *et al.*, 1984; Evans, *et al.*, 1987; Hart, 1990).

Electrical

The rapid development of electrical gradients has been reported in gravistimulated roots and shoots (Tanada & Vinten-Johansen, 1980; Behrens, *et al.*, 1982; Björkman & Leopold, 1987b). First reported by Brauner (1927), the "geo-electric" effect was discounted when it was found to be an artefact of the measuring probes (Wilkins, 1984; Pickard, 1985). However, recent studies have found a correlation between rapid changes in a transverse current and polarization events at the cellular level in the columella (Sievers, *et al.*, 1984; Behrens, *et al.*, 1985). It has been suggested that these electrical changes correspond to changes in intracellular calcium concentrations (Sievers, *et al.*, 1984; Behrens, *et al.*, 1985; Hepler & Wayne, 1985; Björkman & Leopold, 1987a,b). Although current development is rapid, its persistence for periods of more than 10 minutes supports the hypothesis that it is a consequence of later transduction events (Pickard, 1985; Behrens, *et al.*, 1985). However, it has also been proposed that a voltage difference may directly regulate hormone movement from the stele into the surrounding tissue (Bandurski, *et al.*, 1990a,b).

Although action potentials and electrophoresis are also possible means of intercellular communication, their role in gravitropism is doubtful (for a discussion, see

Björkman, 1988). Action potentials have been shown to play a role in leaf folding in *Mimosa* and *Dionea* (Pickard, 1973a). However, the measurement of action potentials in gravitropically responding plants has been equivocal (Berry & Hoyt, 1943; Behrens & Gradmann, 1985). Moreover, calcium and auxin are redistributed against the electrical gradient (Lee, *et al.*, 1983; Slocum & Roux, 1983; Mertens & Weiler, 1983; Bandurski, *et al.*, 1983), supporting the view that electrophoresis is not involved in the redistribution of these presumed effectors.

Summary

Calcium, calmodulin, phosphoinositides, auxin, and electrical gradients may all contribute to the transmission of the gravitropic signal from the site of perception to the site of response. The ordering of these events, as well as the discovery of additional events, will provide valuable information for the identification of the gravity receptor. However, the relationships between these components remain to be established.

Response

In response to the gravitropic signal, growth patterns are established across the plant organ which allow it to achieve and maintain a particular orientation with respect to the gravity vector. The study of gravitropism typically involves measurements of the curvature which results when an organ is displaced from its preferred orientation. In both roots and shoots, this curvature is the consequence of growth inhibition on the concave side and, at the same time, growth stimulation on the convex side of the curving organ (MacDonald, *et al.*, 1983; Bandurski, *et al.*, 1984; Mueller, *et al.*, 1984; Jaffe, *et al.*, 1985; Selker & Sievers, 1987).

In the 1920s, the Cholodny-Went hypothesis was proposed as a possible explanation for the regulation of differential growth in phototropic and gravitropic organs. In this model, a directional stimulus induced lateral transport of auxin across the organ tip, and the resulting asymmetrical distribution of auxin was longitudinally transported to the region of the growth response. The higher regional concentration of auxin resulted in a local alteration of growth. Early investigators detected auxin gradients across both phototropically and gravitropically stimulated coleoptiles (Went, 1929; Dolk, 1929), and these results were later confirmed (for examples, see Gillespie & Thimann, 1961; Goldsmith & Wilkins, 1964). However, the reliance on bioassays, inadequate statistical analyses, and the possible influence of wounding on the experimental results prevented general acceptance of the hypothesis (for a discussion, see Hart, 1990).

Several lines of evidence have cast further doubt on the Cholodny-Went hypothesis. First, the ability of the relatively small auxin gradients to induce significant differential growth has been questioned based on auxin dose-response curves (Digby & Firn, 1976). Second, phototropic and gravitropic curvature has been observed in the absence of the coleoptile tip (Firn, *et al.*, 1981; Franssen, *et el.*, 1982), although the tip was required for the complete development of the gravitropic growth pattern (Hart & MacDonald, 1984; MacDonald & Hart, 1985a,b). Third, compatibility between the time required for development of an auxin gradient and the observed lag times for curvature development has not been demonstrated (Digby & Firn, 1976). Fourth, gravitropic curvature has been observed in the absence of a detectable auxin gradient (Jackson & Barlow, 1981; Mertens & Weiler, 1983). Finally, the simplicity of the Cholodny-Went hypothesis may not adequately explain the complex patterns of differential growth observed in curving roots and hypocotyls (Selker & Sievers, 1987; MacDonald & Hart, 1987a).

Even with some doubts surrounding the Cholodny-Went hypothesis, curvature induced by the asymmetrical application of exogenous auxin (Ullrich, 1978; Clifford, *et al.*, 1985; Moore & Evans, 1986; Clifford, 1988; Young, *et al.*, 1990) and the observed differential increase in SAURs upon gravistimulation (McClure & Guilfoyle, 1989) suggest that auxin regulates differential growth. In addition, evidence is either lacking or contradicts the involvement of abscisic acid (ABA), gibberellins (GA), or ethylene. Neither ABA nor GA induced curvature when applied asymmetrically to roots or shoots (Mertens & Weiler, 1983; MacDonald & Hart, 1987a). Gradients of ABA were not found in gravitropically stimulated roots (Jackson & Barlow, 1981; Mertens & Weiler, 1983), and GA gradients were detected relatively late in the response (Phillips, 1972). ABA-deficient mutants and seedlings treated with ABA inhibitors remained gravitropic in the absence of ABA (Feldman & Sun, 1986; Moore & Smith, 1984; Moore & Smith, 1985; Weyers, 1985). Although inhibitors of ethylene production were reported to reduce gravitropism in tomato hypocotyls (Wheeler & Salisbury, 1981), subsequent studies failed to show any increase in ethylene production during gravitropism, and the rate of gravitropic curvature was not affected by varying levels of exogenous ethylene (Harrison & Pickard, 1986). In addition, increased ethylene synthesis was not observed in grass nodes until more than 5 hours after gravistimulation (Kaufman, et al., 1987).

Several alternatives to the Cholodny-Went hypothesis have been proposed to describe the regulation of differential growth by auxin. The release of auxin from physical or chemical compartmentalization (Mertens & Weiler, 1983; Kaufman, *et al.*, 1987), calcium-enhanced transport of auxin (Pickard, 1985; Evans, *et al.*, 1986; Moore & Evans, 1986), and calcium-auxin plasmalemmal symport (Hertel, 1983) are proposals consistent with the observed auxin and calcium gradients. Changes in tissue sensitivity (Clifford, *et al.*, 1985; Moore & Evans, 1986; Salisbury, *et al.*, 1986; Meudt, 1987; Clifford, 1988; Salisbury, *et al.*, 1988) or differences in the responsiveness of epidermal and sub-epidermal tissues to auxin (Brummell & Hall, 1980; Kutschera & Briggs, 1987; Kutschera & Briggs, 1988) could also account for large growth differentials without requiring lateral movement of auxin across an entire organ (Firn & Digby, 1977; MacDonald & Hart, 1987a,b). Based on the failure of bilateral excision of the epidermis and the cortex to eliminate gravitropism in maize roots, Björkman and Cleland (1988) suggested that the transport of and response to auxin involves the stele and the endodermis. However, curvature was eliminated when the roots were girdled, supporting the hypothesis that the gravitropic signal moves through the outermost cell layers (Yang, *et al.*, 1990).

Summary

Although the Cholodny-Went hypothesis may not fully explain the regulation of differential growth, it seems likely that auxin is involved. Questions about how auxin differentials are established, the relationship between auxin and calcium, and the identity of the responding tissue remain unanswered.

INTERACTIONS BETWEEN LIGHT AND GRAVITY SENSORY SYSTEMS

Gravitropism is influenced by several environmental factors such as temperature, mechanical stimuli, and light (for a review, see Hart, 1990). For example, the direction of the gravitropic response is temperature-dependent in some species (Bell & Coombe, 1976), and can be completely masked by thigmotropic responses (Darwin, 1896; Edwards & Pickard, 1987). Light has also been shown to affect the direction and the kinetics of gravitropism (Wilkins & Goldsmith, 1964; Wilkins, 1965; Nick & Schäfer, 1988; Leopold & LaFavre, 1989), and to compete with gravitropism through the induction of phototropism (Shen-Miller & Gordon, 1967; Pickard, 1969). Differences in the gravitropic response of dark-grown and light-grown seedlings suggests that light may also affect gravitropic sensitivity (Scholdeen and Burström, 1960; Olsen and Iversen, 1980; Britz & Galston, 1982).

The effects of light on gravitropism kinetics, direction, and sensitivity have been attributed to phytochrome-mediation of gravitropism effectors (Wilkins & Goldsmith, 1964; Wilkins, 1965; Feldman & Briggs, 1987; Woitzik & Mohr, 1988a). Irradiation with red light increases the gravitropic response in both roots and shoots (Feldman, 1984; Pickard, 1985; Woitzik & Mohr, 1988a), and induces a change in response direction from plagiogravitropic to orthogravitropic in some maize roots (Wilkins, 1964; Wilkins, 1979; Mandoli, *et al*., 1984; Nick & Schäfer, 1988; Leopold & LaFavre, 1989). The effect on the gravitropic response depends on the direction of the red light irradiation relative to the gravity vector (Woitzik & Mohr, 1988a), an observation which may help explain the conflicting data of earlier studies (for example, see Blaauw, 1961, and Wilkins & Goldsmith, 1964).

The phytochrome-mediated events responsible for these phenomena have not been identified. Growth regulator synthesis (Juniper, 1976; Suzuki, *et al*., 1979; Feldman, 1984), growth regulator distribution (Blaauw, 1963; Kang & Burg, 1972), calcium distribution (Perdue, *et al*. 1988; Leopold & LaFavre, 1989), and starch synthesis (Audus, 1975) have all been suggested as possible gravitropism effectors susceptible to phytochrome mediation. Rapid changes in protein phosphorylation upon red light irradiation have been observed in roots of the Merit corn cultivar (McFadden & Poovaiah, 1988), suggesting that activation or deactivation of some unknown enzyme may be involved in light-induced changes in direction. However, until the primary events of transmission and response are identified, it is unlikely that the role of phytochrome in gravitropism will become more clearly defined.

Light is naturally encountered in varying stimulus strengths, and the ability to perceive such variation may be important to survival. In contrast, gravity is naturally encountered at a constant stimulus strength of 1 *g*. Thus, the ability to perceive variations in stimulus strength may be less developed or nonexistent in gravitropism.

Based on the differences in the natures of gravity and light, differences in certain elements of transduction for gravitropism and phototropism would be expected. Differences in the distribution of curvature along the responding organs, and in the effects of decapitation and auxin transport inhibitors, suggested that different mechanisms may control stem curvature in phototropism and gravitropism (Britz & Galston, 1983). Independence of the early transduction events was demonstrated by the simple additivity of the two responses when sub-threshold stimulations were applied simultaneously (Nick & Schäfer, 1988).

However, since gravitropism and phototropism both culminate in differential growth, other elements of signal transduction may be similar or identical. This prediction has precedence in the commonality found between gravitropism, phototropism, and chemotropism in a series of mutants of *Phycomyces* (Russo, 1980). In *Zea mays*, simple additivity of phototropic and gravitropic response was not observed when the seedlings received red-light pretreatments (Nick & Schäfer, 1988), suggesting that red light exerts its influence at the same transduction event in both response pathways. Similarly, hypocotyls of *Sesamum indicum* exhibited the same response kinetics for gravitropism and phototropism when irradiated with very low fluence red light (Woitzik & Mohr, 1988a,b).

In summary, although it seems clear that phytochrome mediates the gravitropic response, the mechanisms through which that mediation is accomplished are unknown. At least one of the phytochrome-mediated events appears to be common to both phototropism and gravitropism. However, with regard to other elements of transduction, the two pathways appear to be independent. The overall extent of commonality and independence of the two pathways is unknown.

GENETIC SYSTEMS IN SENSORY PHYSIOLOGY

The dissection of biochemical and sensory pathways has been accomplished through combinations of physiological, biophysical, biochemical, and genetic methods. The methods which prove most useful depend on the nature of the pathway and its components. For example, the identification of phytochrome and related transduction events began with biochemical and biophysical analyses. The description of red and far-red reversibility in seed germination (Borthwick, *et al.*, 1952) led to crude extracts which exhibited photoreversible changes in optical density (Butler, *et al.*, 1959). Spectrophotometric and biochemical studies (Siegelman & Butler, 1965) eventually led to the isolation and molecular description of the chromophore (Rudiger & Correll, 1969) and the apoprotein (Briggs & Rice, 1972; Pratt, 1982; Vierstra & Quail, 1983). Subsequent genetic and molecular genetic studies promise to identify sensory transduction events involved in responses to red light (for reviews, see Schäfer, *et al.*, 1986; Quail, *et al.*, 1987).

In some biochemical and sensory pathways, the lack of a readily extractable quality has made genetic dissection the most feasible approach. The identification of events involved in such phenomena as phototropism and carotenogenesis in *Phycomyces* (Ootake, *et al.*, 1974; Galland & Lipson, 1987), bacterial chemotaxis (Macnab, 1979; Macnab, 1984), bacterial thermosensing (Imae, 1985), and swimming behaviors in *Paramecium* (Hinrichsen, *et al.*, 1985) has been enhanced through the study of mutants. Collections of GA-deficient mutants were essential to the description of the GA biosynthesis pathway in plants and fungi (Phinney & Spray, 1982; Bearder, 1983; Spray, *et al.*, 1984; for a review, see Reid, 1990). In addition, mutants have helped increase our understanding of other aspects of plant biochemistry and physiology, such as photosynthesis and nitrogen fixation (for a review, see Blonstein & King, 1986).

A number of mutants exhibiting altered gravitropic responses have been described in several species (for a review, see Roberts, 1987). An influence of light on the gravitropic response was demonstrated using mutants of pea (Scholdeen & Burström, 1960; Olsen & Iversen, 1980), and the genetic separability of shoot and root responses was suggested in mutants of pea and *Arabidopsis* (Scholdeen & Burström, 1960; Maher & Martindale, 1980). Conversely, commonality between the transduction pathways in roots and shoots was demonstrated using the dwarf (*Dwf*) mutant of *Arabidopsis* (Mirza, *et al.*, 1984). The role of growth regulators has been examined using corn, pea, and *Arabidopsis* mutants with aberrant gravitropic responses

(Pilet, 1983; Woods *et al.*, 1984; Bell & Maher, 1990), and the hypothesis that ABA was directly involved in gravitropism was rejected based on the normal gravitropic response of ABA-deficient mutants of corn and tomato (Moore & Smith, 1985; Weyers, 1985).

Although controversy over the direct involvement of ABA in gravitropism was resolved using genetic analyses, the same cannot be said for the controversy over the role of starch in gravitropism. A positive correlation between amyloplast starch content, amyloplast sedimentation, and gravicurvature in the *amylomaize* mutants was consistent with the amyloplast hypothesis for gravity susception (Hertel, *et al.*, 1969). However, a starchless mutant in *Arabidopsis* deficient in plastid phosphoglucomutase exhibited a near-normal gravitropic response in light-grown seedlings (Caspar & Pickard, 1989). Conflicting interpretations of the significance of plastid sedimentation in this starchless line have confounded the resolution of this controversy (Caspar & Pickard, 1989; Kiss, *et al.*, 1989). Although starch is clearly not necessary for gravitropism in *Arabidopsis*, it was proposed that the starchless plastid remained capable of gravity susception, and that starch contributed to full gravitropic sensitivity by enhancing the plastid's susceptibility to the gravity vector (Kiss, *et al.*, 1989).

One possible explanation for the often conflicting information obtained in studies of gravitropism mutants is the lack of a comprehensive collection of gravitropism mutants in a single species. The advantages of establishing such a genetic system in *Arabidopsis thaliana* are well documented (Redei, 1975; Koornneef, *et al.*, 1983; Estelle & Somerville, 1986; Meyerowitz, 1987; Finkelstein, *et al.*, 1988; Meyerowitz, 1989), and a genetic system for the study of phototropism in *Arabidopsis* has been described (Khurana, *et al.*, 1989a). Thus, the development of a genetic system for the study of gravitropism in *Arabidopsis* was undertaken. This necessitated the identification of mutants with alterations in gravitropism, followed by the genetic, physiological, and biophysical analysis of these mutants.

A DIRECT SCREENING PROCEDURE FOR GRAVITROPISM MUTANTS IN *ARABIDOPSIS THALIANA* (L.) HEYNH.[1]

ABSTRACT

In order to isolate gravitropism mutants of *Arabidopsis thaliana* (L.) Heynh. var Estland for the genetic dissection of the gravitropism pathway, a direct screening procedure has been developed in which mutants are selected on the basis of their gravitropic response. Variability in hypocotyl curvature was dependent on the germination time of each seed stock, resulting in the incorrect identification of several lines as gravitropism mutants when a standard protocol for the potentiation of germination was used. When the protocol was adjusted to allow for differences in germination time, these lines were eliminated from the collection. Out of the 60,000 M2 seedlings screened, 0.3 to 0.4% exhibited altered gravitropism. In approximately 40% of these mutant lines, only gravitropism by the root or the hypocotyl was altered, while the response of the other organ was unaffected. These data support the hypothesis that root and hypocotyl gravitropism are genetically separable.

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[1] Bertha L. Bullen, Therese R. Best, Mary M.Gregg, Sara-Ellen Barsel, and Kenneth L. Poff (1990) Plant Physiol. 93: 525-531.

INTRODUCTION

Gravitropism was described nearly 100 years ago by Charles Darwin (1896). Research since then has increased our understanding of the phenomenon (Audus, 1975; Juniper, 1976); yet, the unequivocal identification of even one step in the pathway remains unaccomplished. Collections of mutants have been used successfully in the dissection of various biochemical pathways (Phinney & Spray, 1982), and data from collections of gravitropism mutants would be expected to yield similar insights. Gravitropism mutants have already been described in several species (Roberts, 1987); however, there has been no concerted effort to establish a collection of gravitropism mutants in a single plant species.

As part of our effort to identify gravitropism mutants in *Arabidopsis thaliana* (L.) Heynh. var Estland, we have developed a three-part procedure to screen directly for mutants with altered gravitropism. Direct screening results in the isolation of mutants unobtainable through screening procedures based, for example, on the role of starch or on the role of a particular phytohormone in gravitropism. Additionally, direct screening increases the potential for saturation of the gravitropism pathway with mutations, since inclusion in a collection of gravitropism mutants does not require that additional criteria, such as starch content or phytohormone response, be met. This approach will eventually enable us to estimate the number of transduction steps involved in gravitropism, and ultimately to identify specific steps in the gravitropism pathway.

MATERIALS AND METHODS

General Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. var Estland were used throughout the present study (gift from C. Somerville, Michigan State University, East Lansing, MI). Seedlings were grown as previously described (Khurana & Poff, 1989).

Light Sources

White light used for potentiation of germination was provided by General Electric Delux white fluorescent tubes at 50 μmol m^{-2} s^{-1} (General Electric Co., Cleveland, OH). Red light was from either a General Electric Red F30T12-R-RD tube or a Sylvania Gold F15T12-GO tube (GTE Products Corp., Danvers, MA) filtered through a red cellophane (Highland Supply Corp., Highland, IL) transmitting light in the 560 to 720 nm range with peak transmission at 630 nm.

Mutagenesis

Seeds were treated with 3% ethyl methane sulfonate (EMS) for 16 h and washed for 4 h with distilled water (Somerville & Ogren, 1982). The EMS-treated M1 seed was sown, and M2 seeds were harvested as previously described (Khurana & Poff, 1989).

Characterization of Gravitropism in the Wild-Type Parent

Wild-type seeds were surface-sterilized by soaking for 5 min in 30% (v/v) commercial bleach (Patterson Laboratories, Inc., Detroit, MI, 5.25% sodium hypochlorite by weight) and 0.02% (v/v) Triton X-100. The seeds were rinsed five times with sterile distilled water, and then sown at 10 mm intervals in square, gridded 100 x 100 x 15 mm^3 Integrid Petri dishes (Becton Dickinson Labware, Lincoln Park, NJ) with a nutrient salts solution (Haughn & Somerville, 1986) solidified with 1% (w/v) Bacto-Agar (Difco Laboratories, Detroit, MI). The dishes were sealed with Parafilm (American Can Co., Greenwich, CT) to maintain high humidity. Seed sterilization and sowing were carried out in room light.

To potentiate germination, the dishes of seeds were placed in darkness at 4°C for 4 d, followed by white light at 25°C for 30 h (Shropshire, *et al.*, 1961). The dishes were then placed on edge in darkness at 25°C and 80% relative humidity for 21 h. Because some enhancement of gravitropic curvature by red light irradiation has been reported in *Pisum sativum* (Britz & Galston, 1982), the seedlings were irradiated with red light for 1 h, and then returned to darkness for an additional 10 h. Seedlings grew along the surface of the agar as previously described (Caspar & Pickard, 1989), thus permitting the simultaneous measurement of root and hypocotyl gravitropic responses.

Three different treatments were applied to different sets of wild-type seedlings during the 32 h following the white light exposure described above. One set was maintained in the original vertical orientation for the entire 32 h. Another set of seedlings was placed horizontally at the end of the first 22 h by rotating the dishes on edge by 90°, and this horizontal orientation was maintained for the remaining 10 h. A third set was placed on a horizontal clinostat, presenting gravity compensation of 10^{-6} to 10^{-5} g, for the entire 32 h.

The seedlings were then shadowgraphed using a photographic enlarger. The angles of deviation from the original vertical vector were measured from these shadowgraphs with a protractor. When the dishes were rotated by 90°, the new downward direction was to the left of the original vertical vector. Positive curvature values were assigned when the root or hypocotyl was to the left of the original vertical vector, and negative curvature values were assigned when the organ was to the right of the original vertical vector. Frequency distribution histograms for the roots and the hypocotyls of each population of seedlings were plotted.

Screens for Possible Hypocotyl Gravitropism Mutants

Primary Screen

M2 seeds were sown under room light in strips of microassay wells as previously described (Khurana, *et al.*, 1989a). After 3 d in darkness at 4°C, the seeds were exposed to continuous white light at 25°C for 30 h. The strips of wells were then transferred to darkness at 25°C and 100% relative humidity for 42 h. After irradiation with red light for 1 h, the strips were placed on edge to orient the seedlings horizontally.

After 8 h in the horizontal orientation, seedlings in which the hypocotyl did not curve upward were designated as possible hypocotyl gravitropism mutants. Each possible mutant was assigned a mutant line number, allowed to green under white light for 3 to 4 d, transferred to soil medium in a plastic pot, and allowed to mature under standard growth conditions. The M3 seeds were harvested from the M2 plants as previously described (Khurana & Poff, 1989). Approximately 40,000 M2 seedlings were subjected to this primary screen.

Secondary Screen

Approximately 140 M3 seeds from each possible mutant line were treated as in the primary hypocotyl screen, except that the seedlings were transferred to transparent tape and shadowgraphed after the 8 h of horizontal orientation. Angles of curvature were measured with a protractor. Possible mutant lines were identified from frequency distribution histograms of the curvature for each M3 line. Subsequent tests were performed on M3 through M5 generations.

Screens for Possible Root Gravitropism Mutants

Primary Screen

M2 seeds were surface-sterilized, sown, and germinated as described above for the wild-type parent. After the dishes of germinating seeds had been on edge in darkness for 20 h, the seedlings were positioned horizontally for 22 h by rotating the dishes on edge by 90°. Seedlings in which the root did not curve downward were identified as possible root gravitropism mutants. Each possible mutant was assigned a mutant line number, and an M3 generation was obtained as in the hypocotyl screen. About 20,000 M2 seedlings were subjected to this primary screen.

Secondary Screen

Approximately 50 M3 seeds from each M2 line were treated as described for the primary root screen, except that images of the plants were traced using a photographic enlarger. The angle of deviation of the root from the original vertical vector was measured from the tracings with a protractor. Positive and negative values were assigned as described above for the wild-type parent. Possible mutant lines were identified from frequency distribution histograms of the curvature for each M3 line. Subsequent tests were performed on M3 through M5 generations.

Tertiary Screen

Subsets of the possible mutant lines were subjected to a two-part tertiary screen.

Germination Time

Fifty seeds from each line were surface-sterilized as previously noted and sown as described for the wild-type parent. After 3 d in darkness at 4°C, the dishes were placed in white light, and the number of germinating seeds was noted a 2 h intervals. The time of 50% germination (t_{50}) was estimated by interpolation from a linear plot of germination as a function of time.

Gravitropism Phenotypes

At least 50 seeds per line were surface-sterilized as before, sown, and germinated as described for the wild-type parent, except that the white light irradiation was terminated 1 to 2 h after the t_{50} for each line. After 21 h in darkness followed by 1 h of red light, the seedlings were positioned horizontally in darkness for 10 h. Both roots and hypocotyls were shadowgraphed by placing the dishes in a photographic enlarger, and the angle of deviation from the original vertical vector was measured from the enlarged image with a protractor. Positive and negative values were assigned as described for the wild-type parent. Separate frequency distribution histograms were plotted for the root curvature and for the hypocotyl curvature of each line. Mean curvatures and standard deviation (SD) were used to categorize the phenotypes.

Assay for Root Cap Starch

Ten surface-sterilized M3 seeds from each possible hypocotyl gravitropism mutant line were sown as described for the wild-type parent, except that the nutrient salts solution was supplemented with 1% (w/v) sucrose. After 2 to 3 d in darkness at 4°C, the dishes were placed on edge in white light at 25°C for 3 d, such that the root tips did not penetrate the agar. The dishes were then maintained horizontally in room light while the seedlings were covered for approximately 20 min with an aqueous iodine-potassium iodide solution (43.4 mM KI + 5.7 mM I). Dark purple or black globules were visible in the root caps of the wild-type parent when viewed under a dissecting microscope. This method did not permit visualization of starch in the hypocotyls. The Estland wild-type parent was used as the positive control, and the starchless mutant, TC7 (Caspar, *et al.*, 1985), was used as the negative control.

RESULTS

The response of wild-type seedlings to gravity is illustrated in Figure 1. If the seedlings were maintained vertically, their roots (Figure 1C) and their hypocotyls (Figure 1D) grew parallel to the vertical vector. If the seedlings were rotated by 90°, the roots curved downward (Figure 1E) and the hypocotyls curved upward (Figure 1F). One predicted mutant phenotype would result from failure to perceive and/or respond to gravity. This condition was simulated using a horizontal clinostat which achieved a gravity compensation of 10^{-6} to 10^{-5} *g*. The slowly rotating clinostat presented the seedlings with a constantly changing gravity vector, thus avoiding presentation of a

FIGURE 1: Frequency distribution histograms for gravitropic curvature by the Estland wild-type parent. Mean curvature (X), standard deviation (SD), and total number of individuals (N) are given on each distribution. Positive and negative curvature values were assigned as described in "Materials and Methods." Panels A, C, and E represent the root response and panels B, D, and F represent the hypocotyl response. (A,B), Seedlings grown for 32 h on horizontal clinostat; (C,D), Seedlings maintained in vertical position for 32 h; (E,F), Seedlings grown in vertical orientation for 22 h, then in horizontal orientation for 10 h.

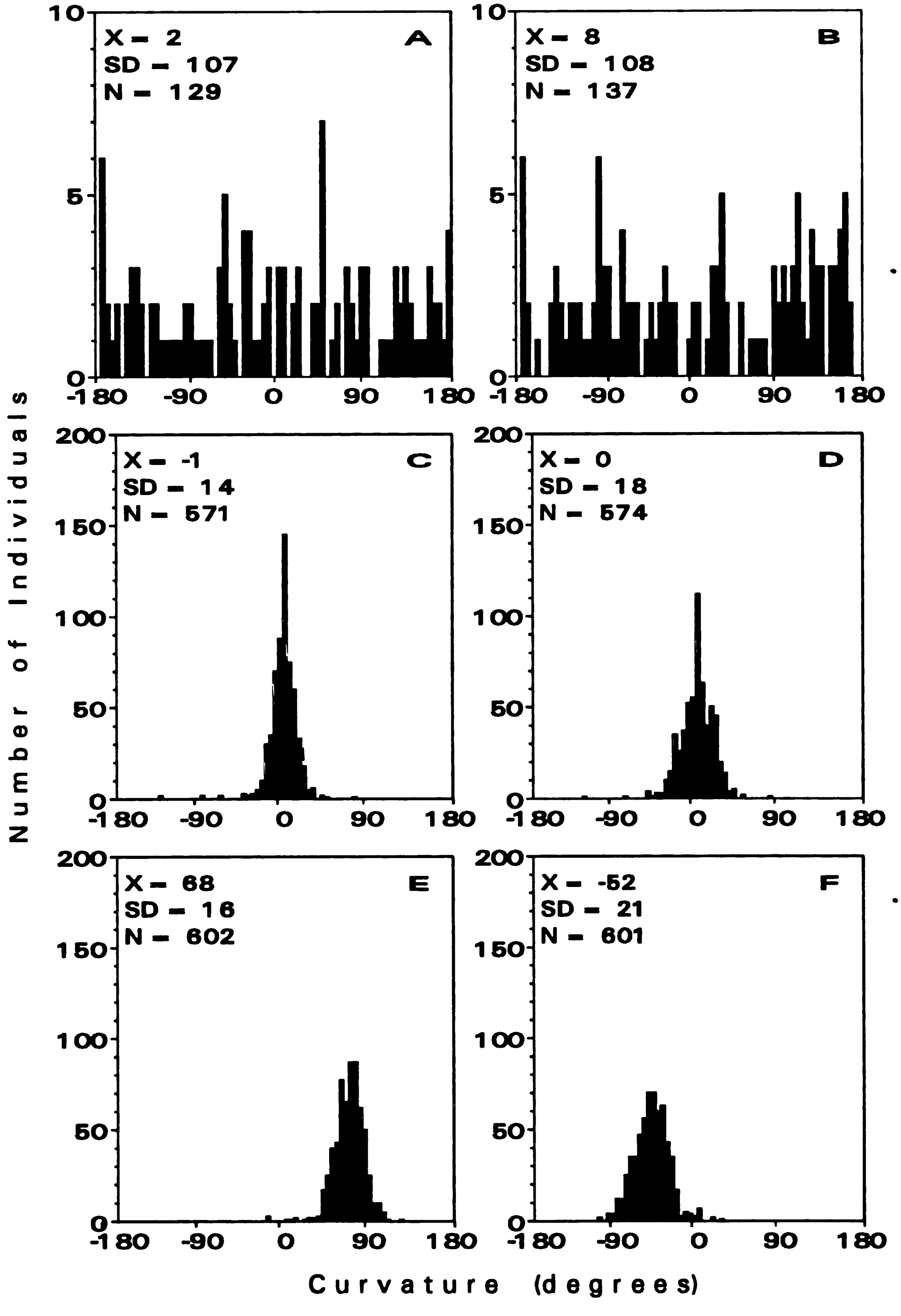
X = 2
SD = 107
N = 129
A
X = 8
SD = 108
N = 137
B
X = -1
SD = 14
N = 571
C
X = 0
SD = 18
N = 574
D
X = 68
SD = 16
N = 602
E
X = -52
SD = 21
N = 601
F
0
5
10
50
100
150
200
-180
-90
0
90
180
Number of Individuals
Curvature (degrees)

FIGURE 1

unilateral stimulus. The frequency distribution histograms for seedlings grown on the clinostat (Figure 1, A and B) were distinct from the frequency distribution histograms for either vertically (Figure 1, C and D) or horizontally (Figure 1, E and F) placed seedlings.

Hypocotyl curvature was dependent on the timing of gravistimulation with respect to germination time. If the white light used for the initiation of germination was terminated approximately 2 h after t_{50}, the population of wild-type seedlings curved in a nearly homogeneous manner (Figure 2A). However, nonhomogeneous hypocotyl curvature resembling that of several possible mutants, was obtained with wild-type seedlings when the white light treatment was terminated more than 10 h after t_{50} (Figure 2B). The tertiary screen, in which the protocol was adjusted to allow for differences in germination times, eliminated those lines which would otherwise have been incorrectly identified as gravitropism mutants.

At the end of the primary screens, 1 to 2% of the seedlings were chosen as possible mutants. The secondary screens reduced this to approximately 0.4% (Table I). The tertiary screen was conducted on possible mutant lines which exhibited the same gravitropism phenotype in repeats of the secondary screen. Two of the 50 hypocotyl gravitropism lines did not contain wild-type levels of starch in the root cap according to the assay described

FIGURE 2: Frequency distribution histograms for gravitropic hypocotyl curvature of the Estland wild-type parent after 10 h in a horizontal orientation. Mean curvature (X), standard deviation (SD), and total number of individuals (N) are given for each population. Curvatures were assigned positive or negative values as described in "Materials and Methods." Germination was potentiated by (A) 15 h and (B) 30 h of white light. The t_{50} for the population was 14 h.

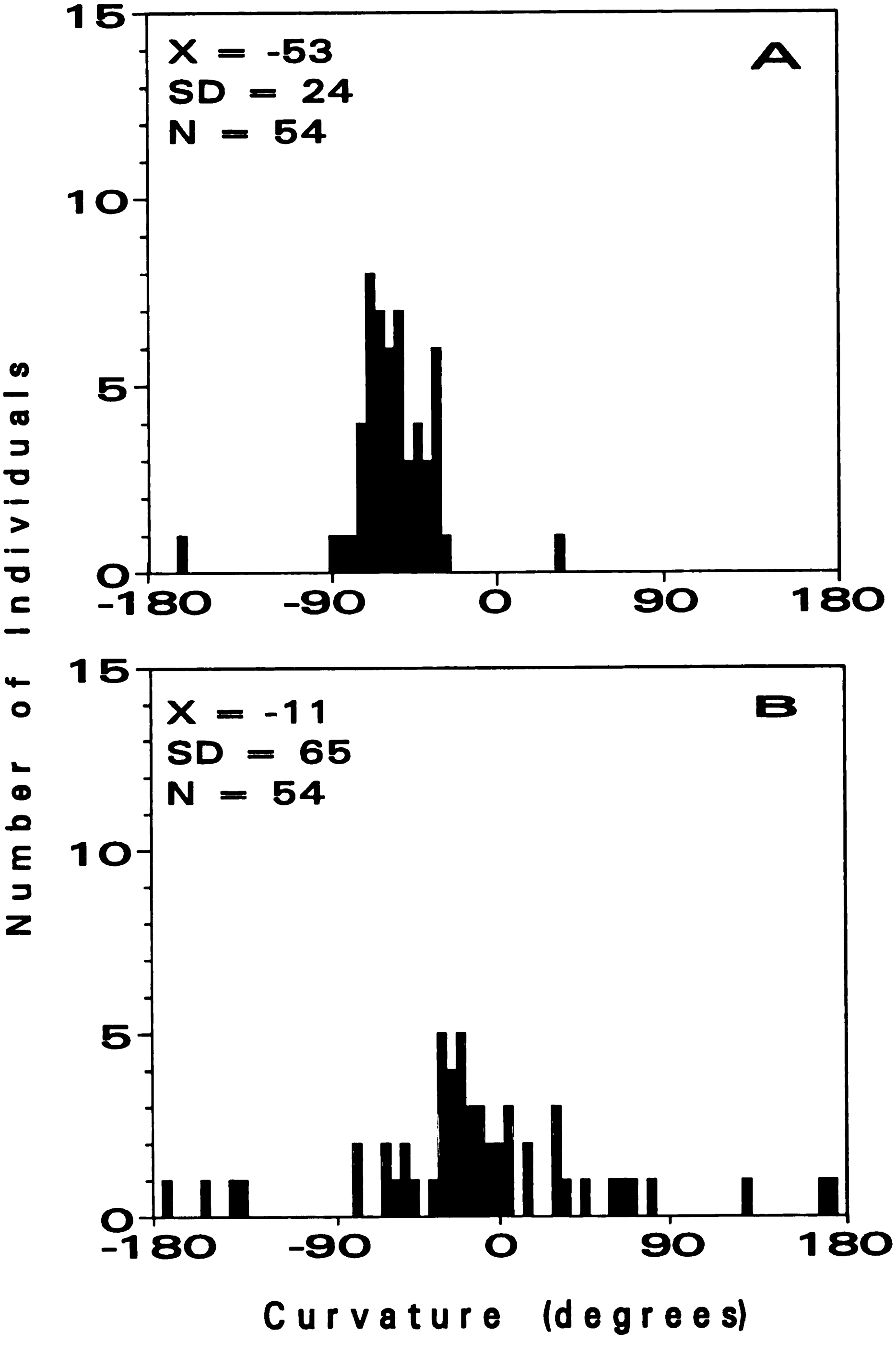
X = -53
SD = 24
N = 54
A
X = -11
SD = 65
N = 54
B
15
10
5
0
-180
-90
0
90
180
Number of Individuals
Curvature (degrees)

FIGURE 2

TABLE I: Recovery of Possible Mutants Identified Using a Three-Stage Screening Procedure

Screen	No. Seedlings Screened		No. Possible Mutants	
	Hypocotyl	Root	Hypocotyl	Root
Primary	40,000	20,000	421	495
Secondary	421	495	152	74
Tertiary	63	12	50	9

above. All nine of the root gravitropism lines contained wild-type levels of starch. However, the sensitivity of the assay does not permit quantitation of the starch deficiency in the lines. Future studies will address this issue, as well as examine uniformity of the starch phenotype throughout the plant.

The phenotypes of the root and hypocotyl were determined separately for each line selected from the tertiary screen. Mean curvatures and SD for each line were compared against wild-type values to define four phenotype categories. If the mean curvature of the line exceeded the wild-type mean by about 15°, and the SD were approximately equal, the line was designated a high bender (Figure 3B). If the mean curvature of the line was less than the wild-type mean by about 15°, and the SD were approximately equal, the line was designated a low bender (Figure 3C). If the mean curvature of the line was less than the wild-type mean by about 15°, and the SD exceeded the wild-type SD by three to six times, the line was designated as altered (Figure 3D). Finally, if the mean curvature of the line was less than 20°, and the SD exceeded the wild-type SD by more than six times, the line was designated as random (Figure 3E). In Figure 3, representative frequency distribution histograms for roots are presented. Similar distributions were obtained for hypocotyls.

The mutant phenotype which was simulated using a clinostat (Figure 1, A and B) corresponds well with the

FIGURE 3: Representative frequency distribution histograms for gravitropic root curvature after 10 h in a horizontal orientation. Mean curvature (X), standard deviation (SD), and total number of individuals (N) are given for each population. Curvatures were assigned positive or negative values as described in "Materials and Methods." (A) Wild-type; (B) high bender; (C) low bender; (D) altered; and (E) random.

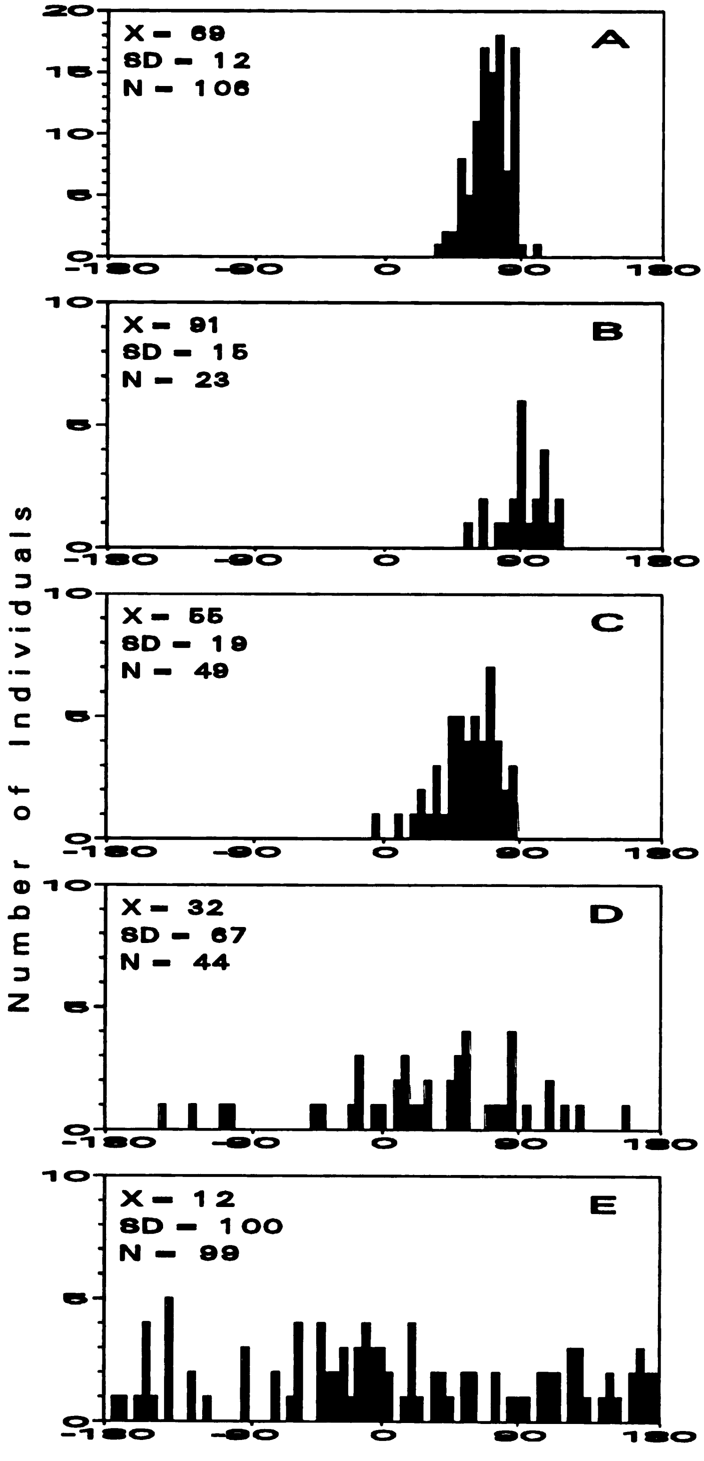
X = 69
SD = 12
N = 106
A
X = 91
SD = 15
N = 23
B
X = 55
SD = 19
N = 49
C
X = 32
SD = 67
N = 44
D
X = 12
SD = 100
N = 99
E
20
15
10
5
0
-180
-90
0
90
180
Number of Individuals
Curvature (degrees)

FIGURE 3

random category obtained in the screen (Figure 3E). Therefore, mutant lines in this category may carry lesions which affect the graviperception mechanism. The remaining three phenotype categories (Figure 3, B, C, and D) were not simulated prior to the screen. However, these categories were not unanticipated, since these phenotypes might be expected with alterations in transduction events, such as phytohormone translocation or signal amplification.

Some special considerations must be noted in the analysis of random distributions (Figure 3E). First, a random distribution does not approximate a normal distribution; so, the SD of a random population cannot be compared with the SD of a normal distribution. Second, the use of an abscissa from -180° to +180° is convenient, but arbitrary. With polar coordinates, -180° and +180° would be at the same point, not at the opposite ends of the abscissa. Therefore, it is necessary to plot frequency distribution histograms, rather than rely on the statistics alone, to distinguish true random lines from lines with large SD resulting from the linearity of the abscissa (Burke, *et al.*, 1988).

The categories of root and hypocotyl phenotypes within a line were not always the same. Thirty-seven of the 59 lines were affected in both root and hypocotyl, but only 16 of these exhibited the same phenotype category in both organs. In 22 lines, gravitropism was affected in either the root or the hypocotyl, but was normal in the other organ.

DISCUSSION

Direct screening for mutants with altered gravitropism avoids biases inherent in screens based on phenotypes presumably related to gravitropism, and results in the isolation of mutants unobtainable in other screens. For example, the screen for hypocotyl gravitropism mutants resulted in the recovery of two starch-deficient lines. These two lines would presumably have been isolated in a primary screen for starchless mutants, and subsequent examination of these mutants would have revealed their aberrant gravitropism. However, two of the random lines contain levels of starch and exhibit growth habits which are not grossly different from the wild-type. These two mutants certainly would not have been identified in a screen for the starchless phenotype.

Direct screening also increases the probability that the gravitropism pathway will be saturated with mutations at all transduction steps. This is highly unlikely with screening procedures in which mutants are eliminated from a collection on the basis of a presumably related phenotype, such as starch content or phytohormone response.

Identification of hypocotyl mutants was complicated by the variability of the wild-type gravitropism phenotype. The position of the hypocotyl hook with respect to the gravity vector has been shown to be one source of variability in curvature in response to short stimulation times (Khurana, *et al.*, 1989b). However, it is unlikely that this was the source of variability here since, by 10 h of gravistimulation, no correlation between curvature and hook position was observed (data not shown).

The homogeneity of hypocotyl curvature by populations of wild-type seedlings was dependent on the duration of exposure to the germination potentiating white light treatment (Figure 2). This variability in wild-type gravitropism could be due to subtle differences in hypocotyl length at the time of gravistimulation, in growth rate during curvature development, or in attenuation of the graviperception mechanism with seedling age. Since homogeneity of curvature was one criterion used in identifying possible mutants, it was necessary to address this variability of response during the screening procedure. By adjusting the protocol in the tertiary screen to allow for differences in germination, the physiological age of mutant and wild-type populations was standardized. As a result, lines which otherwise would have been incorrectly identified as gravitropism mutants were eliminated in the tertiary screen.

The overall frequency of gravitropism mutants can only be estimated at this point in the screen. Out of 226 possible mutant lines from the secondary screen, a total of 75 lines was tested in the tertiary screen, and about 20% of these were eliminated. Hence, 80% of the remaining 151 untested lines would be expected to maintain their mutant phenotypes. Therefore, the expected total number of gravitropism mutants is 179, and the estimated overall frequency is 0.3%. This is higher than the 0.1% reported for starchless mutants (Caspar, *et al.*, 1985), and, if significant, could indicate that the gravitropism pathway is more complex than that of starch metabolism. However, since the lines subjected to the tertiary screen were chosen on the basis of the repeatability of their phenotype, it is feasible that a larger percentage of the untested lines would be eliminated in a tertiary screen, thus reducing the overall frequency. In addition, direct comparisons of these frequencies with those reported by Caspar, *et al.* (1985) are not possible without some comparison of mutagenicity in the two separately-mutagenized seed stocks.

Thirty-seven percent of the lines exhibited mutant phenotypes in either the root or the hypocotyl. This genetic separability was reported previously in *Pisum sativum ageotropum* (Scholdeen & Burström, 1960), and supports the hypothesis that some gravitropism transduction events are not shared by the two organs. However, collections of mutants resulting from direct screens will permit us to estimate the number of steps shared by the roots and hypocotyls. Such an estimate requires that the lines represent mutations in different loci. This can be determined by crossing each line with each of the other lines, and examining the progeny for evidence of complementation. If the progeny exhibit a wild-type gravitropism phenotype, complementation has occurred and the lines probably represent mutations at different loci. However, if the gravitropism phenotype of the progeny remains mutant, the lines probably represent mutations in the same locus. Although the occurrence of intra-allelic complementation would result in overestimation, these guidelines afford a first approximation of the number of unique loci present in a collection. Combining this type of information from several collections obtained using a variety of mutagens, it is feasible that mutations could be obtained in each step of the gravitropism pathway. Hence, the number of steps in the pathway could be estimated for each organ, and the identification of individual steps would be facilitated.

In summary, the direct screening procedure presents us with data and opportunities unavailable through previously used procedures. First, mutants unobtainable through biochemically based screens can be isolated through a direct screening procedure. For example, 48 of the 50 hypocotyl gravitropism lines are apparently wild type with regard to starch content. Second, collections of mutants which saturate the pathway with mutations would allow determination of the number of steps involved in transduction, and elucidate the relationship(s) and possible differences between the root and hypocotyl gravitropic responses. Such collections of mutants are only possible through repeated direct screening. However, direct screening for gravitropism mutants must include a means for addressing the variability of the gravitropism phenotype introduced by the variability in other physiological parameters.

GENETIC ANALYSIS OF GRAVITROPISM MUTANTS IN *ARABIDOPSIS THALIANA*

ABSTRACT

Five gravitropism mutants from the collection described by Bullen, *et al.* (1990) were chosen for further study on the basis of their gravitropism phenotypes and apparent starch contents. Backcrosses of these lines to the wild-type parent and analyses of F2 segregation ratios were used to determine the mode of inheritance for both phenotypes. Complementation analyses were used to estimate the number of lesions represented by the five lines, and a dihybrid analysis was used to examine the relationship between the gravitropism and starch phenotypes. The data support the hypothesis that each phenotype is the consequence of single recessive nuclear mutations in all five lines. The three gravitropism phenotypes represent two complementation groups, and all three starch-deficient lines appeared to carry mutations in phosphoglucomutase (*pgm*). The starch and gravitropism phenotypes were separable at frequencies from 5% to 20%, suggesting that the gravitropism lesion is tightly linked to *pgm*. Given this linkage, the role of starch in gravitropism must be re-examined.

INTRODUCTION

Although numerous physiological and genetic studies of gravitropism have been conducted in a variety of species (Audus, 1975; Juniper, 1976; Roberts, 1987), such analyses have often involved mutants which were not initially isolated on the basis of their gravitropism phenotype (for examples, see: Hertel, *et al.*, 1969; Mirza, *et al.*, 1984). A direct screening method developed to generate a large collection of gravitropism mutants in *Arabidopsis thaliana* was expected to reveal mutants not found in screens based on biochemical or physiological parameters presumed to be related to gravitropism (Bullen, *et al*, 1990).

Five mutant lines from the collection described by Bullen, *et al.* (1990) were chosen for further study on the basis of their gravitropism phenotypes and their qualitative starch content. Three of the lines chosen exhibited the "random" phenotype which would be expected in gravity susception or perception mutants. One of these lines was also qualitatively starch-deficient. Two other apparently starch-deficient lines, each exhibiting different gravitropism phenotypes in their roots and hypocotyls, were also chosen for further analysis. This cross-section of phenotypes should allow for the preliminary analysis of starch's role in gravitropism and the genetic separability of root and hypocotyl gravitropism.

The purposes of this paper are to quantify the starch content of the three starch-deficient mutants, to determine the genetic characteristics of the gravitropism and starch phenotypes, and to use this information to examine the relationships between starch and gravitropism, and between root and hypocotyl gravitropic responses.

MATERIALS AND METHODS

General Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. var Estland used in this study were obtained through the direct screening procedure described in Bullen, *et al.* (1990). Seedlings were grown and permitted to set seed under previously described conditions (Khurana & Poff, 1989).

Light Sources

White light was provided by General Electric Delux white fluorescent tubes at 50 μmol m^{-2} s^{-1} (General Electric Co., Cleveland, OH). Red light was from a General Electric Red F30T12-R-RD tube filtered through a red cellophane (Highland Supply Corp., Highland, IL) transmitting light in the 560- to 720-nm range with peak transmission at 630 nm. The fluence rate of the red light source was 1.2 μmol m^{-2} s^{-1} as measured using a datalogger with quantum sensor (LI-COR, Inc., Lincoln, NE).

Measurement of Gravitropism

Seeds were surface-sterilized and sown, and gravitropism response was measured as described for the tertiary screen in Bullen, *et al.* (1990). The angle of deviation from the original vertical vector was measured directly from the image of the seedlings using a CCD camera (Cohu, Inc., San Diego, CA) and image analysis software (Jandel Scientific, Corte Medera, CA). Positive and negative values were assigned as described previously (Bullen, *et al*., 1990).

For the classification of the mutant lines, the analysis of F1 progeny of all crosses, and the analysis of F2 segregation in MG-32 and MG-212, separate frequency distribution histograms were plotted for root and hypocotyl curvature for each line. Mean curvatures and the standard deviation (SD) of curvature were used to categorize each population phenotype as previously described (Bullen, *et al*., 1990).

For the analysis of F2 segregation in MG-20, MG-65, and MG-421, an estimate of the gravitropic response was obtained by comparing the percentage of roots or hypocotyls in each quadrant of a superimposed Cartesian coordinate system (Figure 4) at the end of a gravistimulation period. For example, all of the roots in a population of gravistimulated wild-type seedlings should rest in quadrant III. However, only 25% of the roots would be expected to rest in this quadrant in an agravitropic "random" population. The remaining 75% of the roots would be distributed among quadrants I, II, and IV. Based on these expectations, the F3 populations from backcrosses of MG-20, MG-65, and MG-421 were categorized according to percentage

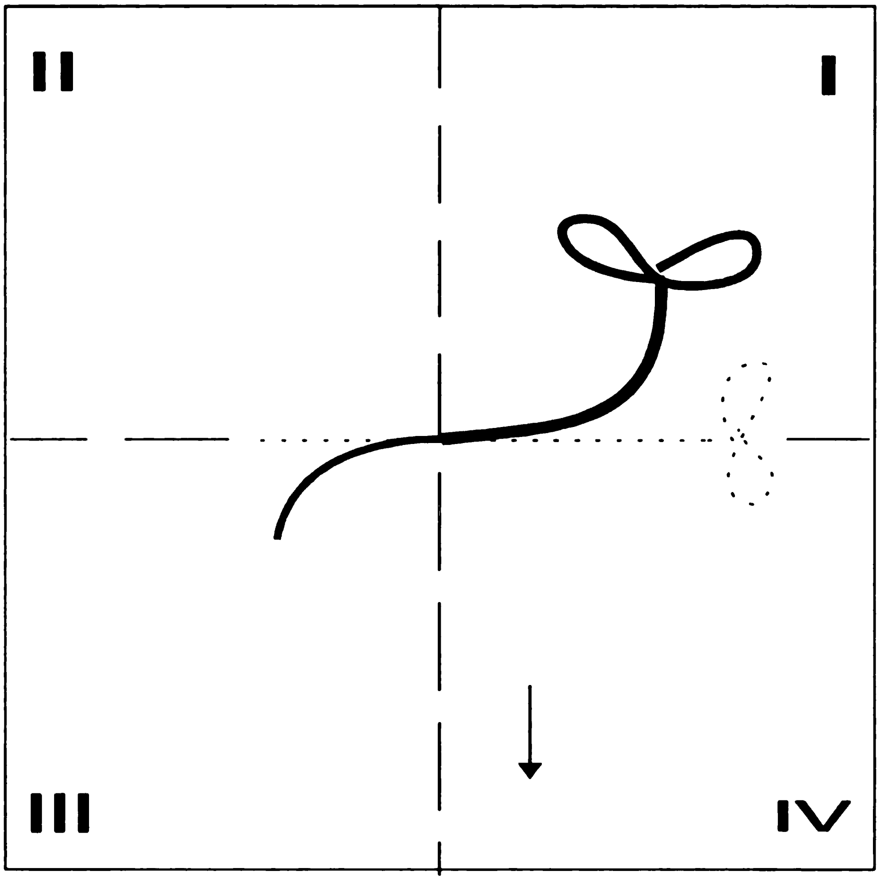

FIGURE 4: Diagram of wild-type gravitropism relative to a superimposed Cartesian-coordinate measurement system. The arrow represents the direction of gravistimulation.

of roots or hypocotyls outside the quadrant predicted for the wild-type parent.

Starch Content Assays

Quantitative Assay

Seeds of the wild-type parent and of each mutant line were sown and seedlings were grown as previously described (Khurana and Poff, 1989). Leaves of 4 wk old plants were harvested at the end of 12 h of white light and immediately frozen in $N_2(l)$. The frozen leaves were stored at -80°C until used.

Starch content was quantified using a previously described assay (Lin, *et al.*, 1988). The only experiments reported are those in which at least 90% of an internal standard was recovered.

Qualitative Assays

Two qualitative assays for starch deficiency were used. One assay examined the starch content of root tips, and was described previously (Bullen,*et al.*, 1990). In the other qualitative assay, individual leaves from 2- to 3-wk-old seedlings were placed in the wells of tissue culture plates (Sargent-Welch Scientific Co., Chicago, IL) with one leaf assayed per seedling. The leaves were covered with 95% ethanol, and the plates were sealed with Parafilm (American Can Co., Greenwich, CT). After 16-24 h, the ethanol was decanted and the leaves were covered with an acidic iodine-potassium iodide (IKI) solution (43.4 mM KI + 5.7 mM I + 0.2 N HCl). After approximately 0.5 h under room light the IKI solution was decanted. Leaves containing starch stained dark purple or black, while leaves lacking starch appeared yellow or orange. The Estland wild-type parent was used as a positive control, and the starchless mutant, TC-7 (Caspar, *et al.*, 1985), was used as the negative control.

Genetic Analyses

Mode of Inheritance

At 4 to 6 wks of age, seedlings of each mutant line were reciprocally backcrossed with the wild-type parent by manually transferring pollen from one plant to the stigma in an emasculated bud of the other plant. Buds chosen as pollen recipients contained mature stigmas but apparently immature pollen, minimizing the potential for self-pollination.

After pollen transfer, the stigma was covered with a small cellophane tent for 2 to 3 d, and a plastic sheet was wrapped around the clay pot to minimize the potential of air-borne pollen reaching the stigma. The resulting silique was allowed to elongate and mature. Each silique was harvested before dehiscence, and the seeds were permitted to after-ripen for a minimum of 2 wks.

F1 seeds were sown as described above for the measurement of gravitropism, and the gravitropism phenotype of the F1 population was measured using the image analysis system. The plates were then placed under white light for 2 to 4 d to allow the seedlings to green. A portion of each F1 population was destructively sampled to determine starch content using the qualitative IKI test on roots. The agar and the remaining F1 seedlings were transferred to clay pots containing the potting mixture previously described (Bullen, *et al.*, 1990). The F1 plants were allowed to self-pollinate, producing F2 seeds. The F2 seeds were bulked and allowed to after-ripen for a minimum of 2 wks.

The F2 segregation ratio for the starch phenotype was determined by assaying starch content in the bulked F2 population using the qualitative IKI tests. Other F2 seeds were treated as described for the measurement of gravitropism. After the gravitropism measurement, the plates were placed under white light for 2 to 4 d to allow the seedlings to green. The agar surrounding each seedling was then cut, and the agar plugs containing individual seedlings were transferred to plastic pots, and each seedling was assigned a unique identification number. In this manner, between 30 and 110 F2 families were generated from each backcross. Each F2 plant was allowed to self-pollinate, and the resulting F3 seeds were after-ripened for at least 2 wks before further use.

The gravitropic response of each F3 population was measured using the gravitropism measurement technique described above. The gravitropism phenotype of the F3 population was used to deduce the phenotype of the parental

F2 plant. The starch content of each F2 family was verified through qualitative assays of each F3 population.

Complementation Analysis

Seedlings of each mutant line were grown and reciprocally crossed at 4 to 6 wks of age. The starch phenotype of the F1 progeny was determined using the qualitative assays described above. The gravitropism phenotype was determined using the measurement of gravitropism, and frequency distribution histograms, mean curvatures, and SD of curvature were compared for each population.

RESULTS

Mutant Phenotypes

Mutant lines were chosen for further study based on their gravitropism and/or starch content phenotypes. The mutants MG-32, MG-65, and MG-212 exhibited the "random" gravitropism phenotype in both roots (Figure 5C,D,E) and hypocotyls (Figure 6C,D,E). The mutant MG-20 exhibited a wild-type gravitropism phenotype in roots (Figure 5B), and an "altered" gravitropism phenotype in hypocotyls (Figure 6B). The mutant MG-421 exhibited an "altered" gravitropism phenotype in roots (Figure 5F), and a "random" phenotype in hypocotyls (Figure 6F). These data are consistent with the genetic separability of root and hypocotyl gravitropism suggested previously (Scholdeen & Burström, 1960; Maher & Martindale, 1980; Bullen, *et al.*, 1990). The mutants MG-20, MG-212, and MG-421 also appeared to be starch-deficient

FIGURE 5: Frequency distribution histograms of the gravitropic response in roots of wild-type and mutant lines of *Arabidopsis thaliana*. Response was measured as degrees of deviation from the original vertical vector after 10 h gravistimulation at 1 *g*. A) Wild-type; B) MG-20a; C) MG-32; D) MG-65; E) MG-212; and F) MG-421.

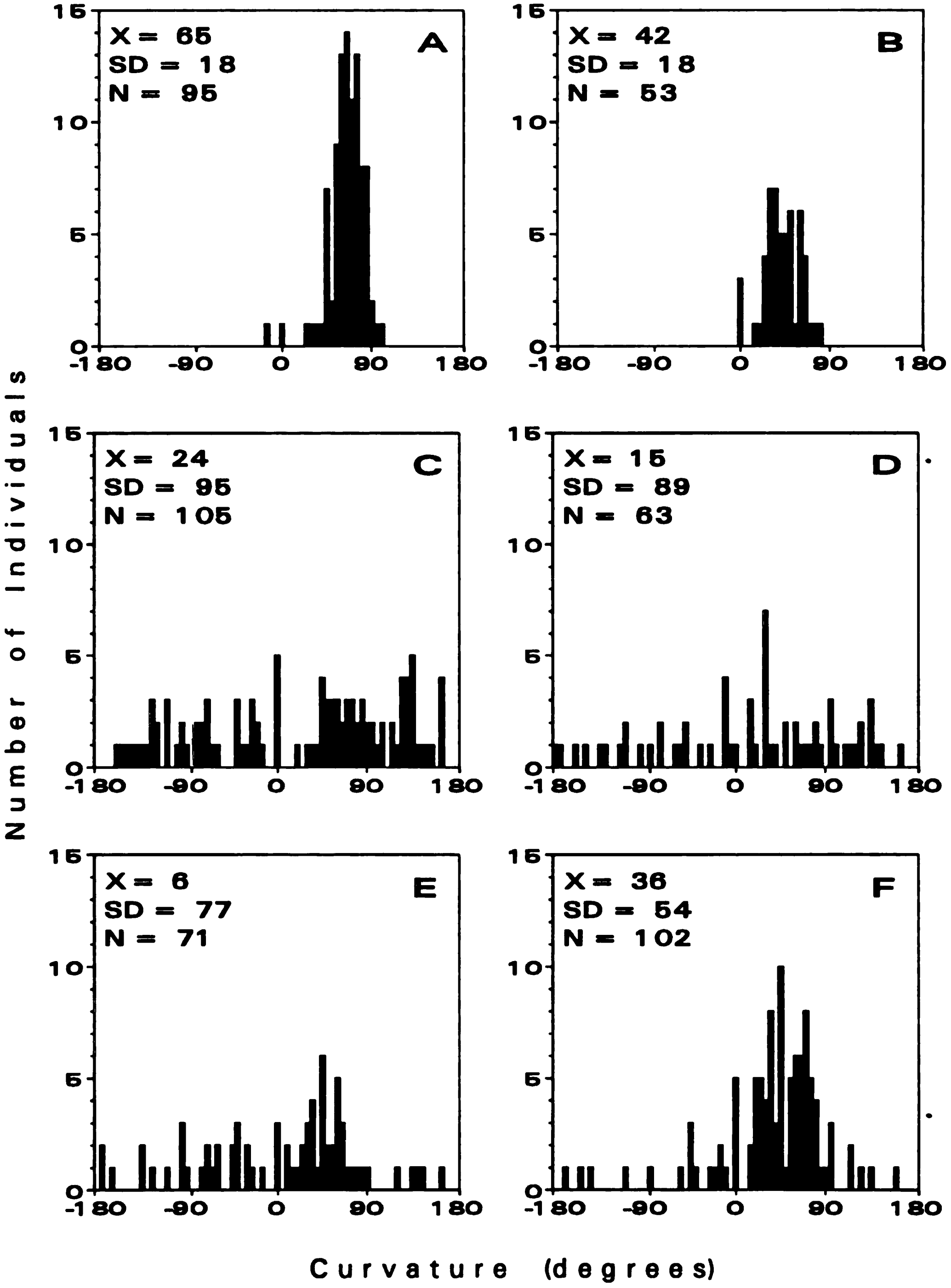
X= 65
SD = 18
N = 95
A
X= 42
SD = 18
N = 53
B
X= 24
SD = 95
N = 105
C
X= 15
SD = 89
N = 63
D
X= 6
SD = 77
N = 71
E
X= 36
SD = 54
N = 102
F
0
5
10
15
-180
-90
0
90
180
Number of Individuals
Curvature (degrees)

FIGURE 5

FIGURE 6: Frequency distribution histograms of the gravitropic response in hypocotyls of wild-type and mutant lines of *Arabidopsis thaliana*. Response was measured as degrees of deviation from the original vertical vector after 10 h gravistimulation at 1 *g*. A) Wild-type; B) MG-20a; C) MG-32; D) MG-65; E) MG-212; and F) MG-421.

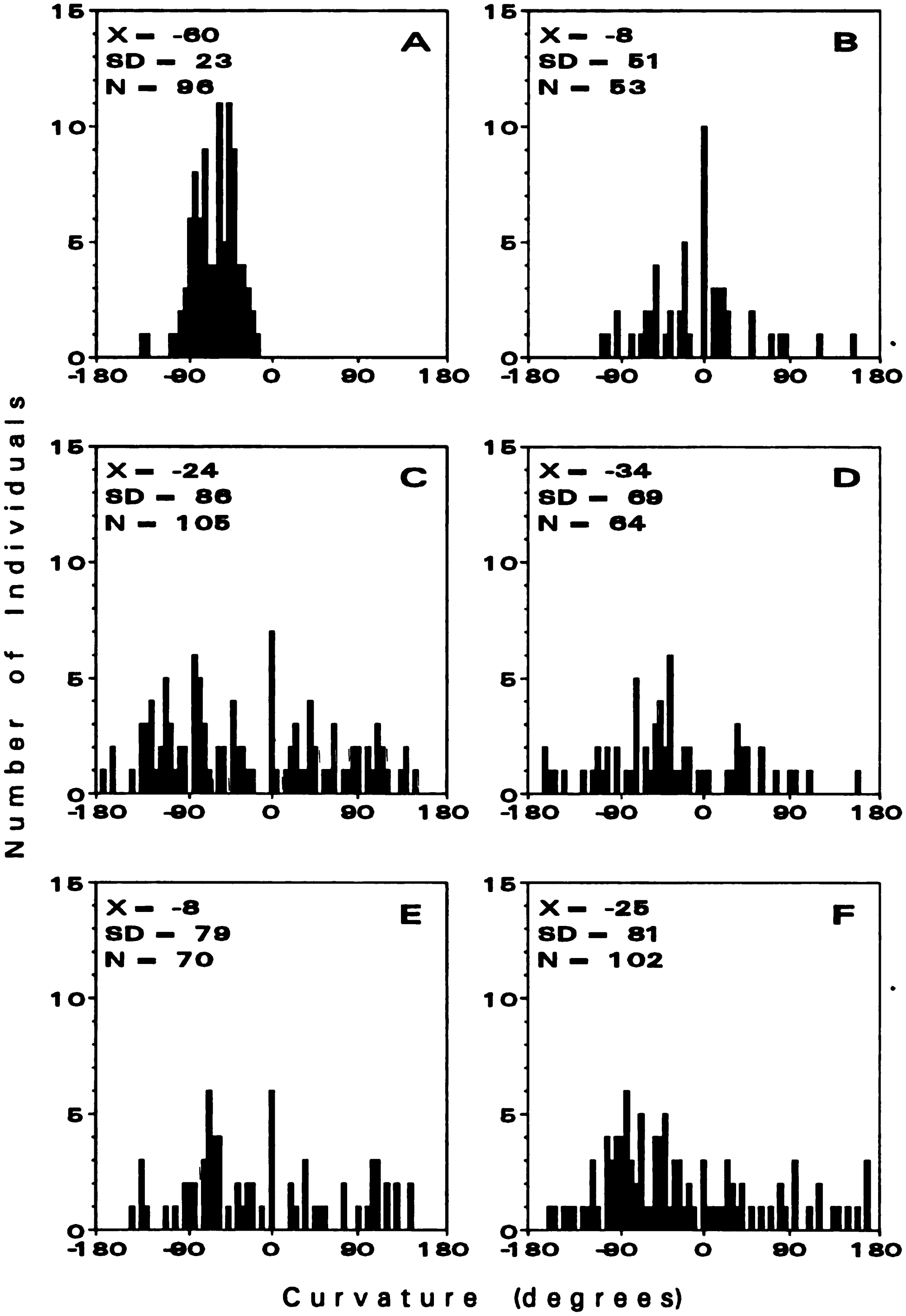
X = -60
SD = 23
N = 96
A
X = -8
SD = 51
N = 53
B
X = -24
SD = 86
N = 105
C
X = -34
SD = 69
N = 64
D
X = -8
SD = 79
N = 70
E
X = -25
SD = 81
N = 102
F
15
10
5
0
-180
-90
0
90
180
Number of Individuals
Curvature (degrees)

FIGURE 6

according to qualitative tests for root starch. Thus, there was no apparent correlation between starch content and gravitropism phenotype.

With each subsequent generation, populations of MG-20 contained increasing numbers of individuals which were not qualitatively starch-deficient. These sub-populations were separated by growing starch-deficient seedlings (MG-20a) and normal seedlings (MG-20b) separately. Gravitropism tests of these sub-populations revealed no difference in the gravitropism phenotype (data not shown). These data suggested that the starch and gravitropism phenotypes of MG-20 were due to two different lesions, both of which were present in the MG-20a populations.

The gross morphology of all five lines was comparable to the wild-type parent.

Quantitation of Starch Content

Quantitative analysis of starch content showed that MG-212, MG-20a, and MG-421 contained approximately 10%, 7%, and 3% of wild-type levels, respectively. Although MG-32 was not qualitatively starch-deficient, its starch content was quantified to verify the results of the qualitative assay. MG-32 and MG-20b both contained wild-type levels of starch. The starch content of MG-65 was not measured. Three independent replicates of this assay yielded similar results.

Modes of Inheritance

Gravitropism

Reciprocal crosses yielded F1 progeny which exhibited gravitropic responses similar to the wild-type parent (Table II). Since the phenotype could only be observed at the population level, it was necessary to deduce the F2 segregation ratio from the behavior of F3 populations. Crosses using either MG-20a or MG-212 as the pollen recipient yielded few progeny which survived to produce F3 seeds. Therefore, the results reported in Table II do not include families from these crosses. All lines exhibited F2 segregation ratios which approximated a 3:1 ratio. Therefore, gravitropism phenotypes in all five lines appear to be due to single recessive nuclear mutations.

Starch-deficiency

Reciprocal crosses yielded F1 progeny which exhibited wild-type phenotypes according to the qualitative assay for starch (Table III). The F2 segregation ratios were determined by assaying individuals from the bulked F2 seed stock. MG-20a and MG-212 exhibited ratios which were not significantly different from a 3:1 ratio. MG-421 exhibited an F2 segregation ratio which was different from 3:1 at the 24% level, but not at the 5% level. Therefore, the starch deficiency exhibited in these three lines appeared to be due to a single recessive nuclear mutation.

TABLE II: Gravitropism phenotypes of F1 and F2 progeny of reciprocal backcrosses between gravitropism mutants (M) and the wild-type parent (WT). Values reported are average curvature (X) and standard deviation (SD) for the population tested. Except for WTxMG20a and MG20axWT, values reported are for roots. The average gravitropic response in roots of the WT was X = 61° and SD = 19°.

Crosses ♀ x ♂	M X(SD)	F1 X(SD)	F2 (WT:M)	Chi^2 (3:1)	Prob
WTxMG20a	42(18)	71(11)	13:3	0.333	0.564
MG20axWT		70(8)	n/d		
WTxMG32	24(89)	69(14)	136:48	0.116	0.733
MG32xWT		66(14)			
WTxMG65	1(105)	50(15)	132:38	0.635	0.426
MG65xWT		51(14)			
WTxMG212	34(80)	61(23)	69:18	0.862	0.353
MG212xWT		66(16)	n/d		
WTxMG421	36(55)	58(18)	97:36	0.303	0.582
MG421xWT		42(21)			

TABLE III: Starch-deficiency in F1 and F2 populations from reciprocal backcrosses between gravitropism mutants and the wild-type parent. Values listed are the percentage of individuals within a population which contain starch in their leaves (for crosses involving MG-20a and MG-421), or in their roots (for crosses involving MG-212). Wild-type populations contain 100% by this measure.

Crosses ♀ x ♂	Mutant (%)	F1 (%)	F2 (WT:M)	Chi^2 (3:1)	Prob
WTxMG20a	0	100	18:6	0.000	1.000
MG20axWT		100			
WTxMG212	0	100	40:13	0.006	0.938
MG212xWT		100			
WTxMG421	12	100	96:40	1.412	0.235
MG421xWT		100			

Complementation Analysis

Gravitropism

The results of the complementation analysis suggested that at least two complementation groups are represented by these mutants (Table IV). One complementation group is comprised of MG-32 and MG-65, the two "random" mutants which do not exhibit starch-deficiency. The second complementation group contains MG-20a, MG-212, and MG-421 as well as the Columbia race mutant TC-18. Although MG-20a and MG-212 did complement, MG-421 did not complement either line, and TC-18 did not complement MG-20a or MG-421. The simplest explanation for this observation is that MG-20a and MG-212 may have exhibited intra-allelic complementation, and that the three lines belong to the same complementation group.

Starch-deficiency

The results of the complementation analysis with respect to starch-deficiency were not conclusive based on MG-20a, MG-212, and MG-421 alone (Table V). This could be due to error in the qualitative starch assay when applied to low numbers of seedlings, to low levels of contamination by airborne wild-type pollen, and/or to undetected self-pollination.

In addition to reciprocal crosses using the five mutant lines, a known starchless mutant in the Columbia race of *A. thaliana*, TC-18, was used as a pollen recipient.

TABLE IV: Complementation analysis of root/hypocotyl gravitropism. Data are presented as root phenotype/hypocotyl phenotype fitting the categories of wild-type (WT), mutant (M), or similar to MG-20 (20).

♀↓ / ♂→	MG-20	MG-32	MG-65	MG-212	MG-421
MG-20	--	WT/WT	WT/WT	WT/WT	20/20
MG-32	WT/WT	--	M/M	WT/WT	WT/WT
MG-65	WT/WT	M/M	--	WT/WT	WT/WT
MG-212	WT/WT	WT/WT	WT/WT	--	M/M
MG-421	20/20	WT/WT	WT/WT	M/M	--
TC-18	M/M	n/d	n/d	n/d	M/M

TABLE V: Complementation analysis of the starch-deficiency phenotype. Results were categorized according to starch content phenotype as wild-type (WT), mutant (M), or intermediate (I) between WT and M.

♀↓ / ♂→	MG-20a	MG-212	MG-421
MG-20a	--	M	I
MG-212	WT	--	M
MG-421	M	I	--
TC-18	M	M	M

TC-18 carries the TC-7 phosphoglucomutase (*pgm*) mutation (Caspar, *et al.*, 1985) and a glabrous morphological marker.

All three starch-deficient lines failed to complement TC-18 (Table V), indicating that all three are *pgm* mutants. Preliminary results using previously described assays for total *pgm* activity (Levi & Preiss, 1978) suggested reductions in activity of 25% to 60% compared with the wild-type parent (data not shown). Assays specific for plastid *pgm* activity were not conducted.

Dihybrid Analysis of Gravitropism and Starch-Deficiency

A dihybrid analysis was conducted using the F2 families generated from backcrosses to the wild-type parent (see page 59, this dissertation).

The results of the dihybrid analysis for the gravitropism and starch-deficiency phenotypes in MG-20a, MG-212, and MG-421 were not consistent with the 9:3:3:1 ratio expected in independently segregating mutations (Table VI). The 3:1 ratio expected if both phenotypes were attributable to one mutation was also not observed. The shift in ratios toward the parental types (G+S+ and G-S-) was consistent with the hypothesis that the starch and gravitropism phenotypes are due to two linked genes. In addition, recombinants (G+S- and G-S+) appeared at frequencies suggesting that the two lesions may be closely linked. Although these analyses are not conclusive, there

TABLE VI: Dihybrid analysis from crosses between the wild-type parent and lines exhibiting both aberrant gravitropism and starch phenotypes. Phenotypes are categorized as wild-type gravitropism (G+), mutant gravitropism (G-), wild-type starch content (S+), and starch deficient (S-).

Mutant	G+ S+	G+ S-	G- S+	G- S-
MG20	13	0	2	1
MG212	69	0	1	17
MG421	75	11	5	24

was no indication in these data of epistasis or other gene interactions.

The frequency with which recombinants were observed varied between the three starch-deficient lines. For example, sixteen recombinants were observed in the 115 seedlings analyzed for MG-421, while only one recombinant was observed in the 87 seedlings analyzed for MG-212 (Table VI). The reasons for and significance of this variability remain unclear.

DISCUSSION

The analyses presented here support four conclusions regarding the five mutant lines under study. First, both the gravitropism and the starch-deficient phenotypes are due to single recessive nuclear mutations. Second, the five lines represent two gravitropism mutations. Third, three lines are quantitatively starch deficient, and are independent isolates of the previously described phosphoglucomutase (*pgm*) mutant (Caspar, *et al.*, 1985). Finally, each of the starch-deficient lines appeared to carry two closely linked lesions; one affecting starch content and the other affecting gravitropism.

It is worth noting that, since none of the five mutant lines carried morphological markers, the slight deviation of observed phenotypic ratios from those expected could be due to self-pollination or contamination by airborne pollen. The possibility of such events, as well as the error inherent in the qualitative starch assay when applied to small populations, have been considered in this analysis.

Genetic separability of root and hypocotyl gravitropism has been suggested previously, based largely on the different phenotypes observed in roots and hypocotyls of the same line (Scholdeen & Burström, 1960; Maher & Martindale, 1980, Bullen, *et al.*, 1990). If these different phenotypes are due to two distinct gravitropism pathways, separation of the phenotypes should be observed in reciprocal backcrosses to the wild-type parent. For example, if the phenotypes are due to two unlinked genes, approximately 19% of the F2 progeny from the reciprocal backcross of a mutant line with "random" roots and "altered" hypocotyls should exhibit "random" roots with "wild-type" hypocotyls. Similarly, another 19% of the progeny should exhibit "wild-type" roots with "altered" hypocotyls. If the genes are linked, the frequency of recombination should decrease according to the degree of linkage.

Recombination of the root and hypocotyl phenotypes was not observed (data not shown). Although the possibility that the phenotypes are due to two tightly-linked genes has not been eliminated, these results suggest that the root and hypocotyl gravitropism responses may not be genetically separable in these lines. Since tissue-specific differences in phytohormone responses are well documented (Thimann & Schneider, 1938; Sachs, *et al.*, 1959; Pilet, *et al.*, 1960; Kutschera, *et al.*, 1987), it would not be surprising to find that gravitropism gene products affect the two tissues differently, resulting in different gravitropism phenotypes within the same line. Thus, previous conclusions regarding genetic separability may need to be re-examined.

Dihybrid analysis revealed genetic separability between gravitropism and *pgm*, suggesting that these lines carried two lesions. The consistent mutation of two closely associated sites is not unexpected in EMS-mutagenized material since EMS acts on sections of open or single-stranded DNA (Brock, 1971). The failure of TC-18 to complement either the starch or the gravitropism phenotypes of MG-20a, MG-212, or MG-421 implies that TC-18 also carries two similar lesions. Thus, conclusions regarding the relationship between starch and gravitropism in the TC-7 or TC-18 mutant (Caspar & Pickard, 1989; Kiss, *et al.*, 1989) and in other starch-deficient mutants (Kiss & Sack, 1989; Kiss & Sack, 1990) should be re-examined.

Since a dihybrid analysis is not equivalent to a test cross, the recombination frequency can only be estimated here. Additional error may also be introduced by the lack of morphological markers mentioned above, the relatively low number of individuals studied, and the variability of recombination observed in MG-212 and MG-421. However, data for MG-421 are sufficient for a first approximation of the distance between the two mutations. Out of 115 lines in the dihybrid analysis, sixteen (16) recombinants were observed (Table VI), representing a recombination frequency of 14%. Given the uncertainty in these measurements, a conservative estimate for the distance between *pgm* and the gravitropism loci of 20 map units is proposed. Thus, based on the location of *pgm* relative to morphological markers described for *A. thaliana* (Koornneef, 1989), a gene involved in gravitropism is located on chromosome 5.

The data presented here also illustrate that backcrossing as a means of eliminating background mutations is not reliable. Although 97% of the independently segregating genetic background is replaced after five backcrosses, linked genes can be carried along with the gene of interest indefinitely if the linked gene is not detected and selected against. The more tightly linked the two genes are, the greater the probability that progeny carrying both genes will be chosen for subsequent crosses and studies. The two phenotypes could appear to be due to a single lesion, and invalid cause-and-effect relationships assumed.

PHYSIOLOGICAL CHARACTERIZATION OF FIVE GRAVITROPISM MUTANTS IN *ARABIDOPSIS THALIANA*

ABSTRACT

A physiological analysis of five gravitropism mutants was conducted to eliminate the possibility that the abnormal gravitropic response was due to changes in general physiological parameters. Phototropism was used to demonstrate that each line was capable of differential growth. Elongation growth rates were measured, and no correlation between these growth rates and the gravitropism phenotypes was found. Although red light enhanced gravitropic curvature by 10% in roots and by 35% in hypocotyls, there was no measurable effect of red light irradiation on elongation growth rates. In two lines, the organs were significantly shorter than in the wild-type seedlings at the time of gravistimulation. The gravitropic response in these lines increased slightly when gravistimulation was administered to organs comparable in length to the wild-type parent. However, the increase was not sufficient to explain the gravitropism phenotypes. The data support the hypothesis that the gravitropism phenotypes observed are due to lesions specific to

transduction events of the gravitropism pathway, and not to alterations in differential or elongation growth.

INTRODUCTION

Gravitropic curvature results from the differential growth of a plant organ relative to the gravity vector. Since the phenomenon involves growth (Digby & Firn, 1979; MacDonald, *et al.*, 1983), alterations in growth parameters might be expected to affect the observed gravitropic response without necessarily being specific to the gravitropism transduction pathway. Indeed, a reduced elongation growth rate was cited as the explanation for the slightly reduced gravitropic response of a starch-deficient mutant in *Arabidopsis thaliana* (Caspar and Pickard, 1989). Plants incapable of differential growth would also appear to be agravitropic, yet would additionally be incapable of phototropism. For this reason, phototropism has often been used as a control in studies of gravitropism to demonstrate differential growth in agravitropic lines (Filner, *et al.*, 1970).

Phototropism may also be useful in estimating the location of gravitropism lesions based on the assumption that gravitropism and phototropism share some elements of signal transduction (Russo, 1980; Nick & Schäfer, 1988). Thus, phototropically normal gravitropism mutants would presumably carry lesions in the gravitropism pathway prior to the confluence of the gravitropism and phototropism pathways.

In this paper, two physiological parameters which could affect the expression of gravitropic curvature response are examined in five gravitropism mutants. First, phototropism is used to examine differential growth in each mutant line, and to estimate the location of the gravitropism mutation relative to the presumed confluence of the two sensory pathways. Second, elongation growth rates during the period of curvature development and organ lengths at the time of gravistimulation are examined as potential explanations for the observed gravitropic responses.

MATERIALS AND METHODS

General Growth Conditions

Seed of *Arabidopsis thaliana* (L.) Heynh. var Estland used in this study were obtained through a direct screening procedure (Bullen, *et al*. 1990). Seedlings were grown and seed set occurred under previously described conditions (Khurana and Poff, 1989). The gravitropism phenotypes and starch content of the five mutant lines selected for this study have been described elsewhere (page 61, this dissertation).

Light Sources

White light was provided by General Electric Delux white fluorescent tubes at 50 μmol m^{-2} s^{-1} (General Electric Co., Cleveland, OH). Red light was from a General Electric Red F30T12-R-RD tube filtered through a red cellophane (Highland Supply Corp., Highland, IL) transmitting light in the 560- to 720-nm range with peak transmission at 630 nm. The fluence rate of the red light source was 1.2 μmol m^{-2} s^{-1} as measured using a datalogger with quantum sensor (LI-COR, Inc., Lincoln, NE). Unilateral blue light was from a light source consisting of a Sylvania 300-W ELH tungsten halogen lamp (GTE Products, Danvers, MA) used in combination with a 12-cm layer of aqueous cupric-sulfate solution (1.25%, w/v) and a 450-nm interference filter with a half-band width of 10 nm (PTR Optics, Waltham, MA).

Measurement of Phototropism

Seedlings were tested for phototropic response to 5 flashes of 450-nm light as described previously (Khurana, *et al.*, 1989a).

Elongation Growth

Seedlings were grown according to the protocol for the measurement of gravitropism in dark-grown seedlings (page 55, this dissertation). Time was measured from the moment the seed were placed in darkness at 25°C following the germination-potentiating white light irradiation. At 21 h, the seedlings were exposed to either 1 h red light or kept in darkness. Plates containing the elongating seedlings were removed at intervals between 22 h and 50 h, and the lengths of roots and hypocotyls were measured directly from the agar plates using a CCD camera (Cohu, Inc., San Diego, CA) and image analysis system (Jandel Scientific, Corte Medera, CA). Growth curves were generated by plotting the measured length as a function of time.

Measurement of Gravitropism

Seed were surface-sterilized, sown, and tested for gravitropic response as previously described (page 55, this dissertation).

RESULTS

Phototropism

All five mutant lines exhibited phototropic responses similar to the wild-type parent (data not shown).

Elongation Growth and Growth Rates

Elongation growth exhibited a similar pattern in all lines examined (Figure 7), although growth rate and initial lengths varied.

Growth rates were calculated for the period between 22 h and 32 h (Table VII). This period coincided with the time during which gravistimulation and curvature development occurred. Three of the five mutant lines exhibited growth rates different from the wild-type parent. In MG-32 and MG-65, the hypocotyl growth rates were reduced relative to the wild-type parent by approximately 50%, while the root growth rates were comparable to the wild-type rate. Conversely, the root growth rate of MG-421 was reduced to less than 50% of the wild-type rate, while the hypocotyl growth rate was comparable.

Since irradiation with red light immediately prior to gravistimulation increases the magnitude of root and hypocotyl curvature in the wild-type parent by 10% and 35%, respectively, the effect of red light on growth rates was

FIGURE 7: Elongation growth curves for roots and hypocotyls of five gravitropism mutants. Growth curves for the Estland wild-type parent are included as dashed lines in each of the panels. A) Wild-Type; B) MG-20a; C) MG-32; D) MG-65; E) MG-212; F) MG-421.

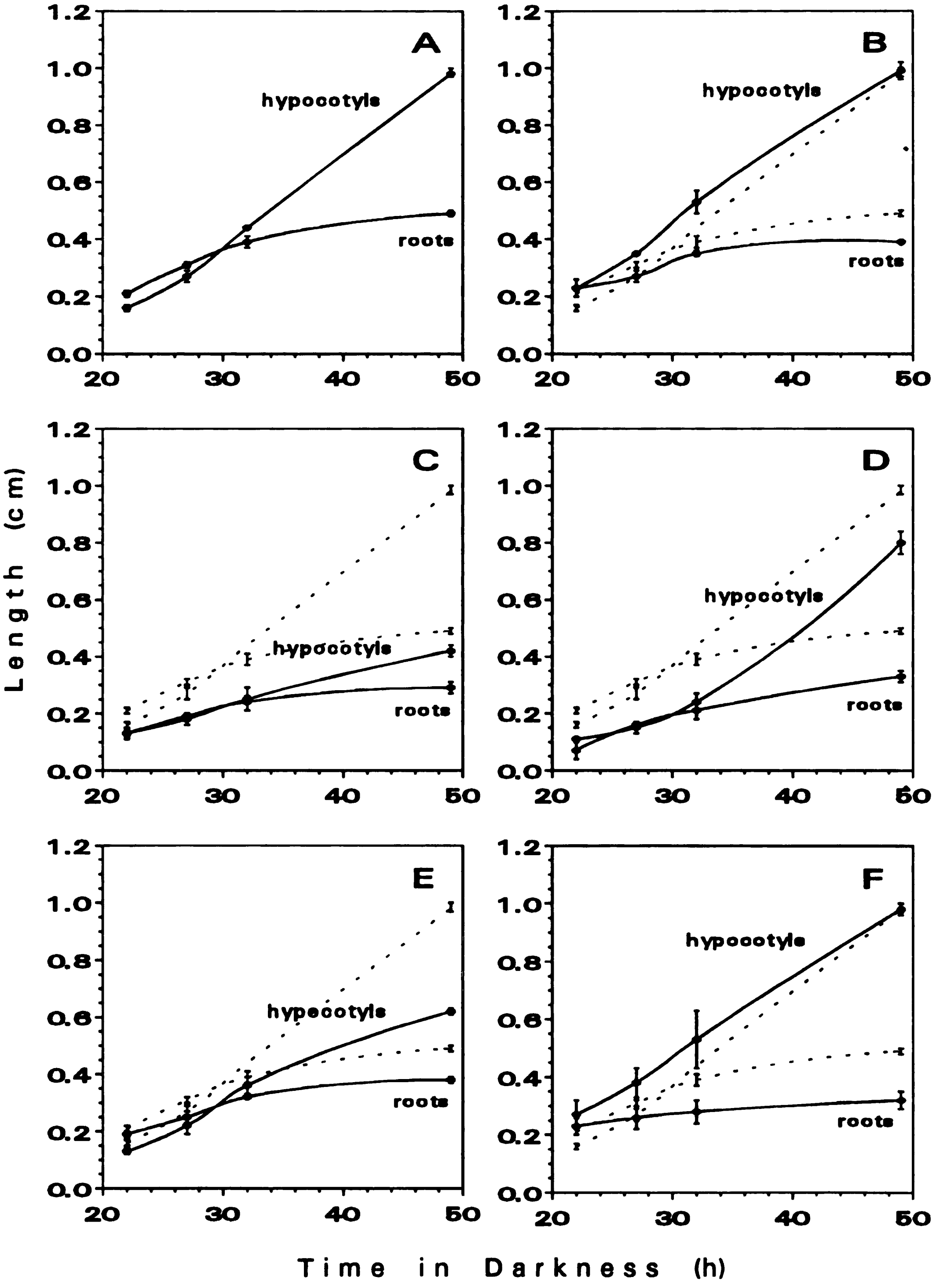
A
B
C
D
E
F
hypocotyls
roots
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
30
40
50
Length (cm)
Time in Darkness (h)

FIGURE 7

TABLE VII: Growth rates between 22 h and 32 h. Seedlings received either no red light treatment (No RL), or 1 h red light (RL) beginning at 21 h. Growth rates are expressed in mm h^{-1}. Numbers in parentheses represent standard error.

	ROOTS		HYPOCOTYLS	
Lines	No RL	RL	No RL	RL
Wild-Type	0.18 (0.03)	0.18 (0.02)	0.28 (0.04)	0.25 (0.05)
MG-20a	0.12 (0.04)	0.11 (0.06)	0.30 (0.07)	0.31 (0.13)
MG-32	0.11 (0.04)	0.09 (0.04)	0.12 (0.05)	0.14 (0.04)
MG-65	0.14 (0.06)	0.14 (0.05)	0.13 (0.03)	0.22 (0.02)
MG-212	0.13 (0.03)	0.14 (0.05)	0.23 (0.04)	0.26 (0.05)
MG-421	0.05 (0.07)	0.09 (0.06)	0.26 (0.15)	0.25 (0.16)

also examined (Table VII). With the exception of MG-65 hypocotyls, no difference in elongation growth rates was observed between seedlings receiving red light and those kept in darkness.

Only lines MG-32 and MG-65 were significantly shorter than the wild-type parent at 22 h, the time at which gravistimulation occurred. Extrapolating from the growth curves (Figure 7C,D), the roots of MG-32 and MG-65 would reach approximately the same length as the 22-h-old wild-type roots by 30 h and 32 h, respectively. Similarly, the hypocotyls of MG-32 and MG-65 would require 31 h and 27 h to reach wild-type lengths. By appropriately adjusting the experimental protocol, the gravitropic response to a 3-h gravistimulation in seedlings of comparable lengths was measured (Table VIII). A slight increase in curvature was observed in the older mutant seedlings. However, curvature remained reduced compared to wild-type seedlings.

DISCUSSION

Mutants incapable of differential growth would not be expected to curve in response to a directional stimulus such as unilateral blue light. However, the phototropic response in all five mutant lines indicated that the gravitropism phenotypes cannot be attributed to a general inability of the seedlings to curve. In addition, the presence of a phototropic response implies that, if gravitropism and phototropism share elements of signal

TABLE VIII: Average curvature of roots and hypocotyls in seedlings of comparable lengths. Stimulation of 3 h was administered at the time indicated. Numbers in parentheses represent standard error.

	Roots		Hypocotyls	
Lines	Time (h)	Curvature (degrees)	Time (h)	Curvature (degrees)
Wild-Type	22	38 (2.9)	22	26 (3.8)
MG-32	22	4 (1.5)	22	2 (0.7)
	30	17 (4.0)	31	12 (2.2)
MG-65	22	2 (1.3)	22	2 (1.0)
	32	15 (2.9)	27	9 (1.7)

transduction, the lesions in these gravitropism mutants affect events not involved in phototropism.

Two lines of evidence support the hypothesis that differences in elongation growth rates during curvature development do not account for the gravitropic phenotypes. First, there was no apparent relationship between growth rates and the gravitropism phenotype. "Random" gravitropism phenotypes were found in both roots and hypocotyls of MG-32, MG-65, and MG-212, and in the hypocotyls of MG-421. However, the elongation growth rate was reduced in the hypocotyls of only MG-32 and MG-65. Similarly, the "altered" gravitropism phenotype was exhibited by the hypocotyls of MG-20a and the roots of MG-421 while only the roots of MG-421 exhibited a reduced growth rate. Second, there was no correlation between growth rates and red-light enhanced gravitropic curvatures since the red light irradiation did not affect elongation growth rates (Table VII).

Starch-deficiency as represented by MG-20a, MG-212, and MG-421 does not directly influence elongation growth. Although all three starch-deficient lines belong to the same complementation group (page 75, this dissertation), only the roots of MG-421 exhibited a reduced growth rate. This reduction in growth may be a consequence of an additional mutation. Since these lines have not been extensively backcrossed, it is reasonable to expect the presence of lesions in addition to those directly affecting gravitropism and starch content.

Organ length at the time of gravistimulation may influence the gravitropism phenotype in MG-32 and MG-65. However, it seems unlikely that this provides a complete explanation for the gravitropism phenotype observed. Gravitropic curvature increased when MG-32 and MG-65 were allowed to grow longer before gravistimulation (Table VIII). However, the magnitude of the response remained at no more than 50% of the wild-type parent, indicating that the gravitropic response was impaired even in the longer organs.

In summary, the gravitropism phenotypes observed in the five mutant lines cannot be adequately explained by alterations in either differential or elongation growth. Although other unrelated lesions may be present, the lines appear to carry lesions specific to events in the gravitropism transduction pathway.

STIMULUS-RESPONSE RELATIONSHIPS OF GRAVITROPISM IN LIGHT-GROWN AND DARK-GROWN MUTANTS OF *ARABIDOPSIS THALIANA*

ABSTRACT

Relationships between gravitropic curvature and stimulation time were examined in light-grown and dark-grown seedlings of five gravitropism mutants in *Arabidopsis thaliana*. In both light-grown and dark-grown seedlings, the five lines exhibited shifts in presentation times relative to the wild-type parent by at least one order of magnitude. This suggests that the mutants carry lesions affecting the perception or other early transduction events in gravitropism. Compared with dark-grown seedlings, gravitropic curvature in light-grown seedlings was enhanced. The absence of this light-enhancement in MG-65 suggests that the mechanism may be altered in this line. Based on the response in starch-deficient lines, there was no difference in the relative magnitude of light-enhancement of gravitropism with respect to starch content. Thus, it is proposed that light-enhancement of gravitropism is not the result of an increased starch content.

INTRODUCTION

The enhancement of gravitropism by light has been reported in several plant species (Lake & Slack, 1961; Blaauw and Blaauw-Jansen, 1974; Pilet, 1979; Britz and Galston, 1982), and in the sporangiophores of *Phycomyces* (Dennison, 1964). The phenomenon is probably best known in certain cultivars of *Zea mays* in which a red light irradiation enables the primary roots to change from a diagravitropic response to an orthogravitropic response (Shen-Miller, 1974; Leopold & LaFavre, 1989). The mechanisms through which light-induction or light-enhancement of gravitropism operate remain unknown.

Britz and Galston (1982) examined the influence of red light irradiations on presentation time in *Pisum*. They concluded that the observed increase in curvature and decrease in presentation time was attributable to increased gravity perception. Kiss and Sack (1990) studied a starch-deficient mutant of *Nicotiana sylvestris* which was gravitropic when light-grown, but exhibited reduced curvature and starch content when dark-grown. They suggested that the reduced starch content in these dark-grown seedlings affected the gravity perception mechanism by altering the sedimentation characteristics of the amyloplasts.

The purpose of this paper is to examine the relationship between presentation time and response in light-grown and dark-grown roots of five previously described gravitropism mutants of *Arabidopsis thaliana* (page 61, this dissertation). The presence or absence of the light-enhancement phenomenon in each mutant is also examined. The role of starch in light-enhancement of gravitropism is also examined by comparing the response in starch-deficient lines with that of lines containing wild-type levels of starch.

MATERIALS AND METHODS

General Growth Conditions

Seed of *Arabidopsis thaliana* (L.) Heynh. var Estland used in this study were obtained through a direct screening procedure (Bullen, *et al.* 1990). Seedlings were grown and seed set occurred under previously described conditions (Khurana and Poff, 1989). The gravitropism phenotypes and starch content of the five mutant lines selected for this study have previously been described (page 61, this dissertation).

Light Sources

White light was provided by General Electric Delux white fluorescent tubes at 50 μmol m^{-2} s^{-1} (General Electric Co., Cleveland, OH). Red light was from a General Electric Red F30T12-R-RD tube filtered through a red cellophane (Highland Supply Corp., Highland, IL) transmitting light in the 560- to 720-nm range with peak transmission at 630 nm. The fluence rate of the red light source was 1.2 μmol $m^{-2}s^{-1}$ as measured using a datalogger with quantum sensor (LI-COR, Inc.,Lincoln NE).

Measurement of Stimulus-Response for Gravitropism

Dark-Grown Seedlings

Seed were surface-sterilized and sown as previously described (Bullen, *et al.*, 1990). Germination was potentiated by a white light irradiation terminating 1 to 2 h after the t_{50} for each line. After 21 h in darkness followed by 1 h of red light, the seedlings were positioned horizontally in darkness for the time period designated. Curvature was then allowed to develop for 1.5 h in darkness on a clinostat rotating at 0.5 rpm and presenting a gravicompensation of 10^{-5} *g*. The actual curvature of each organ was then measured relative to the organ's original orientation using a CCD camera and image analysis software (page 56, this dissertation).

Light-Grown Seedlings

Seed were surface-sterilized and sown as above, except that after 4 d in darkness at 4°C, the plates were placed on edge under white light at 25°C for 48 h, or until the roots were approximately 3 to 5 mm in length. The plates were then placed on edge in darkness at 25° C for 1 h, after which the seedlings were gravistimulated. The curvature response was allowed to develop on a clinostat, and curvature was measured as described above for the dark-grown seedlings.

RESULTS

Stimulus-response curves for roots of light-grown and dark-grown seedlings are presented in Figure 8.

FIGURE 8: Stimulus-response curves for root gravitropism in dark-grown and light-grown seedlings. Average curvature is plotted as a function of stimulation time in sec. Each point represents the average curvature of the roots relative to the roots original orientation. Error bars represent standard error. A) Wild-type parent; B) MG-20a; C) MG-32; D) MG-65; E) MG-212; and F) MG-421.

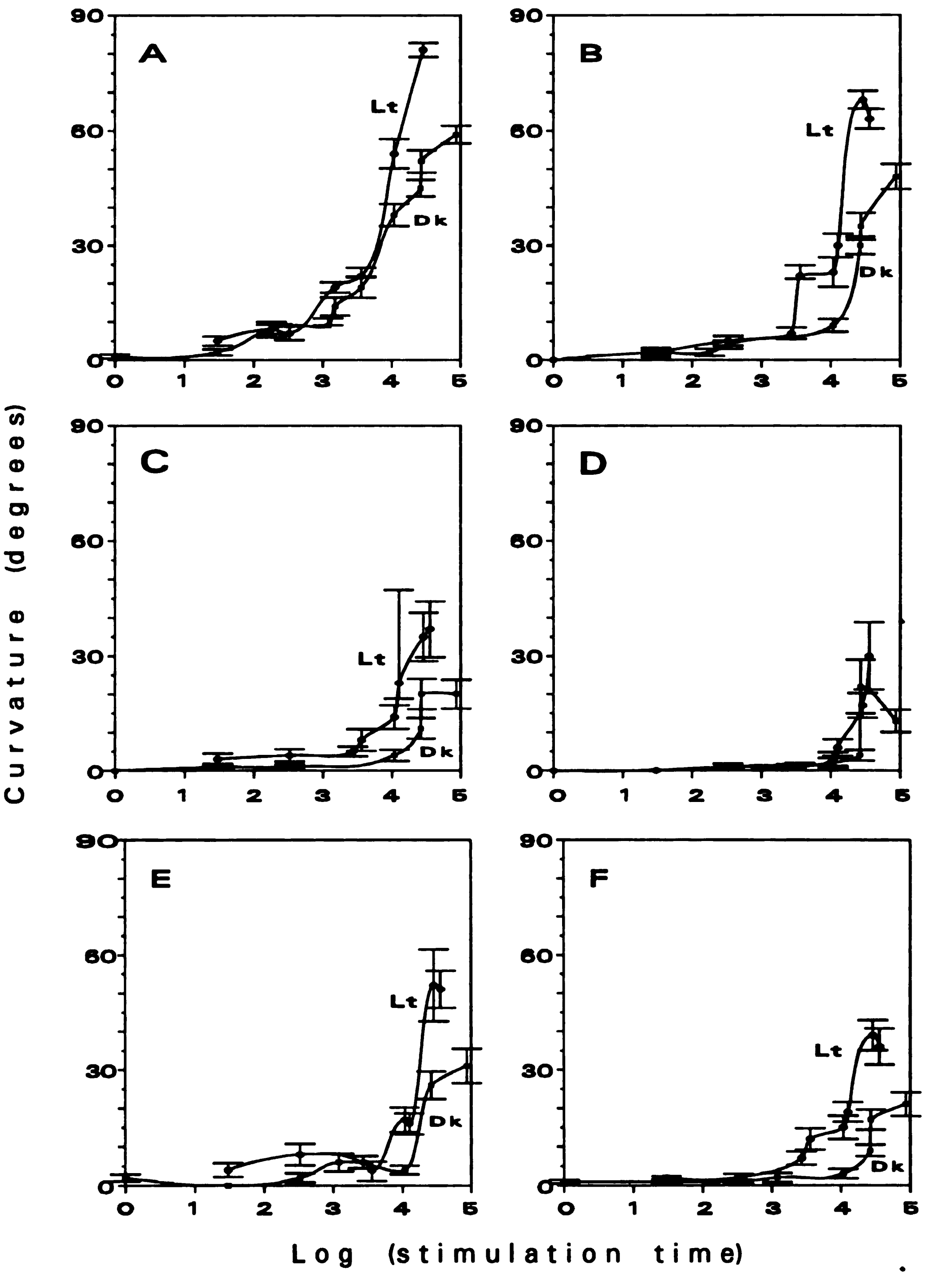
A
B
C
D
E
F
Lt
Dk
90
60
30
0
0
1
2
3
4
5
Curvature (degrees)
Log (stimulation time)

FIGURE 8

Light-grown hypocotyls exhibited greatly reduced gravitropic curvature compared to dark-grown hypocotyls, with gravitropic curvature not exceeding 14° in light-grown wild-type hypocotyls. Thus, although the general shape of the stimulus-response curves was similar to that obtained for roots, the small range of response rendered the results for hypocotyls more difficult to interpret. Therefore, only the results obtained for roots are presented here.

Presentation times were estimated using the method described elsewhere (page 118, this dissertation). In all five mutant lines, the presentation time for dark-grown roots was shifted to a longer time relative to the dark-grown wild-type roots. Similarly, the presentation time for roots of light-grown mutant seedlings was shifted to a longer time relative to the light-grown wild-type roots. These shifts were close to or exceeded one order of magnitude (Table IX).

In all lines except MG-65, curvature of light-grown seedlings was increased over that observed in dark-grown seedlings at stimulation times exceeding 1,000 sec (Figure 8). The difference between the curvature responses of light-grown and dark-grown seedlings was also similar in all lines except MG-65 (Figure 9). This light-enhanced curvature was accompanied by a slight reduction in presentation time to between one-half and one-fourth of the values obtained for dark-grown seedlings (Table IX). As

TABLE IX: Approximate presentation times for light-grown and dark-grown seedlings.

Line	Presentation Time in Roots Dark-Grown (sec)	Presentation Time in Roots Light-Grown (sec)
Wild-Type	600	200
MG-20a	7,000	2,000
MG-32	9,000	2,000
MG-65	20,000	20,000
MG-212	10,000	3,000
MG-421	9,000	1,500

FIGURE 9: Difference between the stimulus-response curves for root gravitropism in light-grown and dark-grown seedlings. Mutant lines represented are MG-20a (■), MG-32 (♦), MG-65 (▼), MG-212 (•), and MG-421 (▲). The solid line represents the difference in wild-type seedlings, and the error bars represent the standard error of this difference at the designated stimulation times.

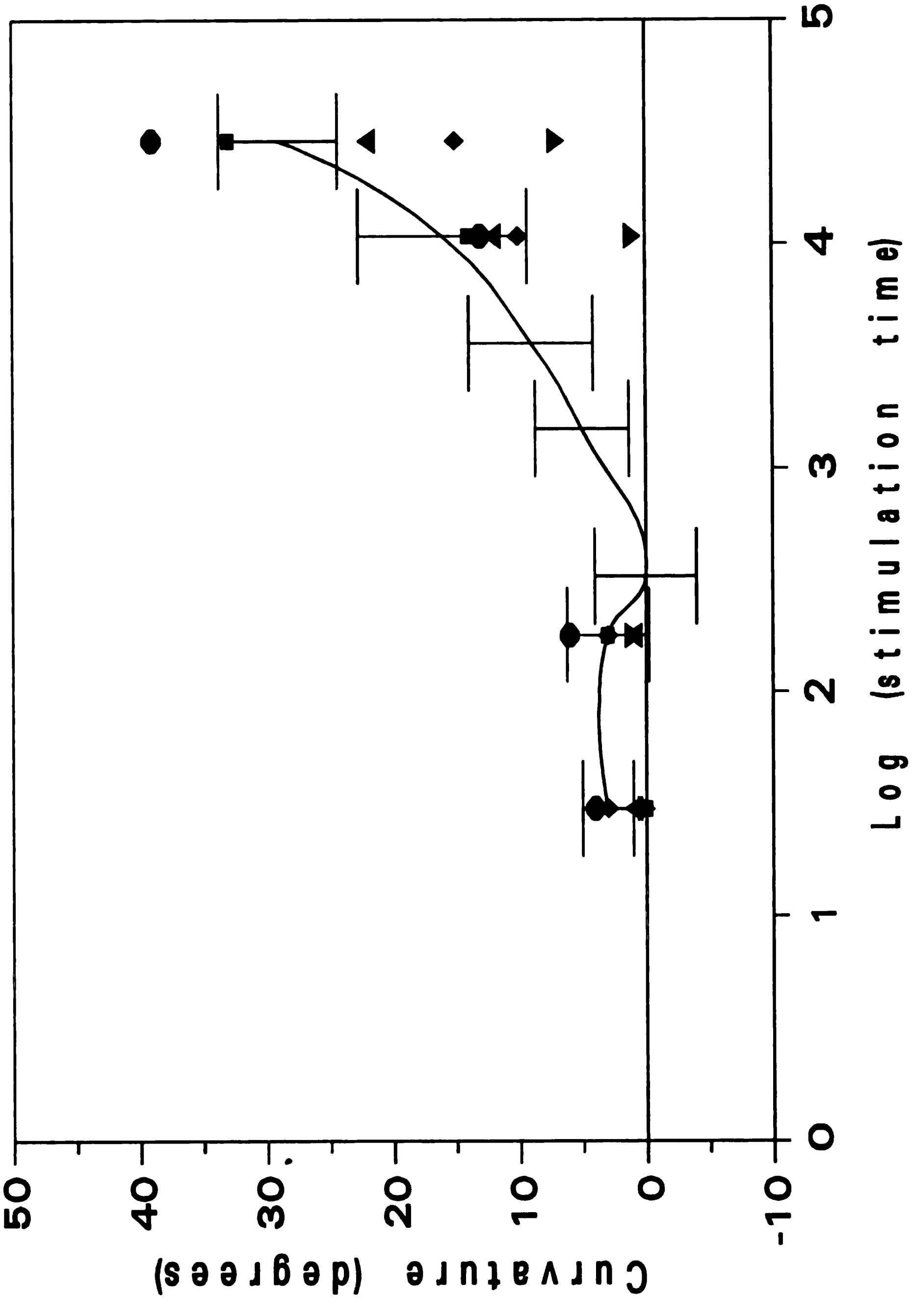
Curvature (degrees)
50
40
30
20
10
0
-10
Log (stimulation time)
0
1
2
3
4
5

FIGURE 9

with curvature, the presentation time in MG-65 was the same in light-grown and dark-grown seedlings.

DISCUSSION

When stimulus receptors are decreased in number or sensitivity, the threshold dose or the minimum dose required to elicit a response is increased. Similarly, when the receptors are increased in number or sensitivity, the threshold dose is decreased. When the law of reciprocity is valid (that is, when the response to a dose is independent of the stimulus strength or duration), the magnitude of such shifts in threshold dose indicate the severity of the change in receptor function. Although reciprocity is not valid for gravitropism by the roots of *Arabidopsis*, the effect of stimulation time is independent of stimulus strength (page 113, this dissertation). Thus, the large shifts in presentation time for the mutants relative to wild-type values are consistent with the hypothesis that all five mutant lines carry lesions directly affecting perception or other early transduction events (Table IX).

The apparent absence of light-enhanced gravitropism in MG-65 suggests that this line may carry a lesion which affects the light-enhancement mechanism. This mutant may prove useful in both the study of this mechanism, and in the study of gravitropism in the absence of such stimulus interactions.

Light-grown seedlings might be expected to respond to lower doses of gravistimulation because of a light-enhanced increase in amyloplast starch content. This hypothesis was examined most recently using a phosphoglucomutase (*pgm*) mutant of *Nicotiana sylvestris* in which dark-grown seedlings exhibited reductions in both curvature and starch content compared with light-grown seedlings (Kiss and Sack, 1990). However, the difference between the response in dark-grown and light-grown seedlings reported here was the same whether the line contained wild-type levels of starch or was starch-deficient (Figure 9). In addition, the only mutant exhibiting a lack of light-enhancement, MG-65, was not apparently altered with respect to starch content. Thus, it is proposed that light-enhancement of gravitropism cannot be attributed to alterations in starch content alone.

DISCUSSION

At the onset of this work, no comprehensive genetic system existed for the dissection of the gravitropism transduction pathway. In addition, although extensive data have been collected regarding the phenomenology of gravitropism, none of the transduction events leading to the gravitropic response had been unequivocally identified. Even the mechanisms of the response itself were not understood.

By screening without regard to any particular model for gravitropism, the genetic system established in *Arabidopsis thaliana* could potentially include mutants representing alterations in each of the transduction events. Additional direct screening using alternative mutagens would aid in further saturating the transduction pathway with mutations. This approach strives to remove as many assumptions about the transduction pathway as possible, and allows the validity of those assumptions to be examined.

Two assumptions commonly made in the study of gravitropism were brought into question by this work. First, reduced elongation growth rate has often been cited as probable cause for reduced gravitropic response, especially in starchless or starch-deficient mutants (for an example, see Caspar & Pickard, 1989). However, no correlations were found between elongation growth, starch content, and gravitropic response. Second, it was often assumed in earlier works that aberrant gravitropic response in starchless or starch-deficient mutants represented cause-and-effect; that is, the starch and gravitropism phenotypes were presumed to be the consequence of a single gene mutation (for an example, see Kiss & Sack, 1989; Kiss, *et al.*, 1989). The genetic separability of the *pgm* and gravitropism lesions demonstrated here for *Arabidopsis* suggests that this assumption needs to be reexamined.

Based on the assumed validity of the amyloplast hypothesis, or at least some role for starch in gravitropism, starch content has been suggested as one possible explanation for the light-induced enhancement of gravitropism (for an example, see Kiss & Sack, 1990). However, in addition to the questions mentioned above regarding starch-deficient mutants, correlations between light-induced enhancement of gravitropism and starch content were not found in this work. Thus, other explanations for the apparent amplification of the gravity signal by light must be sought.

Several directions for future research are suggested. If mapping verifies the possible location of one gravitropism gene near *pgm*, cloning of a gene in the transduction pathway may now be within reach. Additional direct screens for gravitropism mutants under alternative growth conditions could identify mutants useful for the study of specific features of gravitropism. For example, mutants with alterations in the light-enhancement mechanism could be identified by altering the light conditions preceding gravistimulation. Additionally, this work suggests that the expected phenotype of gravity susception or perception mutants is not simply agravitropism, but rather agravitropism in both light-grown and dark-grown seedlings. Thus, screening under both conditions would facilitate the identification of mutants with lesions in these early transduction events.

Although the goal of establishing a genetic system for the study of gravitropism was met, more questions about the gravitropism transduction pathway were raised by this work than were answered. No conclusions can be offered regarding the role of starch in gravitropism or the validity of the plastid-based hypothesis for gravity susception. However, with the demonstration of linkage between *pgm* and the "gravitropism" lesion, at least one possible source for the conflicting data reported in the literature has been identified. Additional questions which have been raised by this work could serve to resolve the controversies surrounding starch and plastid involvement as well. For example, it is interesting to note that all of the starch-deficient lines identified in the screening procedure carried lesions in *pgm*. Is there some biological significance in this, or is it simply a consequence of an unidentified bias in the screening procedure? Further, could there be some physiological or evolutionary significance to the tight linkage between the *pgm* and gravitropism loci?

Given the success of genetic dissection in other sensory pathways (for example, see Galland & Lipson, 1987; Macnab, 1984), it is anticipated that the genetic system described here, and others like it, will likely lead to the identification of actual gravitropism transduction events in the future.

APPENDIX

APPENDIX A

THE STIMULUS THRESHOLD FOR GRAVITROPISM IN *ARABIDOPSIS THALIANA*

ABSTRACT

To permit comparisons between the stimulus-response relationships of gravitropism mutants and the wild-type parent, the presentation time for gravitropism in the wild-type line was determined. Reciprocity was examined using centrifugal forces of 2 and 4.7 *g*. The force or stimulus strength showed no effect on the magnitude of the response. The response depended only on the time of stimulation. Thus, for forces of 1 to 4.7 *g*, reciprocity was not valid. Presentation times were estimated from stimulus-response curves by extrapolating from the point of transition between the two phases of the biphasic curves. The presentation time for roots was estimated at 600 sec, and the presentation time for hypocotyls was estimated at 800 sec.

INTRODUCTION

The study of stimulus-response mechanisms often begins with the measurement of a dose-response curve, a log-linear plot of response *vs.* stimulus dose. Such curves have been used to study the effectiveness of one stimulus form *vs.* another (Sachs, 1864; Parker, *et al.*, 1946), or the relationship between the stimulus and its receptor (Withrow, *et al.*, 1957; Baer, 1981). Although gravity is a physical stimulus which does not offer variations of form comparable to wavelengths of light or isomers of chemical compounds, the relationship between gravity and the gravity receptor can still be examined using stimulus-response curves to measure stimulus thresholds.

The definition of gravitational dose and stimulus thresholds has varied throughout the study of gravitropism (for a review, see Volkmann & Sievers, 1979), making direct comparisons of stimulus-response analyses difficult. The three most frequently used definitions for threshold are: 1) threshold dose, 2) presentation time, and 3) perception time. Threshold dose is the minimum dose required to elicit a response, where dose is dependent on the stimulus strength or force. Variations in gravitational stimulus strength have been delivered as centrifugal force, and under the normal circumstances of 1 *g*, dose is equivalent to presentation time. Presentation time is the minimum time required to affect a visible response. Presentation times have been measured in several species, and vary from 12 to 30 sec (Johnsson, 1971; Iversen & Larsen, 1973;). In contrast, perception time is the minimum time required to affect perception, and may not elicit a visible response. Perception time has typically been studied through some form of intermittent stimulation (Fitting, 1905a,b; Pickard, 1973b), and thresholds as low as 0.5 sec have been reported (Pickard, 1973b).

The gravitropism stimulus-response curve is biphasic, with the first phase consisting of a small curvature response increasing slowly over the lower dose or time range. The second phase begins as curvature increases rapidly and linearly throughout the higher stimulus range. Some investigators have defined the stimulus threshold as the dose or time which gives the first measurable curvature above zero (Johnsson, 1965). Others, arguing that nutational movements in plants may be responsible for the first phase of the stimulus-response curve, have extrapolated the threshold from the point of intersection between the two phases of the response (Shen-Miller, 1970).

However estimated, shifts in threshold under different conditions or in different mutant lines suggest that perception of the stimulus has been affected. This assumes that the stimulus-response curve reflects the interaction between a "quantized" stimulus and a single receptor, or that the "law of reciprocity" is valid (Bunsen and Roscoe, 1862).

Simply, the law of reciprocity states that the response is constant for a given stimulus dose. Because dose is defined by stimulus strength x time, reciprocity is valid when the individual values of stimulus strength and time do not affect the response. When the law of reciprocity fails, the stimulus could be interacting with more than one receptor/transduction pathway, all leading to the same response. Other models for the failure of the law of reciprocity which do not require invoking multiple receptors and pathways have been described (Poff, *et al.*, 1992).

In this paper, stimulus thresholds for the roots and the hypocotyls of *Arabidopsis thaliana* are measured. To accomplish this, the system is tested for valid reciprocity with variations in stimulus strength achieved using a centrifuge to administer gravity as a centrifugal force greater than 1 *g*. Based on the results of these studies, presentation time is selected as the appropriate measure of stimulus threshold to be used in subsequent studies. Finally, the stimulus-response curves are used to estimate the presentation time of a wild-type line of *Arabidopsis thaliana*.

MATERIALS AND METHODS

General Growth Conditions

Seed of *Arabidopsis thaliana* (L.) Heynh. var Estland used in this study were from the previously described wild-type parent seed stock (Bullen, *et al.* 1990). Seedlings were grown and seed set occurred under previously described conditions (Khurana and Poff, 1989).

Light Sources

White light was provided by General Electric Delux white fluorescent tubes at 50 μmol m^{-2} s^{-1} (General Electric

Co., Cleveland, OH). Red light was from a General Electric Red F30T12-R-RD tube and was filtered through a red cellophane (Highland Supply Corp., Highland, IL) transmitting light in the 560- to 720-nm range with peak transmission at 630 nm. The fluence rate of the red light source was 1.2 μmol m^{-2} s^{-1} as measured using a datalogger with quantum sensor (LI-COR, Inc., Lincoln, NE).

Measurement of Stimulus-Response Curves

Seed were surface-sterilized and sown as previously described (Bullen, *et al.*, 1990), and germination was potentiated by 15 h of white light. After 21 h in darkness followed by 1 h of red light, the seedlings were gravistimulated for the designated period of time at a constant force. Forces of 1 *g* were administered by placing the plates on edge in darkness on a table surface. Higher forces of 2 *g* and 4.7 *g* were administered by placing the plates in a custom-built centrifuge calibrated using a digital tachometer (Fisher Scientific, Pittsburgh, PA). Following gravistimulation, curvature was allowed to develop for 1.5 h in darkness on a clinostat rotating at 0.5 rpm and presenting a gravicompensation of 10^{-5} *g*. The curvature of each organ was then measured using a CCD camera and image analysis software (page 94, this dissertation).

The centrifugal forces reported throughout this paper represent the centrifugal force along a vector sum. Since all earth-bound experiments are conducted under 1 *g*, the horizontal vector presented by the centrifuge is affected by this ubiquitous vertical vector. The sum of these two vectors is the force applied to the plants. Thus, the angle of gravitational stimulation is not 90° for the centrifuged plants, but rather described by the trigonometric equation $90^\circ - \sin^{-1}$(vertical vector/vector sum). Based on this calculation, the angles of gravitational stimulation were 60° and 78° for the 2 *g* and the 4.7 *g* forces, respectively.

Measurement of Stimulation Angle Effects

Seed were surface-sterilized, sown, and treated as previously described for the measurement of gravitropism (page 55, this dissertation), except that the plates were placed in darkness at 25°C on a clinostat presenting gravicompensation of 10^{-5} *g* following the 15 h white light treatment. This resulted in randomly oriented seedlings (Bullen, *et al.*, 1990). After 21 h, the plates were irradiated for 1 h with red light. Immediately following the red light treatment, the seedlings were gravistimulated by removing the plates from the clinostat and placing them on edge on a table top in darkness at 25°C for the designated time period.

The angle of stimulation was measured from the initial orientation of each organ relative to the gravitational vector, and the angle of curvature was measured for each organ relative to this initial orientation. The correlation between the angle of curvature and the angle of stimulation was examined.

RESULTS

The stimulus-response relationships for roots and for hypocotyls of dark-grown wild-type seedlings were similar in both shape and magnitude of response (Figure 10, 11). Reciprocity was examined first by changing dose through a variation in the gravitational or centrifugal force. When gravity was applied as a centrifugal force of 2 *g*, reciprocity was valid up to approximately 1,500 *g* x sec in roots (Figure 10A), and up to 21,600 *g* x sec in hypocotyls (Figure 10B). Reciprocity was not valid in either organ when gravity was applied as a centrifugal force of 4.7 *g* (Figure 10A,B).

The influence of force on presentation time was also examined by plotting response against the duration of the stimulus (Figure 11). At both 2 *g* and 4.7 *g*, force showed no affect on the response up to approximately 18,000 sec in hypocotyls (Figure 11B). In roots, the response was not affected up to 330 sec when gravity was applied as a centrifugal force of 4.7 *g* (Figure 11A). At 2 *g*, force did not affect the response throughout the range of stimulation times tested (Figure 11A,B).

Presentation time was estimated from the stimulus-response curves (Figure 11) by extrapolating from the point of intersection of the two phases of the curve. This

FIGURE 10: Stimulus-response curves for determining threshold dose. Dose is expressed as force x time, with variations in force of 1 *g* (■), 2 *g* (*), and 4.7 *g* (x). Panels represent the response in A) roots and B) hypocotyls.

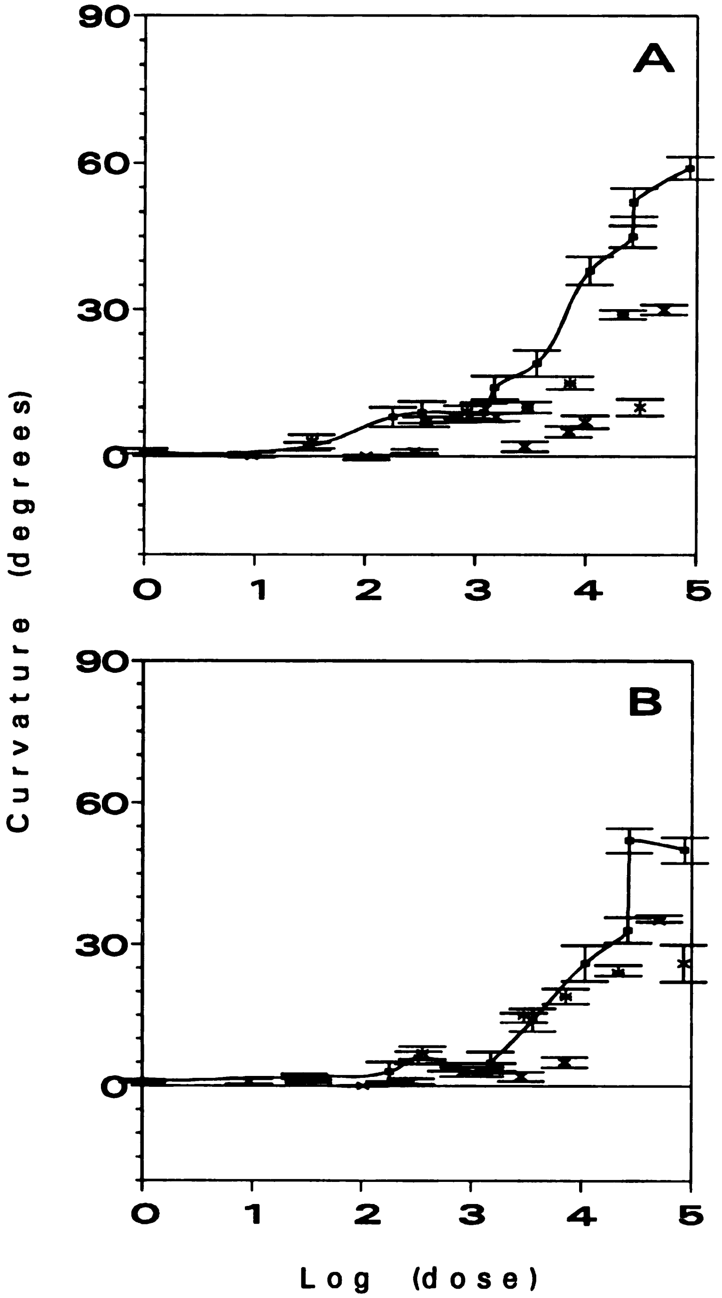

FIGURE 10

FIGURE 11: Stimulus-response curves for determining presentation time. Stimulus is expressed as the duration of stimulation in seconds. Variations in force of 1 *g* (■), 2 *g* (*), and 4.7 *g* (x) are indicated. Panels represent the response in A) roots and B) hypocotyls.

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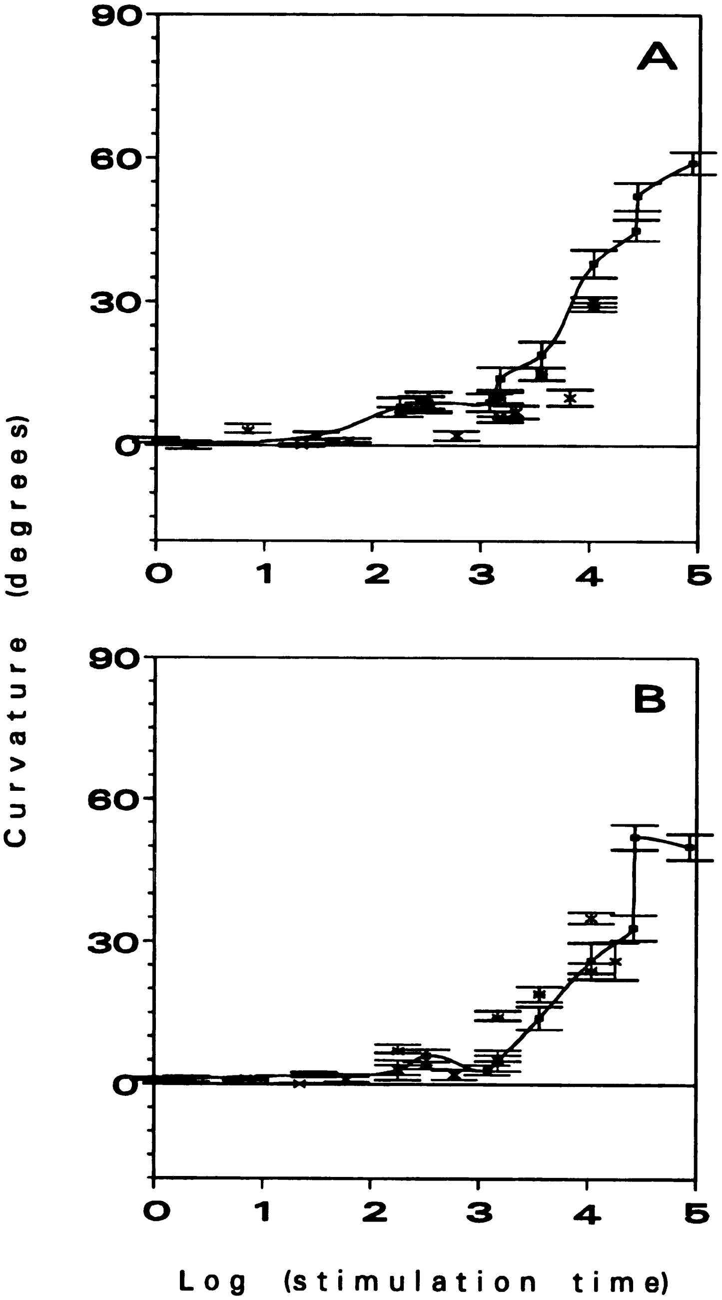

FIGURE 11

method yielded presentation time estimates of 600 sec for wild-type roots, and 800 sec for wild-type hypocotyls.

The angle of stimulation increasingly affected the curvature response as the duration of gravistimulation increased (Figure 12). In roots, curvature increased with increasing stimulation angle (Figure 12E). Curvature in hypocotyls also increased with increasing stimulation angle up to 120°, beyond which curvature decreased with increasing stimulation angle (Figure 12F). In both organs, the correlation between stimulation angle and curvature response increased as stimulus duration increased.

DISCUSSION

The extrapolation method for presentation time was selected for this study of gravitropism since it allowed for the possible influence of nutational movements on the small initial angles (Shen-Miller, 1970). In addition, responses in the first phase of the gravitropism stimulus-response curve were typically less than 10°. Since the measurement error in the system used was between 5° and 10°, determination of the time at which the response increased significantly above 0° was equivocal. Thus, the extrapolation method was appropriate for the estimation of presentation times.

Previous studies have shown reciprocity in gravitropism to be valid over a wide range of doses (Audus, 1962; Wilkins, 1966). However, these studies and others examining the gravitropic response under increased stimulus

FIGURE 12: Correlations between curvature and angle of stimulation in roots and hypocotyls. Each point represents an individual seedling. Periods of gravistimulation were A,B) 4 h, C,D) 8 h, and E,F) 22 h. Panels A,C, and E represent root responses. Panels B,D, and F represent hypocotyl responses.

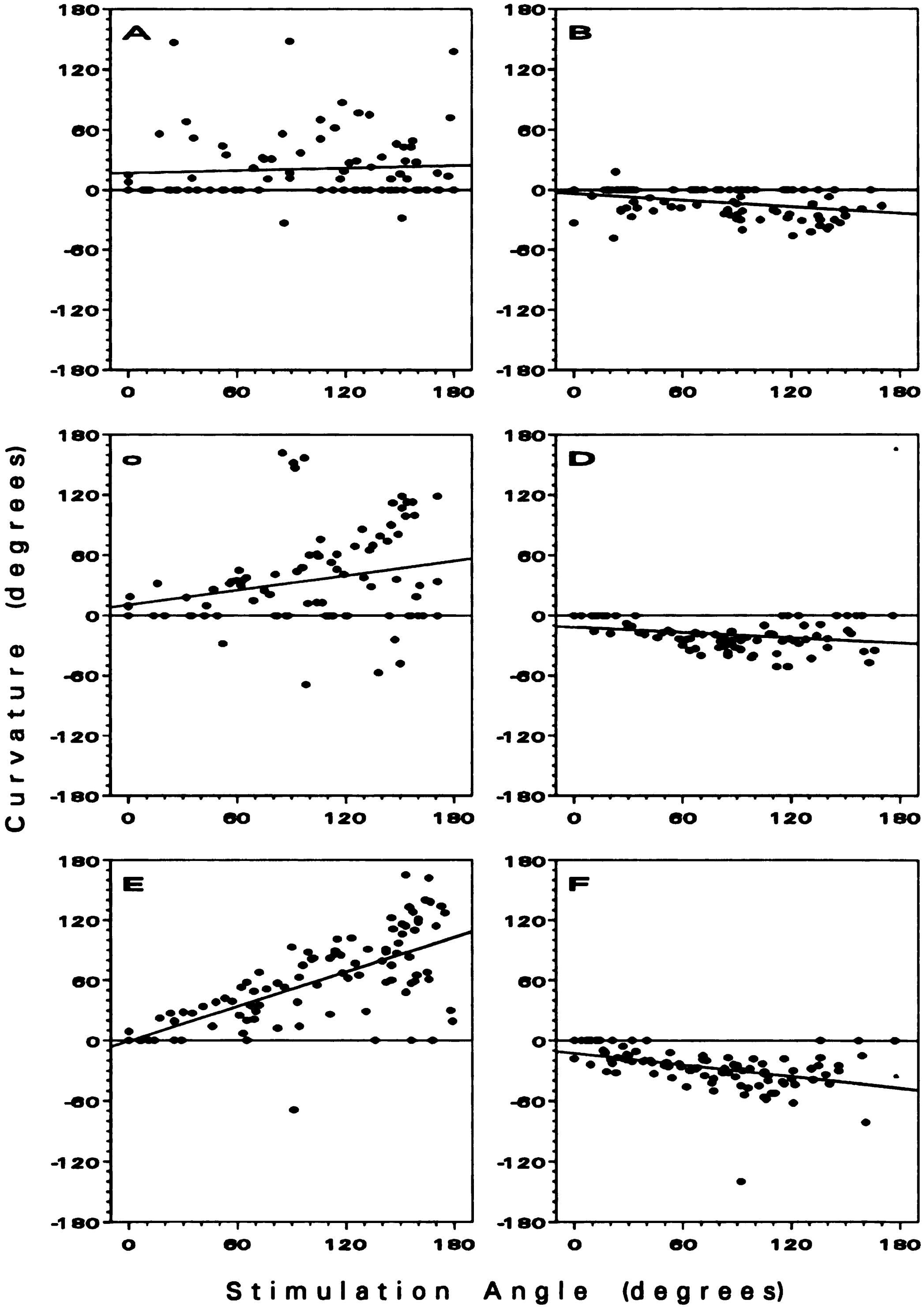
A
B
C
D
E
F
180
120
60
0
-60
-120
-180
0
60
120
180
Curvature (degrees)
Stimulation Angle (degrees)

FIGURE 12

strength used forces in excess of 10 *g* (for an example, see Hild and Hertel, 1972). The effects of applying such forces to plant tissue have been questioned (Mitchison, 1981). However, other investigators have proposed that high centrifugal forces have no appreciable effect on the plants ability to grow (Waldron and Brett, 1990).

The failure of reciprocity in this system is not due to differences in the stimulation angle at the three forces applied (Figure 12). As explained in Materials & Methods above, the stimulation angle presented at 2 *g* is approximately 60° while the stimulation angle presented at 4.7 *g* is 78°. Thus, the angle of stimulation at 4.7 *g* is closer to the 90° presented under 1 *g*. If this variation in stimulus angle was the cause of the failure of reciprocity, the response observed at 4.7 *g* would more closely approximate the curvatures observed at 1 *g*. However, the converse was observed with the responses at 2 *g* more closely approximating the 1 *g* response.

The failure of reciprocity observed here is similar to the failure of reciprocity observed in second positive phototropism (for a review, see Briggs, 1963). A recent study of phototropism in *Arabidopsis* proposed that the failure of reciprocity in second positive phototropism could be attributed to a time threshold, or minimum duration of exposure required for expression of the response (Janoudi and Poff, 1990). Since force or stimulus strength did not affect the gravitropic response over a wide range of stimulation times, gravitropism appears to be solely a function of the stimulus duration.

This seems reasonable within the context of the natural world. Throughout evolution, the predominant stimulus strength has been 1 *g*. Under unusual conditions such as high velocity winds, plants may briefly experience forces greater than 1 *g* over short time intervals. A gravitropic response to such temporary changes in the gravitational vector would not likely help, and could possibly hinder, the plant's ability to survive. More important to survival would be the response to more permanent changes in the gravitational vector, presumably exceeding the time thresholds of 600 to 800 sec.

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