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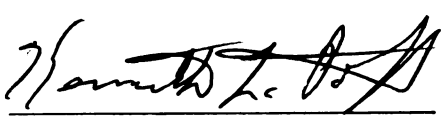

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A STUDY OF GRAVITROPISM MUTANTS IN *ARABIDOPSIS THALIANA*

By

Elizabeth S. Rosen

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ABSTRACT

A STUDY OF GRAVITROPISM MUTANTS IN *ARABIDOPSIS THALIANA*

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As exposure to light can alter the response of *Arabidopsis thaliana* seedlings to gravity, a direct screen for gravitropism mutants in this study utilized two seedling growth protocols that differ in light conditions. This approach was designed to identify mutants that show light-modulated gravitropism, and mutants that show light-independent gravitropism. Several mutant lines were identified and the variety of phenotypes associated with gravitropism in this study was assessed. Genetic analysis of a subset of the mutant collection identified five complementation groups, each due to a single locus recessive mutation. Two mutant groups were allelic to the previously identified mutants *agr*1 and *aux*1, and two unique hypocotyl gravitropism mutants were identified. These hypocotyl mutants were given the names *hgr*1 and *hgr*2.

Blue light phototropism of the auxin-resistant mutant allelic to *aux*1 was not significantly different from phototropism of the parental wild-type. Thus, this auxin-response mutation is distinct to gravitropism, and shows that auxin-resistant mutants may not represent a general alteration in auxin-regulated differential growth.

The *hgr*2 mutant showed increased phototropic curvature

for all fluences greater than the threshold of first positive phototropism. In addition, the *hgr*2 mutant showed no resistance to growth hormone induced root or hypocotyl stunting, but exhibited aberrant cotyledon hook opening in the presence of ethylene. This suggests that ethylene-response in differential growth could be independent from ethylene inhibition of cell elongation, and that some elements of ethylene-response may be necessary for gravitropism, but serve to reduce or moderate phototropism.

The chemical cross-linking subtractive hybridization technique was used to identify differences in RNA transcript abundance between seedlings of *hgr*1, *agr*, and the parental wild-type stimulated to induce gravitropism. Preliminary studies identified four transcripts, and a theoretical analysis of possible roles for these transcripts in gravitropism is presented on the basis of sequence identity to known genes. These data suggest that this could be an effective technique for the identification of gene regulation events associated with gravitropism.

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Chapter 3

List of Symbols, Abbreviations, or Nomenclature

aux : auxin-resistant mutant

axr : auxin-resistant mutant

CCLS : Chemical cross linking subtraction

cop4 : constitutively photomorphogenic mutant

hgr1 : hypocotyl gravitropism mutant 1

hgr2 : hypocotyl gravitropism mutant 2

l : liter

M : molar

m : meter

mg : milligram

pgm : starchless phosphoglucosemutase mutant

s : second

SSLP : Simple sequence length polymorphism

ul : microliter

umol : micromole

2,4-D : 2,4-dichlorophenoxyacetic acid

Literature Review:

The use of directed growth responses known as tropisms allow plants to adapt to environmental conditions to maximize viability. Gravity is a constant factor and has a profound effect on plant morphology, inducing positive gravitropism or downward growth of roots, and negative gravitropism of shoots or hypocotyls (Bjorkman 1988). Lateral branches and roots can have modified perpendicular responses to gravity, and this gravity mediated horizontal growth combined with positive and negative gravitropism allows gravity to have a fundamental role in the determination of plant structure (Digby and Firn 1995).

Directional growth in response to gravity contributes independently to the spatial orientation and structural development of the plant and appears to moderate responses to other environmental factors. An example of this is the intermediate growth vector that occurs when maize hypocotyls are exposed to antagonistic light and gravity stimulation that simultaneously induce directional growth in two different directions (Nick & Schaffer 1988). Both gravitropism and thermotropism contribute to directional growth to determine the final maize root orientation (Fortin and Poff 1991). A study of maize hydrotropism and gravitropism showed an interactive effect of both responses (Takahashi and Scott 1991). In a study of response to

touch, light and gravity, it was found that all of these stimuli could be integrated by a growing plant organ to determine its final orientation (Okada and Shimura 1992). Based on the interactive nature of plant responses to a variety of environmental factors, the direction of final growth may be said to represent a simultaneous response to all stimuli.

Perception

The mechanisms used by plants to perceive gravity and to respond to changes in a gravity vector are still not completely understood. Many comprehensive reviews (Masson 1995, Bjorkman 1988, Firn and Digby 1980) have been written to summarize the interesting although inconclusive research in this area. Primarily it is thought that a susceptor, or a cellular component that is directly affected by gravity either by rising or falling within a cell, is the first step in the perception of gravity (Sack 1991). A second component of perception would be the receptor, which would be deformed as a consequence of stretching or compression through interaction with the susceptor (Sack 1991). This is a very general model for a cellular mechanism to convert changes in cellular orientation to chemical or physical changes that are perceived as directional stimuli.

Starch filled amyloplasts have historically been proposed as a mechanism for the perception of gravity as

they are present in most gravity responsive tissues and move within the cell in response to changes in the gravity vector (reviewed in Audus 1962). The gravity mediated movement of amyloplasts has been found to resemble the activity of the barium statocyte in Chara, that is necessary for gravity perception in some single celled plants (Sievers and Volkmann 1979). Studies with destarching (Iverson 1969), starchless mutants (Kiss et al 1989) and starch deficient mutants (Kiss et al 1996) have shown a strong correlation between starch content of plastids, the sedimentation of plastids, and full sensitivity to gravity, which. The columella cells in the root cap have been suggested as the most likely statocytes in root gravitropism as they are distinctly polar cells that undergo the most visible gravity affected change in cell orientation (Hensel 1989), and are located in the region of the root most sensitive to gravity (Evans et al 1986). Although other studies have shown that regions of the root tip that do not contain columella cells can also perceive gravity (Poff and Martin 1989).

Because starch is susceptible to diamagnetic force induced movement within a nonuniform magnetic field, this property has been used to relocate amyloplasts and to show that amyloplast movement induces differential curvature and determines plant growth direction (Kuznetsov and Hasenstein 1996). Although this technique can effectively eliminate

the contribution of gravity to directional growth, it has yet to be shown that diamagnetic forces are not altering other elements of the plant cell.

Another theory of gravity sensing is that the weight of the protoplast itself directly induces deformation of the plasma membrane and results in gravity sensing (Wayne et al 1992). This is observed in characean cells where differences in applied pressure on opposite sides of a cell mimic the effects of changing the gravity vector (Staves et al 1992). So far this effect has been observed in single celled organisms or algae with large cells, and there is some question about whether the change in protoplast pressure in the much smaller higher plant cells would be sufficient to be perceived by the plasma membrane (Bjorkman 1988).

The plasmalemma control center model has been proposed as a method by which stretch activated ion channels in the plasma membrane and the cytoskeleton could interact in the perception of gravity (Pickard 1994). The study of giant algal cells has shown that gravity induced membrane changes have sufficient energy to open stretch-activated calcium channels (Wayne et al 1990), and similar forces are thought to result from the interaction of multiple small *Arabidopsis* cells (Sinclair et al 1996).

Cholodny-Went Theory

A major component of gravitropism is the transformation of the gravity signal into differential growth that produces curvature. The most commonly accepted mechanism to explain this is the Cholodney-Went theory of auxin transport. A concise summary of this theory states that "Growth curvatures....are due to an unequal distribution of auxin between the two sides of the curving organ. In the tropisms induced by light and gravity the unequal distribution is brought about by a transverse polarization of the cells which results in lateral transport of auxin" (Went and Thiman 1937)

This has been interpreted as the establishment of an auxin gradient (or a growth inhibitor gradient) in the root cap, and the maintenance of this lateral asymmetry as growth regulators are transported to the growing region (Jackson and Barlow 1981). It has also been interpreted as unequal lateral transport of active IAA into the stele from stored inactive IAA conjugates in the cortex (Bandurski et al 1984).

Auxin Transport and Sensitivity

To determine if redistribution could account for differential growth, auxin levels were assessed in seedlings during gravitropism. The distribution of radiolabled IAA corresponded to the changes in growth rate required for curvature in maize coleoptiles (Hild and Hertel 1972).

Combinations of maize decapitation and auxin application supported the theory that auxin transport is necessary for gravitropism (Iino 1995), and application of auxin transport inhibitors at concentrations that slightly reduce growth was found to effectively eliminate gravitropism (Muday and Haworth 1994).

Changes in tissue sensitivity to auxin have been proposed as another possible element to mediate auxin action in gravitropism (Rorabaugh and Salisbury 1989). Auxin sensitivity is increased in *Avena sativa* pulvini during gravitropism, and the authors suggest that this is achieved through regulation of auxin binding affinity (Kim and Kaufman 1995).

There is some debate over the effective timing of auxin redistribution and whether it sufficiently preceeds development of curvature. A forum on the Cholodney Went theory presents a discussion among scientists of the modern interpretations of this theory, and whether it is sufficient to explain tropistic responses (Trewavas ed. 1992).

Auxin Conjugation and Metabolism

In addition to auxin transport, auxin conjugates and metabolites of auxin have been proposed as a method of controlling the concentration of active auxin (Bandurski et al 1994). When radiolabled IAA conjugates were applied to bean stems, the rate of stem bending was found to correlate

to the rate of conjugate hydrolysis (Cohen et al 1988). A number of enzymes involved in auxin conjugation have been identified, and the gene that controls the first step in the biosynthesis of IAA conujugates, IAA-Glc has been cloned from maize (Szerzen et al 1994). Transgenic tobacco plants that overexpress this gene display altered apical dominance and weak gravitropism (Normanly et al 1995), showing a direct link between *in vivo* manipulation of auxin and gravitropism.

Genetic Analysis of Gravitropism

Gravitropism mutations have been observed in a variety of plant species, and a number of morphological/developmental characteristics have been associated with gravity response mutants (Roberts 1987). Although the majority of gravitropism mutants in *Arabidopsis* have also been found to be altered in response to exogenous auxin, there are additional mutant phenotypes. Several mutants impaired in starch biosynthesis or accumulation have been shown to be altered in gravitropism (Kiss et al 1996). The *Arabidopsis agr* mutant is altered in primary root gravitropism, but has unaltered plastid starch content and intact response to exogenous auxin (Bell and Maher 1990). Two *Arabidopsis* mutants with impaired gravitropism in the primary root and hypocotyl of young seedlings were found to contain full plastid starch although response to auxin has

not been assessed (Bullen 1992).

Gravitropism can occur in inflorescence stems, and three shoot gravitropism mutants, have been found in *Arabidopsis thaliana*. These mutants are the *sgr*3 mutant, which shows unimpaired seedling gravitropism, and the *sgr*1 and *sgr*2 mutants which show reduced response to gravity in the root and hypocotyl of young seedlings (Fukaki et al 1996). These mutants have been found to be unaltered in auxin induced stem elongation (Fukaki et al 1996), although other aspects of hormone response have not yet been determined.

*Cop*4 was identified on the basis of the constitutive photomorphogenic mutant phenotype, which is the appearance of light regulated development in etiolated seedlings, and this mutant was found to be altered in hypocotyl and root seedling gravitropism(Hou et al 1993). Perhaps this mutation affects a signal transduction element common to the gravity response and to light regulated development.

Auxin Resistant Gravitropism Mutants

Mutant analysis has provided a great deal of genetic evidence that alterations in seedling response to auxin can affect seedling response to gravity. The first mutants with an altered response to both exogenous auxin and gravity were the *aux*1 mutant and the DWF mutant (Mizra et at 1984). The dominant DWF mutant produces severely dwarfed plants with

greatly impaired gravitropism and apical dominance, while the recessive *aux* mutants produced a gravitropism mutant phenotype only in the root, and showed slightly reduced hypocotyl growth (Mizra et al 1984). Later studies of the *aux* mutants found them to be resistant to exogenous ethylene as well as to auxin (Picket et al 1990).

The *axr* class of mutants were identified on the basis of resistance to root growth inhibition when seedlings area grown on media containing toxic concentrations of exogenous auxin. The *axr*1 mutant is altered in gravitropism (Lincoln et al 1990), and has unusual leaf, root and flower morphology (Estelle and Sommerville 1987). The *axr*2 mutant is a dominant agravitropic mutant, and is resistant to auxin, ethylene, and abscisic acid (Wilson et al 1990). This is a severely dwarfed pleiotropic phenotype and could represent an alteration in a secondary messenger that is active in signal transduction in all of these hormones (Wilson et al 1990), or a growth determining factor in root development that is not affected by hormone metabolism. Because the phenotype examined in these hormone resistance studies is root growth, it is possible that the *axr*2 gene is necessary for a non-specific stress response that reduces root growth in a toxic environment. Characterization of mutant root length under conditions of salt stress or heavy metal toxicity would be useful to determine if *axr*2 is affected only in auxin mediated growth. Examination of root

elongation induced by low concentrations of exogenous auxin has provided some evidence that this mutation does not represent a specific auxin response. These studies show that the kinetics of root growth induced by auxin and the magnitude of that growth are identical in the wild type and the *axr*2 mutants (Evans et al 1994).

The *axr*3 mutant is altered in gravitropism and response to exogenous auxin, but a published characterization of this mutant is not available (Hobbie and Estelle 1995). The *axr*4 mutant is unusual in that it is resistant to auxin but not to other plant growth hormones, while all previously identified *axr* and *aux* mutants have all been shown to have resistance to multiple hormone classes (Hobbie and Estelle 1995). The identification of more specific mutants should help resolve the differences between an interactive response to many hormones and a specific auxin response.

The *rgr*1 mutant is another gravitropic mutant that is axuin resistant (Simons et al 1995). This mutant is resistant to polar transport inhibitors and shows an alteration in root waving (Simons et al 1995). The formation of root waves has been previously shown to occur in *Arabidopsis thaliana* seedling roots as the result of undulations in growth (Okada and Shimura 1992), and the alteration of both traits in the *rgr*1 mutant suggests that a common genetic element exists for root waving and gravitropism subsequent to polar auxin transport.

Auxin Responsive Gene Expression

A tool in the study of hormone regulated components of gravitropism is auxin regulated gene expression. Since it was first observed that application of exogenous auxin could induce a general increase in RNA synthesis as well as up-regulation of specific genes (Walker and Key 1982), a number of auxin regulated gene families have been characterized. Although not all of the auxin regulated genes have been associated with tropisms, this is not unexpected given the numerous aspects of plant growth and development that are affected by auxin. An important factor to consider in the interpretation of auxin up-regulated genes is that the concentration of exogenous auxin used to induce these genes is frequently in the 50 micromoler range (Abel and Theologis 1996). This is 500 times greater than the concentration of applied auxin that will induce 100% inhibition of *Arabidopsis* root growth (Timpte et al. 1995) It is not surprising given this near toxic level of auxin application that some families of auxin up regulated genes are also up regulated by heat shock and heavy metals (Abel and Theologis 1996).

The most direct association between gravitropism and auxin-regulated gene expression comes from spatial correlation between localization of the gene products and the faster growing region of an organ undergoing tropism induced differential growth. The promoter region of the

auxin up regulated gene AtAux2-11 has been fused to a reporter gene to determine gene expression in transgenic plants. Asymmetric reporter gene expression was found in those plants undergoing gravitropic curvature, although this localized to a region closer to the cotyledons than the zone of curvature (Wyatt et al 1993) suggesting that this gene may not be directly involved with growth, but with other aspects of establishing or maintaining an auxin gradient. This gene does not show strong sequence identity to previously studied genes, but contains a nuclear localization signal as well as a DNA binding region and could be a direct link between auxin distribution and alterations in gene expression (Abel et al 1996). Another auxin up-regulated gene, PS-IAA6, displays changes in transcription during gravitropism to favor message abundance on the more rapidly growing side of the hypocotyl in a region that stretches from beneath the cotyledons to the root junction (Wong et al 1996).

The SAUR gene family (Small Auxin Up-RNAs) messages are found at much higher levels in auxin treated tissue than in untreated tissue. Members of this family have not been shown to be up-regulated in response to other hormones or to other stress inducing stimuli (McClure and Guilfoyle 1987). A strong asymmetry of RNA levels is observed in tissue prints such that greater abundance of message is found on the lower, more rapidly growing region of a hypocotyl

undergoing gravitropism. This asymmetry develops within 20 minutes of stimulation and persists throughout curvature (McClure and Guilfoyle 1989) thus providing temporal and spacial correlation of gene expression with gravitropism. Work with a SAUR promoter region and a reporter gene showed that the SAUR promoter induced transcription in the more rapidly growing tissue during phototropism and gravitropism (Li et al 1991). Although a function has not been proposed for these genes, the study of gene regulation shows that this gene family is likely to be specifically responsive to auxin.

Auxin Response Genes in Auxin Resistant Mutants

The auxin resistant gravitriopism mutants have been useful in the study of auxin regulated genes. The auxin induced expression of a SAUR gene was determined in the *aux*1 mutant, and was found to be 50% of the wild type (Gil et al 1994). The same SAUR gene was examined in the *axr* mutant and also found to be maintained at lower levels (Timpte et al 1995). SAUR transcript accumulation was assessed for *aux*1 and *axr*1 mutants in a number of tissue types ranging from seedlings to rosette leaves, and in all mutant plant tissue examined, the message level induced by exogenous auxin application was lower than in the similarly treated wild-type tissue (Timpte et al 1995). This shows that these genetic elements of auxin perception or metabolism altered

in these gravitropism mutants is necessary for standard regulation of the auxin response genes.

A study of the spatial regulation of auxin up-regulated genes during gravitropism in auxin resistant mutants and other gravitropism mutants could differentiate between alterations in the establishment of hormone gradients and alterations in the response to those gradients. Starch deficient mutants are unimpaired in auxin response and could be used to assess whether auxin response genes have a specific role in differential growth. Comparative analysis of gene regulation difference between gravitropism mutants could be used to determine the sequential order in which the mutations affect the gravitropism/auxin response pathway. An additional strength of these auxin resistant mutants is that *aux*1 is specifically altered in root gravitropism but has no other strong phenotype while the *axr*1 mutant is altered in gravitropism and in several other auxin regulated morphological features (Timpte et al 1995). This could be useful to determine if auxin response genes are active in different auxin regulated components of plant development. Clearly there is great potential for the use of gravitropism mutants to explore the role of auxin in gravitropism.

Genes Altered in Auxin Resistant Gravitropism Mutants

Recently two novel genes were cloned from two different auxin-resistant gravitropism mutants in *Arabidopsis*. The

AXR1 gene was found to have sequence identity to a ubiquitin activation enzyme, although it lacks the active domain for this function (Layser et al 1993). The identification of this gene allows further research to determine the role of auxin and ubiquitin in plant growth and development. An obvious function for this gene would be to increase degradation of the PS-IAA6 auxin induced gene product, which is a highly unstable protein (Abel et al 1994). Increased stability of this protein in the *axr*1 mutant would provide a convincing connection between gravitropism and auxin induced genes. When transgenic *Arabidopsis* strains that overproduce IAA were crossed to the *axr*1 mutant, those progeny homozygous for both traits showed the *axr*1 mutant phenotype and lacked all phenotypic effects of auxin overproduction including alterations in apical dominance and stem elongation (Romano et al 1995). This confirms that *axr*1 has a role in later steps of auxin metabolism or in responses induced by auxin. The abundance of unconjugated IAA in the double mutant was identical to that found in *axr*1, which is three times higher than the wild-type, and three quarters of the level found in the auxin overproducing line (Romano et al 1995). This suggests a role for the AXR1 gene product in the establishment or maintenance of free IAA levels.

The AUX1 gene was also cloned and was found to have sequence identity to an amino acid permease (Bennett et al

1996). As auxin has great structural similarity to tryptophan, these authors have suggested that this permease could have a role in the transport of auxin. Analysis of gene expression through in-situ localization of RNA has shown that the AUX1 transcript is most abundant in the root apex and in the epidermal cells (Malcolm et al 1996). These localization data were unexpected as it has been demonstrated that gravitropism is uneffected in maize roots that have been completely stripped of the epidermis from the growing root tip through the elongation zone (Bjorkman & Cleland 1991). As mutant analysis shows that an intact copy of this gene is necessary for gravitropism, this observation may indicate that stripped seedlings are able to compensate for the loss of the epidermis through AUX1 gene expression in other tissues, or that tissue specificity is different for gravitropism in maize and *Arabidopsis*. There are studies that support a role for the AUX1 gene product in auxin uptake or transport. The phenotype of the *aux* mutants is very similar to the appearance of wild type seedlings treated with auxin transport inhibitors (Muday and Haworth 1994). Detailed analysis of low IAA concentrations that induce root growth show that the lag time between auxin application and the initiation of root growth is twice as long in the *aux*1-7 mutant as it is in the wild type or the *aux*1 and *aux*2 mutants (Evans et al 1994).

Ethylene in Gravitropism

Although less well documented than the effects of auxin, there is some evidence that ethylene plays a role in gravitropism. Both *aux*1 and *axr*1 mutants have been found to be resistant to ethylene as well as auxin (Timpte et al 1995). The *eir* (Ethylene Insensitive Root) gravitropic mutant has wild type response to exogenous auxin, but the root tissue is resistant to exogenous ethylene (Roman et al 1995). Auxin induced ethylene production is reduced in the tomato diageotropica mutant, and the mutant gravitropism phenotype is eliminated when ethylene is applied to mutant plants (Zobel 1973).

Direct evidence of ethylene mediated gravitropism is provided by the observation that applied ethylene attenuates curvature in *Zea mays* seedlings (Lee et al 1990). Ethylene was shown to be the primary factor that eliminates gravitropism in submerged rice and pea roots (Hoson et al 1996). And work with ethylene inhibitors in snapdragon spikes showed that elimination of an ethylene gradient greatly reduced or eliminated gravity induced inflorescence bending (Philosoph-Hadas et al 1996). These results must be interpreted carefully, as it has been shown that auxin induces ethylene biosynthesis (Abel and Theologis 1996), and that ethylene regulates auxin transport (Osborne and Mullins 1969) so it can be difficult to determine that a response is specific to one hormone.

Cytoskeleton

The role the cytoskeleton plays in the establishment and maintenance of cellular polarity (Seagull 1989) make it an ideal candidate to be an intermediate step in the perception or amplification of a gravity stimulus. Increases in cytosolic calcium increase the tension of the actin network, demonstrating that the cytoskeleton has the potential to undergo subtle changes in response to known signal transduction molecules (Grabski and Schindler 1996). Directional re-arrangement of microtubules has been found to be correlated with tropism induced bending in maize and sunflower and (Nick et al 1991).

The cytoskeleton is primarily composed of actin microfilaments, microtubules made of tubulin, and intermediate filaments (Godard et al 1994). Because of the heterogeneity of the networks that comprise the cytoskeleton, an effort has been made to examine the elements individually. Cellular localization has shown that specific, developmentally regulated networks of microtubules and microfilaments form in different types of cress root cells (Hensel 1989).

Chemical inhibitors that destroy or disrupt these networks have been used to elucidate the role of the cytoskeleton in various aspects of cell growth. In cress cells all cellular polarity is eliminated by disruption of the cytoskeleton (Hensel 1985). The use of toxins to

destroy microfilaments in chara rhizoids has been shown to disrupt the positioning of statoliths and to eliminate the ability of the rhizoid to reorient in response to an altered gravity vector (Sievers et al 1991). This suggests that intact microfilaments are necessary for gravitropism, but in higher plants the role of microfilaments has not been well defined.

The use of two anti-microtubule drugs showed a correlation between disassembly of microtubules and lack of phototropism and gravitropism (Nick et al 1990). The use of two different toxins that destroy microtubules had no effect on gravitropism in the root (Baluska et al 1996b). It is difficult to reconcile these contradictory experiments. However there is evidence that there are 10 actin genes in *Arabidopsis* (Mcdowell et al 1996), as well as many alpha and beta tubulin genes (Godard et al 1994), and it may be that individual toxins act to disrupt some network elements and not others.

Clearly the use of drugs that disrupt the cytoskeleton is too destructive to accurately determine the role of the cytoskeleton in gravitropism. Now that so many genes for the cytoskeleton subunits have been identified in *Arabidopsis*, it should be possible to determine which actins and tubulins are present in gravitropically responding tissue, and which cytoskeleton components are required for gravitropism.

Calcium and Calmodulin in Gravitropism

Calcium has been shown to have a role as a secondary messenger in many aspects of plant growth and development (Trewavas and Knight 1994). Spatial correlation between gravitropism and calcium redistribution was shown with calcium dependent fluorescent dyes that detect an increase in cytosolic calcium on the lower side of a gravity stimulated maize coleoptile within 3 minutes of alteration of the gravity vector (Ghering et al 1991). Calmodulin is a calcium binding protein that has been found to be necessary for calcium induced up regulation of protein phosphorylation (Veluthambi and Poovaiah 1984). Some varieties of maize show orthogravitropism, or growth perpendicular to the gravity vector in dark grown roots (Feldman and Briggs 1989). Calmodulin activity in orthogravitropic plants is three times lower than in other varieties, but red light treatment simultaneously induces positive root gravitropism and raises calmodulin activity to the level found in other varities (Stinemetz et al 1987). In the *Arabidopsis* gravitropism mutant *agr*-3, a half hour exposure to an altered gravity vector reduced calmodulin mRNA levels, while similar stimulation in the wild-type increased calmodulin message abundance (Sinclair et al 1996).

Extracellular calcium has been proposed to have a role in controlling cell growth as a wall hardening agent that is

removed by cell wall acidification (Arif and Newman 1993). Manipulation of extracellular calcium levels through addition of exogenous calcium or calcium chelators has been shown to stimulate or inhibit root growth (Lee et al 1983), suggesting that calcium distribution could be a regulatory factor in differential growth. In gravitropically responding oat seedlings there is an apoplastic calcium redistribution that results in accumulation of calcium in the epidermal cells with slight increase in the upper side of the coleoptile (Slocum and Roux 1983). The root cap mucilage has also been shown to have a role in cell elongation that is dependent on calcium content (Baluska et al 1996a), which may be an external mechanism for calcium regulation in plants.

Acid Growth Theory

The regulation of plant cell elongation has been explained by the acid growth theory which proposes that acidification of the apoplast leads to cell wall loosening and cell expansion (Rayle and Cleland 1970). While there is symmetric proton efflux in a vertically growing maize root, those horizontally placed roots undergoing gravitropic curvature showed inhibition of proton efflux on the slowly growing lower side, and increased efflux on the more rapidly growing upper side (Mulkey and Evans 1981). In an analysis of a maize root undergoing curvature, the transverse section

of the root that showed maximum assymetry in proton flux corresponded to the region of maximum differential growth (Versel and Pilet 1986). More specific measurements in the apoplast of maize epidermal cells has shown that proton efflux results in significant change in the apoplast pH (Taylor et al 1996). Additional evidence for a specific role of the epidermis in gravity stimulated proton efflux is seen in epidermal peels of tulip peduncles that show positionally directed changes in efflux that are not found in the corresponding cortex cells (Hejnowicz and Sievers 1995).

Experiments with proton efflux inhibitors, auxin transport inhibitors, and exogenous auxin show a correlation between sunflower hypocotyl gravitropism, proton efflux and levels of available auxin (Wright and Rayle 1983). The interaction of proton efflux and calcium displacement in the cell wall could indicate that the mechanism for acidification induced cell wall loosening is through moderation of calcium (Arif and Newman 1993).

Gravitropism and Light Conditions

In addition to interaction of light directed growth and gravity directed growth, there are additional effects of light on gravitropism. A comparison of etiolated *Arabidopsis* seedling roots with light grown seedling roots shows that gravity perception is more sensitive in light

grown seedlings (Bullen 1992).

Some maize cultivars show a phytochrome mediated light requirement such that positive root gravitropism will only occur after exposure to inductive light (Feldman and Briggs 1989). A red/far red photo-reversible light treatment introduces an element of randomness in gravitropism of *Arabidopsis* hypocotyls, producing a less uniform seedling response (Hangarter and Liscum 1993). The role of phytochromes in this phenomenon was extensive analysed with the use of mutants in phytochrome A and B, the phyAB double mutant, and transgenic plants that overexpress PHYA and PHYB and it was found that both phytochromes can mediate this randomness (Robson and Smith 1996).

Summary

Although much progress has been made to improve understanding of gravitropism, some unresolved questions remain. Advances in technology including molecular characterization of the components of the cytoskeleton, isolation of gravitropism mutants that aren't altered in hormone content or metabolism, and continued molecular analysis of hormone regulated genes necessary for gravitropism should resolve some of the contradictory gravitropism data. Further study of the cytoskeleton could contribute to the evaluation of models of gravity perception through identification of specific interactions with the

plasma membrane or the amyloplasts and the effect of altered subunit abundance on gravitropism. The role for stretch activated ion channels proposed in the plasmalemmal control center model can be determined directly when molecular mechanisms to specifically manipulate different classes of ion channels becomes available.

The involvement of multiple plant growth hormones and non-specific secondary messengers in gravitropism show that eventually this plant response will have to be considered in the more global context of simultaneous plant responses to many environmental factors. The phytochrome mediated randomness may represent a mechanism for reducing the response to gravity to increase the response to other stimuli. The gene altered in the *cop*4 mutant could represent a similar function. Further study of all tropisms should determine if plants directly prioritize the response to some stimuli, or if the interactive response to multiple stimuli is mediated by common signal transduction components in different responses.

This work was initiated to identify genetic componants of gravitropism through the use of a direct screen for gravitropism mutants. A screen that identifies mutants on the basis of gravity response was chosen as it has the potential to identify many genetic element of gravitropism and could be used to evaluate the frequency with which certain types of gravitropism mutants occur. The use of two

light protocols to study gravitropism was adopted to increase the understanding of the role of light conditions in gravitropism, and to evaluate the effect of light on different gravitropism mutants. The analysis of genes expressed at different levels in gravitropism mutants than in the parental wild-type seedlings, was another approach to identify elements of gravitropism. These genes have the potential to be components of the response to gravity.

References

Abel S, Oeller PW, Theologis A (1994) Early auxin-induced genes encode short-lived nuclear proteins. Proceedings of the National Academy of Sciences. 91:326-330

Abel S, Theologis A (1996) Early genes in auxin action. Plant Physiology 111:9-17

Arif I, Newman IA (1993) Proton efflux from oat coleoptile cells and exchange with wall calcium after IAA or fusicoccin treatment. Planta 189:377-383

Audus LJ (1962) The mechanism of the perception of gravity by plants. Symposium of the Society of Experimental Biology 16:197-226

Baluska F, Folkmann D, Hauskrecht M, Barlow PW (1996a) Root cap mucilage and extracellular calcium as modulators of cellular growth in postmitotic growth zones of the maize root apex. Botanica Acta 109:25-34

Baluska R, Hauskrecht M, Barlow PW, Sievers A (1996b) Gravitropism of the primary root of maize: a complex pattern of differential cellular growth in the cortex independent of the microtubular cytoskeleton. Planta 198:310-318

Bandurski RS, Reinecke DM, Cohen JD, Slovin JP (1995) Auxin biosynthesis and metabolism *IN* Plant Hormones: Physiology, Biochemistry and Molecular Biology. PJ Davies ed. Kluwer Academic Press. Dordrecht, The Netherlands. pp35-57

Bandurski RS, Schulze A, Dayanandan P, Kaufman PB (1984) Response to gravity by *Zea mays* Seedlings. Plant Physiology 74:284-288

Bell CJ, Maher EP (1990) Mutants of *Arabidopsis thaliana* with abnormal gravitropic responses. Molecular and General Genetics 220:289-293

Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schultz B, Feldmann KA (1996) Arabidopsis AUX1 gene: A permease-like regulator of root gravitropism. Science 273:948-950

Bjorkman T(1988) Perception of gravity by plants. *IN* Advances in Botanical Research Vol. 15 Academic press limited.

Bjorkman T, Cleland RE (1991) Root growth regulation and gravitropism in maize roots does not require the epidermis.

Planta 185:34-37

Bullen B(1992) Development of a genetic system for the study of gravitropism in *Arabidopsis thaliana*. PhD Dissertation

Cohen JD, Slovin JP, Bialek K, Chen C-H, Derbyshire M (1988) Mass spectrometry genetics and biochemistry: Understanding the metabolism of indole-3-acetic acid *IN* Biomechanisms Regulating Growth and Development. GL Steffens, TS Rumsey eds. Beltsville Symposia in Agricultural Research Volume12. Kluwer Academic Publishers. Dordrecht, The Netherlands pp229-241

Digby J, Firn RD (1995) The gravitropic set-point angle (GSA): the identification of an important developmentally controlled variable governing plant architecture. Plant, Cell and Environment 18:1434-1440

Estelle MA, Somerville C (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Molecular and General Genetics 206:200-206

Evans ML, Ishikawa H, Estelle MA (1994) Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: Comparison of wild type and auxin-response mutants. Planta 194:215-222

Evans ML, Moore R, Hassenstein K-H (1986) How roots respond to gravity. Scientific American 255:112-119

Feldman LJ, Briggs WR (1987) Light-regulated gravitropism in seedling roots of maize. Plant Physiology 83:241-243

Firn RD, Digby J (1980) the establishment of tropic curvature in plants. In the Annual Review of Plant Physiology 31:131-148

Fortin M-C, Poff KL (1991) Characterization of thermotropism in primary roots of maize: Dependence on temperature and temperature gradient, and interaction with gravitropism. Planta 184:410-414

Fukaki H, Fujisawa H, Tasaka M, (1996) SGR1, SGR2, and SGR3: Novel genetic loci involved in shoot gravitropism in *Arabidopsis thaliana*. Plant Physiology 110:945-955

Gehring CA, Williams DA, Cody SH, Parish RW (1990) Phototropism and geotropism in maize coleoptiles are spatially correlated with increases in cytosolic free calcium. Nature 345:528-530

Gil P, Liu Y, Orbovic V, Verkamp E, Poff KL, Green PJ (1994)

Characterization of the auxin inducible SAUR-AC1 gene for use as a molecular genetic tool in *Arabidopsis*. Plant Physiology 104:777-784

Goddard RH, Wick SM, Silflow CD, Snustad DP (1994) Microtubule components of the plant cell cytoskeleton. Plant Physiology 104:1-6

Grabski S, Schindler M (1996) Auxins and Cytokinins as antipodal modulators of elasticity within the actin network of plant cells. Plant Physiology 110:965-970

Hejnowicz Z, Sievers A(1995) Proton efflux from the outer layer of the peduncle of Tulip in gravitropism and circumnutation. Botanica Acta 108:7-13

Hensel W(1985) Cytochalasin B affects the structural polarity of statocytes from Cress roots(*Lepidium sativum* L.) Protoplasma 129:178-187

Hensel W(1989) Tissue Slices from living root caps as a model system in which to study cytodifferentiation of polar cells. Planta 177:296-303

Hild V, Hertel R (1972) Initial phases of gravity-induced lateral auxin transport and geotropic curvature in Corn coleoptiles. Planta 108:245-258

Hobbie L, Estelle M (1995) The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. The Plant Journal 7:211-220

Hoson T, Kamisaka S, Masuda Y (1996) Suppression of gravitropic response of primary roots by submergence. Planta 199:100-104

Hou Y, von Arnim AG, Deng X-W (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. The Plant Cell 5:329-339

Iino M (1995) Gravitropism and phototropism of maize coleoptiles: Evaluation of the Cholodny-Went theory through effects of auxin application and decapitation. Plant and Cell Physiology 36:361-367

Iverson T-H (1969) Elimination of geotropic responsiveness in roots of cress(*Lepidium sativum*) by removal of statolith starch. Physiologia Plantarum 22:1251-1262

Jackson MB, Barlow PW (1981) Root geotropism and the role of growth regulators from the cap: a re-examination. Plant,

Cell and Environment 4:107-123

Kim D, Kaufman PB (1995) Basis for changes in the auxin-sensitivity of *Avena sativa* (Oat) leaf-sheath pulvini during the gravitropic response. Journal of Plant Physiology 145:113-120

Kiss JZ, Hertel R, Sack FD(1989) Amyloplasts are necessary for full gravitropic sensitivity in roots of *Arabidopsis thaliana*. Planta 177:198-206

Kiss JZ, Wright JB, Casper T (1996) Gravitropism in roots of intermediate-starch mutants of *Arabidopsis*. Physiologia Plantarum 97:237-244

Kuznetsov OA, Hasenstein KH (1996) Intracellular magnetophoresis of amyloplasts and induction of root curvature. Planta 198:87-94

Lee JS, Mulkey TJ, Evans ML (198?) Reversible loss of gravitropic sensitivity in maize roots after tip application of calcium chelators. Science 220:1375-1376

Lee JS, Chang W-K, Evans ML (1990) Effects of ethylene on the kinetics of curvature and auxin redistribution in gravistimulated roots of *Zea mays*. Plant Physiology 94:1770-1775

Leyser HMO, Lincoln CA, Timpte C, Lammer D, Turner J, Estelle M (1993) *Arabidopsis* auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. Nature 362:161-164

Li Y, Hagen G, Guilfoyle TJ (1991) An auxin-responsive promoter is differentially induced by auxin gradients during tropisms. The Plant Cell 3:1167-1175

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr*1 mutants of *Arabidopsis*. The Plant Cell 2:1071-1080

Liscum E, Hangarter RP (1993) Genetic evidence that the red-absorbing form of phytochrome B modulates gravitropism in *Arabidopsis thaliana*. Plant Physiology 103:15-19

Masson PH (1995) Root gravitropism. BioEssays 17:119-127

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Molecular Biology 9:611-623

McClure BA, Guilfoyle T (1989) Rapid redistribution of

Auxin-regulated RNAs during gravitropism. Science 243:91-93

Mcdowell JM, Huang SR, Mckinney EC, An YQ, Meagher RB (1996) Structure and evolution of the actin gene family in *Arabidopsis thaliana*. Genetics 142:587-602

Mirza JI, Olsen GM, Iversen T-H, Maher EP (1984) The growth and gravitropic responses of wild-type and auxin-resistant mutants of *Arabidopsis thaliana*. Physiologia Plantarum 60:516-522

Muday GK, Haworth P (1994) Tomato root growth, gravitropism, and lateral development: Correlation with auxin transport. Plant Physiol. Biochemistry 32(2):193-203

Mulkey TJ, Evans ML (1981) Geotropism in corn roots: Evidence for its mediation by differential acid efflux. Science 212:70-71

Nick P, Bergfeld R, Schafer E, Schopfer P (1990) Unilateral reorientation of microtubules at the outer epidermal wall during photo- and gravitropic curvature of maize coleoptiles and sunflower hypocotyls. Planta 181:162-168

Nick P, Schafer E (1988) Interaction of gravi- and phototropic stimulation in the response of maize (*Zea mays*) Coleoptiles. Planta 173:213-220

Nick P, Schafer E, Hertel R, Furuya M (1991) On the putative role of microtubules in gravitropism of maize coleoptiles. Plant Cell Physiology 32:873-880

Normanly J, Slovin JP, Cohen JD (1995) Rethinking auxin biosynthesis and metabolism. Plant Physiology 107:323-329

Okada K, Shimura Y (1992) Aspects of recent developments in mutational studies of plant signaling pathways. Cell 70:369-372

Osborne DJ, Mullins MG (1969) Auxin, Ethylene and kinetin in a carrier-protein model system for the polar transport of auxins in petiole segments of *Phaseolus vulgaris*. New Phytology 68:977-991

Philosoph-Hadas S, Meir S, Rosenberger I, Halevy AH (1996) Regulation of the gravitropic response and ethylene biosynthesis in gravistimulated snapdragon spikes by calcium chelators and ethylene inhibitors. Plant Physiology 110:301-310

Pickard BG (1994) Contemplating the plasmalemmal control center model. Protoplasma 182:1-9

Pickett FB, Wilson AK, Estelle M (1990) The *aux*1 mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiology 94:1462-1466

Poff KL, Martin HV (1989) Site of graviperception in roots: a re-examination. Physiologia Plantarum 76:451-455

Rayle DL, Cleland R (1970) Enhancement of wall loosening and elongation by acid solutions. Plant Physiology 46:250-253

Roberts JA (1987) Mutants and gravitropism. *IN* Developmental Mutants in Higher Plants. H Thomas and D Grierson eds. Cambridge University Press. Cambridge pp135-153

Robson PRH, Smith H (1996) Genetic and transgenic evidence that phytochromes A and B act to modulate the gravitropic orientation of *Arabidopsis thaliana* hypocotyls. Plant Physiology 119:211-216

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR (1995) Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: Five novel mutant loci integrated into a stress response pathway. Genetics 139:1393-1409

Romano CP, Robson PRH, Smith H, Estelle M, Klee H (1995) Transgene-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy*6-1 hypocotyl elongation and *axr*1 auxin-resistant mutants. Plant Molecular Biology 27:1071-1083

Rorabaugh RA, Salisbury FB (1989) Gravitropism in higher plant shoots: VI changing sensitivity to auxin in gravistimulated soybean hypocotyls. Plant Physiology 91: 1329-1338

Sack FD (1991) Plant gravity sensing. International Review of Cytology 127:193-252

Seagull RW (1989) The plant cytoskeleton. CRC Critical Reviews in Plant Science 8:131-167

Sievers A, Kramer-Fischer M, Braun M, Buchen B (1991) The polar organization of the growing Chara rhizoid and the transport of statoliths are actin-dependent. Botanica Acta 104:103-109

Sievers A, Volkmann D (1979) Gravitropism in single cells. *In* Encyclopedia of Plant Physiology. New Series Volume 7 W Haupt and ME Feinleib eds. Springer-Verlag Berlin pp567-572

Simons C, Migliaccio F, Masson P, Caspar T, Soll D (1995) A novel root gravitropism mutant of *Arabidopsis thaliana*

exhibiting altered auxin physiology. Physiologia Plantarum 93:790-798

Sinclair W, Oliver I, Maher P, Trewavas A (1996) the role of calmodulin in the gravitropic response of the *Arabidopsis thaliana agr*-3 mutant. Planta 199:343-351

Slocum RD, Roux SJ (1983) Cellular and subcellular localization of calcium in gravistimulated oat coleoptiles and its possible significance in the establishment of tropic curvature. Planta 157:481-492

Staves MP, Wayne R, Leopold AC (1992) Hydrostatic pressure mimics gravitational pressure in characean cells. Protoplasma 168:141-152

Stinemetz CL, Kuzmanoff KM, Evans ML, Jarrett HW (1987) Correlation between calmodulin activity and gravitropic sensitivity in primary roots of maize. Plant Physiology 84:1337-1342

Szerszen JB, Szczyglowski K, Bandurski RS (1994) iaglu, a gene from *Zea mays* involved in conjugation of a growth hormone indole-3-acetic acid. Science 265:1699-1701

Takahashi H, Scott TK (1991) Hydrotropism and its interaction with gravitropism in maize roots. Plant Physiology 96:558-564

Taylor DP, Slattery J, Leopold AC (1996) Apoplastic pH in corn root gravitropism: A laser scanning confocal microscopy measurement. Physiologia Plantarum 97:35-38

Timpte C, Lincoln C, Pickett FB, Turner J, Estelle M (1995) The AXR1 and AUX1 genes of *Arabidopsis* function in separate auxin-response pathways. The Plant Journal 8:561-569

Trewavas AJ ed.(1992) Forum: What remains of the Cholodny-Went theory? Plant Cell and Environment 15:759-794

Trewavas A, Knight M (1994) Mechanical signalling, calcium and plant form. Plant Molecular Biology 26:1329-1341

Veluthambi K, Poovaiah BW (1984) Calcium-promoted protein phosphorylation in plants. Science 223:167-169

Versel J-M, Pilet P-E (1986) Distribution of growth and proton efflux in gravireactive roots of maize (*Zea mays* L.) Planta 167:26-29

Walker JC, Key JL (1982) Isolation of cloned cDNAs to auxin-responsive poly(A)+RNAs of elongation soybean hypocotyl.

Proceedings of the National Academy of Science 79:7185-7189

Wayne R, Staves MP, Leopold AC (1990) Gravity-dependent polarity of cytoplasmic streaming in *Nitellopsis*. Protoplasma 155:43-57

Wayne R, Staves MP, Leopold AC (1992) The contribution of the extracellular matrix to gravisensing in characean cells. Journal of Cell Science 101:611-623

Went FW, Thimann KV (1937) Phytohormones p154-157 NY Macmillan 294

Wilson AK, Pickett FB, Turner JC, Estelle M (1990) A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Molecular and General Genetics 222:377-383

Wong LM, Abel S, Shen N, de la Foata M, Mall Y, Theologis A (1996) Differential activation of the primary auxin response genes, PS-IAA4/5 and PS-IAA6, during early plant development. The Plant Journal 9:587-599

Wright LZ, Rayle DL (1983) Evidence for a relationship between H+ excretion and auxin in shoot gravitropism. Plant Physiology 72:99-104

Wyatt RE, Ainley WM, Nagao RT, Conner TW, Key JL (1993) Expression of the *Arabidopsis* AtAux2-11 auxin-responsive gene in transgenic plants. Plant Molecular Biology 22:731-749

Zobel RW (1973) Some physiological characteristics of the ethylene-requiring tomato mutant diageotropica. Plant Physiology 52:385-389

Chapter I

Use of gravitropism mutants to evaluate the role of light in gravitropism: An analysis of models of gravity response

Abstract:

To isolate genetic components of gravitropism that are affected by light conditions and that are independent of light, an M2 population of *Arabidopsis thaliana* seedlings was screened for altered gravitropism under two growth protocols that vary in levels of light exposure. The mutants identified in this screen were found to be pleiotropic, and were placed into groups based on the additional phenotype associated with gravitropism. These associated traits were evaluated to determine the possible functional significance of these characteristics in gravitropism. A starch-deficient mutant isolated in this study was found to exhibit growth-protocol dependent changes in gravitropism unlike the effects of light on a previously identified starchless gravitropism mutant. Gravitropism of two hormone-response mutants was observed under each growth protocol, and it is suggested that light-regulated change in plant growth hormones could be a mechanism for the effect of light on gravitropism. Phototropism was examined for all mutants to determine if aberrant gravitropism is the result of a differential growth impairment, or a specific gravitropism alteration. All mutants examined, including the auxin-resistant mutant, were found to have phototropism

similar to the parental wild-type which suggests that auxin may have different functions in phototropism and gravitropism.

Introduction

Exposure to light can affect the ability of plants to perceive gravity and can alter the magnitude of gravitropic curvature (Woitzik and Mohr 1988). As plants have multiple photoreceptor pigments that exhibit distinct but overlapping wavelength specificity in light absorbance (Kendrick and Kronenberg 1994), light mediated changes in gravitropism could be the result of several independent regulatory factors. This introduces an element of ambiguity that may explain why different studies have attributed light-regulated changes in gravitropism to both blue and red light, contributing to controversy about the number of photoreceptors that modulate gravitropism.

The phytochrome gene family encodes five separate photoreceptors in *Arabidopsis* each showing different but integrated effects on plant development (Pratt 1996). Both phytochrome A and phytochrome B can mediate a light induced increase in random hypocotyl orientation, and lack of both phytochromes leads to increased uniformity of *Arabidopsis* hypocotyl response (Robson and Smith 1996). Phytochrome has a different effect on root gravitropism as red light irradiation has been shown to increase sensitivity to gravity and does not cause seedling orientation to become less homogeneous (Bullen 1992).

In addition to changes induced by short pulses of red light, there is some evidence that a long duration red light

irradiation can cause a photomorphogenic change that affects gravitropism. Sesame seedlings grown for five days under red light exhibited an increase in hypocotyl gravitropism that was not reversed by far-red treatment (Woitzik and Mohr 1988). The constitutively photomorphogenic *Arabidopsis* mutant *cop*4 shows altered gravitropism in the root and hypocotyl, and provides genetic support for a phytochrome-regulated developmental program that affects gravitropism (Hou et al 1993).

Several blue light photoreceptors are present in *Arabidopsis thaliana* (Ahmad and Cashmore 1996). Because phytochrome can absorb blue light as well as red light, it is necessary to carefully separate phytochrome-regulated events from blue-light specific events. The blue light inhibition of gravitropism in maize coleoptiles shows a fluence response curve similar to that observed for induction of phototropism (Hild 1977). This could indicate that blue light inhibits gravitropism because some signal transduction elements are common to the two tropisms. Alternatively, blue light may directly induce a response that is antagonistic to gravitropism. Mutants altered in both phototropism and gravitropism show that some genetic elements are necessary for both responses (Khurana and Poff 1989). In roots, blue light but not red light has been found to induce directional growth that can be separated from gravitropism (Okada & Shimura 1992). This root

phototropism may be an independent phenomenon, or may represent the mechanism through which blue light affects gravitropism.

This study has used a combination of etiolated and light grown seedlings to determine the response of gravitropism mutants to changes in light conditions. Mutants in known genetic traits associated with gravitropism, such as hormone-resistance and starch content, have been examined under both growth protocols to compare the effect of light on mutant gravitropism to the previously established effect of light on starch biosynthesis and hormone action. Analysis of gravitropism mutant phenotypes found in this study has been undertaken to determine the spectrum of traits associated with gravitropism. Phototropism has been evaluated for some gravitropism mutants and is proposed as a method to analyze mutant specificity. Results with phototropism of an auxin-resistant mutant suggests that all auxin-resistant mutants could be examined for phototropism to determine whether they are altered in some aspect of differential growth, or in a component that has a unique role in gravitropism.

Materials and Methods:

Mutagenesis:

Dry seed of *Arabidopsis thaliana* were exposed to gamma

irradiation from a ^{60}Co source at the chemistry department at Michigan State University. Using the 1990 calibration report, it was determined that the dosage applied was 1320 R/minute. An exposure time of 60 minutes was selected as this irradiation induced a mortality rate of 60% in small test populations. Approximately 10,000 seed were exposed to this dosage. These seed were then planted in flats filled with a peat/perlite mix and placed in a growth chamber at 23° C with an 18 hour light/6 hour dark photoperiod, where they were allowed to grow and produce seed. All seed produced by these plants were harvested as one bulk M2 population for use in this study.

An additional 250,000 dry seed were exposed to fast neutron irradiation by Dr H. Brunner at the Plant Breeding Unit of the International Atomic Energy Agency in Vienna Austria. These seed were given a total dosage of 6,000 rad. The mutagenized seed were divided into 29 sub-populations of approximately 8,600 seed and grown on 29 individual flats in a growth chamber. Seed produced from each mutagenized sub-population were harvested separately and screened individually for gravitropism mutants.

Screen:

Two protocols were used to measure gravitropism of seedlings. The two protocols were chosen to represent conditions under which the rate of hypocotyl growth was

approximately equal. One protocol induced gravitropism in etiolated seedlings, and the other gravitropism in seedlings grown under white light. In both cases, seeds were surface sterilized using a 20 minute exposure to a 30% bleach solution with 0.1% of a 20% Triton X-100 solution, followed by 6 rinses with sterile distilled water. Seeds were planted on the surface of Petri dishes containing plant mineral media (Haughn and Somerville 1986) supplemented with 1% agar. These dishes were then placed in darkness at 4° C for 3 days to potentiate germination. At this time the seeds were treated according to one of the two growth protocols to measure gravitropism.

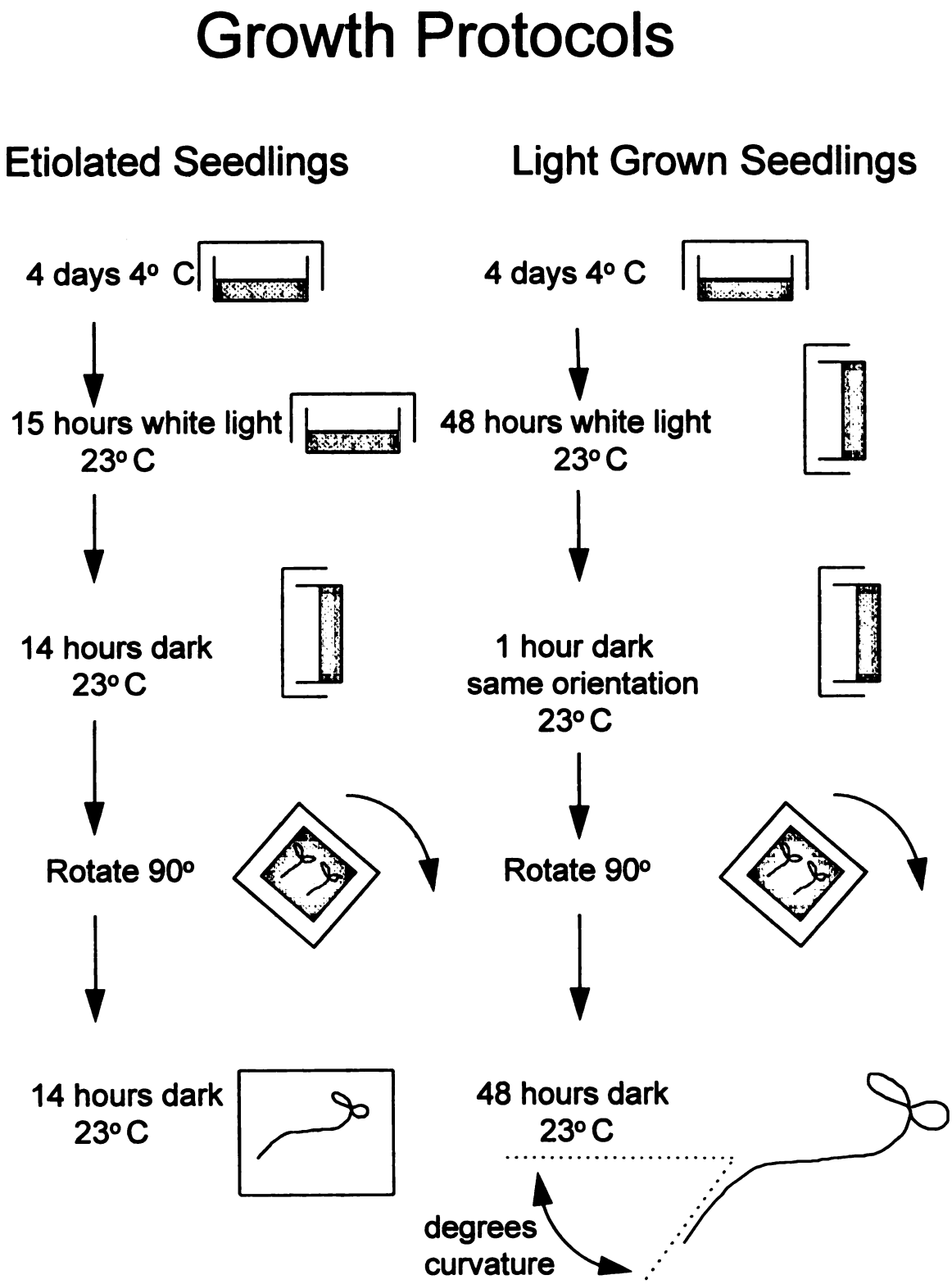
a. Etiolated-Growth Protocol

The planted Petri dishes were placed horizontally at 25° C under white light at a fluence rate of 50 uMoles $m^{-2}s^{-2}$ for 15 hours. This time had been previously determined to induce germination in approximately 50% of the *Arabidopsis* seeds. After germination, the plates were placed vertically in darkness at 25° C for 14 hours. Seedlings were then rotated 90° in complete darkness, and allowed to develop curvature for an additional 14 hours (Figure 1).

b. Light-Growth Protocol

The planted Petri dishes were placed vertically under white

Figure 1. Schematic drawing of seedling growth protocols utilized to observe gravitropism.



light at a fluence rate of 50 uMoles $m^{-2}s^{-2}$ at 25° C for 48 hours, by the end of the light treatment time seedlings had emerged and the hypocotyls had begun to green. Seedlings were then placed in the dark at 25° C in the same orientation for one hour. The Petri dishes were then rotated 90° in darkness and seedling curvature was allowed to develop for an additional 48 hours (Figure 1).

Seed Generation

The M2 seed was collected and utilized for a primary screen of seedlings under one of the two growth protocols. Each selected M2 seedling was transplanted from the agar Petri dish into a pot of *Arabidopsis* soil mix and placed in the growth chamber. These plants were allowed to self-pollinate to produce M3 families. The M3 families were the subject of the secondary screen.

Genetic Studies

Crosses were performed between each mutant line and the parental wild-type. The mutant line was used as a pollen donor to emasculated flowers of the parental wild-type line. The F1 seedlings were planted and allowed to produce F2 seed. A population of approximately 50 seedlings in the F2 generation were examined for most lines to determine if the phenotypes associated with gravitropism could be genetically separated. The auxin-resistant mutant was allowed to cross

pollinate *aux*1 from the *Arabidopsis* Biological Resource Center (The Ohio State University). The auxin-resistant line isolated in this study was used as a pollen donor, and F1 seed from two separate pollination events were examined for gravitropism under the etiolated-growth protocol.

Determination of Curvature

Seedlings were traced using a photographic enlarger, and orientation was determined relative to a grid imprinted on the petri dish. The seedling image was then analyzed using a CCD Camera (Cohu, Inc.) and image analysis software (Jandel Scientific) to measure the angle of curvature. Similar seedling manipulation and data analysis were described previously (Bullen 1992). This method of data collection allows the simultaneous measurement of hypocotyl and root gravitropism. The angle of curvature represents the angle formed between the plant organ and the original gravity vector (Figure 1).

Phototropism

Seeds were planted in microtitre wells prepared from Falcon 3911 microtest III flexible assay plates (Becton Dickinson Labware) containing 1.0mM KNO_3 and 0.8% Difco-Bacto agar. One seed was placed in each well, and the strips were placed in a transparent plastic container sealed with Parafilm (American Can Company) and lined with

moistened paper to maintain a high humidity environment. Following four days in darkness at 4°C, seedlings were placed under white light at 24°C for 4 days to induce germination. Seedlings were then allowed to grow in complete darkness at 24°C and 90% relative humidity for an additional 42 hours. Phototropism was induced with 5 pulses of 450nm blue light at fifteen minute intervals (Steinitz and Poff 1986). These pulses were at $0.15uMm^{-2}s^{-2}$ for a total fluence of 0.75 $uMolm^{-2}$. Curvature was allowed to develop for 30 minutes in darkness, and seedlings were mounted on clear tape and placed in a photographic enlarger to allow seedling orientation to be traced. These shadowgraphs were then analyzed using the system described above.

Light Sources

For phototropism experiments, the light source was a projector equipped with a Sylvania 300W ELH tungsten halogen lamp wise with a 450 nm interference filter with a half-band width of 10nm (PTR Optics) The fluence rate was measured with a model LI-185A radiometer (Li-Cor), and the duration of exposure was controlled with a Uniblitz shutter (Vincent Associates)

Starch Measurement

Seeds were surface sterilized and plated on agar media

as described previously, and placed under florescent light at 50uMoles $m^{-2}s^{-2}$ for 4 days. Whole seedlings were removed from the plates, and pigments were extracted by boiling in 95% ethanol for 10 minutes. A stain of 5.7 mM iodine and 43.4 mM potassium iodine in 0.2N HCl was applied for 30 minutes to allow the visualization of stained starch granules. The intensity of stain was used as a qualitative measure of starch quantity (Casper et al 1985).

Auxin Response

Petri dishes containing *Arabidopsis* mineral media (Haughn and Somerville 1986) were prepared with 8% Bacto agar (Sigma) and 20% sucrose (Sigma). A concentration of 10^{-7}M synthetic auxin, 2,4-dichlorophenoxyacetic acid, was added to some of these plates after the medium was autoclaved. Seeds were surface sterilized as described earlier, and allowed to germinate and grow along the surface of medium containing and lacking synthetic auxin. When *Arabidopsis* seedlings are grown on plates containing high levels of 2,4-dichlorophenoxyacetic acid, there is severe reduction in root length and great increase in root hair abundance (Lincoln et al 1990). For the purpose of this study, mutant seedlings exhibiting visibly greater root length than the parental wild-type seedlings were considered to be resistant to auxin-induced root stunting.

Ethylene Response

Seedlings were prepared as described for the gravitropism response assay. After 14 hours of white light to induce germination, the Petri dishes were placed in sealed containers with either air, or 1uL/L ethylene and allowed to grow in darkness for 3 days (Bleeker et al 1988). The addition of ethylene caused a reduction in hypocotyl length, and root length in the parental wild-type seedlings. Any mutant seedling line exhibiting less growth reduction than parental wild-type seedlings was considered to be resistant to ethylene-induced stunting.

Statistical Analysis

Mean curvature of gravitropism mutants were compared to the parental wild-type response using a two-tailed t-tests(Steel and Torrie 1981). This test was also used to compare mean gravitropic curvature between the two growth protocols for each seedling line.

The F-test was used to determine homogeneity of variance between gravitropism in the two growth protocols for each plant line. This test was also used to analyze variance between the wild-type parental line and each mutant mutant line under each light growth protocol(Steel and Torrie 1981). All seedling populations used for characterization consisted of 80 to 100 individuals.

Results:

Generation of Mutants

A primary mutant screen examined 63,000 light-grown M2 seedlings from the gamma-irradiated population. This identified 200 individual seedlings that exhibited altered gravitropism. The M3 seed produced by each putative mutant was subjected to a secondary screen in which seedlings were grown under either the light-growth protocol or the etiolated-growth protocol. A total of 65 seedling lines showed altered gravitropism under one or both of the growth protocols. These mutant lines were subjected to additional characterization.

A total of 103,000 seedlings from the 29 fast neutron mutagenized sub-populations was subjected to a primary screen for gravitropism mutants using the light growth protocol. Three hundred and twenty four seedlings with an altered response to gravity were identified in this screen. The M3 seed were generated for each line, and examined in a secondary screen as described for the gamma-irradiated population. Fifty five M3 families with altered gravitropism were identified.

Mutant Phenotype Groups

A large number of mutants were generated from both screens, and criteria were developed to identify mutant lines with common characteristics (Table 1). This resulted

Table 1. The distribution of characteristics associated with gravitropism mutants. Determination of characteristics was performed as described in the materials and methods.

Phenotype group	Number of lines
High mortality mutants: Poor germination or poor seed set led to death of seedlings in these lines.	43
Rootless or stunted root mutants: Altered hypocotyl gravitropism was observed in some seedlings with impaired root growth.	32
Auxin-resistant mutants: Gravitropism mutants resistant to root growth stunting by high concentrations of synthetic auxin.	23
Not heritable: Gravitropism mutant phenotype not detected in the F2 or F3 generation following cross-pollination with the parental wild-type.	13
Ethylene-resistant mutants: Gravitropism mutants resistant to ethylene induced reduction of root and hypocotyl growth.	5
Constitutively wavy root mutants: Gravitropism mutants exhibit small undulations along the length of the root causing a wave-like growth appearance.	2
Starch deficient mutant: Gravitropism mutant with reduced starch content in light-grown primary leaves.	1
No other phenotype mutant: Gravitropism mutant with no detected associated phenotype.	1
Total number of mutant lines	120

in the generation of eight phenotype groups. Some groups consisted of phenotypes previously associated with gravitropism such as starch content (1 line) and response to plant growth hormones (23 auxin-resistant lines, 5 ethylene resistant lines), while others showed a variety of phenotypes associated with seedling growth and development.

Characterization of Gravitropism

A representative mutant was selected from three of the phenotype groups for descriptive characterization of mutant gravitropism under the two growth protocols (Table 1). These mutant lines were g101 which was shown to be resistant to auxin-induced root stunting (Chapter 2 Table 4), g2 which was shown to be altered in starch content (Chapter 2 Figure 2) and 17-3 which was shown to be resistant to root stunting induced by ethylene (Chapter 2 Table 4). This auxin-resistant was shown to be allelic to *aux*1. A population of 46 F1 seed from two siliques were examined for etiolated root gravitropism, and found to have an average curvature of 23, and a standard deviation of 63, which is a response characteristic of the auxin-resistant line (Table 2).

The parental wild-type seedlings showed strong gravitropism in both the hypocotyl and the root under both growth protocols (Figures 2 and 3). Average curvature in the wild-type roots is greater in the etiolated growth protocol (X=71) than the light growth protocol (X=80), and

Table 2. Comparison of effect of light and dark growth protocols on the gravitropism response of the wild-type and gravitropism mutants. Mean values were compared by t-test, variances were compared by F tests for homogeneity of means.

		wild type		starch-deficient		auxin-resistant		ethylene-resistant	
		light	dark	light	dark	light	dark	light	dark
root	X	80	71**	31	58**	27	27	35	34
	SD	14	14**	27	23	44	54	45	46
hypocotyl	X	35	65**	29	10**	36	46	40	55
	SD	33	17**	43	37	63	60	55	72*

*, ** indicate significantly different from wild-type at p<= 0.05, and P<= 0.01 respectively. ns indicates no significant difference was found.

Figure 2. Graphic presentation of hypocotyl gravitropism under both growth protocols for the auxin-resistant mutant, the ethylene-resistant mutant, and the starch deficient mutant.

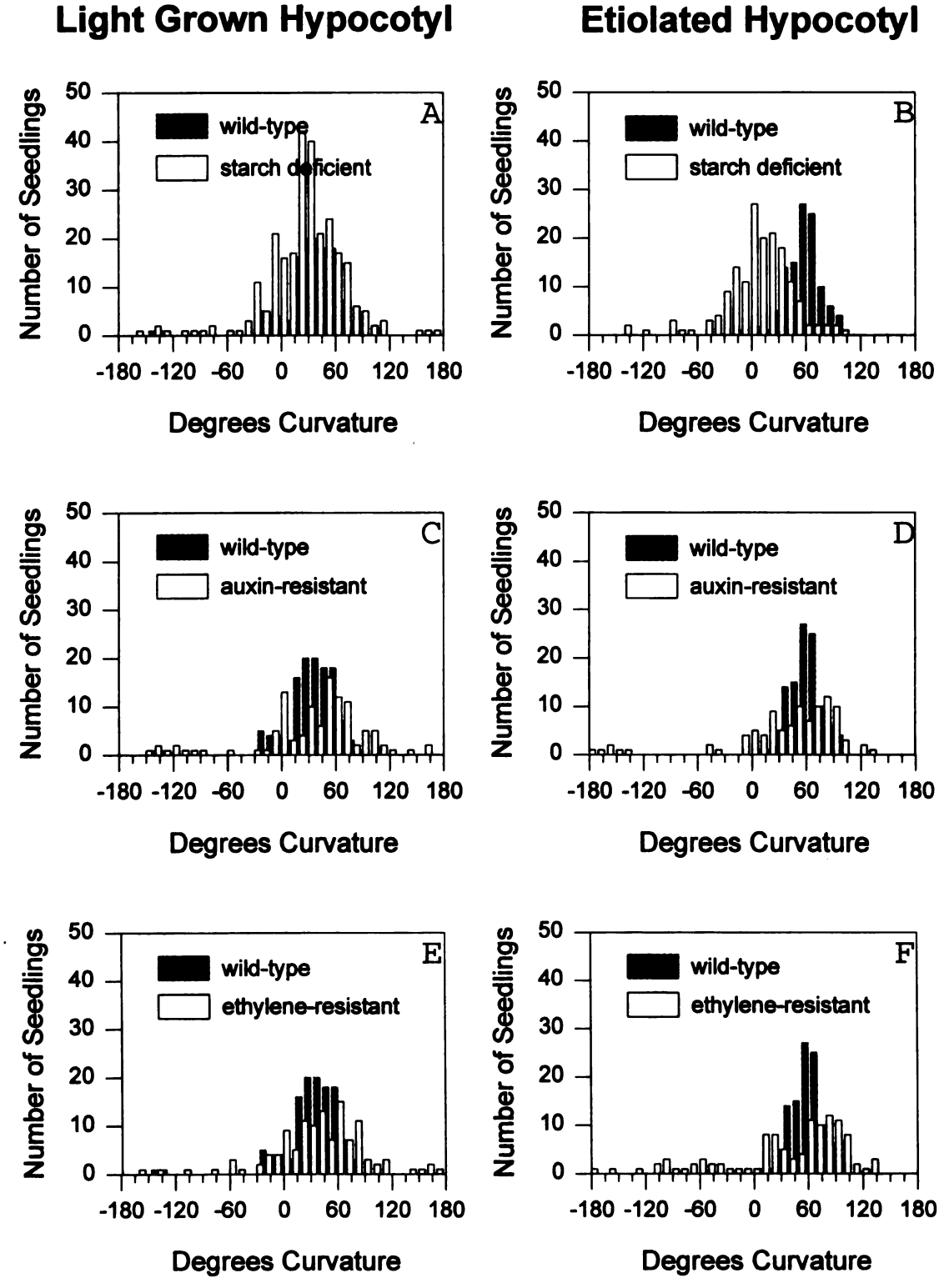
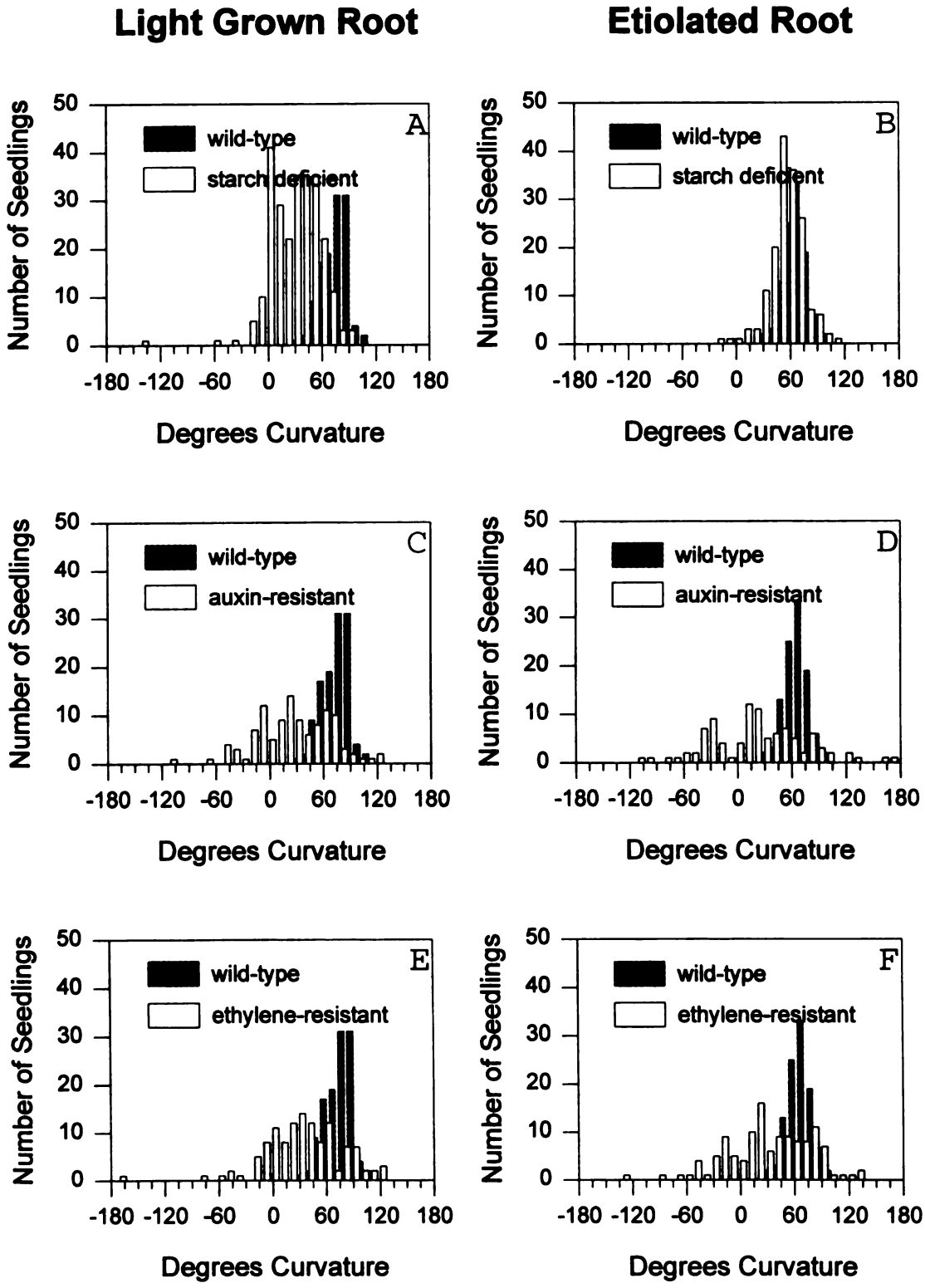


Figure 3. Graphic presentation of root gravitropism under both growth protocols for the auxin-resistant mutant, the ethylene-resistant mutant, and the starch deficient mutant.



this difference is statistically significant at P=0.01 (Table 2). The average wild-type hypocotyl curvature is greater in the etiolated seedings (X=65) than the light grown seedlings (X=35), while gravitropism in etiolated seedlings exhibits greater variance (Table 2).

The starch deficient mutant root response (Figure 3A and 3B) exhibits greater average curvature under the etiolated-growth protocol (X=58) than the light-growth protocol (X=31), a significant difference in curvature (Table 2). The variance of gravitropic curvature in the starch deficient mutant roots is not affected by growth protocol (Table 2). The hypocotyls of the starch deficient line (Figure 2A and 2B) show greater average curvature in the light-growth protocol (X=29)than under the etiolated growth protocol (X=10), but no significant change in variance (Table 2).

Root gravitropism of the auxin-resistant mutant (Figure 3C and 3D) showed low average curvature and high variance in curvature under both the etiolated (X=27 SD=44), and the light (X=27 SD=54) growth protocols (Figure 3C and 3D). There was no significant change in either aspect of gravitropism between the two growth protocols (Table 2). The hypocotyl of the auxin-resistant mutant (Figure 2C and 2D) showed no significant difference between seedlings in the two growth protocols (Table 2).

Root gravitropism of the ethylene-resistant mutant

Table 3. Mean curvature and variance for phototropism and gravitropism for each plant line. Mean values of mutant response were compared to wild-type response by t-test, variances were compared by F tests for homogeneity of means.

	wild-type	starch deficient	auxin-resistant	ethylene-resistant
etiolated root gravitropism	X 71 SD 14 n 120	X 58** SD 23** n 161	X 27** SD 54** n 100	X 34** SD 46** n 121
light-grown root gravitropism	X 80 SD 14 n 108	X 31** SD 27** n 204	X 27** SD 44** n 110	X 35** SD 45** n 119
etiolated hypocotyl gravitropism	X 65 SD 17 n 116	X 10** SD 37** n 162	X 46 SD 60** n 100	X 55 SD 72** n 122
light-grown hypocotyl gravitropism	X 35 SD 33 n 104	X 29 SD 43* n 207	X 36 SD 63** n 110	X 40 SD 55** n 119
five-pulse blue light phototropism	X 30 SD 31 n 85	X 28ns SD 22ns n 116	X 30ns SD 21ns n 72	X 28.8ns SD 21ns n 55

*, ** indicate significantly different from wild-type at p<= 0.05, and P<= 0.01 respectively. ns indicates no significant difference was found.

(Figure 3E and 3F) exhibited a high variance of curvature under both the light growth protocol (SD= 45) and the etiolated growth protocol (SD=46), and no significant difference was found between root gravitropism under either light treatment protocol (Table 2). The hypocotyls of ethylene-resistant gravitropism mutants (Figure 2E and 2F) showed gravitropic curvature with a large variance. Hypocotyl gravitropism in this mutant line was found to have a significantly greater variance in etiolated seedlings (SD=72) than in light grown seedlings (SD=55) although the mean curvature was not altered by differences in growth protocol (Table 2).

Gravitropism of these mutants was compared to that of the parental wild-type for both roots and hypocotyls under each growth protocol (Table 3). Root gravitropic curvature of the starch deficient mutant was significantly lower, and the standard deviation of gravitropism significantly higher than the wild-type parent under both growth protocols (Table 2). The response of the etiolated hypocotyls of this mutant was significantly different from the etiolated hypocotyls of the wild-type parent, but the light-grown hypocotyl response of the mutant was similar to the light-grown response of the wild-type hypocotyls (Table 2). Root gravitropism in both the auxin-resistant mutant and the ethylene-resistant mutant was significantly different from the parental wild-type root gravitropism under both growth protocols with both mutants

showing lower average curvature and greater standard deviation than the wild-type parent (Table 2). Average hypocotyl gravitropism of the hormone-response mutants was not significantly different from the wild-type response for either growth protocol, although the standard deviation of hypocotyl response was greater for the mutants than the parental wild-type (Table 2).

Characterization of Phototropism

The five pulse treatment with blue light was designed to induce significant phototropic curvature in response to a total fluence within the range of first positive phototropism. This treatment induced similar response in the parental wild-type seedlings, and in both hormone-response mutant seedlings and in the starch deficient mutant seedlings (Table 2).

Discussion

Traits associated with gravitropism

A large number of general alterations in growth and development were found to be associated with gravitropism. The common occurrence of high seedling mortality in this gravitropism mutant collection could indicate that poor seedling vigor generally reduces gravitropism, or that some genetic components necessary for gravitropism are also necessary for seed germination and seedling growth. The presence of an altered root was shown to influence gravitropism, although it could not be determined how the altered morphology contributed to the gravitropism mutant. Altered root development could directly affect hypocotyl gravity response, or an apparent change in gravitropism could develop if rootless seedlings are less securely anchored, and orientation changes result from seedlings sliding on the agar surface. A direct connection between altered root waving and altered gravitropism has been shown for the *aux*1 and *agr*1 mutants (Okada and Shimura 1992), but the mutants identified in this study suggested that constitutive root waving occurs in addition to gravitropism. The two mutants that showed constitutive root waves displayed less uniform seedling orientation than the parental wild-type seedlings, but still exhibited directional growth in response to gravity.

Although auxin-resistant mutants constitute the third

largest group of mutants, this does not show that genes necessary for auxin response represent the third largest genetic component of gravitropism. In this study, auxin-resistant mutants display a greatly altered mutant gravitropism phenotype under both growth protocols, which means that these mutant lines are more likely to be detected than less severe gravitropism mutants. A large number of auxin-resistant gravitropism mutants have been previously isolated (Fukaki et al 1996), and while this may reflect the genetic complexity of the auxin response component of gravitropism, it may also result from the use of the auxin-resistance phenotype to isolate mutants (Hobbie and Estelle 1995). Since the stunted root phenotype can be detected more easily than changes in average gravitropic curvature, screens for this trait are quicker and more efficient than screens for gravitropism mutants.

A much larger number of mutants were found to be resistant to auxin, than were found to be resistant to root inhibition by exogenous ethylene, but unaltered in response to auxin (Table 1). The gravitropism response characterized for the ethylene-resistant phenotype group was similar to that of the auxin-resistant mutant (Figure 2 and 3), and it is somewhat surprising that so many more auxin resistant mutants were isolated in this study than ethylene-resistant mutants (Table 1). This may be an indication of the relative genetic complexity of these two hormone response

systems, or it may result because the majority of the auxin-resistance mutants found in this study were not tested for additional hormone responses. As most auxin-resistant mutants are also resistant to several classes of plant growth hormones (Hobbie and Estelle 1995), further study of these auxin-resistant mutants may show that some are altered in response to other plant hormones.

Only one mutant line in this study was found to be altered in starch content (Table 1). As four different mutations have been shown to affect both starch content and gravitropism (Kiss et al 1996), it is likely that control of starch abundance will be found to represent a large genetic component of gravitropism. The small number of starch deficient mutants identified in this study may be attributable in part to the use of a primary screen that identified gravitropism in light grown seedlings. As light-grown starchless *pgm* mutant seedlings exhibit less impaired gravitropism (Casper & Picard 1989) than etiolated seedlings (Kiss et al 1989), the light-growth protocol used in this study would cause the *pgm* starchless mutant to show a less apparent mutant gravitropism phenotype. This light effect could affect other starch deficient mutants in a similar manner, and cause such mutant to be less noticeable in the primary screen.

Growth protocol-dependent gravitropism

As previously observed (Bullen 1992), wild-type seedlings of *Arabidopsis thaliana* in the Estland ecotype show greater curvature in root and hypocotyl gravitropism under a light-growth protocol (Figure 3). The increased randomness of hypocotyl orientation in the light-growth protocol (Figure 2) confirms previous data regarding the effect of light on hypocotyl gravitropism (Robson and Smith 1996). No consistent light-modulated change in gravitropism was observed for the three mutants examined.

Although the starch-deficient mutant in this study, and the *pgm* mutant both exhibit light-mediated changes in gravitropism, the response of these mutants to light is very different. The *pgm* mutant shows a light-mediated increase in root gravitropism, while the starch deficient mutant in this mutant collection shows a light-mediated decrease in root gravitropism. As both starch synthesis (Casper et al 1985) and root gravitropism are increased by light, greater starch accumulation could be a mechanism to enhance gravity response. Previous studies had suggested that this is unlikely as the *pgm* mutant shows similar plastid starch content under all light conditions (Casper et al 1985), yet shows gravitropism that is altered by light exposure. This study also does not support a direct role for starch content in light mediated gravitropism, as such a mutant would be expected to show the same response under the two growth protocols, or to show "unenhanced" gravitropism identical to

etiolated wild-type seedlings, under both protocols. Gravitropism of the starch deficient mutant in this study is significantly different from the parental wild-type response only in etiolated hypocotyls and light-grown roots (Figure 2 and 3). Thus, the requirement for wild-type levels of starch for expression of full gravitropism appears to be both organ-dependent and light-dependent. This may indicate an organ specific effect of light occurs to amplify the effect of reduced starch, or that these different phenotypes represent changes in the role of starch under different environmental conditions.

The auxin-resistant mutant shows little change between gravitropism in root or hypocotyl under the two light regimes (Table 2). Some studies show that auxin transport is affected by light treatment (Scott and Wilkins 1969), and work with the auxin-resistant/auxin transport inhibitor-resistant gravitropism mutant *rgr*1, shows that the etiolated mutant seedlings are more resistant to root inhibition by exogenous auxin than light-grown seedlings (Simons et al 1995). The lack of light-mediated gravitropism may show that this mutant is altered in a light regulated component of auxin response/perception or possibly in later response stages of gravitropism subsequent to the light response.

The ethylene-resistant mutant line selected for characterization was found to show a gravitropism mutant phenotype that was very similar to that of the auxin-

resistant mutant (Figure 2 and 3). Several studies have proposed a link between light treatment and ethylene accumulation. A red light pulse was shown to reduce ethylene accumulation in intact pea seedlings (Goeschl et al 1967). Temporal correlation between maximum gravitropic enhancement and greatest light induced ethylene reduction was observed in pea stem sections (Kang & Burg 1972). Because this ethylene-resistant mutant does not show a strong difference between gravitropism of light grown seedlings and etiolated seedlings (Table 2), it may be a useful tool for studying the role of light regulated ethylene reduction in gravitropism.

Lack of change in gravitropism between the two growth protocols in the two hormone-response mutants suggests that hormone regulation could be the light-modulating component of gravitropism. As previously isolated gravitropism mutants have been found to be responsive to light (Bullen 1992), the light-modulation of gravitropism is not commonly altered in gravitropism mutants. Based on the response of these mutants, and the known effects of light on ethylene and auxin, a hypothetical model for light mediated changes in gravitropism could be proposed. These mutants are altered in some genetic component of gravitropism that is responsive to auxin and/or ethylene, and is necessary for gravitropism under all light conditions. As light treatment reduces ethylene & increases auxin transport, this results

in a general increase in differential growth or signal transduction, and results in a more uniform seedling response to gravity, and increases gravitropic curvature. Mutants that are altered in steps of gravitropism prior to hormone regulation should be responsive to light induced changes in hormone balance, while mutants affected in later steps of gravitropism should not be affected by light conditions. Although the elimination of light-modulated increase in gravitropism in two mutant lines suggests that this could be a general hormone-response trait, it may be that further study will show that this response is specific to ethylene. As complementation data of the auxin resistant mutant has shown that it is allelic to *aux*1 , a mutant that is resistant to both auxin and ethylene (Picket et al 1990), the common element of these two mutants is altered response to ethylene.

Phototropism:

Because both phototropism and gravitropism involve differential growth, it has been proposed that they use similar mechanisms to regulate growth. The examination of phototropism can be useful to distinguish general alterations in differential growth from specific alterations in gravitropism. The exhibition of unaltered phototropism by all gravitropism mutants in this study indicates that all of these mutants are specific to gravitropism. Full

phototropism in the auxin-resistant mutant is surprising, as previous models of tropisms have proposed that auxin plays a similar role in establishment of differential growth in all tropisms (Went and Thiman 1937). As the auxin-resistant mutant examined in this study is primarily affected in root gravitropism, a conservative interpretation of this result would suggest that the root and hypocotyl have some organ-specific mechanisms of hormone response. Alternatively, this could imply that the role of auxin in gravitropism is fundamentally different from the role of auxin in phototropism. The study of phototropism in *axr2*, an auxin-resistant mutant that is altered in hypocotyl gravitropism could determine if auxin response should be separated into two tropism specific components, or into organ specific components that are common to both tropisms

Unimpaired phototropism in the starch deficient mutant supports previous studies in the conclusion that starch content has a specific role in gravity response. If reduced starch content diminished response to the environment through a general metabolic impairment such as reduction of seedling growth or alteration of sucrose balance, it would be expected that phototropism would exhibit a reduced response similar to gravitropism.

The use of these two protocols to study the effect of light on gravitropism mutants has provided evidence that hormone regulation could be a fundamental component of

light-mediated enhancement of gravitropism. The use of phototropism is suggested as a phenotype to evaluate the role of genetic elements suggested to have a general role in differential growth in all tropisms. Unimpaired phototropism in both hormone-response mutants is an unexpected finding, and shows that phototropism and gravitropism can be separated at the level of hormone response. The study of phototropism in additional auxin-resistant mutants would be useful to evaluate the role of auxin response in differential growth, and could separate tropism specific effects of auxin from organ specific effects that are common to both tropisms.

References:

Ahmad M, Cashmore AR (1996) Seeing blue-the discovery of cryptochrome. Plant Molecular Biology 30:851-861

Bleeker AB, Estelle MA, Somerville S, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241:1086-1089

Bullen B (1992) Development of a genetic system for the study of gravitropism in *Arabidopsis thaliana*. PhD dissertation

Casper T, Pickard BG (1989) Gravitropism in a starchless mutant of *Arabidopsis*. Planta 177:185-197

Casper T, Huber SC, Somerville C (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucosemutase activity. Plant Physiology 79:11-17

Fukaki H, Fujisawa H, Tasaka M (1996) SGR1, SGR2, and SGR3: Novel genetic loci involved in shoot gravitropism in *Arabidopsis thaliana*. Plant Physiology 110:945-955

Goeschl JD, Pratt HK, Bonner BA (1967) An effect of light on the production of ethylene and the growth of the plumular portion of etiolated pea seedlings. Plant Physiology 42:1077-1080

Haughn GW, Somerville CR (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Molecular and General Genetics 204:430-434

Hild V (1977) Wirkung von Vorbestrahlung mit Rot- oder Blaulicht auf die geotropische Empfindlichkeit von Maiskoleoptilen. Planta 133:309-314

Hobbie L, Estelle M (1995) The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. The Plant Journal 7:211-220

Hou Y, von Arnim AG, Deng X-W (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. The Plant Cell 5:329-339

Kang BG, Burg SP (1972) Regulation of phytochrome-enhanced geotropic sensitivity to ethylene production. Plant Physiology 50:132-135

Kendrick PE, Kronenberg GHM (1994) Photomorphogenesis in Plants. Kluwer Academic Press. Dordrect, the Netherlands

Kiss JZ, Hertel R, Sack FD (1989) Amyloplasts are necessary for full gravitropic sensitivity in roots of *Arabidopsis thaliana*. Planta 177:198-206

Kiss JZ, Wright JB, Casper T (1996) Gravitropism in roots of intermediate-starch mutants of *Arabidopsis*. Physiologia Plantarum 97:237-244

Khurana JP, Poff KL (1989) Mutants of *Arabidopsis thaliana* with altered phototropism. Planta 178:400-406

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr1* mutant of *Arabidopsis*. The Plant Cell 2:1071-1080

Okada K, Shimura Y (1992) Mutational analysis of root gravitropism and phototropism of *Arabidopsis thaliana* seedlings. Australian Journal of Plant Physiology 19:439-448

Pickett FB, Wilson AK, Estelle M (1990) The *aux*1 mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiology 94:1462-1466

Pratt LH (1995) Phytochromes: Differential properties, expression patterns and molecular evolution. Photochemistry and Photobiology 61:10-21

Robson PRH, Smith H (1996) Genetic and transgenic evidence that phytochrome A and B act to modulate the gravitropic orientation of *Arabidopsis thaliana* hypocotyls. Plant Physiology 110:211-216

Scott TK, Wilkins MB (1969) Auxin transport in roots. IV. Effects of light on IAA movement and geotropic responsiveness in *Zea* roots. Planta 87:249-258

Simmons C, Migliaccio F, Masson P, Casper T, Soll D (1995) A novel root gravitropism mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. Physiologia Plantarum 93:790-798

Steel RGD, Torrie JH (1981) Principles and procedures of statistics: A biometrical approach. McGraw-Hill International Book Co pp577-585

Steinitz B, Poff KL (1986) A single positive phototropic response induced with pulsed light in hypocotyls of *Arabidopsis thaliana* seedlings. Planta 186:305-315

Woitzik F, Mohr H (1988) Control of hypocotyl gravitropism by phytochrome in a dicotyledonous seedling (*Sesamum indicum* L.) Plant, Cell and Environment 11:663-668

Went FW, Thimann KV (1937) In Phytohormones. NY Macmillan Volume 294 pp154-157

Chapter II

Analysis of Gravitropism Mutants

Abstract:

A direct screen for gravitropism mutants in *Arabidopsis thaliana* was initiated to identify novel genetic components necessary for gravitropism. Mutant lines found in this study were examined for characteristics previously associated with gravitropism to evaluate established models of gravity perception and response. Genetic analysis resulted in the establishment of five complementation groups, each due to a single locus recessive mutation. Three complementation groups exhibited phenotypes similar to known gravitropism mutants, and two of these three groups were shown to be allelic to previously identified mutants. Two complementation groups isolated in this study appear to represent novel genetic elements of gravitropism. These mutant alleles are altered in hypocotyl gravitropism and have been given the names *hgr*1 and *hgr*2. Comparison of mutant gravitropism under the two growth protocols showed that both hypocotyl mutants are capable of some response to gravity under some conditions. Neither hypocotyl mutant is resistant to growth inhibition by high levels of growth hormones, although *hgr*2 exhibits unusual cotyledon hook development in the presence of ethylene. Hypocotyls of *hgr*2 show greater phototropic curvature in response to blue light

than wild-type or *hgr*1 hypocotyls at all fluences beyond the threshold of perception. This shows that the gene altered in *hgr*2 has a role that limits or inhibits phototropic curvature, but is necessary for full gravitropism. This suggests that the interaction of phototropism and gravitropism is not a general function of gravity response acting to "straighten" phototropic curvature, but the result of specific genetic elements that are active in both tropistic responses. As cotyledon hook formation and hook response to ethylene, as well as phototropism are altered in *hgr*2, it is proposed that mediation between two tropism-induced growth vectors could be related to ethylene response.

Introduction:

The use of *Arabidopsis thaliana* as a genetic system for the study of tropisms has greatly contributed to gravitropism research. Accessibility of existing mutants, and the ease of mutant isolation has permitted the specific analysis of individual elements of gravitropism perception/response models, and has been used to test the accuracy of the models. This study has used a direct mutant screen for aberrant seedling gravitropism to identify additional genetic components of the response. An emphasis has been placed on finding mutants that are not affected in previously identified aspects of gravitropism, so that further study of these mutants will provide insight into the mechanism of perception & response to gravity.

As differential growth is required for gravitropism, a number of plant growth hormones have been proposed to have a specific role in this response (Reviewed in Pickard 1985). Analysis of gravitropism mutants has provided genetic evidence that alterations in response to hormones has a direct affect on response to gravity. Several mutants in *Arabidopsis* have been identified that are altered in both hormone response and gravitropism. Five mutants isolated on the basis of resistance to auxin-induced root inhibition are impaired in gravitropism, and have been found to be altered in response to at least two additional classes of hormones (Hobbie and Estelle 1995). One gravitropism mutant has been

shown to be exclusively altered in response to auxin (Hobbie and Estelle 1995) and another has been found to be altered in response to auxin and to auxin transport inhibitors (Simons et al 1995). Transgenic *Arabidopsis* plants that overproduce IAA haven't been examined for gravitropism, however IAA overproduction in tobacco has no affect on gravitropism (Sitbon et al 1992). Auxin conjugation has been altered in transgenic *Arabidopsis* plants and been found to slightly reduce gravitropism (Normanly et al 1995).

Abscisic acid has been proposed to have a role as a growth inhibitor in gravitropism (reviewed in Wilkins 1984), but ABA deficient mutants (Moore and Smith 1985) and ABA insensitive mutants have not been shown to be impaired in gravitropism (Finkelstein and Zeevaart 1994). The study of hormone response gravitropism mutants has shown that growth hormones have a complex and interactive role in the establishment of differential growth.

The sedimentation of starch filled amyloplasts has been proposed as the initial step in gravity perception (Juniper 1976). The generation of mutants with reduced amyloplast density through manipulation of plastid starch content has been used to test this model. It was found that amyloplasts lacking starch were capable of reduced sedimentation, and that gravity perception was impaired in starchless mutants (Kiss et al 1989) and reduced starch mutants (Kiss et al 1996).

Environmental light conditions have been shown to play a role in gravitropism in several plant species. Molecular analysis of phytochrome A and phytochrome B, has shown that either phytochrome can serve to moderate gravitropism (Robson and Smith 1996). The constitutively photomorphogenic mutant *cop*4 is also altered in gravitropism and has been shown to be epistatic to phytochrome mutants (Hou et al 1993). This suggests that *cop*4 may represent a step of the phytochrome initiated developmental pathway that is interactive with gravitropism.

This work was undertaken to isolate novel gravitropism mutants in *Arabidopsis thaliana*. Two unique hypocotyl gravitropism mutants have been identified. The mutant *hgr*1 is not altered in starch content or response to exogenous hormones, and the hypocotyl gravitropism mutant *hgr*2 is not resistant to hormone induced growth stunting, but shows altered apical hook formation when treated with ethylene. A number of mutants that are allelic to previously identified mutants have also been found. All mutants have been subjected to genetic characterization and phenotypic analysis, to evaluate the possible function of these mutant genes in gravitropism.

Materials & Methods

Gravitropism:

Seeds were surface sterilized in by soaking for 20

minutes in a solution of 30% (v/v) commercial bleach (5.25% Sodium hypochlorite by weight) (Patterson laboratories, Inc.) and 0.02% (v/v) Triton X-100 (Sigma) followed by 5 rinses in sterile distilled water. Seedlings were planted on square gridded 100 x 100 x15 mm^3 integrid Petri dishes(Becton Dickinson Labware) containing 1% Bacto-Agar (Difco Laboratories) in *Arabidopsis* mineral nutrient media (Haughn & Somerville 1986). Similar seedling preparation is performed in most *Arabidopsis* gravitropism studies (Kiss et al 1996). Gravitropism was examined under both growth protocols described in the previous chapter. Angles of curvature were determined from seedling shadowgraphs drawn with the aid of a photographic enlarger. A CCD camera (Cohu, Inc.) and image analysis software (Jandel Scientific) were used to analyze the curvature in the shadowgraphs.

Phototropism:

Seeds were planted in microtitre wells prepared from Falcon 3911 microtest III flexible assay plates (Becton Dickinson Labware) containing 1.0mM KNO_3 and 0.8% Difco-Bacto agar. One seed was placed in each well, and the strips were placed in a transparent plastic container sealed with Parafilm (American Can Company) and lined with moistened paper to maintain a high humidity environment. Following four days in darkness at 4°C, seedlings were placed under white light at 24°C for 4 days to induce

germination. Seedlings were then allowed to grow in complete darkness at 24°C and 90% relative humidity for an additional 42 hours. Phototropism was induced with 5 pulses of 450nm blue light at fifteen minute intervals (Steinitz and Poff 1986). These pulses were at $0.15uMm^{-2}s^{-2}$ for a total fluence of 0.75 $uMolm^{-2}$. Curvature was allowed to develop for 30 minutes in darkness, and seedlings were mounted on clear tape and placed in a photographic enlarger to allow seedling orientation to be traced. These shadowgraphs were then analyzed using the system described above. The fluence response curves generated for hgr1 and hgr2 were similar to this, but fluence rates used were $0.23uMm^{-2}s^{-2}$. Seedlings were given a single light exposure, and duration was varied to produce the desired total fluence dose for each treatment group.

Genetic Crosses :

Genetic analysis was conducted to determine the pattern of inheritance of each mutant line on the basis of cross pollination with the parental wild type line, and to determine allelism based on cross pollination between two mutant lines. For all crosses, plants were propagated in a growth chamber with a photoperiod of 18 hours light and 6 hours dark, under conditions of high humidity. Plants were grown in clay pots in a soil mixture containing equal parts of perlite, sphagnum, and vermiculite, and were fertilized

with Hoaglands solution. For each cross, pollen from one homozygous plant was applied to an unopened flower of another genotype which had been dissected to remove all floral parts except the pistil. These hand pollinated flowers were wrapped in Sealwrap (Borden) to protect them from desiccation and from exposure to additional pollen, and allowed to produce seed. The F1 families were characterized for gravitropism, and the seedlings were then planted and allowed to grow to produce F2 seed. Individual F2 seed were planted, and the F3 families of seed were collected from each plant and gravitropism was examined under the growth protocol determined to most effectively identify the parental gravitropism mutant phenotype.

Segregation analysis:

To study segregation analysis, the growth protocol that had been found to give the most distinct difference between the mutant gravitropism and the parental wild-type gravitropism was used. For the auxin-resistant mutant and the ethylene-resistant mutant, the gravitropism of etiolated roots was examined. For the starch deficient mutant, and *hgr*1 and *hgr*2, the gravitropism of etiolated hypocotyls was examined. This allowed the parental wild-type response to be easily separated from the mutant response when a populations of 40 individual seedlings was examined for each F3 family. Mock heterozygote populations were generated

from 50 randomly selected mutant seedlings and 150 randomly selected wild type seedlings for each mutant line, and these mock populations were used as a guide for the expected average gravitropic curvature of a heterozygous population. In most cases, the F3 families were easily categorized as showing gravitropism similar to the parental wild-type, the parental mutant line, or an intermediate response that was considered to indicate a heterozygous population.

For the auxin resistant mutant and the *hgr*2 mutant, F3 populations were not available, and segregation analysis was conducted with F2 seedlings. A previously developed technique (Bullen 1992) was used to estimate the frequency of mutant seedlings in an F2 population. Response to gravity was divided into four 90° quadrants in a superimposed Cartesian coordinate system. The parental wild-type response was found to fall almost exclusively into the quadrant that represented 0° to 90° curvature. A population of homozygous mutant seedlings was examined, and the percent of mutant seedlings that fall within the 0° to 90° curvature quadrant was determined. A population of F2 seedlings was examined for gravitropism, and the number of seedlings with a gravitropic response within the other 3 quadrants was determined. Because some mutant seedlings do show curvature within the 0° to 90° range, the number of seedlings outside of this range was considered to represent a percentage of the total number of mutant seedlings. For

an estimate of the total number of mutants within the F2 population, the number of actual seedlings with a response in the 90° to 360° quadrant was divided by the previously determined percentage of mutants in these quadrants. For example, if 60 seedlings are found to have curvature between 90° to 360°, and 50 percent of the mutant population has been shown to fall within this range, these 60 seedlings are estimated to represent 50% of a total mutant population of 120 seedlings.

Starch Content:

Surface sterilized seed were planted on the surface of petri dishes containing *Arabidopsis* mineral media. These seed were placed in the dark at 4° C for four days, and then moved to 23°C under white light for four days to allow the seedlings to grow. These seedlings were then stained with an IKI solution containing 43.4mM KI and 5.7mM I in 0.2N HCl to identify the presence or absence of starch in the root tips (Casper et al 1985). Seedlings were incubated in the dye for approximately 30 minutes or until the presence of starch was indicated by darkly stained black grains in the root. The starchless mutant TC7 was used as a negative control, and was found to stain a very light brown or yellow in the presence of the dye.

Auxin Response:

Arabidopsis mineral media was supplemented with 8g/Liter Agar, and 20g/Liter Sucrose. After autoclaving, 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma)was added in incremental concentrations ranging from 10^{-7}Molar to 10^{-11}Molar (Lincoln et al 1990). Seedlings were surface sterilized as described earlier and plated in a single line on the agar surface. These plates were wrapped in Parafilm and placed in the dark at 4°C for four days to potentiate germination. The plates were then placed on the vertical edge and seeds were allowed to germinate under white light at 23°C and grow along the agar surface. Following two days of seedling growth, root length was measured for all seedlings, and those lines with less root inhibition than the parental wild-type seedlings were considered to be resistant to this treatment.

Ethylene Response:

Seedlings were prepared as described for the gravitropism response assay. After 14 hours of white light treatment to induce germination, the Petri dishes were placed in sealed containers with either air, or 1uL/L ethylene and allowed to grow in darkness for 3 days (Bleeker et al 1988). The addition of ethylene caused a reduction in hypocotyl and root length in the parental wild-type seedlings. Any mutant seedling line exhibiting less growth

reduction than parental wild-type seedlings was considered to be resistant to ethylene-induced stunting.

Genetic Mapping:

The MapPairs kit (Research Genetics) of primer pairs was used for chromosome mapping of the gravitropism mutations. This kit contains simple sequence length polymorphism (SSLP) primers that are able to differentiate Estland, the parental ecotype of the gravitropism mutants, from other ecotypes based on previously mapped co-dominant molecular markers (Bell and Ecker 1994). When a primer set is used in polymerase chain reaction with genomic DNA, a different sized product is amplified from each ecotype and this size difference can be identified through agarose gel electrophoresis. The gravitropism mutants were mapped by crossing a homozygous mutant line to both the Landsburg Erecta and the Columbia ecotypes. Gravitropism was assessed for F3 families from this cross and the distribution of mutant gravitropism was compared to the segregation of the ecotype specific markers to determine linkage.

Two bulked populations were prepared by the combination of 15 F3 families with either the mutant or the wild-type response, and these were used to simplify the initial mapping step. When an SSLP marker is genetically linked to the mutation, the bulked mutant population will display exclusively, or primarily the Estland marker, while the

bulked wild-type population will favor the marker of the other ecotype. Analysis of individual F3 families was used to more accurately determine the chromosome location of the mutation.

Light Sources:

For phototropism experiments, the light source was a projector equipped with a Sylvania 300W ELH tungsten halogen lamp wise with a 450 nm interference filter with a half-band width of 10nm(PTR Optics) The fluence rate was measured with a model LI-185A radiometer (Li-Cor), and the duration of exposure was controlled with a Uniblitz shutter (Vincent Associates).

Results:

Genetic analysis:

Segregation analysis of the gravitropism mutations in the F1 and F3 generation showed that each gravitropism mutant is due to a single locus recessive mutation (Table 1). This was based on an analysis of the ratio of mutant populations to full response populations in the F3 families, and a comparison of this ratio to the expected segregation ratios calculated for a single locus mutation (Dellart 1980). The data collected for 17-2, and 13-8, showed the gravitropism mutation segregation closely resembled the

Table 1. Segregation analysis based on the patterns of inheritance of the gravitropism mutant phenotype in F1, F2, and F3 seedlings.

pollen donor	gravitropism F1 population		phenotypic ratios[a]	single locus model X^2
			F3 populations	
17-2 *agr*	n=34	+[b]	4:9:4	0.009ns
g2 starch deficient	n=40	+	9:16:12	1.163ns
13-8 *hgr*1	n=37	+	6:8:4	0.666ns
			F2 populations	
18-5 *hgr*2	n=26	+	38:109	0.057ns
g101 *aux*	n=10	+	14:28	1.554ns

a. In segregation analysis of the F2 populations and the F3 families, the growth protocol used to examine gravitropism was the one that induced the greatest difference in phenotype between that mutant line and the parental wild-type. Each F3 family was determined to be heterozygous, homozygous for the mutant response, or homozygous for the wild type response based on comparison with parental seedling response, and on similarity to the response of a simulated heterozygous seedling population. Analysis of both mean curvature and standard deviation was sufficient to determine the identity of the F3 families. In analysis of the F2 population, the average curvature and standard deviation in gravitropism was compared to the expected response of a single locus F2 population using a superimposed Cartesian coordinate system (Bullen 1992).

b. The designation of + indicates that the population showed gravitropic curvature within 10 degrees of that exhibited by the wild-type control seedlings under a growth protocol previously found to show difference in gravitropism between the mutant and the parental wild-type.

expected segregation of a monogenic trait. F3 families were not available for g101 or 18-5, but analysis of populations of F2 seedlings showed that each of these mutants is also due to single locus mutation (Table 1).

To determine the number of different gravitropism genes represented in this study, reciprocal cross-pollinations were performed among all mutant lines (Table 2). Eight different mutant lines were used in this study and the phenotype of the F1 seedlings were evaluated to determine if mutants were allelic. The mutant line 18-5 has not been used in as many crosses as other mutant lines because the initial isolate of this mutant showed reduced root growth, and was difficult to characterize. After cross-pollination with the parental wild-type, F2 seedlings with altered gravitropism and unimpaired root growth were identified for this mutant line, and were selected for further characterization.

Five complementation groups of gravitropism mutants were established from the complementation data (Table 3). These include two allelic mutant lines that are altered in response to exogenous auxin (Table 4), that were shown to be allelic to aux1 (Chapter 1), and three mutants shown to be allelic to *agr*1 based on genetic complementation with a previously isolated agr mutant (Pierre Hilson, personal communication). A single starch deficient mutant was identified (Table 4), and two hypocotyl mutants, 13-8 and

Table 2. Complementation data from reciprocal crosses of gravitropism mutants based on the phenotype of the F1 seedlings

male parent \ female parent	g2	g101	13-8	17-2	17-3	18-5	20-14	21-3
g2		wt	wt	wt	wt	-	wt	wt
g101	wt		wt	wt	wt	-	M	wt
13-8	wt	wt		wt	wt	wt	wt	wt
17-2	wt	wt	wt		M	-	wt	M
17-3	wt	wt	wt	M		wt	wt	M
18-5	wt	wt	wt	-	-		-	wt
20-14	wt	M	wt	wt	wt	-		wt
21-3	wt	wt	wt	M	M	-	wt	

Table 3. Complementation groups showing the identification of five different gravitropism mutations.

Complementation Groups				
Group1	Group2	Group3	Group4	Group5
auxin-resistant	*agr*	*hgr*1	*hgr*2	starch deficient
g101	17-2	13-8	18-5	g2
20-14	17-3			
	17-2			
	21-3			

Table 4. Characterization data for mutant starch content, phototropism, and response to exogenous auxin.

Mutant Characterization

Plant Line	Starch Detection	Auxin induced root stunting	five-pulse blue light phototropism
Wild-Type	+	+	X=30
g2	-	+	X=28
g101(*aux*)	+	resistant	X=30
17-2(*agr*)	+	+	
17-3(*agr*)	+	+	X=28
13-8(*hgr*1)	+	+	X=38
18-5(*hgr*2)	+	+	X=53

18-5 that do not appear to be similar to previously isolated mutants. These hypocotyl mutants are referred to as *hgr*1 and *hgr*2 throughout this study.

The SSLP markers were used to establish the position of two gravitropism mutants on the *Arabidopsis thaliana* genetic map on the basis of two groups of bulked F3 families composed of homozygous mutant lines or homozygous wild-type response lines. It was found that altered gravitropism in the ethylene-resistant mutant is linked to markers on the lower arm of chromosome 5 (Figure 1), which is the established location of *agr*1 (Pierre Hilson, personal communication) . The bulked populations of mutant *hgr*1 showed linkage between the gravitropism mutant phenotype and the ecotype specific markers on the lower arm of chromosome 1 (Figure 1).

Phenotypic analysis:

Members of the five complementation groups were analyzed to determine starch content (Table 4 and Figure 2), and all were found to possess high levels of starch with the exception of g2. This mutant response to the potassium iodine stain was similar to the response found in the TC7 starchless control plants, which may indicate severe alterations in starch content in the g2 mutant line, although this assay is not sufficiently sensitive for use in the accurate determination of relative starch content.

Figure 1. Diagram of the genetic map for *Arabidopsis* (Bell and Ecker 1994) with SSLP markers in bold, and marks to distinguish the relative map positions of *hgr*1, and the *arg* mutant

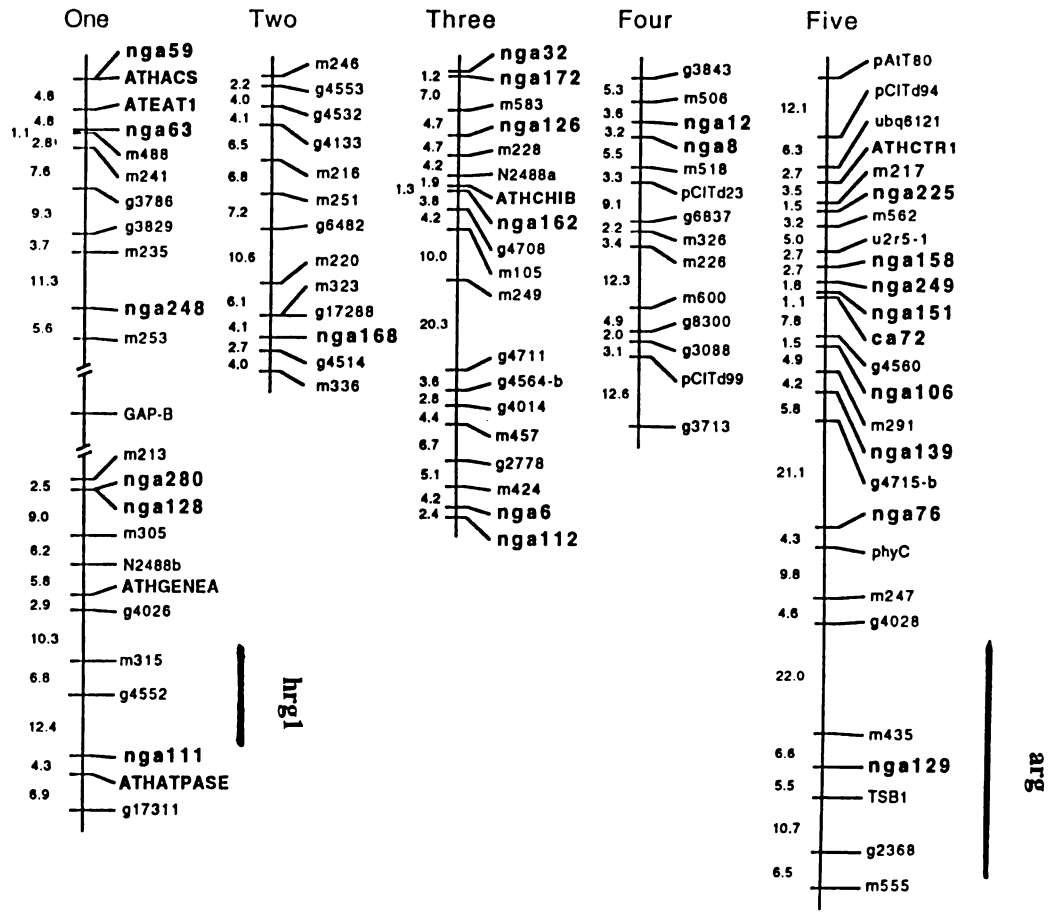
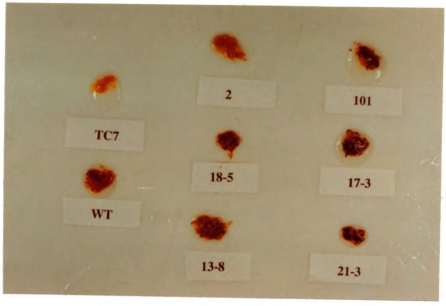


Figure 2. Whole seedlings of gravitropism mutant lines and the wild-type parental line stained for starch with potassium iodine.



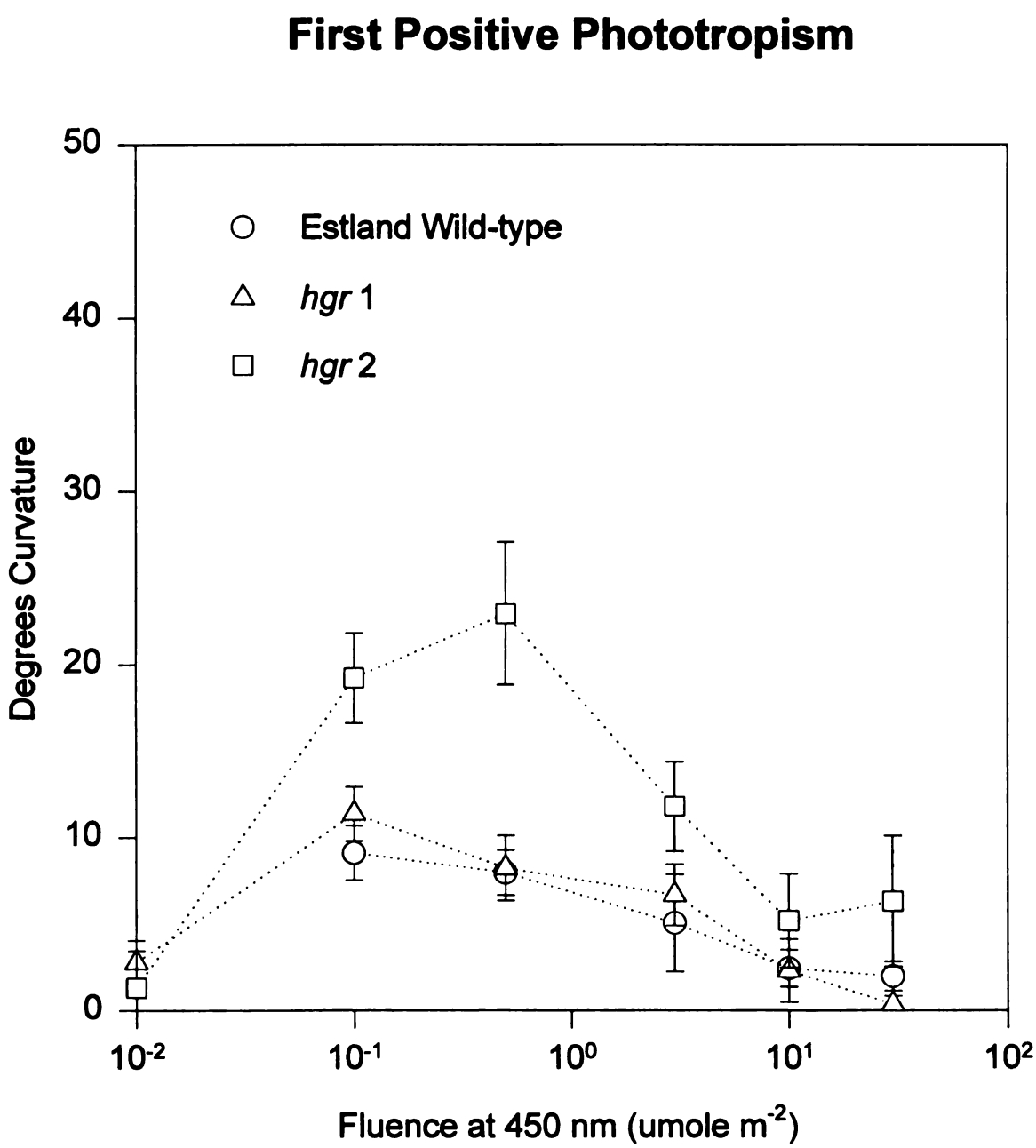
TC7 is an allele of pgm a phosphoglucose mutase mutant (Casper et al 1985).
The mutant lines 2 and 101 are designated g2 and g101 throughout the text.

Analysis of root inhibition in the presence of high concentrations of exogenous auxin showed root stunting for all seedlings with the exception of g101, which showed root growth similar to the *axr*1 negative control seedlings (Table 4).

Phototropism induced by blue light was examined, and all mutants were able to respond to this stimulus. Both *hgr*1 and *hgr*2 showed significantly increased curvature under the multiple pulse light treatment (P=0.05), when t-tests were used to compare this response to the parental wild-type response (Table 4). In the fluence response curve, the *hgr*2 mutant showed significantly greater curvature in a t-test where P=0.05, than the parental wild-type and the hgr1 mutant at all fluences that exceeded the threshold (Figure 3). As the threshold of the response appears unchanged in this mutant, it is likely that perception is not altered. Analysis of this fluence response curve suggests that this mutation affects some element of signal transduction or differential growth.

As phototropism of *hgr*1 is significantly different from the parental wild-type response in five-pulse blue light phototropism, but not significantly different from the wild-type in the range of first positive phototropism (t-test P=0.05), further examination of second positive phototropism, or other multiple pulse treatments would be necessary to establish a consistant phototropism phenotype

Figure 3. Curvature of *hgr*1, *hgr*2, and wild-type seedlings given single pulse blue light in the fluence range of first positive phototropism.



The data points represent average curvature. The error bars represent standard deviation.

for this mutant.

The response of root and hypocotyl to high concentrations of ethylene (Figure 4) was assessed for hypocotyl gravitropism mutants *hgr*1 and *hgr*2. The *hgr*1 mutant and the *hgr*2 mutant exhibited full stunting of root and hypocotyl in response to this treatment. The *hgr*2 mutant was found to develop an unusual opening of the cotyledons in the presence of ethylene (Figure 4). Close observation of the control *hgr*2 seedlings shows that these mutant seedlings also have a slight reduction in hook curvature in the absence of ethylene. This suggests a similarity to the previously isolated hookless mutant (Roman et al 1995), although an altered gravity response has not been observed in these mutants. Change in cotyledon hook development is also similar to the aberrant development of cotyledons observed in *cop*4 (Hou et al 1993), although little phenotypic characterization has been described for this mutant.

For both mutants, the mean curvature and the standard deviation of the response were significantly different from that found in wild-type seedlings in both root and hypocotyl under both growth protocols (Table 5). The *hgr*2 mutant exhibits nearly random hypocotyl orientation in both growth protocols (Figure 5 and Table 5), when compared to a hypothetical phenotype of complete random orientation. Such random seedlings would be expected to show a mean curvature

Figure 4. Seedling growth response of mutants *hgr1*, *hgr2*, *etr1*, and the wild-type parental line grown in the presence and absence of ethylene.

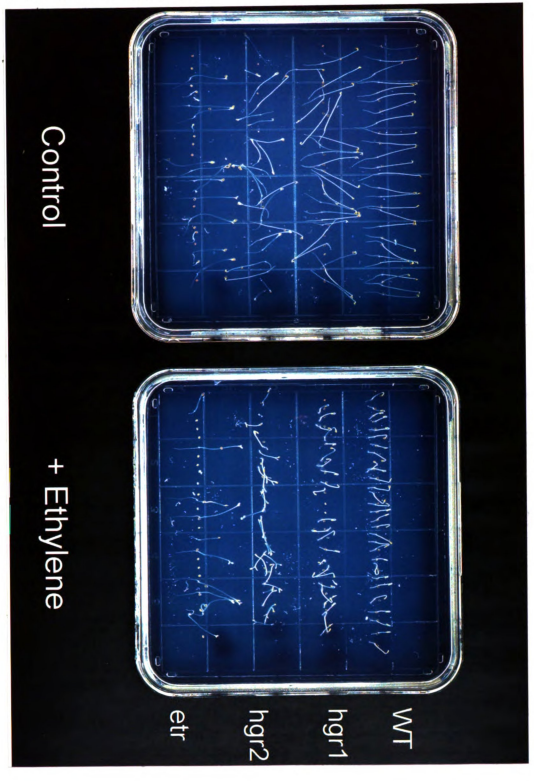
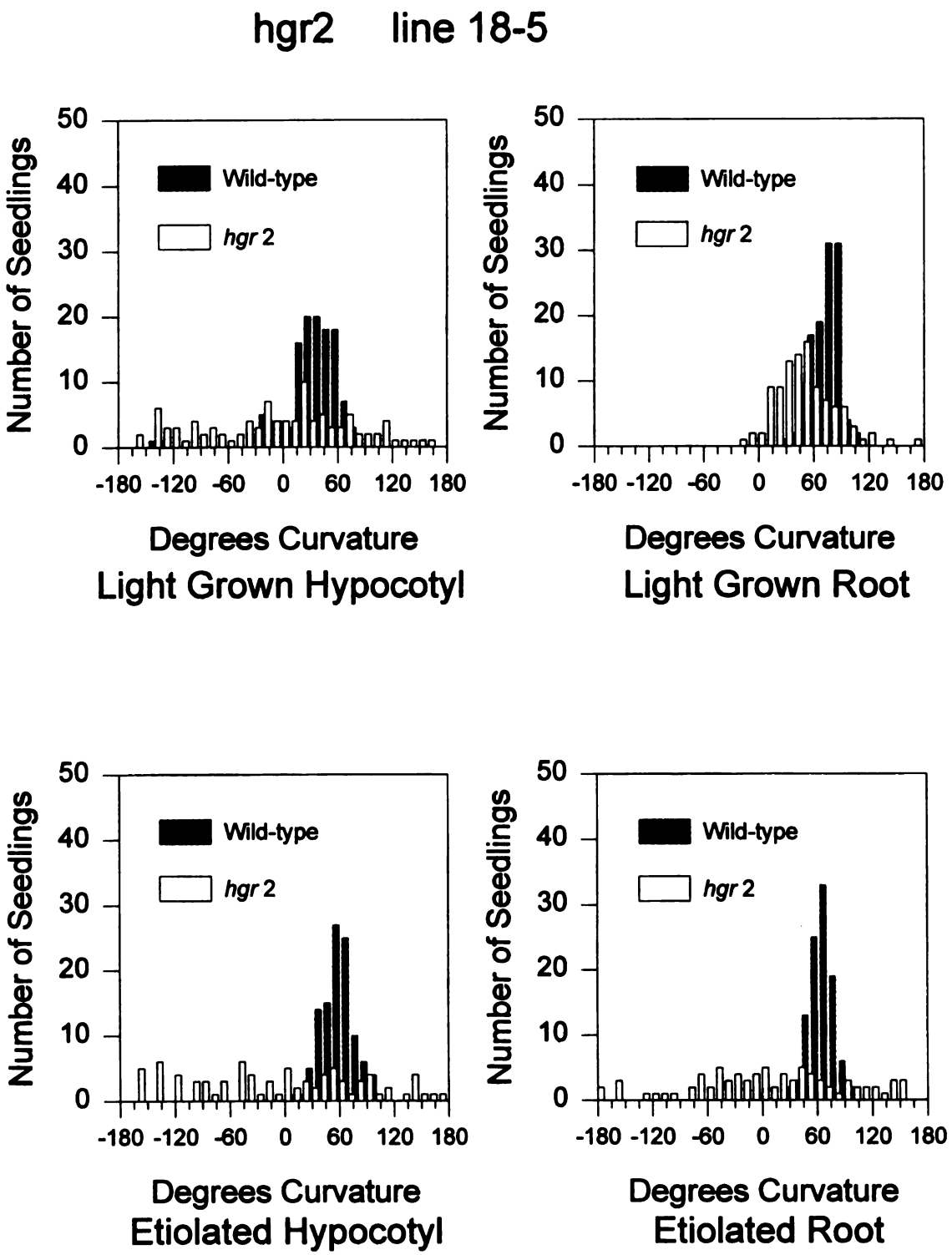


Table 5. Mean curvature and variance for gravitropism for each plant line. Mean values of mutant responses were compared to the wild-type response through t-tests, variances were compared by F tests for homogeneity of variance.

	wild-type	*hgr*1	*hgr*2
light-grown root	X 80 SD 14	X 61** SD 23**	X 35** SD 35**
etiolated root	X 71 SD 15	X 49** SD 43**	X 12** SD 83**
light-grown hypocotyl	X 37 SD 33	X 14** SD 52**	X -4** SD 83**
etiolated hypocotyl	X 65 SD 17	X 22** SD 55**	X -4** SD 91**

*, ** Indicate significant difference at P<= 0.05, and P<= 0.01 respectively.

Figure 5. Graphic presentation of root and hypocotyl gravitropism for mutant *hgr2* under the two growth protocols



of zero degrees with a high standard deviation. Roots of *hgr*2 are more responsive to gravity in light-grown seedlings than in etiolated seedlings (Figure 5 and Table 5). Both roots and hypocotyls of *hgr*1 are less responsive to gravity than the parental wild-type, but show some gravity directed orientation under both growth protocols (Figure 6 and Table 5).

The hypocotyls of *hgr*1 and *hgr*2 do not show significant changes in mean curvature or standard deviation when gravitropism is compared between the two growth protocols, although the roots of both *hgr* mutants show significant increase in variance in gravitropism under the light growth protocol (Table 6). Significantly greater curvature (P=0.01) in the light grown roots of *hgr*1, and the light grown roots of hgr2 show a smaller but significant (P=0.05) increase in curvature over etiolated roots (Table 6).

Analysis of other hypocotyl mutants:

Mutants altered in inflorescence gravitropism have been identified, and *sgr*2 and *sgr*3 show both altered hypocotyl gravitropism and altered inflorescence gravitropism. These mutants are not likely to be allelic to *hgr*1, and *hgr*2, as neither hypocotyl mutant in this study exhibits the altered inflorescence gravitropism, or the altered lateral inflorescence gravitropism described for *sgr* mutants (Fukaki et al 1996). Both hypocotyl gravitropism mutants

Figure 6. Graphic presentation of root and hypocotyl gravitropism for mutant *hgr*1 under the two growth protocols

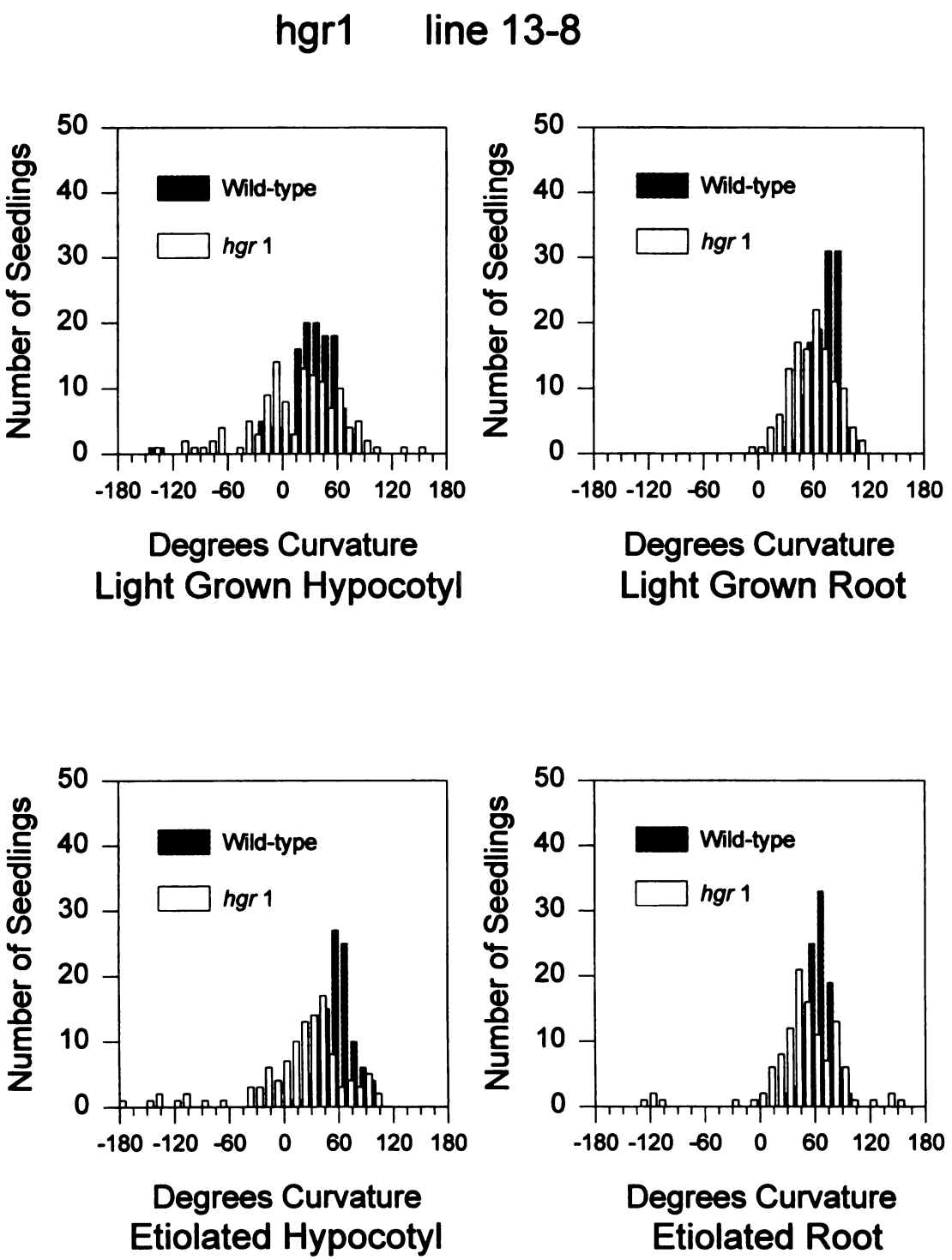


Table 6. Test of effect of light and dark growth protocols on gravitropism response of wild-type and gravitropism mutants. Mean values of mutant responses under the two protocols were compared through t-tests, variances were compared by F tests for homogeneity of variance.

		*hgr*1		*hgr*2	
		light	dark	light	dark
root	X	61	49**	35	12*
	SD	23	43**	35	83**
hypocotyl	X	14	22	-4	-4
	SD	52	55	83	91

*, ** Indicate significant difference at P<= 0.5, and P<= 0.1 respectively

identified in this study showed a gravitropic response of inflorescence stems, which indicate that gravitropism has independent genetic elements active in hypocotyl gravitropism and stem gravitropism.

The *cop*4 mutant is a hypocotyl gravitropism mutant that has not been well characterized. The phenotype of *cop*4 is somewhat similar to *hgr*2 in aberrant hook formation, and complementation analysis would be necessary to show that these mutants are not allelic.

Gravitropism phenotype consistency:

Three repetitions of etiolated hypocotyl gravitropism experiments were performed for wild-type seedlings, and for mutants *hgr*1 and *hgr*2 (Table 7). These were prepared according to the etiolated-growth protocol, but each replicate was initiated on a different day, placed under a different light source to potentiate germination, and stored in a different area of the dark room, to determine the effect of variation in environmental conditions on gravitropism. In a student's t-test (P=0.05), it was found that there was no significant difference between mean curvature of each plant line in the three experiments. For each experiment, a comparison of the parental wild-type gravitropism to that of each mutant, showed significant difference (P=0.05) between mean curvature (Table 7). The mean curvature and standard deviation of gravitropism in

Table 7. Mean curvature and standard deviation for etiolated hypocotyl gravitropism from three experiments using seedlings of *hgr*1, *hgr*2, and the parental wild-type. The t-test was used to compare mean curvature of each mutant to the mean wild-type curvature for each repetition.

	repetition 1	repetition 2	repetition 3
Wild-type	X 49 SD 20 n 78	X 45 SD 19 n 74	X 49 SD 26 n 73
*hgr*1	X 35* SD 38 n 82	X 35* SD 30 n 78	X 28* SD 37 n 77
*hgr*2	X 21* SD 94 n 60	X 19* SD 84 n 63	X 13* SD 76 n 63

* indicates significant difference between the wild-type and the mutant curvature at P<=0.05.
No significant difference was found between mean curvature of each plant line in the three repititions P<=0.05.

these experiments were not identical to the values presented in figures 5 and 6 for either the wild-type seedlings or the mutant seedlings. This suggests that changes in gravitropism are not the result of variations in growth conditions, but may be the result of differences in seed age, or variation between seed lots.

Discussion:

Comparison of Gravitropism under two growth protocols:

Both hypocotyl gravitropism mutants are capable of response to gravity under some conditions. The *hgr2* seedling roots exhibit a distinct light-mediated increase in seedling uniformity, a characteristic measured by changes in the standard deviation of mean curvature (Table 6). This change in orientation uniformity is not exhibited by the wild-type roots, and is difficult to interpret. Perhaps *hgr2* is altered in a genetic component of growth that contributes to both uniform seedling orientation and gravitropism, or seedling orientation may be a general function of gravity response. This suggests a model of seedling response where control elements directly act to increase or reduce random orientation. Change in orientation uniformity for a population could represent a balance between polarity of individual seedlings, and a common growth direction induced by phototropism or gravitropism. The light-mediated increase in random seedling orientation seen in wild-type hypocotyls (Robson and Smith 1996) seems to imply that the uniformity of a seedling population can be directly controlled.

Interaction of Phototropism and Gravitropism:

Only the *hgr2* gravitropism mutant shows consistant alteration in phototropism (Figure 3 and Table 4). Although

a number of phototropism mutants have been identified that are also altered in gravitropism (Khurana and Poff 1989), the majority of gravitropism mutants in this study were not affected in phototropism. As the *hgr*2 mutant is impaired in gravitropism but enhanced in phototropic curvature, there must be a genetic component necessary for gravitropism that is antagonistic to phototropism. This does not show that intact gravitropism generally reduces phototropism, as this phenomenon is not observed for the starch-deficient mutant g2 (Chapter 1), and *hgr*1 shows no change in first positive phototropism (Figure 3). Although this conclusion is based on a simple model for interaction of gravitropism and phototropism, where gravity-induced hypocotyl straightening directly acts to reduce light-induced hypocotyl bending. Thus, first positive phototropism may not develop sufficient curvature to effectively observe slight changes in gravity response.

Since the only gravitropism mutant in this collection that exhibits increased phototropism also shows an unusual response to ethylene, this study provides evidence that some aspect of ethylene biosynthesis or response has an antagonistic role in phototropism, and is necessary for gravitropism. This may not be a general effect, as few ethylene-resistant mutants have been reported to be altered in gravitropism. However, an alteration in the photogravitropic balance might not produce an obvious

phenotype, but might only be visible under conditions where opposing light and gravity vectors are examined. Most ethylene-response mutants have not been examined for phototropism, and if ethylene is active in establishing a balance between opposing growth directions induced by different tropisms, it would be expected that some ethylene insensitive mutants would show increased phototropism.

The ethylene-resistant mutant isolated in this study was found to show unaltered phototropism (Chapter 1). This shows that not all changes in ethylene response affect phototropism, but this mutant not the ideal system for the study of this response as it is resistant to ethylene induced root stunting and not hypocotyl stunting (data not shown). Analysis of a mutant altered in hypocotyl response to ethylene would be necessary to determine if phototropism is affected only in a subset of ethylene-response mutants, or is altered in all ethylene hypocotyl-response mutants.

Previous studies have observed that phototropism and gravitropism combine to determine final seedling orientation, which represents a photogravitropic balance (Hart and Macdonald 1981). When seedlings are irradiated and placed on a clinostat to eliminate directional gravity stimuli, it was found that phototropic curvature developed more slowly (Nick and Schafer 1988). A relation between clinostat treatment and ethylene levels has been shown for tomato plants, where ethylene production doubles within an

hour of clinostat initiation (Leather et al 1972). In addition, light treatment has been shown to decrease ethylene production and decrease tissue sensitivity to ethylene (Goeschl et al 1967), while continuous red light increases phototropic curvature (Nick and Schafer 1988).

Although this is not conclusive, all these experiments seem to support a role for ethylene in the inhibition of phototropism. The isolation of a gravitropism mutant that shows increased phototropism, and an unusual hook response to ethylene provides further evidence that ethylene has an antagonistic role in phototropism, and implies that some function of ethylene response is necessary for gravitropism.

Hook formation has been shown to be the result of differential transport of auxin, and auxin-regulated asymmetric ethylene biosynthesis (Schwark and Bopp 1993). Both the exaggerated hook curvature formed by ethylene treated seedlings, and the hook formed by untreated seedlings are the result of hormone balances within the hook tissue (Guzman and Ecker 1990). The unusual response of *hgr*2 to ethylene treatment suggests that mutant is altered in a hormone regulated component of differential growth. However, as *hgr*2 exhibits growth inhibition by ethylene and auxin similar to the wild-type response, elements of hormone-regulated differential growth appear to be genetically separate from hormone-regulated growth.

Further work would be necessary to elucidate a specific

role for ethylene in phototropism and gravitropism, and to determine if ethylene has a role in mediating conflicting signals for differential growth direction. Some obvious studies could be initiated to understand this phenomenon. In a system where an intermediate growth response results from opposing directions of light and gravity stimulation, manipulation of ethylene levels might be expected to alter the intermediate seedling orientation. As ethylene decreases auxin transport (Lyon 1970), seedlings treated with auxin transport inhibitors might be expected to show increased phototropism, or changes in the photogravitopic balance. Studies of phototropism in ethylene-insensitive or ethylene-resistant mutants would determine if increased phototropism is limited to *hgr*2, if it extends to other hook development mutants, or if it is a general feature of ethylene-response mutants.

This study has identified two interesting hypocotyl gravitropism mutants. The identification of *hgr*1, a mutant not altered in response to exogenous hormones or previously identified traits associated with gravitropism, shows that direct screens for gravitropism can be used to find novel genetic components of gravity response. The identification of *hgr*2, and the study of phototropism in these mutants has suggested a role for ethylene in mediating seedling directional response to gravity and light. As the *hgr*2 mutants show hypocotyl and root stunting in response to

ethylene, a role for ethylene in differential growth appears to be genetically distinct from the role of ethylene in growth regulation.

References:

Bell CJ, Ecker JR (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. Genomics 19:137-144

Bleeker AB, Estelle MA, Somerville S, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241:1086-1089

Bullen B (1992) Development of a genetic system for the study of gravitropism in *Arabidopsis thaliana*. PhD Dissertation

Casper T, Huber SC, Somerville C (1985) Alterations in growth photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucosemutase activity. Plant Physiology 79:11-17

Dellart (1980) Segregation Frequency. Theoretical and Applied Genetics 57:137-143

Finkelstein RR, Zeevaart JAD (1994) Gibberellins and Abscisic Acid. *IN Arabidopsis*. EM Meyerowitz and CR Somerville (eds) Cold Spring Harbor Laboratory Press

Fukaki H, Fujisawa H, Tasaka M (1996) SGR1, SGR2, and SGR3: Novel genetic loci involved in shoot gravitropism in *Arabidopsis thaliana*. Plant Physiology 110:945-955

Goeschl JD, Pratt HK, Bonner BE (1967) An effect of light on the production of ethylene and the growth of the plumular portion of etiolated pea seedlings. Plant physiology 42:1077-1080

Guzman P, Ecker JR (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. The Plant Cell 2:513-523

Hart JW, Macdonald IR (1981) Phototropism and geotropism in hypocotyls of cress (*Lepidium sativum* L.) Plant, Cell, and Environment 4:197-201

Haughn GW, Somerville CR (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Molecular and General Genetics 204:430-434

Hobbie L, Estelle M (1995) The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. The Plant Journal 7:211-220

Hou Y, von Arnim AG, Deng X-W (1993) A new class of

Arabidopsis constitutive photomorphogenic genes involved in regulating cotyledon development. The Plant Cell 5:329-339

Juniper BE (1976) Geotropism. Annual Review of Plant Physiology 27:385-406

Khurana JP, Poff KL (1989) Mutants of *Arabidopsis* with altered phototropism. Planta 178:400-406

Kiss JZ, Wright JB, Casper T (1996) Gravitropism in roots of intermediate starch mutants of *Arabidopsis*. Physiologia Plantarum 97:237-244

Leather GR, Gorrence LE, Abeles FB (1972) Increased ethylene production during clinostat experiments may cause leaf epinasty. Plant Physiology 49:183-186

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr*1 mutants of *Arabidopsis*. The Plant Cell 2:1071-1080

Lyon CH (1970) Ethylene inhibition of auxin transport by gravity in leaves. Plant Physiology 45:644-646

Moore R, Smith JD (1985) Graviresponsiveness and abscisic acid content of roots of carotenoid deficient mutants of *Zea mays*. Planta 164:126-128

Nick P, Schafer E (1988) Interaction of gravi- and phototropic stimulation in the response of maize (*Zea mays* L.) coleoptiles. Planta 173:213-220

Normanly J, Slovin JP, Cohen JD (1995) Rethinking auxin biosynthesis and metabolism. Plant Physiology 107:323-329

Pickard BG (1985) Roles of hormones, protons and calcium in geotropism. *IN* Encyclopedia of Plant Physiology, New Series Volume 11 RP Pharis & DM Reid (eds) pp. 193-281

Robson PRH, Smith H (1996) Genetic and transgenic evidence that phytochromes A and B act to modulate the gravitropic orientation of *Arabidopsis* hypocotyls. Plant Physiology 119:211-216

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR (1995) Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: Five novel mutant loci integrated into a stress response pathway. Genetics 139:1393-1409

Schwark A, Bopp M (1993) Interaction of ethylene and auxin in the regulation of hook growth II. The role for ethylene in different growing regions of the hypocotyl hook of

Phaseolus vulgaris Journal of Plant Physiology 142:585-592

Simmons C, Migliaccio F, Masson P, Caspar T, Soll D (1995) A novel root gravitropism mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. Physiology Plantarum 93:790-798

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens* iaaM and iaaH genes display altered growth an Indoleacetic Acid metabolism. Plant Physiology 99:1062-1069

Steinitz B, Poff KL (1986) A single positive phototropic response induced with pulsed light in hypocotyls of *Arabidopsis thaliana* seedlings. Planta 168:305-315

Went FW, Thimann KV (1937) *IN* Phytohormones. NY Macmillan Volume 294 pp154-157

Wilkins MB (1984) Gravitropism. *IN* Advanced Plant Physiology. MB Wilkins (ed) Pitman. London pp163-185

Chapter III

Gene Expression in Gravitropism Mutants

Abstract:

Two gravitropism mutant lines, *hgr*1 and an *arg* allele, were used in an expression based study to identify genes maintained in mutant seedlings at different levels of transcript abundance from levels in the wild-type parental line. Plant tissue was harvested from a seedling growth stage known to be responsive to gravity in both root and hypocotyl. Prior to harvest, seedlings were given inductive changes in gravity vector to alter expression of potential gravitropism response genes. Several transcripts were identified that showed greater accumulation in either wild-type parental seedlings or mutant seedlings. Based on sequence analysis one transcript was found to have strong identity to Catalase 3, while another transcript showed identity to a Senescence Associated Protein. Both these gene products, could be elements of signal transduction or differential growth, and some proposed roles for these genes in gravitropim are discussed. Although the data collected in this study are preliminary, they suggest that this protocol can be used to identify gene regulation differences between mutants and the parental wild-type in a complicated response system such as gravitropism.

Introduction:

The establishment of patterns of plant development generally corresponds to distinct patterns of gene expression. Some genes show an increase or decrease in transcript accumulation during senescence (Kawakami and Watanabi 1988), while other genes show specific changes in regulation during the induction of flowering (Coupland 1995). The identification of genes associated with a plant growth event can lead to an understanding of the program of genetic changes necessary to establish that event.

Plant growth hormones are powerful regulatory factors of plant development and have been shown to have an effect on gene expression. A number of genes that are directly regulated by abscisic acid have been identified and characterized (Hall et al 1993). Increased accumulation of calmodulin RNA is induced by gibberellic acid, although when added in combination with abscisic acid, gene expression remains at basal levels (Schuurink et al 1996). Cytokinin can act to stimulate accumulation of specific mRNAs (Dominov et al 1992) or can act to reduce transcript levels of genes up-regulated by other hormones (Chaloupkova and Smart 1994). Clearly gene expression can be induced and repressed by various growth hormones, and the identification of antagonistic hormone promoter elements within a single gene (Skriver et al 1991) may show a mechanism by which gene regulation could be responsive to hormone ratios similar to

the way that leaf movement has been found to be responsive to red:far red light ratios (Robson et al 1993).

Auxin is the plant growth hormone that has been most directly linked to the differential growth necessary for gravitropism. A large gene family that is regulated by auxin has been identified and a number of these have been shown to have a link to gravitropism (Abel & Theologis 1966). The first direct association between auxin induced gene expression and gravitropism was found with the SAUR gene family (McClure and Guilfoyle 1987). Examination of the RNA from four of the SAUR genes found that transcript relocalization could be induced by changes in gravity direction, and that transcript accumulation was greatest in the more rapidly growing regions throughout curvature development (McClure and Guilfoyle 1989). The PS-IAA6 gene was also shown to be associated with differential growth, as the promoter region of this gene causes a reporter gene to localize to the region of rapid growth during gravitropism (Wong et al 1996)

Two mutant lines have been used in this study to identify genes that show altered expression patterns in gravitropism mutants. These mutants were primarily selected on the basis of their hormone response phenotype. The *hgr*1 mutant is similar to the wild-type parental line in response to all hormones, and the *agr* mutant shows resistance to ethylene-induced root stunting but is not altered in response to other hormones (chap 2). The use of mutants that are not altered in

auxin perception or response should increase the probablity of identifying changes in gene regulation that are not directly caused by auxin, although some auxin-regulated genes may also be affected by these gravitropism mutations.

Materials & Methods:

Gravitropism mutants used in this study were from F3 generation lines that had been subjected to one genetic cross with the wild-type parental line to reduce the number of unlinked mutations (data not shown). To generate tissue for DNA isolation, seed were surface sterilized by soaking for 20 minutes in 30% (v/v) commercial bleach (5.25% Sodium Hypochlorite by weight) (Patterson Laboratories, Inc.) and 0.002% (v/v) Triton X-100 (Sigma Chemical Co) followed by five rinses with sterile double distilled water. Seeds were sown at a high density on the surface of a Petri dish filled with 1%(w/v) Bacto-Agar (Difco Laboratories) in an *Arabidopsis* nutrient salts solution (Haughn & Somerville 1986) The petri dishes used were square gridded 100x100x15 mm^3 integrid Petri dishes (Becton Dickinson Labware) which allowed for easy vertical placement of the dishes and accurate 90° rotation. Following planting, the dishes were wrapped in Parafilm (American Can Company) and placed in darkness for 4 days at 4° Celsius. The plates were then placed horizontally under white light for 15 hours at 23°C to induce germination. This time was selected to produce early germination events in 50% of the

seeds of both the wild type and the mutant lines. The plates with germinating seeds were then placed vertically in darkness for 14 hours and the seedlings were allowed to grow along the surface of the agar. The plates were then rotated 90° and curvature was allowed to develop for 10 hours. The plates were then rotated an additional 90° (180° from the original orientation) and maintained in that orientation for two hours. This treatment was intended to enhance expression of genes involved in early response to gravity, as well as genes associated with development of curvature. All manipulation of developing seedlings following germination and prior to harvest occurred in physiological darkness. At the completion of the seedling growth, seedlings had become a dense "lawn" on the surface of the plate and could be easily harvested by peeling the mat of tissue from the agar. The final harvest of seedlings occurred under green safe light, and tissue was immediately frozen in liquid nitrogen and stored at -80°C until used for RNA isolation.

Isolation of Nucleic Acids:

Total RNA was isolated as described by (Ausubel et al 1995) with a single modification. Tissue was ground with Liquid Nitrogen with a mortar and pestle instead of in a polytron. Separate populations of Poly(A)$^+$ RNA were isolated from the total RNA from mutants and wild type using a mini-oligo (dT) spin kit(Five Prime Three Prime) according to the

manufacturer's instructions. The generation of cDNA from the Wild Type Poly(A)$^+$ RNA pool was accomplished using Reverse Transcriptase Superscript (Gibco BRL). Following enzymatic generation of cDNA, all contaminating RNA was destroyed using heat and high salt to prevent interference in the later steps of the subtraction (Hampson et al 1992).

Preparation of Subtraction Products:

Subtraction was performed according to Hampson et al 1992. Two sequential rounds of hybridization were induced with 500ng of WT cDNA under high salt conditions and high temperatures for 20 hours. In each round 10 ug of mutant Poly(A)$^+$ RNA was added to the initial population of wild type cDNA. The cross linking chemical diaziridinytl-1, 4-bezoquinue was added to induce covalent bond formation between GC pairs in the cDNA-RNA hybrid molecules. The remaining single stranded cDNA population is enriched for messages unique to the wild type that cannot hybridize to mutant RNA and will not be affected by the chemical crosslinking treatment. The subtraction products were labeled with ^{32}P dCTP (Amersham) using the T7 DNA Polymerase Sequenase II (US Biochemicals) which does not use RNA as a template for DNA synthesis. Two independent subtractions were performed using RNA from each mutant. Following the subtraction, these two pools were combined to generate a single sample enriched in genes expressed more highly in the Wild-type than in one or

both mutants. Half of the combined subtraction products were used to probe a cDNA phage library from the *Arabidopsis* sequencing facility in the lambda zip lox vector (Gibco BRL), while the other half were reserved for a secondary screen of positive plaques.

Screen of Isolated Clones:

About 60,000 plaques were screened and positive plaques were purified according to standard procedures (Ausebel et al 1995). The clones isolated in the primary screen were auto-excised to generate plasmids containing the original cDNA insert. These plasmids were then grown in cultures to allow isolation of plasmid DNA using the boiling mini-prep (Ausebel et al 1995). The isolated plasmid DNA was digested with SAU3A and HINDIII (Gibco BRL) to release the cDNA insert, and southern blots of these samples were prepared on Hibond N+ Membrane (Amersham) according to the manufacture's direction. These southern blots were probed with the other half of the subtraction products used in the secondary screen to confirm that the cDNAs were able to hybridize with the probe. Clones identified in this screen were then used in a tertiary screen in which cDNA from each mutants or the wild type was used to probe one of 3 identical slot blots of total plasmid DNA. Four plasmids out of 16 total plasmids found to show increased hybridization to one or more of the three probes, are discussed in this report.

Characterization of Differentially Regulated Transcripts:

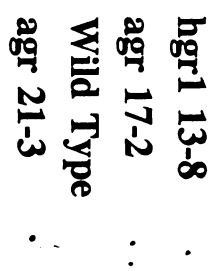
Insert DNA was isolated from each digested plasmid with NuSieve GTC agarose gel separation (FMC BioProducts), and used to probe northern blots of total RNA from each plant line prepared according to standard procedure (Ausebel et al 1995). Tissue for RNA isolation was obtained from mutant and wild type lines subjected to the growth protocol described for the subtraction. No internal control was used for these northern blots, and analysis of loading consistancy was limited to comparison of fluorescence intensity when ethidium bromide stained gels were observed under ultraviolet light.

Sequencing of the 5'end for four of the inserts associated with differentially regulated genes was conducted at the Plant Research Laboratory sequencing facility using the ABI Catalyst 800 (Applied Biosystems) for taq cycle sequencing and the ABI373A sequencer for analysis of the products. Sequence identity of these cDNAs to previously sequenced genes was established using a BLAST program at NCBI (National Center for Biotechnology Information) to evaluate sequence comparison.

Results:

Several cDNA clones were identified based on visible differences in accumulation of their associated RNAs. Transcript 101 was found to be at higher levels in the wild type than in either mutant (Figure 1). Transcript 300 was

Figure 1. Autoradiograph of a northern blot of total RNA from the mutants and the wild-type parental line probed with labled transcript 101



found to be at higher levels in both mutants than in the wild type (Figure 2). Comparison of 21-3 and 17-2 shows that this transcript is more abundant in 17-2 than in 21-3. This is surprising as both are alleles of *agr*, and exhibit a similar gravitropism phenotype. Futher analysis would be necessary to determine if this represents differences in gene regulation between the two alleles, or if this difference is the result of technical error. In all cases sequence analysis was used to determine a possible function for the gene based on sequence identity to previously studied genes. Transcript 101 was found to have over 95% sequence identity to the Catalase 3 gene from *Arabidopsis thaliana* (Figure 3). Transcript 300 was found to have very high sequence identity to a senescence associated protein also from *A. thaliana* (Figure 4). Additional cDNAs were also analyzed, and a transcript with identity to a 14-3-3 protein (data not shown), and one with sequence identity to polygalacturonidase gene (data not shown), are discussed briefly as general examples of the types of genes identified using this subtraction protocol.

Figure 2. Autoradiograph of a northern blot of total RNA from the mutants and the wild-type parental line probed with labled transcript 300

Figure 3. Sequence data for Transcript 101 showing similarity to Catalase 3

cDNA Insert 101

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AGGTCCGGTTCTTCTTGAGGATTATCATTTGATCGAGAAGGTTGCTAA
||||||||||||||||||||||||||||||||||||||||||||||||
AGGTCCGGTTCTTCTTGAGGATTATCATTTGATCGAGAAGGTTGCTAA

TTTCACCAGAGAGAGGATCCCTGAGAGAGTGGTTCATGCTAGAGGAAT
||||||||||||||||||||||||||||||||||||||||||||||||
TTTCACCAGAGAGAGGATCCCTGAGAGAGTGGTTCATGCTAGAGGAAT

CAGTGCTAAGGGTTTCTTTGAAGTCACCCATGACATTTCAAACCTCAC
||||||||||||||||||||||||||||||||||||||||||||||||
CAGTGCTAAGGGTTTCTTTGAAGTCACCCATGACATTTCAAACCTCAC

TTGTGCTGATTTTCTCAGAGCCCCTGGTGTTCAAACTCCGGTTATTGT
||||||||||||||||||||||||||||||||||||||||||||||||
TTGTGCTGATTTTCTCAGAGCCCCTGGTGTTCAAACTCCGGTTATTGT

CCGTTTCTCCACGGTGTCCACGAACGTGCCAGTCCTGAAA
|||||||||||||||||||||||||||||||||||||||
CCGTTTCTCCACGGTGTCCACGAACGTGCCAGTCCTGAAA
```

Transcript #101 has 97% identity over a 233bp region to an *Arabidopsis* Catalase 3 gene.

Accession number U26944

Figure 4. Sequence data for Transcript 300 showing similarity to a Senescence Associated Protein.

cDNA Insert 300

```
AAAGAAGAAGTGAAAATGGAAACCACTGCTTTTAACACAACATCACGAAT
||||||||||||||||||||||||||||||||||||||||||||||||||
AAAGAAGAAGTGAAAATGGAAACCACTGCTTTTAACACAACATCACGAAT

TGGAAACTGGTCATCGGCTATTTCTCCACCTCTACAAACATGTGGTTCTT
|||||||||||||||||||||||||||||||||||||||||||| ||||
TGGAAACTGGTCATCGGCTATTTCTCCACCTCTACAAACATGTCCATCTT

TCAAGTGCCAATTACCAACACGAAGAGGTGTTATTGTAGCTGATCTTCGA
||||||||||||||||||||||||||||||||||||||||||||||||||
TCAAGTGCCAATTACCAACACGAAGAGGTGTTATTGTAGCTGATCTTCGA

AACTCAAACTTCCGATGGAGGAAAGCAACGACAACAAGCA
|||||||||||||||||||||||||||||||||||||||
AACTCAAACTTCCGATGGAGGAAAGCAACGACAACAAGCA
```

Transcript #300 has 99% identity over a 190bp region to an Arabidopsis senescence associated protein. Accession number U26944

Discussion:

Although a strong indication that the chemical cross-linking subtractive hybridization can be useful in the study of gene regulation, these data are preliminary and further study would be necessary to definitively associate these gene regulation events with gravitropism. As these gravitropism mutants had only been crossed with the wild-type parental line a single time, there is a possibility that additional unlinked mutations are present in some of these mutant lines, and that changes in gene regulation are the result of these other mutations. The use of two independently isolated *agr* alleles was intended as a control, as it is unlikely that the same second site mutation should occur in both lines. Also, the evaluation of gene regulation in three gravitropism mutant lines, is intended to further reduce the probability of transcript changes that are induced by unlinked mutations. As no internal control was used in the analysis of transcript abundance, it is not possible to accurately calculate transcript levels from this study. All data should be considered preliminary indications of gene regulation events that may be associated with gravitropism.

As transcripts identified in this study appear to be similarly altered in two different gravitropism mutants, they are discussed as possible common signal transduction elements. Further analysis would be necessary to specifically associate these transcripts with the mutations in *hgr*1 or *agr*, or to

show that they represent genes that are required for full gravitropism.

Higher levels of steady state RNA for Catalase 3 in the wild-type parent compared to both gravitropism mutants, suggests a role for catalase in gravitropism. There are a number of studies involving the physiological role of catalase that can be used to evaluate possible mechanisms for catalase function. Because catalase is an enzyme that converts hydrogen peroxide to O_2 and water (Boldt and Scandalios 1995), this gene could have a role in plant growth though the manipulation of hydrogen peroxide levels in the cell. Some studies with plant response to pathogens have suggested that hydrogen peroxide could have a role as a secondary messenger involved in signal transduction (Chen et al 1993). In this case, the increased concentration of catalase could lead to increased differential growth if the enzyme was found to be asymmetrically localized. Another model that could be useful in interpretation of the role of catalase in gravitropism is the involvement of hydrogen peroxide in cell hardening (Hohl et al 1995) and specifically in oxidative cross-linking of cell wall structural proteins (Bradley et al 1992). As the wild-type parent has higher levels of catalase than the mutant, this suggests that differential growth could have a component of directed cell wall hardening, as opposed to directed cell wall loosening (Rayle DL, Cleland R 1970) which has previously been proposed as the likely mechanism to

regulate differential growth.

The isolation of a gene with strong sequence identity to a senescence associated protein has a number of possible consequences for understanding the molecular mechanism of gravitropism. This gene has been previously isolated on the basis of higher levels of transcript accumulation in dark treated plants than light treated plants and has been implicated as a possible early step in senescence (Azumi and Watanabe 1990). This growth treatment has been associated with senescence in previous studies, where 72 hours dark exposure was shown to induce rapid leaf senescence (Kawakami and Watanabe 1988). Also this gene has been found to be upregulated by ethylene (Azumi and Watanabe 1990) which has been associated with senescence (Davies and Grierson 1989). Changes in RNA accumulation for this gene could be significant for gravitropism on several levels. First it may suggest that ethylene regulated genes are expressed at higher levels in these mutant plants than in the wild-type parental plants. Also, as the *agr* mutant is ethylene-resistant in the roots, it may be that this aberrant response to ethylene leads to altered regulation of ethylene response genes.

Other genes that have been identified in this study have sequence identity to a polygalacturonidase gene and a gene for a 14-3-3 protein (data not shown). There are possible roles for the polygalacturonidase gene in cell wall softening (Crookes and Grierson 1983) and the 14-3-3 protein in signal

transduction (Ferl 1996). Combined analysis of the genes identified in this study suggest that this procedure is most effective in the identification of components of signal transduction or in cell growth. This may be an inherent limitation of this protocol, as regulatory factors are thought to be present at low abundance, and an initial cDNA pool of 10 ug may be insufficient to represent rare messages. This bias towards more abundant messages may also be the result of a tertiary screen in which total RNA was used to probe a southern blot of plasmid DNA, as this technique favors the detection of highly abundant messages.

Since these genes represent fairly abundant messages, and sequence analysis does not suggest a role for any gene isolated in this study as a transcriptional regulator, it is likely that these genes function as later elements of a transduction pathway. The altered expression of some genes in both of the mutants is additional evidence that these genes are active in common later steps of gravitropism. Further analysis of gene expression in other gravitropism mutants would be useful to determine whether expression changes in gene regulation are an integral component of the later stages of gravitropism.

Identification of these genes has provided some very interesting preliminary data that will be useful in the study of gravitropism. Although the transcripts studied in this research are present at different levels in gravitropism

mutant lines than in the parental wild-type, additional research would be necessary to show that they are required for gravitropism. Study of the changes in gene expression would be useful to determine if differences in transcript abundance are constantly maintained in the three plant lines, or if this difference appears following changes in a gravity vector. An understanding of the temporal regulation of these genes in the wild type would be useful to establish a connection between the regulation of these genes and plant response to gravity. Also the use of gene localization studies could determine if asymmetric gene product distribution is associated with differential growth in the response. If these genes prove to be required for signal transduction or response in gravitropism, they will be useful tools to further explore molecular mechanisms of a plant response pathway that is not well understood.

References:

Abel S, Theologis S (1996) Early genes in auxin action. Plant Physiology 111:9-17

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Sturh LK (1995) Current Protocols in Molecular Biology. John Wiley and Sons INC. USA

Azumi Y, Watanabe A (1991) Evidence for a senescence-associated gene induced by darkness. Plant Physiology 95:577-583

Boldt R, Scandalios JG (1995) Circadian regulation of the Cat3 catalase gene in maize (*Zea mays* L.):Entrainment of the circadian rhythm of Cat3 by different light treatments.

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defense response. Cell 70:21-30

Chaloupkova K, Smart CC (1994) the abscisic acid induction of a novel peroxidase is antagonized by cytokinin in *Spirodela polyrrhiza* L. Plant Physiology 195:497-507

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262:1883-1886

Coupland G (1995) Genetic and environmental control of flowering time in Arabidopsis. Trends in Genetics 11:393-397

Crookes PR, Grierson D (1983) Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in cell wall degradation. Plant Physiology 72:1088-1093

Davies KM, Grierson D (1989) Identification of cDNA clones for tomato (*Lycopersicon esculentum* Mill.) mRNAs that accumulate during fruit ripening and leaf senescence in response to ethylene. Planta 179:73-80

Dominov JA, Stenzler L, Lee S, Schwartz JJ, Leisner S (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. the Plant Cell 4:451-461

Ferl FJ (1996) 14-3-3 proteins and signal transduction. Annual Review of Plant Physiology and Plant Molecular Biology. 47:49-73

Hampson IN, Pope L, Cowling GJ, Dexter MT (1992) Chemical cross linking subtraction (CCLS): a new method fo the

generation of subtractive hybridisation probes

Hohl M, Greiner H, Schopfer P (1995) The cryptic-growth response of maize coleoptiles and its relationship to H_2O_2-dependent cell wall stiffening. Physiologia Plantarum 94:491-498

Hull G, Gaubier P, Delseny M, Casse-Delbart F (1993) Absicisic acid inducible genes and their regulation in higher plants. Current Topics in Molecular Genetics 1:289-305

Kawakami N, Watanabe A, (1988) Change in gene expression in radish cotyledons during dark-induced senescence. Plant Cell Physiology 29:33:42

McClure BA, Guilfoyle T (1987) Charactaerization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Molecular Biology 9:611-623

McClure BA, Guilfoyle T (1989) Rapid redistibution of auxin-regulated RNAs during gravitropism. Science 243:91-93

Rayle DL, Cleland R (1970) Enhancement of wall loosening and elongation by acid solutions. Plant Physiology 46:250-253

Robson PRH, Whitelam GC, Smith H (1993) Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiology 102:1179-1184

Schuurink RD, Chan PV, Jones RL (1996) Modulation of calmodulin mRNA and protein levels in barley aleurone. Plant Physiology 111:371-380

Skriver K, Olsen FL, Rogers JC, Mundy J (1991) Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. Proceedings of the National Academy of Science. 88:7266-7270

Wong LM, Abel S, Shen N, de la Foata M, Mall Y, Theologis A (1996) Differential activiation of the primary auxin response genes, PS-IAA4/5 and PS-IAA6 during early plant development. The Plant Journal 9:587-599

Summary

This work has directly addressed the role of light in gravitropism, through the evaluation of gravitropism mutants under different light treatments. The study of phototropism was also addressed to further analyze the interaction of light and gravity, and to identify mutants altered in both tropisms. Two novel hypocotyl gravitropism mutants have been identified, and further study of these mutants should be useful to increase the genetic understanding of gravitropism.

A starch deficient mutant was isolated that showed a significantly more impaired response to gravity in light grown roots than in etiolated roots. The parental wild-type shows increased gravitropic curvature under light, as does a previously isolated starch deficient mutant. Thus, although photo-conditional gravitropism varies between starch deficient mutants, light modulated-gravitropism appears to be a common feature.

Two hormone-response mutants were not shown to respond to variation in light conditions by a change in gravitropism, and it is proposed that some element of hormone response could be an intermediate step in light regulated change of gravitropism.

Two novel hypocotyl gravitropism mutants, *hgr*1 and *hgr*2, were identified and found to show no change in response to hormone induced growth stunting of hypocotyl or root. Study of *hgr*2 showed an alteration of cotyledon hook

formation in the presence of ethylene, and it is suggested that ethylene could play a role in differential growth that is distinct from the role of ethylene in direct growth regulation.

Phototropism was examined for several gravitropism mutants. The auxin resistant mutant in this study that is allelic to *aux*1 was found to show phototropism similar to the parental wild-type. This indicates that auxin-response can have a specific role in gravitropism that does not affect phototropism, and shows that auxin-resistant mutants may not represent general alterations in auxin regulated differential growth.

Phototropism of hgr1 was identical to wild-type phototropism in first positive fluence range, but significantly increased curvature was observed in the five-pulse blue light treatment. Phototropism of hgr2 was increased under the five-pulse light treatment, and greater curvature was shown for all fluences greater than the threshold in first positive. The presence of unaltered phototropism in the starch deficient mutant, and the auxin-resistant mutants show that it is unlikely that gravitropism has a general role in the reduction of phototropic curvature. As *hgr*2 is altered in phototropism and ethylene response, it is suggested that normal ethylene response is necessary for gravitropism, but antagonistic to phototropism.

A number of transcripts were identified that appeared to represent changes in gene regulation between parental wild-type seedlings, *hgr*1, and two alleles of *agr*1. Although this work is preliminary, it is suggested that further investigation of these transcripts could increase the understanding of gene regulation associated with gravity response.

The most interesting mutant in this collection is *hgr*1, as previous mutants have primarily been altered in aspects of hormone-response or starch contents, and this mutant appears to represent an unknown element of gravitropism. Because this mutant was not altered in established factors associated with gravitropism, further study of hgr1 could result in the identification of novel genetic components of gravity response. This mutant would be an excellant candidate for map-based cloning studies. Identification of the gene altered in this mutant would be a molecular clue to the process of gravitropism, and could be used to evaluate current models of gravity response.

The hgr2 gravitropism mutant has an interesting phenotype, and a molecular understanding of the basis of this altered gravity response could explain the interaction of phototropism and gravitropism. Further study of the hookless mutant, other ethylene-response mutants, and the direct effect of ethylene treatment on phototropism, would be useful to explore the role of ethylene in phototropism.

Bibliography

Bibliography

Abel S, Oeller PW, Theologis A (1994) Early auxin-induced genes encode short-lived nuclear proteins. Proceedings of the National Academy of Sciences. 91:326-330

Abel S, Theologis A (1996) Early genes in auxin action. Plant Physiology 111:9-17

Ahmad M, Cashmore AR (1996) Seeing blue-the discovery of cryptochrome. Plant Molecular Biology 30:851-861

Arif I, Newman IA (1993) Proton efflux from oat coleoptile cells and exchange with wall calcium after IAA or fusicoccin treatment. Planta 189:377-383

Audus LJ (1962) The mechanism of the perception of gravity by plants. Symposium of the Society of Experimental Biology 16:197-226

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Sturh LK (1995) Current Protocols in Molecular Biology. John Wiley and Sons INC. USA

Azumi Y, Watanabe A (1991) Evidence for a senescence-associated gene induced by darkness. Plant Physiology 95:577-583

Baluska F, Folkmann D, Hauskrecht M, Barlow PW (1996a) Root cap mucilage and extracellular calcium as modulators of cellular growth in postmitotic growth zones of the maize root apex. Botanica Acta 109:25-34

Baluska R, Hauskrecht M, Barlow PW, Sievers A (1996b) Gravitropism of the primary root of maize: a complex pattern of differential cellular growth in the cortex independent of the microtubular cytoskeleton. Planta 198:310-318

Bandurski RS, Reinecke DM, Cohen JD, Slovin JP (1995) Auxin biosynthesis and metabolism *IN* Plant Hormones: Physiology, Biochemistry and Molecular Biology. PJ Davies ed. Kluwer Academic Press. Dordrecht, The Netherlands. pp35-57

Bandurski RS, Schulze A, Dayanandan P, Kaufman PB (1984) Response

to gravity by *Zea mays* Seedlings. Plant Physiology 74:284-288

Bell CJ, Ecker JR (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. Genomics 19:137-144

Bell CJ, Maher EP (1990) Mutants of *Arabidopsis thaliana* with abnormal gravitropic responses. Molecular and General Genetics 220:289-293

Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schultz B, Feldmann KA (1996) Arabidopsis AUX1 gene: A permease-like regulator of root gravitropism. Science 273:948-950

Bjorkman T(1988) Perception of gravity by plants. *IN* Advances in Botanical Research Vol. 15 Academic press limited.

Bjorkman T, Cleland RE (1991) Root growth regulation and gravitropism in maize roots does not require the epidermis. Planta 185:34-37

Bleeker AB, Estelle MA, Somerville S, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241:1086-1089

Boldt R, Scandalios JG (1995) Circadian regulation of the Cat3 catalase gene in maize (*Zea mays* L.):Entrainment of the circadian rhythm of Cat3 by different light treatments.

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defense response. Cell 70:21-30

Bullen B(1992) Development of a genetic system for the study of gravitropism in *Arabidopsis thaliana*. PhD Dissertation

Casper T, Pickard BG (1989) Gravitropism in a starchless mutant of *Arabidopsis*. Planta 177:185-197

Casper T, Huber SC, Somerville C (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucosemutase activity. Plant Physiology 79:11-17

Chaloupkova K, Smart CC (1994) the abscisic acid induction of a novel peroxidase is antagonized by cytokinin in *Spirodela polyrrhiza* L. Plant Physiology 195:497-507

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262:1883-1886

Cohen JD, Slovin JP, Bialek K, Chen C-H, Derbyshire M (1988) Mass spectrometry genetics and biochemistry: Understanding the metabolism of indole-3-acetic acid *IN* Biomechanisms Regulating Growth and Development. GL Steffens, TS Rumsey eds. Beltsville Symposia in Agricultural Research Volume12. Kluwer Academic Publishers. Dordrecht, The Netherlands pp229-241

Coupland G (1995) Genetic and environmental control of flowering time in Arabidopsis. Trends in Genetics 11:393-397

Crookes PR, Grierson D (1983) Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in cell wall degradation. Plant Physiology 72:1088-1093

Davies KM, Grierson D (1989) Identification of cDNA clones for tomato (*Lycopersicon esculentum* Mill.) mRNAs that accumulate during fruit ripening and leaf senescence in response to ethylene. Planta 179:73-80

Dellart (1980) Segregation Frequency. Theoretical and Applied Genetics 57:137-143

Digby J, Firn RD (1995) The gravitropic set-point angle (GSA): the identification of an important developmentally controlled variable governing plant architecture. Plant, Cell and Environment 18:1434-1440

Dominov JA, Stenzler L, Lee S, Schwartz JJ, Leisner S (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. the Plant Cell 4:451-461

Estelle MA, Somerville C (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Molecular and General Genetics 206:200-206

Evans ML, Ishikawa H, Estelle MA (1994) Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: Comparison of wild type and auxin-response mutants. Planta 194:215-222

Evans ML, Moore R, Hassenstein K-H (1986) How roots respond to gravity. Scientific American 255:112-119

Feldman LJ, Briggs WR (1987) Light-regulated gravitropism in seedling roots of maize. Plant Physiology 83:241-243

Ferl FJ (1996) 14-3-3 proteins and signal transduction. Annual Review of Plant Physiology and Plant Molecular Biology. 47:49-73

Finkelstein RR, Zeevaart JAD (1994) Gibberellins and Abscisic Acid. *IN Arabidopsis*. EM Meyerowitz and CR Somerville (eds) Cold Spring Harbor Laboratory Press

Firn RD, Digby J (1980) the establishment of tropic curvature in plants. In the Annual Review of Plant Physiology 31:131-148

Fortin M-C, Poff KL (1991) Characterization of thermotropism in primary roots of maize: Dependence on temperature and temperature gradient, and interaction with gravitropism. Planta 184:410-414

Fukaki H, Fujisawa H, Tasaka M, (1996) SGR1, SGR2, and SGR3: Novel genetic loci involved in shoot gravitropism in *Arabidopsis thaliana*. Plant Physiology 110:945-955

Gehring CA, Williams DA, Cody SH, Parish RW (1990) Phototropism and geotropism in maize coleoptiles are spatially correlated with increases in cytosolic free calcium. Nature 345:528-530

Gil P, Liu Y, Orbovic V, Verkamp E, Poff KL, Green PJ (1994) Characterization of the auxin inducible SAUR-AC1 gene for use as a molecular genetic tool in *Arabidopsis*. Plant Physiology 104:777-784

Goddard RH, Wick SM, Silflow CD, Snustad DP (1994) Microtubule components of the plant cell cytoskeleton. Plant Physiology 104:1-6

Goeschl JD, Pratt HK, Bonner BA (1967) An effect of light on the production of ethylene and the growth of the plumular portion of etiolated pea seedlings. Plant Physiology 42:1077-1080

Grabski S, Schindler M (1996) Auxins and Cytokinins as antipodal modulators of elasticity within the actin network of plant cells. Plant Physiology 110:965-970

Guzman P, Ecker JR (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. The Plant Cell 2:513-523

Hampson IN, Pope L, Cowling J, Dexter TM (1992) Chemical cross linking subtraction (CCLS): a new method for the generation of subtractive hybridisation

Hart JW, Macdonald IR (1981) Phototropism and geotropism in hypocotyls of cress (*Lepidium sativum* L.) Plant, Cell, and Environment 4:197-201

Haughn GW, Somerville CR (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Molecular and General Genetics 204:430-434

Hejnowicz Z, Sievers A(1995) Proton efflux from the outer layer of the peduncle of Tulip in gravitropism and circumnutation. Botanica Acta 108:7-13

Hensel W(1985) Cytochalasin B affects the structural polarity of

statocytes from Cress roots(*Lepidium sativum* L.) Protoplasma 129:178-187

Hensel W(1989) Tissue Slices from living root caps as a model system in which to study cytodifferentiation of polar cells. Planta 177:296-303

Hild V (1977) Wirkung von Vorbestrahlung mit Rot- oder Blaulicht auf die geotropische Empfindlichkeit von Maiskoleoptilen. Planta 133:309-314

Hild V, Hertel R (1972) Initial phases of gravity-induced lateral auxin transport and geotropic curvature in Corn coleoptiles. Planta 108:245-258

Hobbie L, Estelle M (1995) The *axr*4 auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. The Plant Journal 7:211-220

Hohl M, Greiner H, Schopfer P (1995) The cryptic-growth response of maize coleoptiles and its relationship to H_2O_2-dependent cell wall stiffening. Physiologia Plantarum 94:491-498

Hoson T, Kamisaka S, Masuda Y (1996) Suppression of gravitropic response of primary roots by submergence. Planta 199:100-104

Hou Y, von Arnim AG, Deng X-W (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. The Plant Cell 5:329-339

Hull G, Gaubier P, Delseny M, Casse-Delbart F (1993) Absicisic acid inducible genes and their regulation in higher plants. Current Topics in Molecular Genetics 1:289-305

Iino M (1995) Gravitropism and phototropism of maize coleoptiles: Evaluation of the Cholodny-Went theory through effects of auxin application and decapitation. Plant and Cell Physiology 36:361-367

Iverson T-H (1969) Elimination of geotropic responsiveness in roots of cress(*Lepidium sativum*) by removal of statolith starch. Physiologia Plantarum 22:1251-1262

Jackson MB, Barlow PW (1981) Root geotropism and the role of growth regulators from the cap: a re-examination. Plant, Cell and Environment 4:107-123

Juniper BE (1976) Geotropism. Annual Review of Plant Physiology 27:385-406

Kang BG, Burg SP (1972) Regulation of phytochrome-enhanced

geotropic sensitivity to ethylene production. Plant Physiology 50:132-135

Kawakami N, Watanabe A, (1988) Change in gene expression in radish cotyledons during dark-induced senescence. Plant Cell Physiology 29:33:42

Kendrick PE, Kronenberg GHM (1994) Photomorphogenesis in Plants. Kluwer Academic Press. Dordrect, the Netherlands

Kim D, Kaufman PB (1995) Basis for changes in the auxin-sensitivity of *Avena sativa* (Oat) leaf-sheath pulvini during the gravitropic response. Journal of Plant Physiology 145:113-120

Kiss JZ, Hertel R, Sack FD(1989) Amyloplasts are necessary for full gravitropic sensitivity in roots of *Arabidopsis thaliana*. Planta 177:198-206

Kiss JZ, Wright JB, Casper T (1996) Gravitropism in roots of intermediate-starch mutants of *Arabidopsis*. Physiologia Plantarum 97:237-244

Khurana JP, Poff KL (1989) Mutants of *Arabidopsis thaliana* with altered phototropism. Planta 178:400-406

Kuznetsov OA, Hasenstein KH (1996) Intracellular magnetophoresis of amyloplasts and induction of root curvature. Planta 198:87-94

Leather GR, Gorrence LE, Abeles FB (1972) Increased ethylene production during clinostat experiments may cause leaf epinasty. Plant Physiology 49:183-186

Lee JS, Mulkey TJ, Evans ML (198?) Reversible loss of gravitropic sensitivity in maize roots after tip application of calcium chelators. Science 220:1375-1376

Lee JS, Chang W-K, Evans ML (1990) Effects of ethylene on the kinetics of curvature and auxin redistribution in gravistimulated roots of *Zea mays*. Plant Physiology 94:1770-1775

Leyser HMO, Lincoln CA, Timpte C, Lammer D, Turner J, Estelle M (1993) *Arabidopsis* auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. Nature 362:161-164

Li Y, Hagen G, Guilfoyle TJ (1991) An auxin-responsive promoter is differentially induced by auxin gradients during tropisms. The Plant Cell 3:1167-1175

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr*1 mutants of *Arabidopsis*. The Plant Cell 2:1071-1080

Liscum E, Hangarter RP (1993) Genetic evidence that the red-

absorbing form of phytochrome B modulates gravitropism in *Arabidopsis thaliana*. Plant Physiology 103:15-19

Lyon CH (1970) Ethylene inhibition of auxin transport by gravity in leaves. Plant Physiology 45:644-646

Masson PH (1995) Root gravitropism. BioEssays 17:119-127

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Molecular Biology 9:611-623

McClure BA, Guilfoyle T (1989) Rapid redistribution of Auxin-regulated RNAs during gravitropism. Science 243:91-93

Mcdowell JM, Huang SR, Mckinney EC, An YQ, Meagher RB (1996) Structure and evolution of the actin gene family in *Arabidopsis thaliana*. Genetics 142:587-602

Mirza JI, Olsen GM, Iversen T-H, Maher EP (1984) The growth and gravitropic responses of wild-type and auxin-resistant mutants of *Arabidopsis thaliana*. Physiologia Plantarum 60:516-522

Moore R, Smith JD (1985) Graviresponsiveness and abscisic acid content of roots of carotenoid deficient mutants of *Zea mays*. Planta 164:126-128

Muday GK, Haworth P (1994) Tomato root growth, gravitropism, and lateral development: Correlation with auxin transport. Plant Physiol. Biochemistry 32(2):193-203

Mulkey TJ, Evans ML (1981) Geotropism in corn roots: Evidence for its mediation by differential acid efflux. Science 212:70-71

Nick P, Bergfeld R, Schafer E, Schopfer P (1990) Unilateral reorientation of microtubules at the outer epidermal wall during photo- and gravitropic curvature of maize coleoptiles and sunflower hypocotyls. Planta 181:162-168

Nick P, Schafer E (1988) Interaction of gravi- and phototropic stimulation in the response of maize (*Zea mays*) Coleoptiles. Planta 173:213-220

Nick P, Schafer E, Hertel R, Furuya M (1991) On the putative role of microtubules in gravitropism of maize coleoptiles. Plant Cell Physiology 32:873-880

Normanly J, Slovin JP, Cohen JD (1995) Rethinking auxin biosynthesis and metabolism. Plant Physiology 107:323-329

Okada K, Shimura Y (1992) Aspects of recent developments in mutational studies of plant signaling pathways. Cell 70:369-372

Osborne DJ, Mullins MG (1969) Auxin, Ethylene and kinetin in a carrier-protein model system for the polar transport of auxins in petiole segments of *Phaseolus vulgaris*. New Phytology 68:977-991

Philosoph-Hadas S, Meir S, Rosenberger I, Halevy AH (1996) Regulation of the gravitropic response and ethylene biosynthesis in gravistimulated snapdragon spikes by calcium chelators and ethylene inhibitors. Plant Physiology 110:301-310

Pickard BG (1985) Roles of hormones, protons and calcium in geotropism. *IN* Encyclopedia of Plant Physiology, New Series Volume 11 RP Pharis & DM Reid (eds) pp. 193-281

Pickard BG (1994) Contemplating the plasmalemmal control center model. Protoplasma 182:1-9

Pickett FB, Wilson AK, Estelle M (1990) The *aux*1 mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiology 94:1462-1466

Poff KL, Martin HV (1989) Site of graviperception in roots: a re-examination. Physiologia Plantarum 76:451-455

Pratt LH (1995) Phytochromes: Differential properties, expression patterns and molecular evolution. Photochemistry and Photobiology 61:10-21

Rayle DL, Cleland R (1970) Enhancement of wall loosening and elongation by acid solutions. Plant Physiology 46:250-253

Roberts JA (1987) Mutants and gravitropism. *IN* Developmental Mutants in Higher Plants. H Thomas and D Grierson eds. Cambridge University Press. Cambridge pp135-153

Robson PRH, Smith H (1996) Genetic and transgenic evidence that phytochromes A and B act to modulate the gravitropic orientation of *Arabidopsis thaliana* hypocotyls. Plant Physiology 119:211-216

Robson PRH, Whitelam GC, Smith H (1993) Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiology 102:1179-1184

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR (1995) Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: Five novel mutant loci integrated into a stress response pathway. Genetics 139:1393-1409

Romano CP, Robson PRH, Smith H, Estelle M, Klee H (1995) Transgene-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy*6-1 hypocotyl elongation and *axr*1 auxin-resistant mutants. Plant Molecular

Biology 27:1071-1083

Rorabaugh RA, Salisbury FB (1989) Gravitropism in higher plant shoots: VI changing sensitivity to auxin in gravistimulated soybean hypocotyls. Plant Physiology 91: 1329-1338

Sack FD (1991) Plant gravity sensing. International Review of Cytology 127:193-252

Schuurink RD, Chan PV, Jones RL (1996) Modulation of calmodulin mRNA and protein levels in barley aleurone. Plant Physiology 111:371-380

Schwark A, Bopp M (1993) Interaction of ethylene and auxin in the regulation of hook growth II. The role for ethylene in different growing regions of the hypocotyl hook of *Phaseolus vulgaris* Journal of Plant Physiology 142:585-592

Scott TK, Wilkins MB (1969) Auxin transport in roots. IV. Effects of light on IAA movement and geotropic responsiveness in *Zea* roots. Planta 87:249-258

Seagull RW (1989) The plant cytoskeleton. CRC Critical Reviews in Plant Science 8:131-167

Sievers A, Kramer-Fischer M, Braun M, Buchen B (1991) The polar organization of the growing Chara rhizoid and the transport of statoliths are actin-dependent. Botanica Acta 104:103-109

Sievers A, Volkmann D (1979) Gravitropism in single cells. *In* Encyclopedia of Plant Physiology. New Series Volume 7 W Haupt and ME Feinleib eds. Springer-Verlag Berlin pp567-572

Simons C, Migliaccio F, Masson P, Caspar T, Soll D (1995) A novel root gravitropism mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. Physiologia Plantarum 93:790-798

Sinclair W, Oliver I, Maher P, Trewavas A (1996) the role of calmodulin in the gravitropic response of the *Arabidopsis thaliana agr*-3 mutant. Planta 199:343-351

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens* iaaM and iaaH genes display altered growth an Indoleacetic Acid metabolism. Plant Physiology 99:1062-1069

Skriver K, Olsen FL, Rogers JC, Mundy J (1991) Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. Proceedings of the National Academy of Science. 88:7266-7270

Slocum RD, Roux SJ (1983) Cellular and subcellular localization

of calcium in gravistimulated oat coleoptiles and its possible significance in the establishment of tropic curvature. Planta 157:481-492

Staves MP, Wayne R, Leopold AC (1992) Hydrostatic pressure mimics gravitational pressure in characean cells. Protoplasma 168:141-152

Steel RGD, Torrie JH (1981) Principles and procedures of statistics: A biometrical approach. McGraw-Hill International Book Co pp577-585

Steinitz B, Poff KL (1986) A single positive phototropic response induced with pulsed light in hypocotyls of *Arabidopsis thaliana* seedlings. Planta 186:305-315

Stinemetz CL, Kuzmanoff KM, Evans ML, Jarrett HW (1987) Correlation between calmodulin activity and gravitropic sensitivity in primary roots of maize. Plant Physiology 84:1337-1342

Szerszen JB, Szczyglowski K, Bandurski RS (1994) iaglu, a gene from *Zea mays* involved in conjugation of a growth hormone indole-3-acetic acid. Science 265:1699-1701

Takahashi H, Scott TK (1991) Hydrotropism and its interaction with gravitropism in maize roots. Plant Physiology 96:558-564

Taylor DP, Slattery J, Leopold AC (1996) Apoplastic pH in corn root gravitropism: A laser scanning confocal microscopy measurement. Physiologia Plantarum 97:35-38

Timpte C, Lincoln C, Pickett FB, Turner J, Estelle M (1995) The AXR1 and AUX1 genes of *Arabidopsis* function in separate auxin-response pathways. The Plant Journal 8:561-569

Trewavas AJ ed.(1992) Forum: What remains of the Cholodny-Went theory? Plant Cell and Environment 15:759-794

Trewavas A, Knight M (1994) Mechanical signalling, calcium and plant form. Plant Molecular Biology 26:1329-1341

Veluthambi K, Poovaiah BW (1984) Calcium-promoted protein phosphorylation in plants. Science 223:167-169

Versel J-M, Pilet P-E (1986) Distribution of growth and proton efflux in gravireactive roots of maize (*Zea mays* L.) Planta 167:26-29

Walker JC, Key JL (1982) Isolation of cloned cDNAs to auxin-responsive poly(A)+RNAs of elongation soybean hypocotyl. Proceedings of the National Academy of Science 79:7185-7189

Wayne R, Staves MP, Leopold AC (1990) Gravity-dependent polarity of cytoplasmic streaming in *Nitellopsis*. Protoplasma 155:43-57

Wayne R, Staves MP, Leopold AC (1992) The contribution of the extracellular matrix to gravisensing in characean cells. Journal of Cell Science 101:611-623

Woitzik F, Mohr H (1988) Control of hypocotyl gravitropism by phytochrome in a dicotyledonous seedling (*Sesamum indicum* L.) Plant, Cell and Environment 11:663-668

Went FW, Thimann KV (1937) Phytohormones p154-157 NY Macmillan 294

Wilkins MB (1984) Gravitropism. *IN* Advanced Plant Physiology. MB Wilkins (ed) Pitman. London pp163-185

Wilson AK, Pickett FB, Turner JC, Estelle M (1990) A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Molecular and General Genetics 222:377-383

Wong LM, Abel S, Shen N, de la Foata M, Mall Y, Theologis A (1996) Differential activation of the primary auxin response genes, PS-IAA4/5 and PS-IAA6, during early plant development. The Plant Journal 9:587-599

Wright LZ, Rayle DL (1983) Evidence for a relationship between H+ excretion and auxin in shoot gravitropism. Plant Physiology 72:99-104

Wyatt RE, Ainley WM, Nagao RT, Conner TW, Key JL (1993) Expression of the *Arabidopsis* AtAux2-11 auxin-responsive gene in transgenic plants. Plant Molecular Biology 22:731-749

Zobel RW (1973) Some physiological characteristics of the ethylene-requiring tomato mutant diageotropica. Plant Physiology 52:385-389