AN ABSTRACT OF THE THESIS OF

Andreas Madlung for the degree of

Doctor of Philosophy in Molecular and Cellular Biology presented on June

29, 2000. Title: Interactions of Auxin with Ethylene and Gravity in

Regulating Growth and Development in Tomato (*Lycopersicon esculentum,*

Mill.).

Abstract approved: *Redacted for Privacy*

Terri L. Lomax

Plant growth, development, and environmental responsiveness are

dependent on hormone-induced gene expression. This dissertation reports

multiple interactions between the plant hormones auxin and ethylene and

investigates their contribution to the gravitropic response, elongation

growth, adventitious root formation, callus and tracheary element initiation

and growth, and flower development.

Four mutants of tomato (*Lycopersicon esculentum*, Mill.) altered in

either hormone production or hormone response were used to test the

involvement of ethylene and auxin. These mutants included *diageotropica*

(*dgt*) which is auxin-resistant, *Never-ripe* (*Nr*), which is ethylene-resistant,

epinastic (*epi*), which overproduces ethylene and *lazy-2* (*lz-2*), which

exhibits a phytochrome-dependent reversed-gravitropic response.

Additionally, a double mutant between *Nr* and *dgt* was constructed and tested.

Gravitropism was studied as an exemplary process involving both auxin and ethylene. Mutant analysis demonstrated that ethylene does not play a primary role in the gravitropic response via the currently known ethylene response pathways. However, ethylene can modify the gravitropic response, e.g. the delayed gravitropic response of the *dgt* mutant can be restored with exceedingly low concentrations of ethylene and ethylene synthesis- and ethylene-action inhibitors can partially inhibit the graviresponse.

The role of gravity in tracheary element (TE) production was tested in microgravity (during a space shuttle flight) and in hypergravity (centrifugation). A correlation was found between gravitational force and the production of TEs, with decreased numbers of TEs produced in microgravity and increased numbers produced in response to hypergravity. Increased production of TEs by *dgt* in both increased and reduced gravity indicates that gravity regulates vascular development via a *DGT*-dependent pathway involving auxin.

Combination of both the *Nr* and *dgt* mutations in a double mutant leads to plants which exhibit the reduction of auxin-sensitivity typical of *dgt* as well as a delay in fruit ripening typical of *Nr*. The reduced gravitropic response of the *dgt* mutant was restored to wild-type levels in the double mutant confirming a complex role for ethylene in the gravitropic response.

Abnormal floral organ development was observed in a subset of double mutant flowers.These data demonstrate multiple connections between auxin and ethylene during development and provide further insight into their cellular interactions.

Interactions of Auxin with Ethylene and Gravity in Regulating Growth and

Development in Tomato *(Lycopersicon esculentum,* Mill.).

by

Andreas Madlung

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented June 29, 2000

Commencement June 2001

<u>Doctor of Philosophy</u> thesis of <u>Andreas Madlung</u> presented on <u>June 29, 2000</u>

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Andreas Madlung, Author

ACKNOWLEDGMENTS

A dissertation is never the product of a single person, nor is it the product of a finite number of years that are spent in the process of conducting the research and analyzing its results. This thesis is the culmination of many years of learning, guidance, and support by people that believed in me. For that I am grateful to everyone who shared part of the way. Today, I thank first and foremost my advisor Terri Lomax for providing professional advice, constant encouragement and personal friendship. Terri supported me in finding my own way and let me craft my own unique graduate school experience the way I thought would best serve my interests. I would also like to thank my graduate committee members, Carol Rivin, Barb Taylor, Anita Azarenko, and Mary Slabaugh for their involvement in my project and their support. Meredith Howell, Kari van Zee and Indira Rajagopal shared with me their skill and experience in teaching molecular biology. They provided me with opportunities to do what I really want to do: pass on the knowledge I have gained about the wonders of life and spark passion about science and nature in others. I deeply thank them for their help.

I am thankful for encouragement and guidance I received from my previous mentors. Karl Dörffling helped me find my interest and passion in plant biology. Joseph Schmuckler has played such an important role in my life

as a scientist, educator and human being that I don't know if I would even be a scientist and educator today if it were not for him. I will never forget what he taught me about life.

I am thankful for the friendship of my fellow Botany and MCB students. They have made the time in Corvallis memorable, fun, and endurable when things were going well and when they were not going so well. The same goes for my lab mates. Thank you for friendship and support through ups and downs in the day to day work. I especially thank Virginia and Kwang Chul for reading my manuscripts, for sharing their scientific ideas with me and just being the best lab mates I could wish for. Both Rosie and TJ ran the lab smoothly and were always available for help and advice. I thank them both, knowing well that without them, my research would not nearly have gone as fast. I want to express my thanks to Mary and Fred who taught me my first steps in the world of molecular biology. They were - and are - examples I am looking up to as well as good friends. Mary was the best mentor I could ask for when I rotated in Terri's lab. Only after mentoring students myself do I understand how much time and effort she dedicated to making me feel welcome and teaching me the ropes. Fred had part in many of my scientific projects and I enjoyed working with him as much as I liked sharing a homebrew with him after long days in the lab.

I am thankful to Marlan Carlson for letting me play in the Corvallis Symphony Orchestra during the five years of my residence in Corvallis. As a fine conductor, teacher and musician, he gave me a second home and

encouraged me to use the other half of my brain not only on Monday nights. The orchestra certainly played a major role in keeping me sane while being a graduate student.

I would like to thank my friend Chip for not being a scientist, and for being a wonderful friend. I thank him for thousands of smiles and hundreds of questions and for opening my mind to different ways of thinking.

I want to thank my parents for supporting me in every endeavor I attempted. They have helped me earn this degree more than anyone else. Their patience, love and understanding means more to me than they may ever know.

Most importantly, I want to thank Marion for a little bit of all of the above, and for being my best friend and companion, for love, a shoulder to rest against, the source of my energy and inspiration, and being a true partner in all I do and all I am.

CONTRIBUTION OF AUTHORS

Chapter 2:

All experiments in this chapter were planned, conducted, and evaluated by the primary author. Fred Behringer was involved in the planning of some of the experiments and in some cases conducted similar preliminary experiments with similar results.

Chapter 3:

All experiments in this chapter were planned, conducted, and evaluated by the primary author. Catharina Coenen provided preliminary data published in her thesis (Auxin and Cytokinin Interaction in Tomato, *Lycopersicon esculentum*, Mill., 1996, Oregon State University) that show similar results. Catharina Coenen also worked out tissue culture conditions and the protocol for the tracheary element count. Figure 1 was provided by Catharina Coenen (1C and D) and Terri Lomax (1A, B, E-H).

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DEDICATION

This thesis is dedicated to my parents, Helga and Wolfgang Madlung who encouraged and supported me on every step along the way with patience and love. I hope some day I can pass on what I received from you to your grandchildren.

INTERACTIONS OF AUXIN WITH ETHYLENE AND GRAVITY IN REGULATING GROWTH AND DEVELOPMENT IN TOMATO (*LYCOPERSICON ESCULENTUM,* MILL.)

1. INTRODUCTION

From the earliest stages of embryo development to completion of the plant's life cycle, plant hormones play a major role in guiding virtually all plant developmental processes. This control is initiated by activating the transcription of genes that in turn can affect a multitude of other genes further downstream in various signal transduction pathways. It is amazing that plants, beginning with relatively few hormones and signaling compounds at the origin of the developmental process, can orchestrate the multitude of developmental events necessary to take the plant from a dry seed to maturity. How can intricate and profoundly different processes, such as flower development or vascular differentiation, be guided by the same hormones? How does the plant integrate cues from its environment to yield useful information that will allow it to set cellular processes in motion and achieve optimal growth? Understanding the circuitry of the signal transduction involved in individual responses will aid our understanding of these phenomena.

For more than a century, scientists have studied plant responses to hormones (Darwin, 1880; Went and Thimann, 1937). Early on, it became evident that the interplay of hormones provides the plant with

an important means of fine-tuning its responses to external stimuli. The same hormone, for example, can induce opposite responses depending on the nature of the stimulus. Such differential responses can be regulated by the concentration of the plant hormone, by differential sensitivity of the tissue, or by the interaction of two or more growth regulators. In many cases hormones act as antagonists, resulting in a tug-of-war between opposing stimuli. This tug-of-war results in an optimal plant response. This method of response is important not only with regard to environmental cues, but also during the development and differentiation of various plant tissues.

Historically, hormone action has been studied by applying hormones and studying their effects on specific responses. Further insight has come from the genetic analysis of mutants altered in their responses to hormones or in their production of specific hormones. Molecular genetics has provided tools in recent years to identify the mutant genes and the proteins they encode, thus helping scientists understand the pathways of signal transduction and providing insight as to the integration and amplification points for these signals during the signal transduction processes.

1.1 Auxin and its role in growth regulation and development

Auxin is involved in many aspects of plant development ranging from cell division and cell elongation to cell differentiation. During embryonic development, auxin establishes bilateral symmetry in the

seedling (Liu et al., 1993). In the emerging hypocotyl, auxin has been implicated in cell elongation (Gray et al. 1998). Auxin is also involved in the formation of roots (Jacobs 1952; Matthysse and Scott, 1984); it stimulates root growth at low concentrations and inhibits it at higher concentrations (Evans, 1984). In addition, auxin is implicated in the inhibition of leaf senescence (Osborne and Sargent, 1976), the promotion of cambial activity (Aloni, 1995), and the maintenance of apical dominance (Thimann et al., 1971). Auxin also plays a pivotal role in the orientation of roots and stems in response to gravity or light (Went and Thimann, 1937), induces hyponasty (Lippencott and Lippencott, 1971) and epinasty (Palmer, 1976), and is involved in vascular development.

The involvement of plant growth regulators in vascular development has been well established. Wetmore and Rier (1963) demonstrated that auxin applied to the cut surface of undifferentiated callus tissue could initiate vascular development. Later, Sachs (1981) proposed that the initiation of vascular tissue leads to a "canalization process". In this process, auxin is transported to the end of the least differentiated provascular cell where the hormone aids in the recruitment of the next cell, thus creating a continuous strand of vasculature (for review, see Aloni et al., 1995). Auxin has also been found to induce the transcription of two genes implicated in vascular development, *Athb-8* (Baima et al., 1995) and *ZePel* (Domingo et al., 1998). *Athb-8* was found to be specificly expressed during re-vascularization in tobacco after stem injury (Baima et al., 1995). *ZePel* was found to be expressed

in vascular bundles and shoot primordia (Domingo et al., 1998). These findings further support a major role for auxin in vascularization events. Lincoln et al. (1990) reported "subtle differences" in the vascular development of the auxin-resistant *axr1* mutant of *Arabidopsis*. Similar effects were found in transgenic *Arabidopsis* plants that overproduce auxin-inactivating enzymes (Klee et al., 1987; Romano et al., 1991). Conversely, overproduction of auxin led to an increase in vascularization (Hobbie and Estelle, 1994).

After the transition from vegetative to reproductive development, auxin is involved in patterning flower development (Nemhauser et al., 1998), stimulates fruit set (Gianfagna, 1995), delays fruit and leaf abcission, and fruit ripening (Ludford, 1995), and represses anthocyanin production in post-harvest fruit (Ludford, 1995). However, some of these processes may involve indirect auxin effects or synergistic action of several hormones. For example, auxin may stimulate ethylene production in the reproductive tissue leading to fruit ripening (Reid, 1995).

1.1.2 Auxin perception and signal transduction

Growth in response to auxin has been attributed to several cellular processes. It was noticed early that cellulase activity was promoted by auxins (Ridge and Osborne, 1969; Sargent et al., 1973), which may lead to cell wall loosening and thus allow cell expansion. Cell wall extension in response to auxin was also reported by Matsuda et al.

(1969). The acid growth theory (Cleland, 1995) postulates that auxin results in cell wall loosening due to acidification which allows protoplast expansion.

Despite years of efforts to elucidate the auxin perception and signal transduction mechanisms, relatively little is known today about the molecular events leading from auxin concentration changes in the target tissue to gene expression and growth or developmental responses. In general, hormone perception starts with the binding of the hormone to a specific receptor either directly or in conjunction with a hormone-binding protein. Binding of a ligand to a receptor elicits a cascade of transduction events that culminates in the binding of transcription factors to the promoter or binding of a hormone response element upstream of the inducible gene, thus initiating transcription. However, in the case of auxin the emerging picture is still sketchy.

At least five putative auxin binding proteins have been identified (Löbler and Klämbt, 1985; Hicks et al., 1989; Macdonald et al., 1991; Feldwisch et al., 1992; Zettl et al., 1994). ABP1 may be the best described of these auxin binding proteins. However, analysis revealed a KDEL endoplasmatic reticulum retention motif in ABP1 (Inohara et al., 1989) making it an unlikely candidate for a membrane receptor molecule.

While a *bona fide* auxin receptor remains elusive, the positional cloning of *axr1* (Leyser et al. 1993) opened up speculation about events immediately downstream from a possible membrane receptor. Leyser et al. (1993) showed that *axr1* encodes a protein with high similarity to the

ubiquitin-activating enzyme E1. Ubiquitination usually targets a protein for rapid degradation. In animal systems it has been shown that ubiquitination of the IgE membrane receptor is reversible upon ligand binding (Paolini et al. 1993). If *axr1* played a similar role in auxin signaling, reversible ubiquitination of an auxin receptor or a downstream signaling molecule could have regulatory functions (Leyser et al. 1993). A second gene possibly involved in early signaling is the *aux1* gene which was cloned more recently (Bennet et al., 1996) and found to have high similarity with a permease-like protein. The existence of such a protein suggests the possibility that auxin is transported into the cell via a permease where it - either inside the cell or in transit into the cell - comes in contact with a component relaying the signal downstream.

The involvement of signaling molecules in the downstream transmission of the auxin signal has become much clearer in the past few years. Four major classes of auxin-response genes have been described: *GH3*-like genes (Hagen et al. 1991, Hagen, 1995), small auxin upregulated RNAs or *SAURs* (McClure et al. 1989; Yang and Pooviah, 2000), *Aux/IAA* genes (Theologis et al., 1985; Ainley et al., 1988; Yamamoto et al., 1992; Abel et al. 1995, Nebenführ and Lomax, 2000) and auxin response factors (*ARFs*), (Ulmasov et al., 1997, 1999). *GH3*-like genes have been described in several species. Transcription of these genes is activated within 5 minutes of external auxin treatment (Hagen and Guilfoyle, 1985). Several auxin-responsive elements have been found in the promoter region of these genes and those elements

have been shown to bind to the auxin response factor ARF1 (Ulmasov et al., 1995). The function of *GH3* itself remains unknown.

The second class of fast-responding, auxin-inducible genes are the *SAURs*. Expression of *SAURs* appears to be restricted to the outer tissues of stems (McClure and Guilfoyle, 1989; Gee at al., 1991; Lee at al., 1991). The physiological functions of SAURs are still unknown. However, Yang and Poovaiah (2000) recently reported the isolation of a new gene from maize roots with similarity to *SAURs*, which is involved in binding calcium/calmodulin. The function of this potential signaling intermediate in auxin signal transduction is still unclear.

The third group of auxin-regulated genes, the *Aux/IAA* genes, comprises a large family of auxin-responsive genes that have four distinct conserved domains including a $\beta\alpha\alpha$ motif that has been proposed to be involved in DNA binding (Guilfoyle, 1998). More recently, this motif has been implicated in dimerization, leading to the proposed formation of homo- and heterodimers of the more than 20 described family members (Guilfoyle, 1998). The fourth group of auxin-responsive genes is known as the auxin response factor gene family. ARFs share two of the four domains commonly found in the *Aux/IAA* genes. This has led to some confusion in their nomenclature and assignment of multiple names for the same gene (such as *ARF5/IAA24/MONOPTEROUS*). *ARFs* contain the same dimerization motif as the *Aux/IAA* genes, and Ulmasov et al. (1999) provided first evidence that such dimerization occurs *in vitro*. The potential dimerization between more than 25 *Aux/IAA* genes and more than 10 *ARF* genes opens up the possibility of

a tremendous diversity of signal modifications to auxin stimulus and could be a major point of control in the fine-tuning of auxin responses (Ulmasov, et al., 1999).

1.2.1 Ethylene and its role in growth regulation and development

The effect of ethylene on plant growth was first noticed over one hundred years ago. Exposure of seedlings to ethylene leads to radial swelling, growth reduction, and exaggerated hook curvature, a phenomenon referred to as the triple response (Neljubow, 1901). Unlike auxin, which is synthesized predominantly in the apex, young leaves and seeds, ethylene is synthesized in every plant cell to varying degrees (Abeles, 1992), with the highest content measured in the apex and other tissues with high auxin concentrations (Abeles, 1992). Ethylene inhibits root elongation (Chadwick and Burg, 1967, Andreae et al., 1968), induces root coiling (Woods et al., 1984) and inhibits leaf expansion (Burg and Burg, 1969). Conversely, ethylene has also been noted to initiate lateral root growth and promote adventitious roots from stems, leaves and pedicels (Abeles, 1992). Most noticeable is the involvement of ethylene in the fruit ripening process of climacteric fruits, such as tomato or avocado, where it induces a number of genes proposed to be involved in the ripening process (Christofferson et al., 1984; DellaPenna et al., 1986). Ethylene is further implicated in delaying flower formation in most plants (Metzger, 1995). In contrast, ethylene is known to induce flowering in the Bromeliaceae (Reid, 1995) and may also be involved in

sex determination where it is reported to induce femaleness (Reid, 1995).

1.2.2 Ethylene's role in gene expression and the ethylene signal transduction pathway

Unlike the auxin signal transduction pathway, ethylene signaling today is quite well understood. Many excellent reviews have recently been published on this topic (Fluhr and Mattoo, 1996; Chang and Shockey, 1999, Bleecker, 1999). Briefly, ethylene binds to a membrane receptor with the help of a copper ion. Ethylene acts negatively on this receptor. Downstream of the receptor molecule, which may be one of five currently known members of the ETR gene family, a cytoplasmic signaling molecule, *CTR1,* transmits the signal via a MAP kinase-like pathway (Chang and Shockey, 1999). Mutations in this gene result in a constitutive triple response, typical for plants exposed to ethylene. *CTR1* acts negatively on *EIN2,* a molecule which contains an integral membrane domain and is hypothesized to be located in the nuclear membrane (Alonso et al., 1999). Downstream of *EIN2* are a series of transcription factors (*EIN3, EIL1, EIL2, EIL3*) which induce expression of *ERF1. ERF1* is a regulator that binds to the GCG-box promoter element found in many ethylene-regulated genes. ERF1 is therefore also referred to as an ethylene response element binding protein (EREBP).

1.3.1 Auxin-ethylene interactions

Plant hormones are in many ways different from hormones found in animals. One major difference is that the same phytohormone may be produced in a much wider range of tissues in the plant, while animals produce most hormones in distinct glands and transport the hormone to its target tissue when its action is required for a particular response. For plants, production of hormones in multiple tissues poses two problems: first, the specificity of a response has to be achieved by means other than just the presence or absence of the hormone, and second, constitutive responses to the hormones must be avoided. To achieve both specificity and an inducible response, several options are available: 1) responses may be hormone concentration dependent, 2) second messengers may take the role of fine-tuning responses, or 3) interactions may occur with other early signaling agents, such as other hormones, to achieve discrete, stimulus-specific responses. These interactions may also involve options 1) and 2).

Hormone-hormone interactions may be additive, synergistic or antagonistic. Many possible points of control can be responsible for hormone interactions: Hormone A can be affected by hormone B in its abundance or perception in the target tissue. Signal transduction events can be stimulated or repressed by hormone A, further, they can be modified by hormone B, and transcriptional activation or repression of

responsive genes to hormone A can be altered by hormone B at the transcriptional, translational or posttranslational level (for review, see Coenen and Lomax, 1997).

It is intriguing that many mutants found to be auxin-resistant also display an alteration in their ethylene response. Among those are the *Arabidopsis* ethylene-insensitive and root agravitropic (*eir1*) mutant (Roman et al, 1995) as well as the auxin resistant mutants *axr1*, *axr2* and *axr3* (Hobbie and Estelle, 1994), *aux1* (Pickett et al., 1990) and the tomato mutant *diageotropica* (*dgt*) (Zobel, 1973; Muday et al., 1995). Some of these mutants are auxin and ethylene insensitive, while others, like the *dgt* mutant (Zobel 1973, 1974; Madlung et al. 1999), and the *Arabidopsis nph4* mutant (Harper et al., 2000) can be partially restored with the application of ethylene. It is therefore likely that pathways of these two hormones interact at multiple levels.

Interactions between auxin and ethlyene were noticed as early as 1935 when it was shown that auxin can induce ethylene production in the plant (Zimmermann and Wilcoxon, 1935; Burg and Burg, 1966; Osborne, 1978). It is therefore likely that many physiological effects cannot be attributed to only one of the two hormones. Lieberman et al. (1979) first reported that auxin is responsible for the induction of ACC synthase, an enzyme synthesizing the immediate ethylene precursor aminocyclopropane carboxylic acid (ACC) (Yang and Hoffman, 1984). While auxin can increase the production of ethylene through this pathway, ethylene has been reported to regulate polar auxin transport, which is pivotal to elongation, tropisms, vascular differentiation and

apical dominance (for review see Lomax et al., 1995). It has also been reported that auxin exerts a limited positive feedback on its own transport (de la Fuente, 1970, Hertel and Flory, 1968). Ethylene control over auxin transport may, however, be the key to feedback control of auxin-induced ethylene synthesis. Here, I will focus on four of the most prominent examples of auxin-ethylene interactions: auxin transport, differential growth, vegetative growth, and reproductive development.

1.3.1.1 Auxin Transport

Auxin promotes the synthesis of ethylene, a process which is further enhanced by cytokinins (Abeles, 1992). Through feedback regulation, ethylene can inhibit the polar transport of auxin and thus control auxin concentration in specific tissues (Abeles, 1992 , Schwark and Schierle, 1992). Auxin transport inhibition was found both in tissue sections and in intact stems or petioles treated with ethylene (Abeles, 1992). Since the velocity of auxin transport was unaffected by ethylene (Burg and Burg, 1967), one possible cause for reduced auxin transport is the reduction of the number of transporters by ethylene (Abeles, 1992). Most recently, the gene encoding a candiate protein for such a proposed function was cloned from *Arabidopsis*: mutation in the *EIR1* gene (which is allelic to *AGR1, WAV6-52* and *PIN2*) was reported to confer reduced ethylene sensitivity and root agravitropism to the plant (Luschnig et al., 1998; Utsuno et al., 1998; Chen et al., 1998). *EIR1* appears to encode an auxin efflux carrier involved in polar auxin

transport and regulated by ethylene. These findings suggest that root growth inhibition by ethylene may be achieved by increasing auxin concentrations to inhibitory levels via regulation of auxin efflux. Interestingly, *EIR1* expression is specific for root tissue (Luschnig et al., 1998) and etiolated hypocotyls (Chen et al., 1998). Almost simultaneously, Gälweiler et al. (1998) reported the cloning and characterization of the *AtPIN1* gene. *Pin-formed* or simply *pin* mutants, display an elongated, leafless inflorescence and reduced polar auxin transport. The *PIN1* gene was identified from three independent transposon-generated mutants and found to encode a protein with similarity to carrier proteins found in both bacteria and eukaryotes (Gälweiler et al., 1998). Expression of *pin1* was detected in every tissue in the plant. *In situ* hybridization assays showed localization of *PIN1* RNA to provascular tissues and antibodies raised against *PIN1* protein localized to the basal ends of parenchymous cells in the vascular bundles (Gälweiler et al., 1998). It will be interesting to see how protein levels of EIR1 and PIN1 respond to ethylene application or upregulation of ethylene's precursor ACC. Likewise, it will be interesting to see if EIR1 and PIN1 protein levels are affected by auxin treatment or how loss of ethylene- or auxin-sensitivity affects auxin transport in double mutants.

1.3.1.2 Differential Growth

Gravity and light are important environmental cues that aid plants in orienting themselves optimally to access life-supporting resources

such as water and light. Gravitropism, the process by which plants orient their roots and shoots with respect to gravity, has been studied intensively for over one hundred years (Darwin, 1888). The Cholodny-Went theory (Went and Thimann, 1937) is widely regarded as the leading hypothesis explaining gravitropism. This theory postulates that the plant hormone auxin, which is synthesized in the shoot apex and transported basipetally down the shoot, is asymmetrically redistributed in response to gravistimulation. This redistribution leads to higher auxin concentrations in the lower half of the stem triggering the increased growth response in that region and resulting in upward curvature of the plant.

The role of ethylene in the gravitropic response has been discussed extensively in the literature, and findings can be split in two opposing groups: those indicating that ethylene plays a role in the gravitropic response (e.g. Kang and Burg, 1973; Zobel, 1973; Wheeler and Salisbury; 1980 and 1981; Clifford and Oxlade, 1989; Philosoph-Hadas et al., 1996) and those supporting the opposite view (e.g. Clifford et al., 1983; Kaufman et al., 1985; Harrison and Pickard, 1986; Woltering et al. 1991). Wheeler and Salisbury (1980, 1981) reported that ethylene inhibitors delayed the gravitropic response in cocklebur (*Xanthium strumarium*) and tomato (*Lycopersicon esculentum* Mill.) and suggested that ethylene plays a "prime role in stem gravitropism". Burg and Kang (1993) reported that ethylene at higher than physiological concentrations could redirect 7-week-old tomato plants to grow downwards, rather than upwards when gravistimulated.

Most auxin-resistant mutants exhibit an altered gravitropic phenotype, such as the *Arabidopsis* mutants *aux1* (Bennett et al., 1996), *axr1* (Lincoln et al., 1990), *axr2* (Wilson et al., 1990) and *axr3* (Leyser et al., 1996), as well as the tomato mutant *dgt* (Kelly and Bradford, 1986; Hicks et al., 1989). These auxin-resistant mutants are also resistant to ethylene with the exception of *dgt*, in which small amounts of ethylene have been reported to be capable of restoring a normal gravitropic response (Zobel, 1974; Madlung et al. 1999). These results suggest that ethylene can aid in signal transduction and possibly act downstream of auxin. As with ethylene, many of the auxin-resistant mutants, such as *aux1, axr1* and *axr3* also show reduced sensitivity to cytokinins (Lomax, 1997) indicating multiple interactions not only at the level of hormone abundance but also on the molecular signal transduction level.

It has been reported that light-grown tomato seedlings treated with ethylene and ethylene inhibitors do not show altered gravitropic behavior (Harrison and Pickard, 1986). The same study also concluded that the lack of change in measurable ethylene production during the first 3 h of the gravitropic response was evidence that ethylene does not play a role in the signal transduction cascade of graviresponses. Although Kaufman et al. (1985) reported a sharp increase in ethylene production between 6h and 24h after gravistimulation in *Avena sativa*, they concluded that this increase occured too late to be a causal factor of gravitropism.

Exogenously-applied ethylene is known to inhibit hypocotyl elongation growth in etiolated plants as part of what is known as the triple response (Goeschl and Kays, 1975; Ecker, 1995). This growth inhibition due to high ethylene should lead to decreased tropic responses, as they are by definition dependent on growth. There is evidence that the reduction in elongation caused by ethylene occurs via an interaction between ethylene and auxin. While ethylene production is stimulated by auxin, ethylene can suppress polar transport of the auxin indole-3-acetic acid (IAA) (Schwark and Schierle, 1992) and can also influence asymmetric distribution of auxin (Schwark and Bopp, 1993). Kang and Ray suggested (1969) that ethylene may be necessary for maintaining the apical hook in etiolated seedlings. Peck et al. (1998) suggested that ethylene mediates the formation and maintenance of the apical hook via an unknown component downstream of *CTR1*, a protein kinase which is part of the ethylene signal transduction pathway. Lehman et al. (1996) suggested that the *Arabidopsis HOOKLESS 1* (*HLS1*) gene controls differential cell growth by regulating auxin activity via its N-acetyltransferase activity. The acetylation process itself may also be modulated by ethylene. Physiological effects can in many instances not be attributed to either auxin or ethylene with certainty because of the multiple interactions between the two hormones.

Another example for differential growth regulated by both auxin and ethylene is the phenomenon known as epinasty. Epinasty is defined as downward curling due to the increased growth on the upper surface of a plant tissue. Epinastic growth of petioles in response to ethylene

has been reported as early as 1932 (Crocker et al., 1932, cited in Ursin, 1989). Interestingly, the epinastic response was also found to be influenced by gravity. When plants were gravistimulated or inverted, epinasty was reduced (Crocker et al., 1932) which argued for a role for auxin in the induction of epinasty as gravistimulation leads to redistribution of auxin within the gravistimulated tissue. Further support for a role of auxin in epinastic growth was provided by Lyon (1963), who reported that inhibition of auxin transport also lead to reduction in epinasty. However, doubts were raised whether epinasty is the result of differential auxin distribution when Palmer (1976) reported that induction of epinasty was unaffected by applied auxin to either the upper or the lower side of the petiole. Ethylene was confirmed as a causal factor of epinasty when inhibitors of ethylene perception or ethylene biosynthesis were shown to result in the loss of epinasty under epinasty-inducing conditions, such as flooding (Amrhein and Schneebeck, 1980; Bradford et al., 1982).

1.3.1.3 Vegetative Growth

As described above, the classic response of seedlings to applied ethylene is known as the triple response (Neljubov, 1901; Goeschl and Kays, 1975; Ecker, 1995). While growth inhibition by ethylene has been described mostly for etiolated plants, it has been reported that ethylene promotes cell growth and elongation in light-grown *Arabidopsis* seedlings grown on nutrient-deficient medium (Smalle et al., 1997).

Ethylene promotion of cell growth and elongation in stem tissue has also been reported for the aquatic plant *Ranunculus sceleratus* when submerged in water (Abeles 1992), as well as for ethylene-treated etiolated rice coleoptiles (Satler and Kende, 1985). In the grasses *Poa pratensis* and *Avena sativa*, the ethylene-releasing compound ethephon has been implicated in tiller internode length increase (Abeles, 1992). Thus it appears that ethylene can both inhibit and promote stem growth.

Elongation inhibition of cell growth may be caused by an increase in cellulose fiber deposition and a decrease in xyloglucan turnover (Eisenger, 1983). This results in a decreased acid growth response, a phenomenon that attributes cell elongation to the loosening of the cell wall by acidification, allowing the protoplast to expand (Taiz et al., 1983; Cleland, 1995). Inhibition of proton secretion has been shown to inhibit ethylene-induced lateral expansion (Eisenger, 1983). From these separate observations, the triple response is possibly a result of an ethylene-induced but auxin-mediated growth response.

As mentioned above, many auxin-resistant mutants are also ethylene-resistant. The auxin-response mutants *axr1, axr2,* and *axr3* are affected in root hair growth (Lincoln et al., 1990; Shimura et al., 1994; Wilson et al., 1990; Leyser et al. 1996) and the *ctr1* mutant displays ectopic expression of root hairs (Tanimoto et al., 1995). Chemical inhibitors of ethylene synthesis, such as aminovinylglycine (AVG) have been shown to abolish root hair production while ACC, the immediate precursor to ethylene, promotes root hairs (Masucci and Schiefelbein, 1996, Tanimoto et al., 1995). On the other hand, applied auxin does not

lead to production of ectopic root hairs (Masucci and Schiefelbein, 1996; Tanimoto et al., 1995; Pitts et al. 1998) and the ethylene-insensitive mutants *etr1* and *ein2* have normal root hairs (Masucci and Schiefelbein, 1996). To explain this apparent contradiction, several scenarios are possible. First, *etr1* and *ein2* may be leaky and therefore allow certain ethylene-dependent processes to function normally (Pitts et al., 1998). There is precedence for this notion as members of the ethylene-receptor gene family have been reported to be somewhat leaky when they were crossed into specific background lines of tomato (Yen et al., 1995). Alternatively, and somewhat more likely, normal root hair initiation may require the interplay of several genes, only some of which may be ethylene-dependent. If these genes are redundant, mutations in one of them, such as *ETR1* or *EIN2,* may not necessarily lead to the abolition of the response.

1.3.1.4 Reproductive Development

Ethylene is implicated in delaying flower formation and inducing fruit development in most plants (Metzger, 1995). In contrast, ethylene is well known to induce flowering in the Bromeliaceae (Reid, 1995). Ethylene may also be involved in sex expression and the induction of femaleness (Reid, 1995). Conversely, it has been reported that exogenous auxin treatment transforms females to males in *Mercuralis annua* (Hamdi et al., 1987). Auxin stimulates fruit set (Gianfagna, 1995), and delays fruit and leaf abcission and fruit ripening (Ludford, 1995).

However, these processes may also be enhanced if auxin acts through ethylene (Reid, 1995).

Whether or not auxin transport and ethylene production are intertwined in flower development and sex determination remains unclear. During fruit development in tomato, both auxin and ethylene levels increase sharply before onset of ripening, and taper off later in the ripening process (Gillaspy et al. 1993). Auxin concentrations surge twice during tomato fruit development, once during fruit expansion and once before the mature green stage, extending well beyond the turning of color, or "breaker stage" (Gillaspy et al., 1993). The interactions between auxin and ethylene at this stage of development are unclear. Ethylene production increases slightly after the onset of the auxin surge, supporting the hypothesis that auxin-induced ethylene may be the signal that leads to the initiation of ripening processes. However, the auxin-resistant *dgt* mutant displays normal fruit ripening (Balbi and Lomax, unpublished data). Therefore it seems unlikely that ripening is dependent on events downstream of DGT-dependent auxin signaling. In contrast, the ethylene-insensitive *Nr* mutant, displays greatly delayed and incomplete ripening, suggesting that *Nr*-dependent ethylene perception is necessary only for the ripening processes but not for fruit development or fruit expansion growth.

Mutations in the *ETTIN* and *MONOPTEROUS* genes lead to malformation of floral organs. Both genes alter the number and appearance of sepals, petals, stamens and carpels to varying degrees. While the proposed function of *MONOPTEROUS* is the promotion of

axialization, *ETTIN* is proposed to establish distinct sectors in the floral

meristems, resulting in correct patterning of organ initiation (for review

see Nemhauser et al., 1998). Additionally, vascular patterning is affected

in *MONOPTEROUS*. Other genes resulting in abnormal flowers are

LOPPED1, as well as the *PIN-FORMED* and *PINOID* genes discussed

earlier. All three are implicated directly or indirectly in auxin transport

(Nemhauser et al., 1998). Both *ETTIN* and *MONOPTEROUS* were later

determined to be *ARF* genes, *ARF3*, and *IAA24/ARF5*, respectively

(Ulmasov et al., 1999). Therefore, floral patterning and axialization are

likely regulated by auxin. It remains to be seen if ethylene responses, as

in the case of *eir1*, are affected by these mutations as well.

1.2. Objectives of this work

Interactions between phytohormones are common in a large

number of physiological plant responses. As scientists are beginning to

unravel pieces of the signal transduction puzzle on a molecular level,

how these signals are integrated and differentiated to result in a specific

plant response remain unclear. The goal of the work presented in this

thesis was to analyze specific developmental and physiological

responses with respect to their co-dependency on at least two different

hormones.

While today many aspects of hormone interactions are studied in

Arabidopsis, I chose to expand our understanding of hormone

interactions using the model plant tomato for three major reasons: (1)

tomato is an important agricultural crop plant whose growth and yield are dependent on a well-defined balance of phytohormones, (2) as a climacteric plant, tomato provides a superb system for the study of flower and fruit development especially with respect to the involvement of ethylene and (3) mutants that are altered in specific responses such as the light-dependent reversed-gravitropic response of the *lazy-2* mutant and the extremely pleiotropic response of the *diageotropica* mutant are to our knowledge only available in the tomato system.

Using genetic and physiological tools, three major objectives were pursued:

1) Characterization of a number of important hormone-dependent plant developmental processes, such as the gravitropic response, adventitious root formation, stem elongation, and vascular cell differentiation with respect to the mutual involvement both of auxin and ethylene, as well as both auxin- and ethylene response genes,

2) Achievement of better understanding of the specific interaction of the auxin-response genes *dgt* and the ethylene receptor *Nr*, and

3) Elucidation of the dependence of tracheary element development on gravity and the auxin-response gene *DGT*.

To achieve these goals the following hypotheses were tested:

1) Ethylene plays a primary role during gravitropism, regulating the magnitude, directionality and the kinetics of the response.

2) Ethylene and auxin interactions are crucial for many aspects of normal plant growth and development in tomato.

3) These hormone interactions vary in their importance and their magnitude depending on the developmental response system in question.

4) Tracheary element development is dependent on gravity and mediated by the *DGT* gene.

2. ETHYLENE PLAYS MULTIPLE NON-PRIMARY ROLES IN MODULATING THE GRAVITROPIC RESPONSE IN TOMATO.

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2.1. Abstract

Ethylene is known to interact with auxin in regulating stem growth, yet evidence for the role of ethylene in tropic responses is contradictory. Our analysis of four mutants of tomato (*Lycopersicon esculentum* Mill.) altered in their response to gravity, auxin, and/or ethylene revealed concentration-dependent modulation of shoot gravitropism by ethylene. Ethylene inhibitors reduce wild-type gravicurvature and extremely low (0.0005 - 0.001 μl L^{-1}) ethylene concentrations can restore the reduced gravitropic response of the auxin-resistant *diageotropica* (*dgt*) mutant to wild-type levels. Slightly higher concentrations of ethylene inhibit the gravitropic response of all but the ethylene-insensitive *Never-ripe* (*Nr*) mutant. The gravitropic responses of *Nr* and the constitutive-response mutant *epinastic* (*epi*) are slightly and significantly delayed, respectively, but otherwise normal. The reversal of shoot gravicurvature by red light in the *lazy-2* (*lz-2*) mutant is not affected by ethylene. Taken together, these data indicate that while ethylene does not play a primary role in the gravitropic response of tomato, low levels of ethylene are necessary for a full gravitropic response and moderate levels of the hormone specifically inhibit gravicurvature in a manner different from ethylene inhibition of overall growth.

2.2. Introduction

Gravity and light are important environmental cues, which aid plants in orienting themselves optimally to access life-supporting resources such as water and light. The process by which plants orient their roots and shoots with respect to gravity, gravitropism, has been studied intensively for over one hundred years (Darwin, 1888). The Cholodny-Went theory (Went and Thimann, 1937), widely regarded as the leading hypothesis explaining gravitropism, postulates that the plant hormone auxin, which is synthesized in the shoot apex and transported basipetally down the shoot, is redistributed asymmetrically in response to gravistimulation. This lateral redistribution of auxin leads to higher concentrations of the hormone in the lower half of the stem which triggers an increased growth response in that region and results in upward curvature of the plant.

The role of ethylene in the gravitropic response has been discussed extensively in the literature, with research results split between two opposing groups: those indicating that ethylene plays a role in the gravitropic response (Zobel, 1973; Kang and Burg, 1974; Wheeler and Salisbury, 1980, 1981; Clifford and Oxlade, 1989; Philosoph-Hadas et al., 1996) and those supporting the opposite view (Clifford et al., 1983; Kaufman et al., 1985; Harrison and Pickard, 1986; Woltering 1991). It has also been reported that ethylene, at concentrations of 100 μl L^{-1} can redirect 7-day-old etiolated pea plants to

grow downwards rather than upwards when gravistimulated (Burg and Kang, 1993).

Most auxin-resistant mutants exhibit an altered gravitropic phenotype, such as the *Arabidopsis thaliana* mutants *aux1* (Bennett et al., 1996), *axr1* (Lincoln et al., 1990), *axr2* (Wilson et al., 1990), and *axr3* (Leyser et al., 1996), as well as the tomato (*Lycopersicon esculentum* Mill.) mutant *diageotropica* (*dgt*; Kelly and Bradford, 1986; Hicks et al., 1989). These auxin-resistant mutants are also resistant to ethylene. However, small amounts of ethylene have been reported to restore a normal gravitropic response in *dgt* (Zobel, 1974), suggesting that ethylene may act downstream of auxin in gravitropic signal transduction. Elongation of *dgt* roots is less sensitive to application of ethylene than its isogenic parent cultivar VFN8 (Muday et al., 1995), whereas the sensitivity of *dgt* shoot elongation to ethylene is greatly increased when compared to the wild-type response (Shi and Cline, 1992). In contrast, it has been reported that the gravitropic behavior of light-grown wild-type tomato seedlings treated with ethylene and ethylene inhibitors is not altered (Harrison and Pickard, 1986). Although a transitory burst of ethylene has been observed in tomato seedlings within 2 min of horizontal placement (Harrison and Pickard, 1984), a subsequent study concluded that the lack of measurable changes in ethylene production during the first 3 h of the gravitropic response was evidence that ethylene does not play a role in the signal transduction cascade of graviresponses (Harrison and Pickard, 1986). Kaufman et al. (1985) reported a sharp increase in ethylene production between 6 h

and 24 h after gravistimulation in *Avena sativa* but concluded that this increase occurred too late to be a causal factor for gravitropism. An increase in the production of ethylene on the lower half of gravistimulated dandelion plants has been observed, however, this increase also occurred hours after the gravitropic response had been initiated (Clifford et al., 1983) which led to the conclusion that ethylene may modulate but not initiate the gravitropic response.

Applied ethylene is known to inhibit hypocotyl elongation growth in etiolated plants as part of the 'triple response' phenomenon (Goeschl and Kays, 1975; Ecker, 1995). Growth inhibition due to high ethylene leads to decreased tropic responses, which are by definition dependent on growth. There is evidence that the reduction in elongation caused by ethylene occurs via an interaction between ethylene and auxin; while ethylene production is stimulated by auxin, ethylene can suppress polar (basipetal) transport of the auxin indole-3-acetic acid (IAA; Schwark and Schierle, 1992) and can also influence asymmetric distribution of auxin (Schwark and Bopp, 1993). Ethylene mediates the formation and maintenance of the seedling apical hook via an unknown component downstream of CTR1, a protein kinase which is part of the ethylene signal transduction pathway (Peck et al., 1998) and it has been suggested that the *Arabidopsis HLS1* gene controls differential cell growth during hook formation by regulating auxin activity via its N-acetyltransferase activity (Lehman et al., 1996). The acetylation process itself may also be modulated by ethylene. Recently Luschnig et al. (1998) isolated the *EIR1* gene from *Arabidopsis*. This gene shows

homology to a bacterial membrane transporter and if mutated confers reduced sensitivity to ethylene and agravitropism to roots. Experimental evidence in yeast suggests that *EIR1* may play a role in auxin transport (Luschnig et al., 1998).

While growth inhibition by ethylene has been described mostly for etiolated plants, it has been reported that ethylene promotes cell growth and elongation in light-grown *Arabidopsis* seedlings maintained on nutrient-deficient medium (Smalle et al., 1997). Ethylene promotion of cell growth and elongation in the stem has also been reported for the aquatic plant *Ranunculus sceleratus* when submerged in water (Abeles et al., 1992), as well as for ethylene-treated etiolated rice coleoptiles (Satler and Kende, 1985). In *Poa pratensis* and *Avena sativa,* the ethylene-releasing compound ethephon has been implicated in the increase of tiller internode length (Abeles et al., 1992). Thus it appears that ethylene can both inhibit and promote stem growth. While it is intriguing to compare the multitude of different effects that ethylene has been reported to exert on the gravitropic process, it is important to note that this information has been gathered using a large number of different species. Different species may respond to the same level of ethylene in different ways as the literature clearly demonstrates.

Ethylene may also play a role in the integration of signals from light and gravity. In soybean, R was found to reduce ethylene production by as much as 45% while promoting hypocotyl elongation (Samimy, 1978). Both effects were found to be reversible by FR, suggesting that phytochrome regulates hypocotyl growth via ethylene (Samimy, 1978).

Arabidopsis seedlings, when grown under R, lose their ability to reorient themselves to the gravity vector. This loss is also reversible by FR and has been shown to be controlled by both phytochrome A (phyA) and phytochrome B (phyB; Poppe et al., 1996). In *Arabidopsis*, the plant hormone cytokinin, acting via ethylene, can restore gravitropism in seedlings that were rendered agravitropic by R (Golan et al., 1996).

One approach to elucidating how auxin, ethylene, and light interact in shoot gravitropism is to study the response of mutants that are altered in their response to one or more of these factors. To this end, we used two tomato mutants with altered gravitropic responses: the auxin-resistant *dgt* mutant (Kelly and Bradford, 1986; Hicks et al., 1989) which exhibits a reduced gravitropic response (Lomax et al., 1993), and the *lazy-2* (*lz-2*) mutant in which the direction of shoot gravitropism is reversed in a phytochrome-dependent manner (Gaiser and Lomax, 1993). We also investigated whether two tomato mutants altered in their ethylene physiology, the ethylene-overproducing *epinastic* mutant of tomato (*epi*; Fujino et al., 1988) and the ethylene-insensitive *Never ripe* (*Nr*) mutant (Wilkinson et al., 1995; Yen et al., 1995) exhibit alterations in their gravitropic response mechanism. Taken together the results from these experiments lead us to propose that ethylene plays multiple, but not primary roles in modulating the gravitropic response in tomato.

2.3. Materials and Methods

2.3.1. Plant Material

Wild-type tomato (*Lycopersicon esculentum* Mill.) varieties Ailsa

Craig (AC) and Pearson (P), as well as four mutants, *epinastic (epi)*,

diageotropica (dgt), *Never ripe (Nr)*, and *lazy-2 (lz-2)* were used. The *dgt*

and *lz-2* mutants were maintained in the AC background, whereas *epi*

was in the VFN8 background. *Never-ripe (Nr)* was used in AC as well as

the Pearson isogenic parent line for curvature experiments. Seeds of *lz -*

2, epi, and *dgt, Nr* in AC and AC were originally obtained from C. M.

Rick (UC Davis, CA). Seeds of *Nr* in the Pearson background as well as

wild-type Pearson seeds were kindly provided by Dr. Harry Klee. All

lines were propagated by selfing at the Oregon State University Botany

Farm.

2.3.2. Gravicurvature Experiments

For the gravicurvature measurements (in Fig. 2.1. and Fig. 2.2.)

plants were grown in 10x10 cm pots filled with vermiculite and kept in

darkness at 29°C for 4 to 5 d (*epi* plants were grown for 6 - 7 d).

Seedlings were gravistimulated in their pots in R (General Electric

F40/Pl fluorescent lights filtered through a Roscolux Filter #27, 2.51

μmol m^{-2} s^{-1} measured from 640-680 nm, transmission maximum = 660

nm Rosco, Hollywood, CA) in a plant incubation chamber (Hoffman,

Albany, OR) at 29°C. At the indicated time points, individual representative plants were excised at the vermiculite level, photocopied and the curvature was determined with a protractor. The data presented were pooled from 3 experiments.

2.3.3. Ethylene Evolution Measurements

A Shimadzu GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 122 cm Poropak Q column (Waters, Milford, MA) was used for all measurements of ethylene evolution. Approximately 20 seeds were germinated on 1 mL of 1% agar in 10 mL vials (Fisher Scientific, Pittsburgh, PA) and grown in the dark for 4.5 d at 29°C. Prior to gravistimulation, the vials were capped with an air-tight serum stopper (Fisher Scientific). Care was taken to use plants that were short enough not to reach the top of the vial or the serum stopper to prevent artifactual ethylene production resulting from seedling damage or stress response (Lehman et al., 1996). Upright controls or gravistimulated seedlings which had been reoriented 90° were either exposed to R or kept in the dark. At the indicated time points, a 1 mL head space sample was withdrawn from the vial using a 1 mL tuberculin syringe with a 25G needle (Becton Dickinson & Company, Franklin Lakes, NJ) and injected into the gas chromatograph. Ethylene concentrations were determined from a standard curve and total ethylene evolution was normalized to the fresh weight of the seedlings.

2.3.4. Gravicurvature Response to Applied Ethylene

Plants used for measurements of gravicurvature in response to various ethylene concentrations (Fig. 2.5.) were grown in 3 mL scintillation vials filled with vermiculite and kept in darkness at 29°C for 4 to 5 d (*epi* plants were grown for 6 - 7 d). Plants measuring approximately 1.5 to 2 cm from the root/shoot node to the hook were selected (*epi* plants were generally shorter and measured only 1 - 1.5 cm) and an interval 1 cm down the hypocotyl from the top of the hook was marked with black ink (Steig Products, Lakewood, NJ) to monitor elongation growth during gravistimulation. Subsequently, 6 to 7 vials containing one seedling each were set in a holder with the cotyledons pointing upward. The holders were transferred into one liter Mason jars lined with Whatman 3MM paper and the jars sealed air-tight. Prior to gravistimulation, the jars were injected with ethylene at various concentrations and then reoriented 90°. All plants were kept under R (General Electric 40R red fluorescent tubes filtered through red acrylic, Shinkolite 102 [Argo Plastics Co., Los Angeles, CA] 0.95 μmol m^{-2} s^{-1} measured from 640-680 nm, transmission maximum = 642 nm) during gravistimulation. Fluence measurements were made with a LiCor LI-1800 spectroradiometer. All manipulations of the plants were done under dim green safe light. After gravistimulation, the plants were excised at the vermiculite level and photocopied. The length of the marked interval was measured and curvature was determined with a

protractor. Data were pooled from 3 to 6 independent experiments and the standard error calculated.

2.3.5. Ethylene Inhibitor Studies

For studies with ethylene inhibitors (Fig. 2.4.), plants were grown and gravistimulated in sealed jars as described above for the gravicurvature experiments. Norbornadiene (NBD, Aldrich, Milwaukee, WI) was pipetted onto the filter paper just prior to gravistimulation and allowed to evaporate after the jars were sealed airtight. Aminovinylglycine (AVG, Sigma, St. Louis, MO) was applied by watering upright plants with AVG solutions 5 h before gravistimulation to allow time for uptake of AVG through the root system.

2.3.6. Statistical Analysis

All statistical analyses were performed using Microsoft Excel software. p-values reported reflect those of two-sided Student t-test analyses.

2.4. RESULTS

2.4.1. The *dgt, lz-2, epi,* and *Nr* Mutants of Tomato Exhibit Altered Gravitropic Responses

If ethylene plays an important role in the gravitropic response of plants, then mutants which are altered in ethylene perception or response should exhibit altered gravitropism. Alternatively, analysis of the ethylene physiology of known gravitropic mutants may provide insight into the role of ethylene in plant responses to gravity. In Fig. 2.1., the morphology of four tomato mutants, *epi* (an ethylene-overproducing mutant), *Nr* (an ethylene-insensitive mutant which has been demonstrated to lack an ethylene receptor), *dgt* (an auxin-resistant mutant with a retarded gravitropic response) and *lz-2* (a mutant which exhibits a phytochrome-regulated reversal of the shoot gravitropic response) is compared to that of wild-type seedlings. All seedlings were grown in ambient air and kept either upright in darkness (Fig. 2.1., left), upright in R (Fig. 2.1., center), or gravistimulated in R (Fig. 2.1., right). When vertically oriented, all of the seedlings maintained correct orientation away from the gravity vector with the exception of *lz-2* seedlings which bent downward in R. Gravistimulation, achieved by placing the plants horizontally in the presence of R results in complete upward reorientation of wild-type, *epi*, and *Nr* seedlings, incomplete upward curvature of *dgt* seedlings, and reversed (positive) curvature of *lz-2* seedlings. Under all conditions, seedlings carrying the *epi* lesion

display characteristics of wild-type seedlings treated with high ethylene concentrations, including shortened and thickened hypocotyls. Interestingly, *epi* seedlings achieve correct reorientation even with severely stunted hypocotyl growth.

Kinetic analysis of gravitropic curvature revealed additional alterations in the gravitropic responses of all four mutants (Fig. 2.2.). The normal upward gravitropic response of dark-grown wild-type tomato seedlings was initiated within 15 to 30 min of gravistimulation and completed by 4 h, while the auxin-resistant *dgt* mutant exhibited a slower and incomplete response to gravity, reaching only ca. 50° curvature after 20 h. The gravitropic response of the ethylene overproducer *epi* was also reduced during the first 12 h after gravistimulation but reached wild-type levels (75-80° curvature) by 20 h. Under these experimental conditions, the *lz-2* mutant curved upward during the first 3 h of gravistimulation before it reoriented, and by 20 h had curved about 80° downward. While the *Nr* mutant in the AC background exhibited a nearly normal gravitropic response, the initiation of curvature was slightly delayed in *Nr* plants when compared to the wild-type response. This delay was detected during the first 30 to 60 min after reorientation (Fig. 2.2. A). However, curvature of *Nr* plants reached wild-type levels within 90 min of gravistimulation. Similar results were observed with *Nr* in the Pearson background (data not shown).

Figure 2.1. Phenotype of WT (cv. Ailsa Craig), *diageotropica* (*dgt*), *lazy-2* (*lz-2*), *epinastic* (*epi*), and *Never ripe* (*Nr*) tomato seedlings. Seedlings were germinated in the dark for 4.5 d (*epi* = 6.5 d) and then oriented vertically in darkness (left), vertically in R (center) or horizontally in R (right) for 20 h. Representative seedlings were selected and photographed. Note that the *lz-2* mutant in the upright or horizontal positions bends downward when exposed to R.

Fig. 2.1.

Upright in darkness	Upright in R	Gravistimulated for 20 h in R
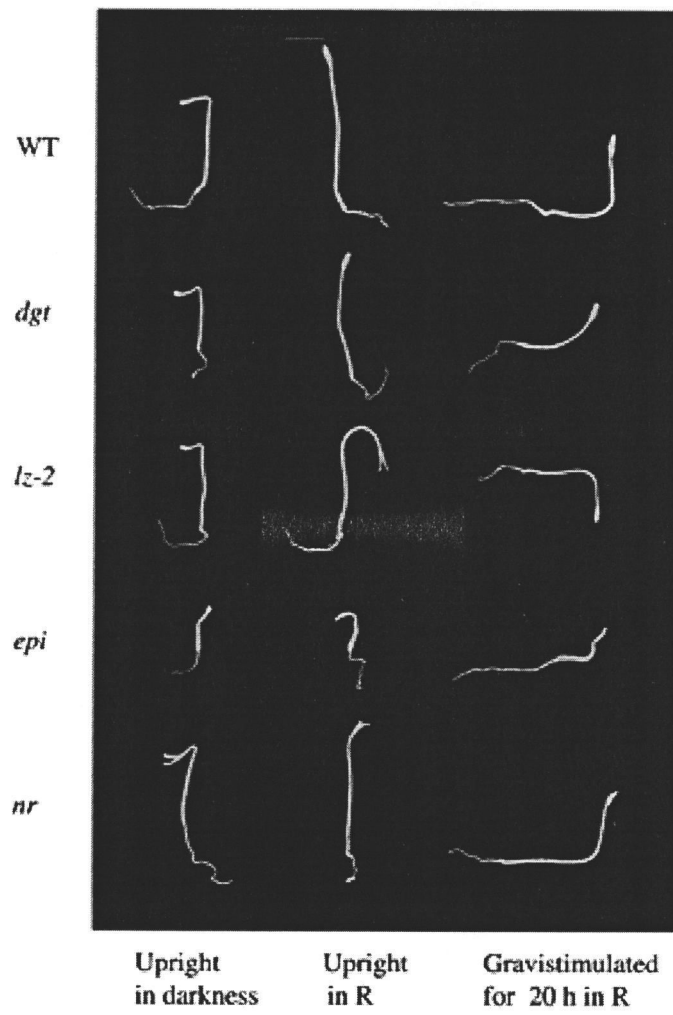
Figure 2.2. The kinetics of gravitropic responses of etiolated auxin- and ethylene- response mutants of tomato in R at 29°C compared with WT. Panel A: Short-term kinetics, panel B: kinetics over 20 h from the same experiments. Symbols are: ▲= WT cv. Ailsa Craig, □= *dgt*, ●= *lz-2*, ▼= *Nr* in AC background, and ◇= *epi*. Plants were grown in darkness for 4.5 d (*epi* = 6.5d) and subsequently transferred to an incubator equipped with fluorescent lights filtered through red Roscolux filters. At the times indicated, representative plants were cut at the vermiculite level and photocopied. The angle of gravicurvature was determined from the photocopies using a protractor. Data were pooled from 3 independent experiments. Error bars reflect SE of the mean, n = 6-21 (with n= 9.3 on average per time point).

Fig. 2.2.
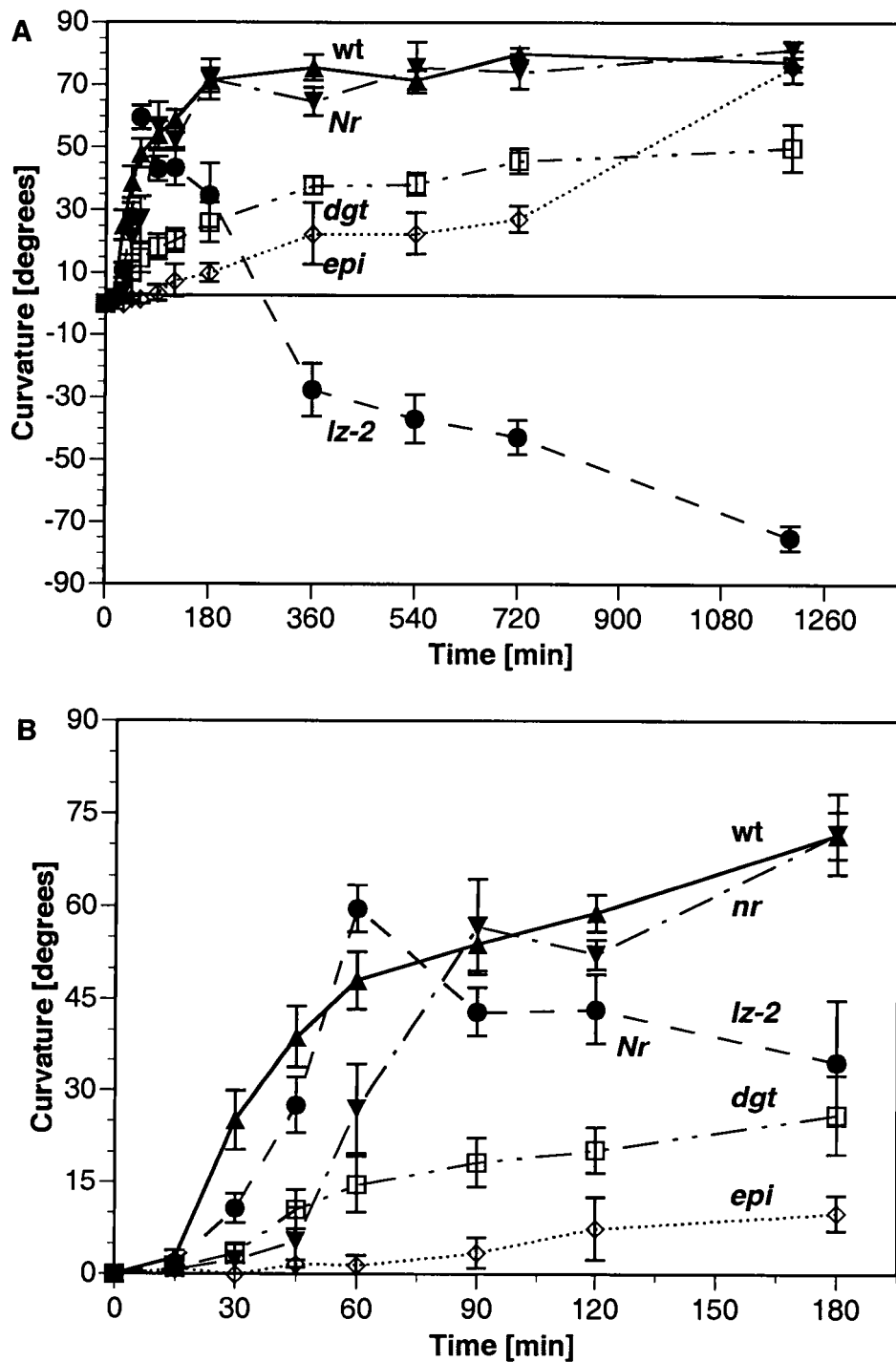
2.4.2. Ethylene Evolution During the Gravitropic Response

As phytochrome mediates the reversal of gravicurvature in the *lz-2* mutant and R has been shown to regulate ethylene concentrations and hypocotyl elongation, we hypothesized that the *lz-2* lesion altered ethylene synthesis or action. We tested this hypothesis by comparing the evolution of ethylene by wild-type, *dgt*, *lz-2*, and *epi* plants which had either been gravistimulated or maintained upright in either darkness or R. Over a 20 h time course, we observed similar rates of ethylene evolution for wild-type, *lz-2* and *epi* plants in both R and darkness and *dgt* plants in darkness (Fig. 2.3.). The exception was a sharp decrease at 20 h in ethylene evolution by *dgt* seedlings which were gravistimulated in R. However, the kinetics of this reduction did not correlate with the reduction in the *dgt* gravitropic response observed as early as 30 min after gravistimulation (compare Fig. 2.2.A and Fig. 2.3. B). It was interesting to note that at 10 h gravistimulated wt and *lz-2* plants as well as both stimulated and unstimulated *dgt* plants showed a plateau in their ethylene production. The rate of ethylene production was increased between 10 and 20 h in wt, *lz-2,* and the ungravistimulated *dgt* mutant, which was in sharp contrast to the gravistimulated *dgt* mutant. Interestingly, with the *epi* mutant, a linear increase in ethylene production was observed for ungravistimulated plants while gravistimulated plants evolved roughly twice as much ethylene as the vertically-oriented plants at 10 h. By 20 h, this difference in ethylene

production between gravistimulated and vertical *epi* plants had disappeared (Fig. 2.3.D).

Figure 2.3. Ethylene evolution is not altered by gravistimulation and/or R. Approximately 20 seeds were planted in each 10 mL vial containing 1mL of 1% agar and incubated at 29°C in the dark for 4.5 d. Vials were capped with serum stoppers prior to treatment. Panel A: wt (AC), panel B: *dgt*, panel C: *lz-2* , panel D: *epi* . Symbols are: Δ= upright plants in RL, ●=gravistimulated plants in darkness, □=gravistimulated plants in RL, ◆=upright plants in darkness. SE bars are shown where larger than the symbol, n = 5-20 vials per time point. Data were pooled from 6 independent experiments.

Fig. 2.3.
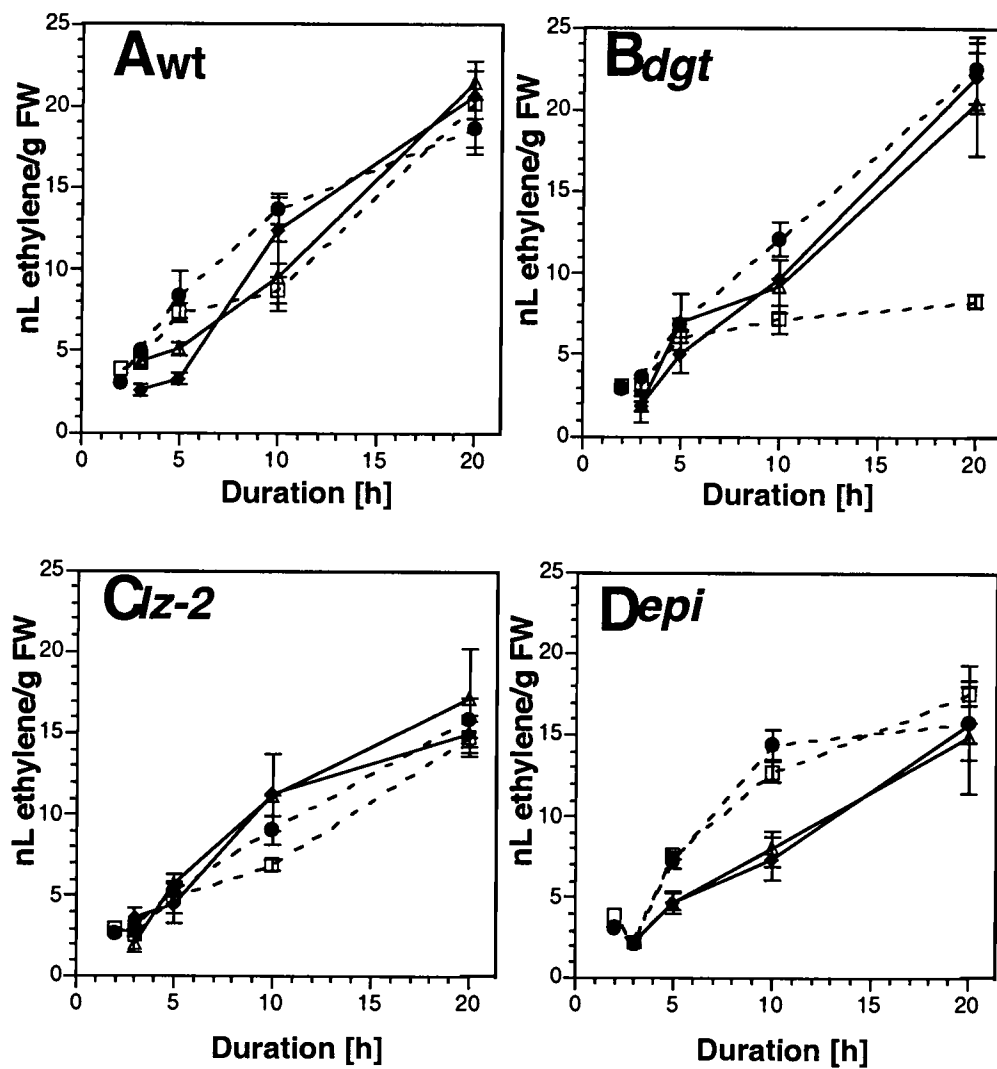
2.4.3. Ethylene Inhibitors Specifically Inhibit the Gravitropic Response

If ethylene is required for a full shoot gravitropic response, then inhibition of ethylene action or synthesis should inhibit gravitropism. Gravicurvature of wild-type plants exposed to varying concentrations of the ethylene action inhibitor NBD was inhibited ca. 50% at 0.87 mM (70 μL) NBD with maximum inhibition (75%) achieved at 1.13 mM (90 μL; Fig. 2.4.) NBD. The overall elongation of the uppermost 1 cm of the hypocotyl was not significantly affected by NBD at these concentrations (Fig. 2.4.). Treatment with the ethylene synthesis inhibitor AVG yielded results similar to those observed with NBD. At the highest concentration tested (100 μM), AVG inhibited curvature 18% (data not shown), which was a statistically significant decrease (p-value 0.01). As with NBD, elongation growth was not affected by concentrations of AVG, which significantly affected gravicurvature (data not shown).

2.4.4. Ethylene has Concentration-Dependent Effects on Curvature

In order to further test the potential of ethylene to modify the response of plants to gravity, we measured gravicurvature in the presence of ethylene concentrations varying from 0.0001 to 0.1 μl L^{-1} (Fig. 2.5.A). The gravitropic response of the ethylene-insensitive Nr mutant was not altered at any ethylene concentrations tested. Seedlings of all other genotypes showed a sharp decrease in curvature in

response to low concentrations of ethylene. Inhibition of the gravitropic response of wild-type and *epi* seedlings was detected at 0.01 μL^{-1} with 50% inhibition at 0.1 μl L^{-1}, while curvature of *lz-2* and *dgt* seedlings was inhibited at even lower ethylene concentrations (significant inhibition was observed at 0.001 and 0.005 μl L^{1}, respectively; Fig. 5B). Treatment with 0.05 to 0.1 μl L^{-1} ethylene resulted in nearly complete inhibition of the *dgt* gravitropic response, but only 50% inhibition for wild-type, *epi*, or *lz-2* plants (Fig. 2.5.B). Thus, all tomato genotypes which retain sensitivity to ethylene exhibit dose-dependent inhibition of their bending behavior in response to gravistimulation.

Interestingly, those ethylene concentrations which significantly inhibited gravicurvature (0.001 μl L^{-1} - 0.1 μl L^{-1}) produced no significant change in overall elongation growth in the 1 cm interval below the hook which included the curvature zone (Fig. 2.5.C). Statistically-significant reduction of elongation growth was observed only for *dgt* and *lz-2* at ethylene concentrations higher than 0.05 μl L^{-1} (p-value 0.01). Those ethylene concentrations are 10- to 50-fold higher than those necessary to significantly inhibit gravicurvature (compare Figs. 2.5.B and 2.5.C). Thus, inhibition of elongation growth and curvature apparently occur independently at the exogenous ethylene concentrations used here.

Figure 2.4. The ethylene action inhibitor NBD inhibits WT gravicurvature but not elongation. 4.5-d-old etiolated WT (AC) seedlings were marked with ink at the top and bottom of a 1cm interval extending from the top of the hook down the hypocotyl. Total hypocotyl length was approximately 1.5 cm. Seedlings in air-tight jars were treated with the indicated concentrations of NBD and reoriented with respect to gravity. After 20 h of gravistimulation, the increase in length of the marked hypocotyl region (% elongation increase of marked interval at 0 h) (●) and hypocotyl curvature (■) were measured. Data are representative of three experiments, n = 6-8 for each point. Error bars represent the SE of the mean.

Fig. 2.4.
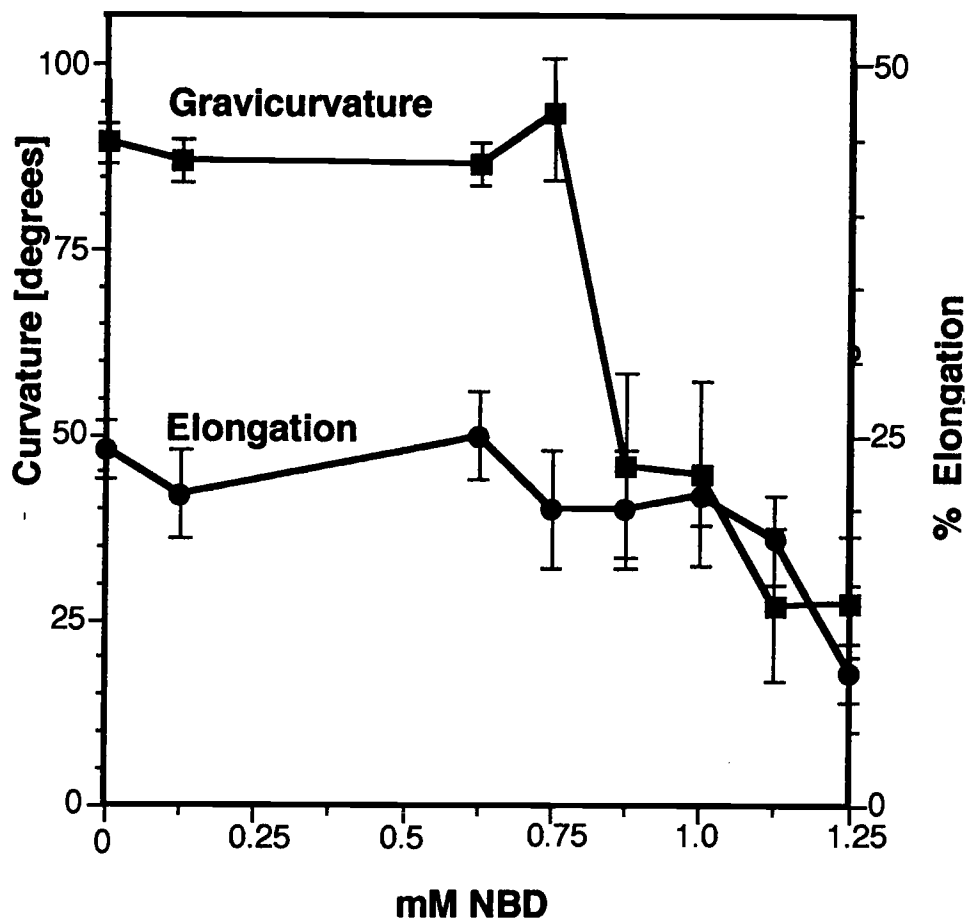
The reduced gravitropic response of the *dgt* mutant is restored by very low levels of ethylene. Fumigation of *dgt* seedlings with ethylene concentrations as low as 0.0005 μl L^{-1} for 20 h resulted in gravicurvature equal to that of wild-type plants (Fig. 2.5.A). This is a 40% stimulation of bending capacity over untreated *dgt* plants (Fig. 2.5.B). To determine whether or not ethylene restored the *dgt* mutant to a full, wild-type gravitropic response, we compared the kinetics of curvature of gravistimulated wild-type and *dgt* seedlings in the presence and absence of the optimal ethylene concentration, 0.0005 μl L^{-1} (Fig. 2.6.). While the wild-type response was similar in either ambient air or ethylene, the gravitropic response of the *dgt* mutant was accelerated by ethylene at this very low concentration. The kinetics of gravicurvature for *dgt* seedlings in the presence of ethylene were, however, not identical to the wild-type gravity response. Curvature of *dgt* seedlings in the presence of ethylene is still slower than that of wild-type seedlings and the acceleration of *dgt* bending by ethylene is not noticeable until 8 h after gravistimulation. By this time, reorientation of wild-type seedlings with respect to gravity was essentially complete. In comparison, the complete reorientation of *dgt* seedlings required 16 h even in the presence of optimal ethylene concentrations. It appears that ethylene enhances and sustains the long-term response of *dgt* hypocotyls to gravity, but does not phenocopy the wild-type gravitropic response (Fig. 2.6.).

Figure 2.5. Ethylene stimulation and inhibition of gravicurvature at concentrations which do not inhibit hypocotyl growth. WT (▲), *lazy-2* (●), *diageotropica* (□), *Never ripe* in AC (▼) and *epinastic* (◇). Gravicurvature (A) and elongation (total length of marked interval after 20 h) (C) were measured after 20 h gravistimulation in R. The bending capacity (B) was derived from the gravicurvature data in (A) as percent change to show differences of sensitivity in the mutants.

Figure 2.5.
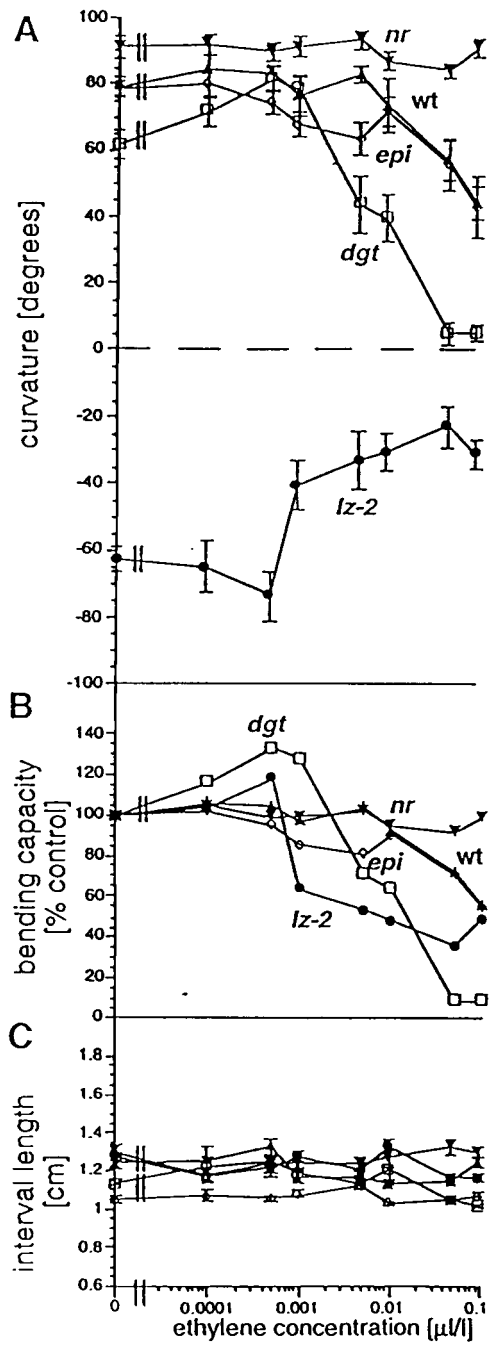
Figure 2.6. Low concentrations of ethylene (0.0005 μl L^{-1}) restore the *dgt* gravitropic response but not with WT kinetics. Plants were grown and treated as for Fig. 5. WT with ethylene (▲), WT without ethylene (△), *dgt* with ethylene (■), *dgt* without ethylene (□). Error bars indicate SE.

Figure 2.6.
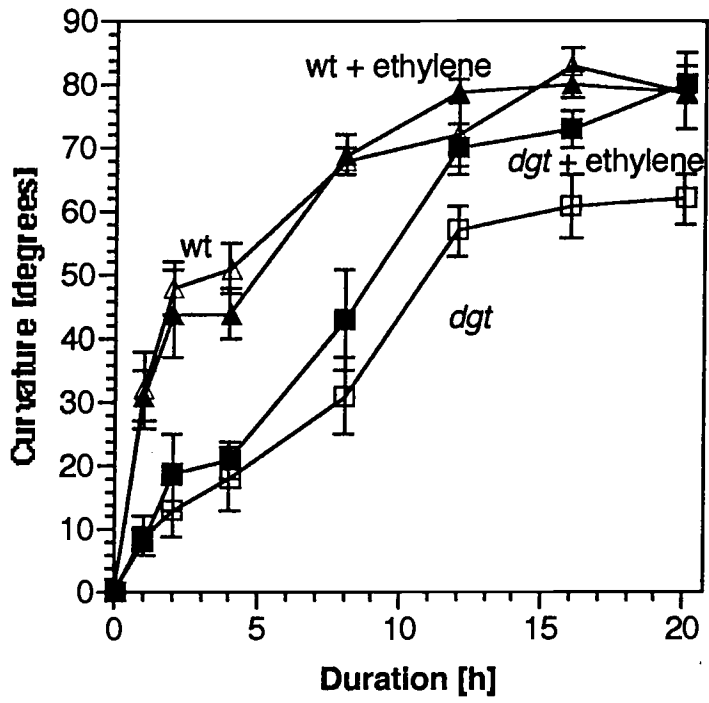
2.5. Discussion

If ethylene plays a primary role in shoot gravitropism as has been proposed (Wheeler and Salisbury, 1980, 1981), then mutants which are altered in ethylene responsiveness should exhibit profound alterations in their gravitropic response. We found that seedlings of the tomato ethylene-response mutants *Nr* and *epi* do exhibit a gravitropic phenotype (Fig. 2.2.). The *epi* mutant has previously been shown to overproduce ethylene (Fujino et al., 1988), while *Nr* is an ethylene-insensitive mutant (Wilkinson et al., 1995). Curvature of both mutants in response to gravistimulation is delayed in comparison with wild-type seedlings, with the *epi* phenotype being much more severe than that of *Nr* (Fig. 2.2.). Both mutants can, however, achieve full reorientation with respect to gravity within 20 h. This relatively minor reduction in the gravitropic response of these ethylene mutants provides strong evidence that ethylene does not play an essential or primary role in the gravitropic response of tomato. Seedlings carrying the *dgt* lesion have a slower gravitropic response and never achieve greater than 50° curvature in ambient air (Fig. 2.2.). The *dgt* lesion has previously been shown to confer greatly reduced auxin sensitivity in hypocotyls (Kelly and Bradford, 1986) and increased sensitivity to ethylene in shoots (Shi and Cline, 1992, and Fig. 2.5.) and it confers a different gravitropic phenotype than either *epi* or *Nr*. The slow but complete gravitropic response of *Nr* and *epi* also did not resemble that of the *lz-2* mutant of

tomato, which, under the R conditions used here, initially curved upward in a manner similar to wild-type seedlings followed by a reversal in the direction of growth resulting in downward curvature (Figs. 1 and 2). The *lz-2* mutant seems to exhibit a biphasic gravitropic response. Similarly, *epi* responds to gravistimulation initially only in an extremely delayed and reduced fashion but curves up rather rapidly after 12 hours, reaching full curvature after 20 h of gravistimulation. Interestingly, this biphasic response in *epi* correlates with an increase in ethylene evolution at 10 h (Fig. 2.3.D).

There are contradictory reports in the literature with regard to ethylene synthesis in response to gravistimulation (Kaufman et al., 1985; Harrison and Pickard, 1986). Treatment with ethylene has been reported to restore normal gravitropic orientation to mature *dgt* plants (Zobel, 1973; Jackson, 1979) and application of ethylene can reverse the direction of the shoot gravitropic response in etiolated pea seedlings (Kang and Burg, 1974; Burg and Kang, 1993) and in mature tomato petioles (Kang and Burg, 1974). However, neither R treatment nor gravistimulation induced changes in the ethylene synthesized by etiolated seedlings of wild-type or any of the mutants tested (Fig. 2.3.). These observations agree with previous reports showing no measurable differences in ethylene production within 3 h of gravistimulation using light-grown wild-type tomato seedlings (Harrison and Pickard, 1986). Overall levels of ethylene evolution by the *epi* and *lz-2* mutants were slightly lower than those observed for wild-type or *dgt* plants. R thus

does not act through increased ethylene evolution in reversing the *lz-2* gravitropic response.

Although *epi* mutants have been characterized as ethylene-overproducing, ethylene synthesis by *epi* seedlings was similar or even reduced compared to wild-type seedlings. This finding is in agreement with observations that hypocotyls are the tissue least affected by the *epi* lesion and the only organ tested that does not overproduce ethylene (Fujino et al., 1988). The morphology of *epi* seedlings is strikingly similar to a constitutive ethylene response in many respects including thicker and shorter hypocotyls (Fig.2.1.; Ursin, 1987). However, it has not been shown that *epi* cosegregates with the constitutive triple response gene (*ctr*) isolated from *Arabidopsis* (Kieber et al., 1993). Although the reduced gravitropic phenotype of *Nr* and *epi* seedlings indicated that normal ethylene responsiveness was necessary for a full gravitropic response, large changes in ethylene synthesis were not observed (Fig. 2.3.).

One exception to the inability of R or gravistimulation to produce changes in ethylene evolution measurable by gas chromatography was the reduction in ethylene synthesis by *dgt* seedlings which had been gravistimulated in the presence of R (Fig. 2.3.B). This suggested that the sluggish gravitropic response exhibited by *dgt* (Fig. 2.2.) may be the result of diminished ethylene production. However, the observed difference in ethylene evolution was significant only after 20 h of treatment, and thus cannot explain the reduction in the *dgt* gravitropic response which was measurable as early as 30 min after

gravistimulation (compare Fig. 2.2. with Fig. 2.3.B). In addition,

curvature of *dgt* seedlings gravistimulated in darkness for 20 h was not

significantly different than that of *dgt* plants gravistimulated in R (data

not shown). While earlier studies have reported that R can cause a

marked deceleration of ethylene production in pea (Goeschl et al., 1967)

and soybean (Samimy, 1978), we observed no effect of either R or

gravistimulation alone on ethylene production in *dgt* or any other tomato

genotype tested. The *dgt* lesion does, however, render ethylene

synthesis sensitive to the simultaneous application of gravity stimulus

and R (Fig. 2.3.B). The basis for this sensitivity remains to be elucidated.

It is interesting to note that increased sensitivity occurred only in *dgt*

shoots, which exhibit increased sensitivity to ethylene (Shi and Cline,

1992; Fig. 2.5.) and resistance to auxin (Kelly and Bradford, 1986;

Muday et al., 1995; Coenen and Lomax, 1998).

The *epi* mutant has been reported to overproduce ethylene in all

tissues except hypocotyls (Fujino et al., 1988). Our results confirm this

finding for the most part. However, at 10 h of gravistimulation we

observed significantly higher ethylene evolution by gravistimulated *epi*

seedlings versus non-gravistimulated *epi* plants. This increased

evolution of ethylene roughly correlates with a somewhat increased rate

of curvature at 10 h in Fig. 2.2., indicating that ethylene may cause (or

be the result of) a second phase of curvature leading to full 90°

curvature after 20 h of gravistimulation. While these correlations are

intriguing, it is important to note that measurements of ethylene

evolution by intact seedlings do not focus on the target tissue that is

affected during gravitropic elongation growth. Therefore, graviresponsiveness or the lack thereof may not necessarily reflect the necessity for ethylene in a normal response in this experiment.

Evidence that ethylene can modulate the gravitropic response is provided by the ability of ethylene action and synthesis inhibitors to reduce gravicurvature in wild-type seedlings. Inhibition of curvature by NBD, an ethylene action inhibitor (Fig. 2.4.), and AVG, an ethylene synthesis inhibitor (data not shown), occurred at concentrations which did not significantly inhibit the overall elongation of the graviresponsive region of the hypocotyl. This confirmed experiments conducted in cocklebur which demonstrated a reduction in gravicurvature by ethylene action and synthesis inhibitors (Wheeler and Salisbury, 1980, 1981) and indicates that low levels of ethylene are necessary for a full gravitropic response. However, a basal gravitropic response still occurred even at high concentrations of NBD. AVG was less effective in inhibiting the gravitropic response than NBD, possibly due to inefficient uptake by the roots.

Further support for the ability of ethylene to stimulate the gravitropic response of tomato seedlings was revealed by the restoration of curvature in *dgt* seedlings to wild-type levels by treatment with extremely low levels of ethylene (Figs. 2.5., 2.6.). These ethylene concentrations are 5- to 10- fold lower that those reported to restore the wild-type gravitropic phenotype of mature *dgt* plants (Zobel, 1973, 1974; Jackson, 1979). However, even in the presence of 0.0005 $\mu l\ L^{-1}$ ethylene, the initial rate of curvature of *dgt* seedlings was much slower

than that of wild-type seedlings. An increase in the curvature rate of ethylene-treated *dgt* seedlings after 12 to 20 h (Fig. 2.6.) subsequently restored the mutant hypocotyls to full curvature. This divergence from wild-type kinetics suggests that low levels of ethylene can stimulate gravicurvature, but not via direct repair of the *dgt* lesion (Fig. 2.6.).

Ethylene was found to play additional concentration-dependent roles in modulating the gravitropic response of etiolated tomato seedlings. While extremely low levels of ethylene appeared to be necessary for a full gravitropic response, intermediate ethylene concentrations (0.005 - 0.1 μl L^{-1}) inhibited wild-type gravicurvature (Figs. 2.5.A and 2.5.B). The gravitropic response of the *dgt* and *lz-2* mutants exhibited increased sensitivity to inhibition by ethylene while the *Nr* gravitropic response was not inhibited by ethylene concentrations as high as 0.1 μl L^{-1}. Inhibition of the reversed gravitropic curvature of *lz-2* plants in R occurred at 0.001 to 0.1 μl L^{-1} ethylene, concentrations that are one thousand-fold lower than those reported to induce reversal of gravitropic orientation of etiolated pea seedlings (Burg and Kang, 1993). In the etiolated seedlings used here, *lz-2* and wild-type seedlings exhibited severe triple response symptoms (stunted growth, radial expansion, and exaggerated hook curvature) at concentrations greater than 1.0 μl L^{-1} (data not shown). Since neither R nor gravistimulation altered ethylene evolution by *lz-2* plants, and ethylene treatment inhibited gravitropism but did not repair the reversed-gravitropic *lz-2*

Ethylene inhibits hypocotyl elongation in a variety of species, including etiolated *Arabidopsis* (Goeschl and Kays, 1975; Ecker, 1995; Peck et al., 1998), yet accelerates hypocotyl growth in light-grown *Arabidopsis* (Smalle et al., 1997) and peanut (Goeschl and Kays, 1975). In this study, we found no significant inhibition or stimulation of overall elongation within the region that includes the gravitropic bending zone by ethylene concentrations that inhibited gravicurvature (Fig. 2.5.C). The lack of correlation between inhibition of gravicurvature and inhibition of growth rates suggests that the differential growth involved in gravicurvature is not regulated in the same manner as overall stem elongation.

If ethylene plays a primary role in gravitropism (Wheeler and Salisbury, 1980, 1981; Clifford and Oxlade, 1989; Philosoph-Hadas, 1996), then an ethylene-insensitive mutant should be agravitropic. Surprisingly, the *Nr* mutant, which is insensitive to ethylene both in seedling and mature stages (Fig. 2.5.; Wilkinson et al., 1995), displayed only a slightly retarded gravitropic response (Fig. 2.2.). Although it is possible that the *Nr* mutation is leaky or that other members of the ethylene receptor gene family can compensate for the missing *Nr* gene product, this does not seem likely because *Nr* is completely insensitive to ethylene with respect to inhibition of the gravitropic response at the concentrations tested in Fig. 2.5.A. The fact that *Nr* seedlings are insensitive to ethylene inhibition of curvature and can attain full reorientation with respect to gravity suggests that inhibition of curvature by ethylene does not play a prominent role in the generation of a normal

gravitropic response. The ethylene-overproducing *epi* mutant could be expected to be either severely inhibited in its graviresponsiveness or enhanced, depending on which part of the ethylene dose-response bell curve is mimicked by the mutation. However, while exhibiting striking similarities to the constitutively ethylene-responding *Arabidopsis ctr* mutant, *epi* did not exhibit an opposite graviresponse to *Nr*. It is possible that higher ethylene concentrations within the tissue lead to a condition that is supraoptimal for the gravitropic response and therefore inhibit or retard the process. However, until the gene products of *dgt*, *epi*, and *lz-2* are identified, these questions cannot be answered unequivocally.

The gravitropic response of the auxin-resistant *dgt* mutant is more severely delayed and reduced than that of *Nr*. Therefore, sensitivity to auxin appears to play a more important role in the gravitropic response mechanism than ethylene sensitivity or synthesis. Application of very low levels of ethylene can, however, compensate for reduced auxin responsiveness by enhancing and sustaining the slower *dgt* response (Fig. 2.6.). The Cholodny-Went hypothesis suggests that tropic curvatures are the result of increased auxin concentrations on one side of a stem (Went and Thimann, 1937). Other studies have provided evidence that gravistimulation results in alterations in auxin sensitivity (MacDonald and Hart, 1987; Rorabaugh and Salisbury, 1989). Our results suggest that ethylene may amplify a signal that either stimulates the asymmetric redistribution of auxin or increases the auxin-sensitivity of the cells in the lower half of a gravistimulated hypocotyl. It has been

proposed that ethylene modulates lateral auxin transport, especially in the apical hook of etiolated seedlings (Schwark and Bopp, 1993; Lehman et al., 1996; Peck et al., 1998). Ethylene may compensate for the reduced auxin responsiveness of the *dgt* mutant by enhancing lateral transport of auxin to target cells in the hypocotyl epidermis. In mutants which have reduced ethylene sensitivity, such as *Nr*, the stimulation of auxin transport by ethylene may be attenuated, leading to a partial or delayed gravitropic response, but not eliminating the basal rate of lateral transport of auxin. This possibility is also supported by the recent finding that *eir-1*, a root-specific, agravitropic, ethylene-insensitive mutant of *Arabidopsis* likely owes its phenotype to a dysfunctional auxin transporter (Luschnig et al., 1998). However, it is possible that stimuli other than ethylene also enhance lateral auxin transport and thus compensate for the inability of *Nr* to respond to ethylene normally. The same argument can also be applied to ethylene stimulation of auxin responsiveness in the *dgt* mutant.

Our genetic analysis has revealed that while ethylene does not play a primary role in the gravitropic response of etiolated seedlings, it can act as a modulator of gravitropism by either stimulating or inhibiting curvature. The mechanism by which ethylene influences gravitropism remains to be elucidated. However, these studies indicate that ethylene is part of a complex feedback mechanism in the gravitropic response similar to that demonstrated for the role of ethylene in elongation growth. Ethylene may play an important role in the initiation or maintenance of differential growth responses which help emerging seedlings detect

obstructive objects and adjust growth rates and direction accordingly. Alternatively, ethylene modulation of the gravitropic response may be a residual interaction resulting from the mechanisms governing hook formation and maintenance. These studies provide testable hypotheses which can be used to elucidate the interaction between auxin and ethylene not only in regulating gravitropism, but also in integrating that information with other environmental cues.

2.6. Acknowledgements:

3. GRAVITY, AUXIN AND THE *DGT* GENE REGULATE THE FORMATION OF TRACHEARY ELEMENTS IN TOMATO CALLUS

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3.1. Abstract

Calli of wild-type tomato (*Lycopersicon esculentum* Mill.) and the auxin-resistant *diageotropica* (*dgt*) mutant of tomato were subjected to a variety of hormone concentrations and gravity environments to study vascular development. Tracheary element (TE) formation is dependent on both cytokinins and auxin. In wild-type calli, the ratio of these hormones to each other is crucial not only for callus growth but also for the development of TEs. We show here that the *dgt* mutant responds to treatment with 2,4-dichlorophenoxy acetic acid (2,4-D) and benzyladenine (BA) with respect to callus growth and TE development. Experiments in microgravity and hypergravity demonstrate that TE differentiation is also dependent on gravity. While adult plants of *dgt* display a poorly developed vascular system, calli of the mutant produce larger numbers of TEs than do wild-type calli. Wild-type calli exposed to microgravity on a 9-day space shuttle flight (STS 95) contained fewer TEs than wild-type calli kept at 1 G. In contrast, *dgt* calli produced even higher amounts of TEs in microgravity than on the ground. When wild-type and mutant calli were subjected to hypergravity (3G) induced by low speed centrifugation, wild-type calli produced higher numbers of TEs than the 1G controls and *dgt* calli showed no significant difference from 1G controls. These results suggest that gravity-dependent cell differentiation during early vascularization is mediated by the DGT gene, likely as part of an auxinpathway, and argue for a regulatory role of gravity during TE development.

3.2. Introduction

Plants growing on Earth have evolved from single cells to become the tallest known organisms in spite of constant exposure to gravity. Life in a gravity environment not only requires strong structural support to stand erect, but also poses the problem of water transport from the roots to the leaves. In some species, water must be transported over 100 meters against the force of gravity. To efficiently transport water, higher plants have evolved a sophisticated network of vessel elements and tracheids, commonly referred to as tracheary elements (TE) that enable the plant to transport greater amounts of water and soluble nutrients to the top of the plant than would be allowed by simple diffusion. These thickened, tubular cells make up the greater part of the xylem in plants and their walls are highly lignified enabling them to withstand high negative pressure caused by the acropetal water flow. In angiosperms, TEs differentiate into vessel members and fibers during secondary growth, while in gymnosperms TEs as tracheids make up by far the greatest portion of the woody tissue. Vessel members mature into strands of water-conducting cells while fiber cells have highly lignified and thickened cell walls but do not conduct water (Esau, 1977).

The involvement of plant growth regulators in vascular development has been well established. Wetmore and Rier (1963) demonstrated that auxin applied to the cut surface of undifferentiated callus tissue could initiate vascular development. Later, Sachs (1981) proposed that the initiation of vascular tissue leads to a "canalization

process", where auxin transported to the end of the least differentiated provascular cell aids in the recruitment of the next cell into a continuous strand of vasculature (for review see Aloni et al., 1995).

During vascular differentiation a set of unique genes is expressed and may specifically be involved in TE differentiation. Genes associated with TE differentiation include *ted3* which encodes a glycine-rich glycoprotein isolated from *Zinnia* (Demura and Fukuda, 1993), the homeobox-containing transcription factor *Athb-8* (Baima et al., 1995) described in *Arabidopsis,* and a pectate lyase (*ZePel*) from *Zinnia elegans* (Domingo et al., 1998). While *ted3* is expressed just hours before provascular cells first differentiate into TEs, *Athb-8* is specifically expressed during re-vascularization in tobacco after stem injury (Baima et al., 1995). *ZePel* is expressed in vascular bundles and shoot primordia (Domingo et al., 1998).

Auxin induces the transcription of both *Athb-8* (Baima et al., 1995) and *ZePel* (Domingo et al., 1998), supporting a major role for auxin in vascularization events. It is therefore likely that auxin-resistant mutants are affected in their vascular development. Lincoln et al. (1990) reported "subtle differences" in the vascular development of the auxin-resistant *axr1* mutant of *Arabidopsis.* TE production was reduced in transgenic *Arabidopsis* plants that overproduce auxin-inactivating enzymes (Klee et al., 1987; Romano et al., 1991), while overproducing auxin led to an increase in vascularization (Hobbie and Estelle, 1994).

The role of gravity in directional growth has been well studied and it is believed that gravitational force leads to a shift in the distribution of

auxin, regulated through changes in auxin transport. Alternatively, the change in the gravity vector may cause a change in tissue sensitivity to auxin (for reviews see Trewawas, 1992, Salisbury, 1993). In experiments conducted in microgravity, a significant reduction in lignin content and the number of lignified cells was found in mung beans and alfalfa seedlings (Cowles et al.,1995; Campbell et al., 1996), indicating a correlation between lignin production and gravity. Previous space flight experiments also showed that the overall production of the auxin indole-3-acetic acid (IAA) by maize karyopses does not change significantly in microgravity when compared to karyopses grown in normal gravity (Schulze et al., 1992). Therefore, a deficiency in the production of auxin is not likely to be responsible for any developmental abnormalities in plants grown in microgravity (Schulze et al. 1992). To our knowledge, it has not been established whether gravity plays a direct role in the formation of TEs.

Our laboratory has used the auxin-resistant *diageotropica* (*dgt*) mutant of tomato to further investigate the role of auxin, cytokinin, and gravity in the development of TEs in undifferentiated callus tissue. The *dgt* mutant is the result of a single-gene, recessive lesion (semi-dominant only with respect to leaf shape, Ursin and Bradford, 1989) but exhibits a pleiotropic phenotype (Zobel, 1973, 1974; Kelly and Bradford, 1986, Lomax et al., 1993; Coenen and Lomax, 1998). The *dgt* lesion reduces both hypocotyl elongation and ethylene production in response to auxin (Kelly and Bradford, 1986), as well as in a reduced gravitropic response, a lack of lateral roots, overproduction of anthocyanins and

chlorophyll, hyponastic leaves, and shortened internodes (Zobel, 1973, 1974, Lomax et al., 1993). In tissue culture regeneration experiments, the *dgt* mutant showed reduced responsiveness to auxin with respect to TE production to auxin but normal sensitivity to cytokinin (Coenen and Lomax, 1998). In calli cultured on a matrix of auxin and cytokinin concentrations for 30 d, the *dgt* mutant was less responsive to the effects of the hormone combinations than wild-type calli (Coenen and Lomax, 1998). The same study revealed an increase in highly differentiated individual vessel members in *dgt* calli when compared to wild-type calli. This increase in in contrast to adult *dgt* plants, which display fewer and less mature TEs than wild-type plants (Zobel, 1974). The goal of this study was to further investigate the role of auxin, cytokinin, and gravity with respect to the development of vascular tissue in tomato.

3.3. Materials and Methods

3.3.1. Plant Material

Seedlings of wild-type tomato (*Lycopersicon esculentum,* Mill, cv. VFN8) and the isogenic *diageotropica* (*dgt*) mutant were grown in a light (16h light/8h dark, 50 uE of PAR m^{-2} s^{-1}) incubator at 29°C in sterile Magenta boxes lined with one layer of wet Kimtowel (Kimberly Clark, Roswell, GA) and covered with one layer of filter paper. Seedlings were

grown for 7-10 days (d) for callus induction. For light microscopy and fluorescence microscopy, plants were grown for 33 d.

3.3.2. Fluorescence microscopy

Thirty-three d old plants (see above) were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.2. Sections were subsequently dehydrated in ethanol, embedded in paraffin (Paraplast, Fort Washington, PA) and sectioned with a rotary microtome. Sections were viewed under fluorescent light or phase contrast without staining.

3.3.3. Tissue Culture

Callus was induced as described elsewhere (Coenen and Lomax, 1998). Briefly, sections of light-grown hypocotyls were sterilized in 5% bleach and cultured on 1x MS medium containing vitamins and minerals (Sigma, St. Louis, MO), 3 μM 2,4 dichlorophenoxyacetic acid (2,4D; Sigma) and 3 μM benzyladenine (BA; Sigma). After two months of induction, the callus was cut away from the original explant and subcultured monthly for the indicated time on induction medium (MS, 3μM BA, 3 μM 2,4D).

For the analysis of hormone interactions, the callus was cut away from the original explant after two months of induction and subcultured monthly for an additional three months on induction medium. At this time, four callus pieces (approximately 0.125 cm^3 in size) were placed

on MS medium containing the indicated concentrations of 2,4D and BA

(see Fig. 3.2. and Table 3.1 and 3.2.). After three additional months of

monthly subculturing on the same hormone concentrations, the callus

diameter was measured at the widest part of the callus. Four pieces of

callus were measured per data point and the values averaged.

Subsequently, cells from each callus were sampled and used for TE

counting. The samples placed on the differential hormone grid did not

contain cells from the original callus, which was readily distinguishable

from new growth. This was done to avoid bias due to previously

differentiated cells (see Fig. 3.3. A).

3.3.4. Tracheary Element Quantitation

For TE counting, the callus was weighed (approximately 0.02 g)

and placed in 5% CrO_3 (w/v) in 5 % HCl (v/v) for 12-24 h. Subsequently,

the callus was passed through a syringe fitted with a 22G needle

(Becton Dickinson &Co, Franklin Lakes, New Jersey) until the tissue was

completely macerated. The cell macerate was diluted 4-fold with distilled

H_2O and centrifuged in a microcentrifuge for 5 minutes at 2000 rpm.

75% of the supernatant was removed and replaced with distilled H_2O.

This process was repeated 4 times. After the last wash, the supernatant

was removed leaving 50 μl in the tube. The pellet was resuspended by

vigorous vortexing and TEs were counted using 7 μl of this dilution in a

BrightLine hemacytometer (Hausser Scientific, Horsham, PA) and a

Leica DMLS light microscope (Bartels and Stout, Seattle, WA) with a

Leica C-Plan 10x objective.

3.3.5. Microgravity experiments

To address the question of whether or not a reduction in gravity

has an influence on TE production, callus was flown on board a space

shuttle. Callus was induced as described above. After 2 months of

induction, callus was cut away from the original explant and subcultured

twice on induction medium for one month each. Callus pieces were cut

from the surface of the callus to ensure a largely undifferentiated state of

the cells (data not shown). Nine days before the space shuttle launch

the samples were flown to Exton, PA, where they were loaded into the

flight hardware by technicians of Instrumentation Technologies

Associates (ITA). Pieces of wild-type and mutant callus were transfered

into 125 μl plastic wells containing induction medium and kept at 20°C in

the dark for the duration of the experiment. The samples were exposed

to microgravity for approximately nine days during the STS-95 space

shuttle flight. An identical set of callus pieces was kept on the ground in

microcentrifuge tubes containing induction medium. The controls were

kept at room temperature (~20°C) in the dark at Oregon State

University.

3.3.6. Hypergravity experiments

Callus was induced as described above. After 2 months of induction, the callus was cut away from the explant and subcultured on induction medium for an additional 3-4 months with monthly transfer to fresh media. Callus pieces were cut from the surface as described above. Three to four pieces of callus were placed on induction medium in 50ml Falcon tubes and placed in a swinging bucket rotor (7-94 GH 3.7) of a Beckmann GPR (Palo Alto, CA) centrifuge and centrifuged at 3G (190 rpm) for 18d in the dark at room temperature (~20°C).

3.3.7. Scanning Electron Microscopy

Upon retrieval of the specimens from the space shuttle 20 hours after landing, the callus was divided into two halves. One half was fixed in 2.5% EM-grade glutaraldehyde (Sigma) for scanning electron microscopy while the other half was placed in CrO_3 and used for TE counts. After fixation in glutaraldehyde overnight, samples were dehydrated in ethanol, chemically dried overnight in hexamethyldisilazane (Ted Pella, Redding, CA) and mounted on aluminum planchettes. The mounts were placed in an Edwards S150B sputter coater and coated with approximately 20 nm of 60/40 wt % gold/palladium. Specimens were viewed using an AmRay 3300 FE scanning electron microscope (KLA Tencor, Bedford, MA). Ground controls were treated in the same manner.

3.4. Results

3.4.1. Vascular development is altered in the *diageotropica* mutant

Hypocotyls from both *dgt* and wild-type were sectioned and viewed using fluorescence microscopy. Vascular bundles autofluoresce due to the lignification of the cell walls. Representative samples of 33 day-old plants are shown in Fig. 3.1. A+B. The xylem is arranged in distinct sectors, the vascular bundles or fascicles. The wild-type hypocotyl (Fig. 3.1. A, G) shows large vessel member cells. The *dgt* mutant displays vessels greatly reduced in number and size as well as an overall reduction in the development, expansion, and organization of the vascular bundles (Fig. 3.1. B, H). Figure 3.1. F+H show a longitudinal section of a 33d-old *dgt* hypocotyl. Arrows point to annular rings or helical spirals of lignin surrounding the vessel members in *dgt* (Fig. 1 F+H), and to lignin in the larger, scalariform vessel members in the wild-type (Fig. 1G). Vessels in the *dgt* hypocotyl are reduced in size, number and developmental stage when compared to wild-type hypocotyls (Fig. 3.1. E+G). Development of most vessel members appears to be at the late protoxylem stage in *dgt* while wild-type sections show that nearly all TEs have developed into metaxylem at 33d (Fig. 3.1.E-H).

Figure 3.1.: The vascular development of the *dgt* mutant is reduced *in planta* and enhanced in two-month-old callus culture. 33 d-old hypocotyls were sectioned and viewed under fluorescent light (A,B,G,H) or using darkfield (E+F). Lignified areas autofluoresce yellow in fluorescent light (A,B,G,H). Callus cells were handsectioned and viewed using light microscopy at 100x magnification (wild-type (C), *dgt* (D, arrow points to TE). Wild-type hypocotyl transverse section, 40x magnification (A); *dgt* hypocotyl transverse section, 40x magnification (B); wild-type hypocotyl longitudinal section, 400x magnification (E,G, arrow in G points to scalariform lignification); *dgt* hypocotyl longitudinal section, 400x magnification (F,H, arrows point to annular rings).

Figure 3.1.
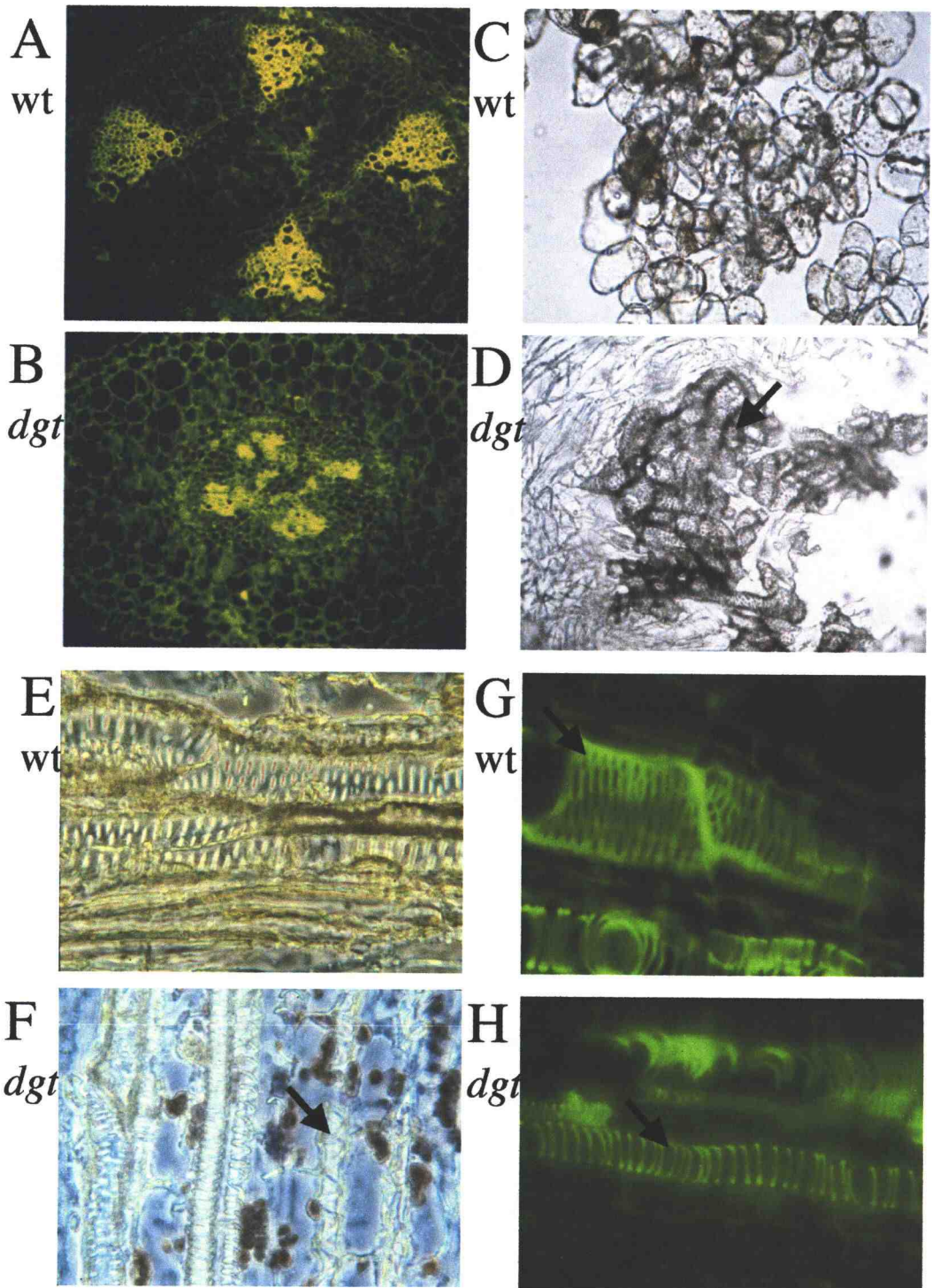
In contrast, in two-month old *dgt* callus grown on 3 μM 2,4D and 3 μM BA numerous well-developed scalariform and pitted vessel members are visible (Fig. 3.1. D, arrow), while wild-type callus displays fewer developed TEs (Fig. 3.1. C).

3.4.2. Cytokinin and auxin affect the growth of both wild-type and *dgt* mutant callus

Previous studies with two-month-old callus have shown that callus induction was dependent on the presence of both auxin and cytokinin in both wild-type and *dgt* tissues (Coenen and Lomax, 1998). The same study revealed optimal hormone concentrations for growth for wild-type callus but no concentration dependence for *dgt* callus growth (Coenen and Lomax, 1998). Here, we have found that in callus, grown over an expanded period of time (2 months induction plus 3 additional monthly transfers), growth of 5-month-old wild-type and *dgt* calli was clearly dependent on both auxin and cytokinin. Wild-type callus displayed (Fig. 3.2.A) optimal growth at equimolar concentrations of BA and 2,4D in the 3-30 μM range. Higher concentrations of auxin inhibited callus growth. This inhibition could be reduced with increasing cytokinin concentrations. Five-month-old *dgt* callus (Fig. 3.2.B) responded to both 2,4-D and BA and displayed optimal growth in the 3-30 μM range, similar to wild-type callus. The responsiveness of both *dgt* and wild-type calli to 2,4-D and BA with respect to growth was statistically significant within a 95% confidence interval when comparing lowest and optimal

hormone concentrations. At 0 and 0.3 μM BA, *dgt* callus was inhibited less by high auxin concentrations than observed for wild-type. Interestingly, callus growth under the conditions tested was more prolific at most hormone combinations in *dgt* than in wild-type.

3.4.3. Cytokinin and auxin affect the development of TEs in both wild-type and the *dgt* mutant

In order to further investigate the observation that *dgt* cultures produce abnormally high amounts of highly-differentiated TEs (Fig. 3.1.), a matrix of auxin and cytokinin identical to that used to assess callus growth was examined for its affect on TE formation in both wild-type and *dgt*. Callus tissue grown for three months on the indicated concentrations of hormones was macerated overnight and TEs were isolated and counted. As expected from the literature, wild-type callus produced no TEs in the absence of either auxin or cytokinin alone and produced more TEs as the concentration of either auxin or cytokinin increased to 3 μM. TE production was inhibited at higher concentrations of auxin and cytokinin (Table 3.1). High levels of TE production was observed in wild-type only in the presence of high concentrations of BA and even the highest concentration of BA tested (30 μM) did not have inhibitory effects (Table 3.1.).

Figure 3.2.: Response of wild-type and *dgt* callus to treatment with auxin and cytokinins with respect to callus growth. Callus was induced on 1x MS media containing 3 μM 2,4-D and 3 μM BA. Fresh callus pieces (0.125 cm^3) were transfered to media containing the indicated hormone concentrations and subcultured monthly. After 3 months the callus diameter was measured. A: wt callus, B: *dgt* callus. Error bars represent SE. ANOVA tests were performed using SAS 8.0. Statistical significance is discussed in the text. Data were pooled from two independent experiments, each data point represents the mean of 8 samples.C: wild-type and *dgt* callus used for growth analysis.

Figure 3.2.
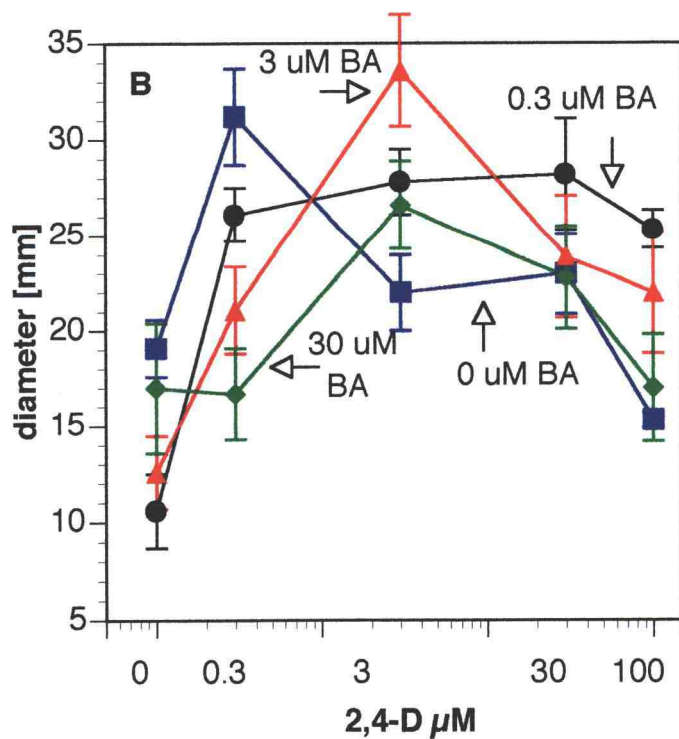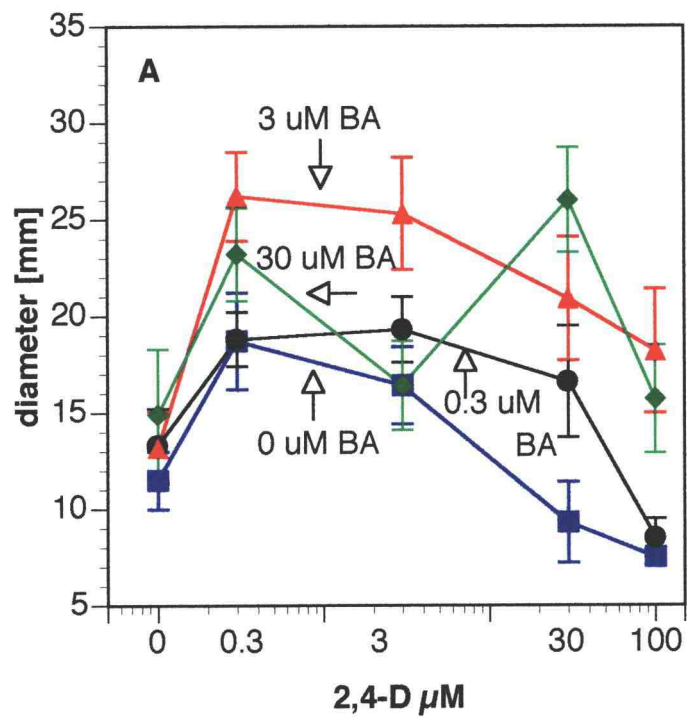
Figure 3.2. C
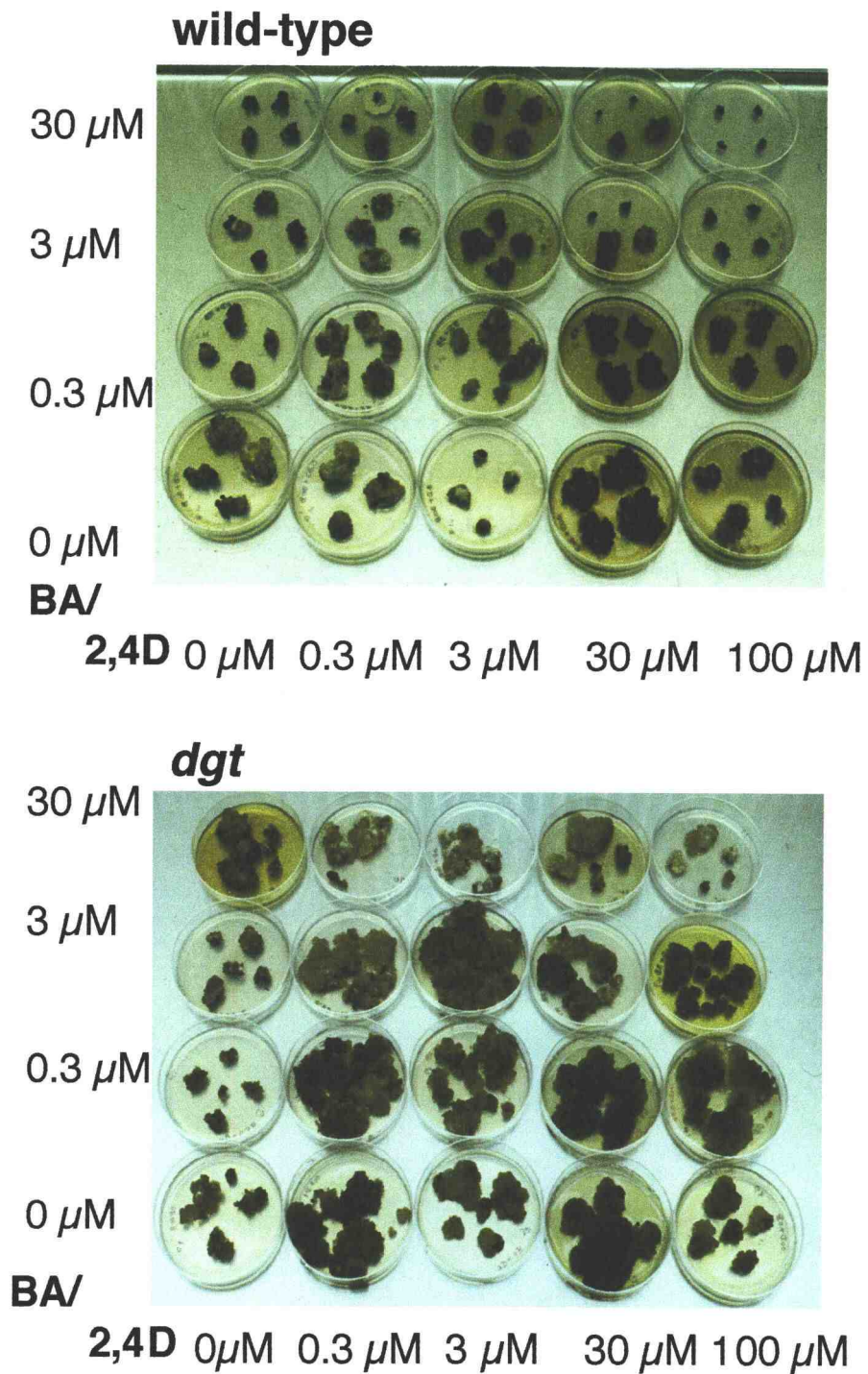
wild-type

30 μM

3 μM

0.3 μM

0 μM

BA/

2,4D 0 μM 0.3 μM 3 μM 30 μM 100 μM

dgt

30 μM

3 μM

0.3 μM

0 μM

BA/

2,4D 0μM 0.3 μM 3 μM 30 μM 100 μM

The dose-response curves of optimal vs. non-optimal auxin concentrations and optimal vs. non-optimal concentrations of BA were statistically significant within a 95% confidence interval when comparing optimal concentrations with non-optimal concentrations.

As observed for wild-type, the *dgt* mutant also produced more TEs at higher BA concentrations and reached its maximum at the highest BA concentrations (Table 3.2.). However, in *dgt* calli, we observed significant growth and production of TEs when both auxin and cytokinin were absent. The dose-response curves of optimal vs. non-optimal auxin concentrations and optimal vs. non-optimal concentrations of BA were statistically significant within a 95% confidence interval when comparing optimal concentrations with non-optimal concentrations. Despite the mutant's resistance to auxin for certain physiological responses, *dgt* responded both to auxin and cytokinin application during the formation of TEs.

Figure 3.3.: Both wild-type and *dgt* callus are responsive to treatment with auxin and cytokinins with respect to TE development. Callus was induced on 1x MS medium containing 3 μM 2,4-D and 3μM BA and subcultured for a total of 5 months. Fresh callus pieces (0.125 cm^3) were transfered to media containing the indicated hormone concentrations and were subcultured monthly. After 3 additional months tissue was sampled, macerated in CrO_3/HCl overnight and TEs were counted in a hemacytometer. Data (tables 3.1. and 3.2.) was collected from portions of the callus as depicted to avoid bias of previously differentiated cells.

Figure 3.3.
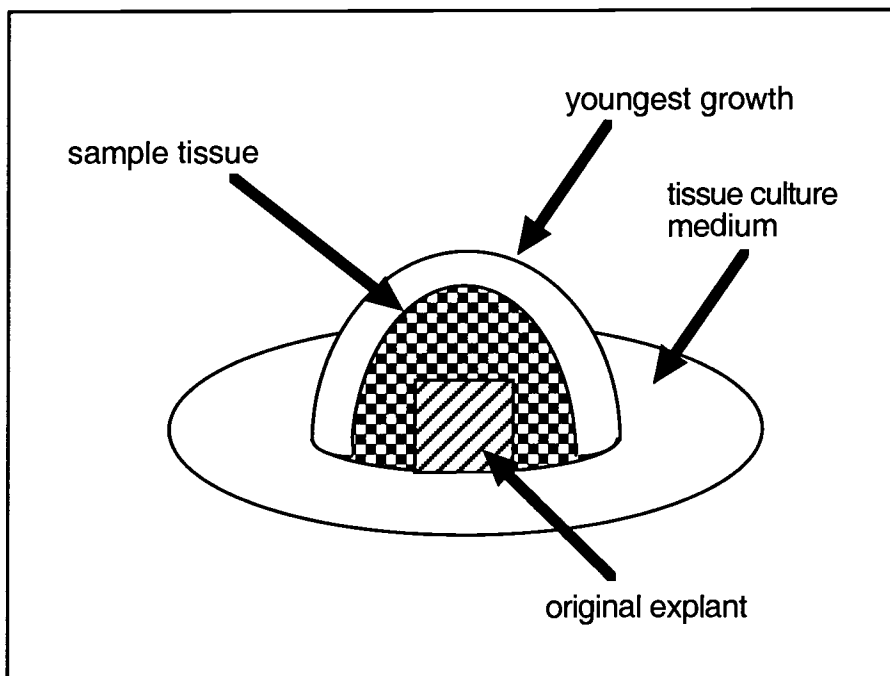
Table 3.1.: TE formation in wild-type callus. TEs were purified and counted as described in Materials and Methods from wild-type callus cultured on the indicated hormone concentrations for 3 months. 4-6 samples from two independent experiments were pooled. Values presented are the means of 4-6 samples from two independent experiments ± SE.

#TEs/gFW

BA \ 2,4D	0	0.3	3	30	100
30	444±203	779±203	943±338	109±47	12±7
3	39±0	210±34	331±118	57±188	8±26
0.3	0±0	216±121	58±24	4±3	0±0
0	0±0	0±0	37±32	0±0	0±0

Table 3.2.: TE formation in *dgt* callus.TEs were purified and counted as described in Materials and Methods from *dgt* callus cultured on the indicated hormone concentrations for 3 months. 4-6 samples from two independent experiments were pooled. Values presented are the means of 4-6 samples from two independent experiments ± SE.
#TEs/gFW

BA \ 2,4D	0	0.3	3	30	100
30	428±216	743±411	131±33	27±14	112±67
3	95±79	115±33	412±138	39±9	56±25
0.3	40±20	436±208	66±21	0±0	1±1
0	150±75	35±9	0±0	0±0	9±5

3.4.4. The effect of gravity on TE development

Gravity plays a pivotal role in the development of structural elements and body shapes of many organisms on Earth. To test the hypothesis that gravity is essential for the development of TEs, we induced callus cells in the laboratory and subcultured them for a total of 4 months (on induction medium with 3 μM auxin and 3μM cytokinin) before exposing them to microgravity for 9d on the Space Shuttle Discovery (STS 95) in the presence of the same hormone concentrations. Upon return of the shuttle, the callus was divided into two halves, one of which was prepared for electron microscopy, the other was prepared for TE counting. Figure 3.4. shows scanning electron micrographs of tissue kept on the ground and tissue exposed to microgravity. Wild-type callus from ground controls produced moderate amounts of TEs (Fig. 3.4.A) while *dgt* callus on the ground produced much higher numbers of TEs, which are arranged in large clusters (Fig. 3.4.B). In both cases, the TEs are highly developed. No TEs could be detected in electron micrographs of wild-type callus exposed to microgravity (Fig. 3.4.C), while TEs were numerous in space-flown *dgt* callus (Fig. 3.4.D).

This visual trend was confirmed when the other half of each callus was macerated overnight and used for TE counting: no TEs were detected in shuttle-flown wild-type tissue, while *dgt* callus exposed to microgravity displayed enhanced production of TEs when compared to ground controls (Fig. 3.5.A). Statistical analysis of the flight experiment

indicates a weak correlation between microgravity and a reduction in the formation of TEs in wild-type callus (p-value of a single sided t-test: 0.119) as well as for the increased production of TEs in the *dgt* mutant (p-value of single sided t-test: 0.156).

Since the spaceflight experiment could not immediately be repeated and the sample size (n = 3-4) of the experiments in microgravity was low, the effects of hypergravity were tested to evaluate a possible role of gravity in TE development in similarly grown callus. Wild-type cells grown for 18 d at 3 G yielded an increased number of TEs (Fig. 3.5.B, p-value of single sided t-test: 0.024), while no statistical difference in TE production was evident in the *dgt* mutant under 3 G gravity conditions when compared with 1G controls (Fig. 3.5.B, p-value of single sided t-test: 0.92).

Figure 3.4.: Microgravity alters TE production in both wild-type and *dgt* callus. Fresh callus was exposed to 9 d of microgravity on the Space Shuttle Discovery (STS95). Upon retrieval, callus was fixed in 2.5% glutaraldehyde and prepared for scanning electron microscopy. Wild-type callus ground control (A), *dgt* callus ground control (B), wild-type callus space-flown (C), and *dgt* callus space-flown (D). Arrows point to TEs.

Figure 3.4.

WT   *dgt*

ground

A   B

space

C   D
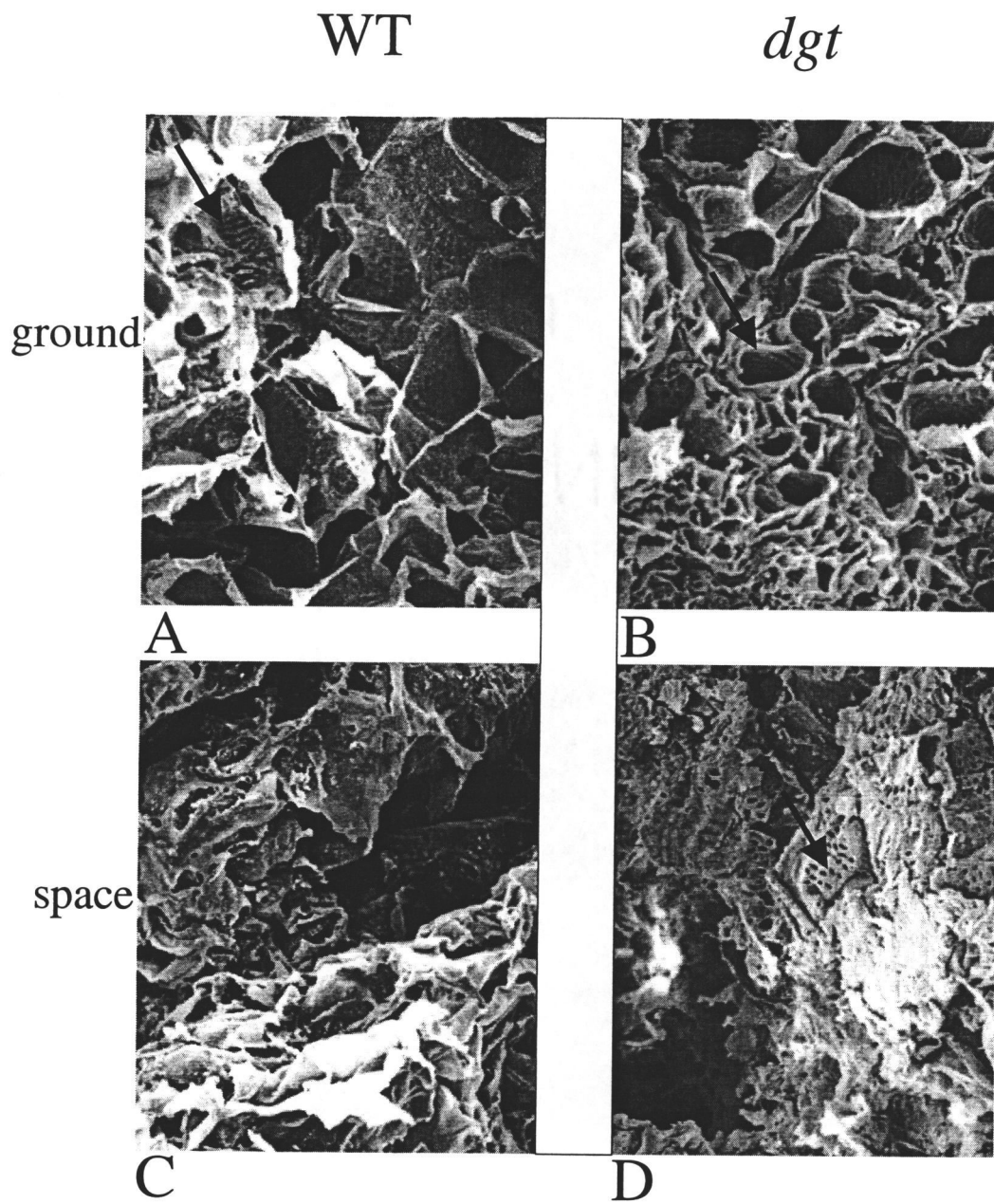
Figure 3.5. A: Microgravity alters TE production in both wild-type and *dgt* callus. Fresh callus was exposed to 9 d of microgravity on the Space Shuttle Discovery (STS 95). Upon retrieval, tissue was weighed, macerated in CrO_3/HCl overnight, and TEs were counted in a hemacytometer. **B:** Hypergravity increases TE count in wild-type callus. Fresh callus was exposed to 18 d of hypergravity (3 G) in a Beckman centrifuge. Upon retrieval, tissue was treated as described above. The experiment was carried out three times and the data pooled. The statistical analysis of varience was calculated (single sided t-tests) and p-values are given in the text. Error bars represent SE of the mean (n=12).

Figure 3.5.
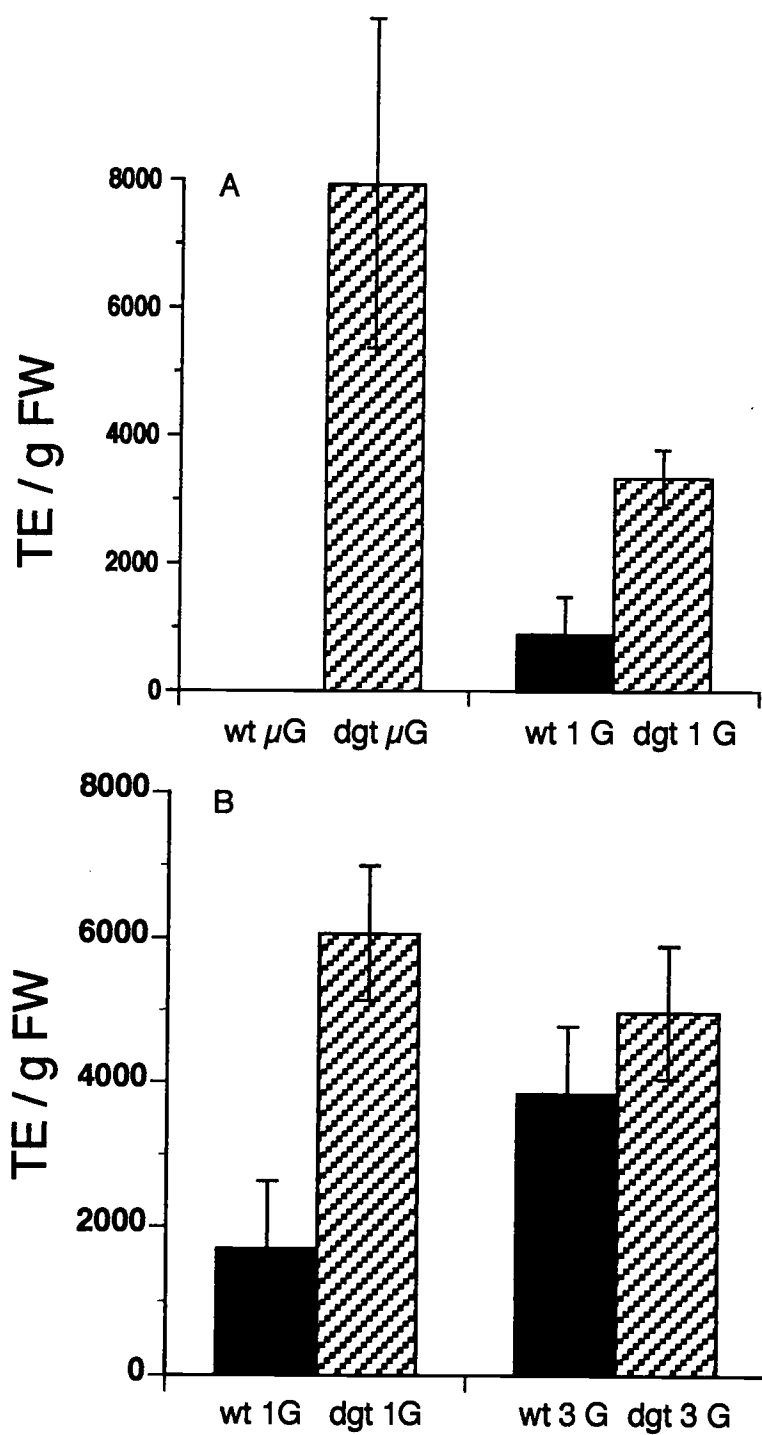
3.5. Discussion

The *dgt* mutation causes a pleiotropic phenotype. One alteration is a reduction in vasculature in adult plants (Zobel 1973, 1974; Fig. 3.1.). The xylem elements are reduced in both number and size. The transition from late protoxylem into early metaxylem is delayed or abolished in *dgt,* which results in thinner, less lignified, and less developed vessels (Fig. 3.1.). To test the hypothesis that this alteration is the result of a change in auxin sensitivity in the *dgt* mutant, we cultured callus from *dgt* and wild-type on a matrix of varying concentrations of auxin and cytokinin.

In tissue culture, both callus growth and TE production are dependent on optimal hormone combinations. Callus growth was found to be largely independent of the DGT gene product in this study. Coenen and Lomax (1998) showed that *dgt c*allus cultured on a hormone matrix for only 30 d was independent of the DGT gene product only for the induction of callus. Subsequent sensitivity hormones with respect to growth was, however, reduced in *dgt* during the first 30 d of culture on varying hormone concentrations (Coenen and Lomax, 1998). In this study, we observed that growth and auxin-sensitivity are normal in *dgt* callus that was pre-cultured for three months and cultured on a hormone matrix for 3 additional months (Fig. 3.2.). Age-dependent differences in sensitivity to auxin in *dgt* has also been shown before with respect to the gravitropic response of hypocotyls. While younger hypocotyls can respond to the gravity stimulus, this ability is lost in adult plants (Lomax et al., 1993). It has been reported that repeated transfer of callus can

lead to habituation and decreased dependence on hormones, such as cytokinins and auxins (Hawes, et al. 1985). Such habituation, however, generally requires subculturing of tissues for more than one year (Christou, 1988) and the results observed here are increased sensitivity to auxin, not decreased.

TE production in both wild-type callus and the *dgt* mutant is influenced by the ratio of auxin and cytokinins (Tables 3.1., 3.2.). In both wild-type and *dgt* callus, sensitivity to high concentrations of 2,4-D is evident at every BA concentration tested, resulting in typical bell-shaped dose-response curves. Similar results were reported earlier for soybean callus (Fosket and Torrey, 1969). The highest concentration of BA tested in our experiment (Table 3.1.) is not high enough to result in inhibition of TE production in wild-type callus. The response of *dgt* callus displays similar trends to the responses seen in the wild-type: a bell-shaped 2,4-D dose-response curve. However, unlike wild-type callus, *dgt* callus was able to produce TEs without the addition of exogenous auxin and cytokinin (Table 3.2.). These results are intriguing given that the *dgt* mutant is known to be resistant in a number of classical auxin responses such as hypocotyl elongation, root inhibition or auxin-induced ethylene production (Gaiser, 1993; Muday et al., 1995; Kelly and Bradford, 1986).

Rice and Lomax (2000) reported normal auxin sensitivity of the *dgt* mutant when auxin was supplied to seedlings growing in a buffer solution. In these experiments, curvature of *dgt* and wild-type plants was equally sensitive to inhibition by increasing IAA concentrations in the

surrounding auxin medium preventing the build-up of an auxin gradient within the bending plant. While the expression of auxin-inducible genes, such as *LeSAUR*, *LeAux,* and some members of the *LeIAA* gene family is reduced in the *dgt* mutant (Mito and Bennet, 1995; Nebenführ and Lomax, 2000), other auxin-response genes, such as *LePar* and a different subset of genes from the *LeIAA* gene family have been reported to elicit a normal response in *dgt* (Mito and Bennet, 1995; Nebenführ and Lomax, 2000). This indicates that in some instances the auxin signal is transduced using a DGT-dependent pathway while other auxin responses do not require mediation by the DGT gene product.

TE production appeared to be hormone dependent both in wild-type and the *dgt* mutant, with only minor shifts in sensitivity. Age-dependent differences in TE production between *dgt* and wild-type at equimolar concnetrations of 3 μM auxin and cytokinin were most pronounced in younger callus (Coenen and Lomax, 1998, Fig. 3.5). In older callus this difference was less pronounced (Tables 3.1.and 3.2.). Thus, we looked elsewhere to understand the increased numbers of TEs observed in *dgt* callus. We exposed *dgt* and wild-type callus to microgravity and hypergravity environments. In microgravity, the production of TEs was all but abolished in wild-type callus under the conditions tested (Fig. 3.4. A+C, Fig. 3.5.A). In contrast, an increase in TE production was observed in *dgt* callus (Fig. 3.4. B+D, Fig. 3.5.A). The small sample size of this experiment and the resulting high p-values bring the significance of the results into doubt. However, results obtained from callus grown in hypergravity (3 G) conditions showing the

opposite response in wild-type (Fig. 3.5.B.) support the notion that gravity regulates TE development in wild-type callus. The slightly enhanced TE production in *dgt* callus observed in microgravity was not statistically significant compared with TE production in *dgt* callus grown in hypergravity conditions. The increase in TE production in wild-type callus at 3 G appears be due to the increased force of gravity exerted on the tissue. To ensure the structural stability of the tissue and the their ability to conduct water, it may be favorable for the plant to increase the number of support structures. In microgravity, however, when wild-type tissue is relieved of the external force of gravity, structural support seems less important. Thesae data agree with previous studies in microgravity in which a decrease in both lignin content (Cowles et al, 1995) and the number of lignified cells (Campbell et al., 1996) during space flight was reported.

The role of the *dgt* gene in TE development seems to be of significance. Vascular development is altered in hypocotyls carrying the *dgt* lesion (Fig. 3.1.), but the *dgt* mutant is responsive to auxin treatment with respect to callus growth and TE differentiation in callus cultures (Fig. 3.2.). While the number of TEs in callus grown at 1 G depends on the auxin and cytokinin concentrations in the medium, vascular development is also influenced by varying the gravity force, at constant amounts of 2,4-D and BA. Our experiments together with reports from the literature (Fosket and Torrey, 1969) suggest that several hormone signals are integrated prior to their effect on TE development but in a DGT-independent manner. This response may be age-dependent in the

dgt mutant, as younger callus showed both auxin-resistance with respect to growth (Coenen and Lomax, 1998) and enhanced production of TEs on 3 μM 2,4-D / 3 μM BA medium (Fig. 3.1.D, Figs. 3.4, 3.5.; Coenen and Lomax, 1998). Gravity appears to exert its effect upstream of TE development. The gravitropic response of stems is the result of a gravity signal mediated by auxin, which results in directional growth away from the gravity vector. Therefore, it appears likely that gravity signals mediated by auxin such as directional growth or TE production are altered in the *dgt* mutant, possibly due to the absence of or defect in the DGT gene product. However, while the ability for directional growth in *dgt* is decreased, TE production in mutant callus is exaggerated. It therefore appears that the DGT gene product negatively regulates TE production while positively regulating other physiological parameters, such as directional growth.

Our data support a model (Fig. 3.6.) in which DGT negatively regulates TE production in callus cells, while the effect of gravity may negatively regulate DGT activity. Whether or not gravity plays a primary role in TE production cannot be unequivocally proven by the data presented here. However, the hypothesis that the wild-type response is modulated by gravity is supported by the experiments conducted in hypergravity (Fig. 3.5.B). In the *dgt* mutant, changes in gravity may not have the same effect (Fig. 3.6.). This notion is supported by the effects of gravity on *dgt* callus and the high p-values in both the microgravity and the hypergravity experiments. To further test our model, it will be informative to compare the expression of auxin-regulated genes

involved in TE development, such as TED3 and ZePel in *dgt* and wild-type under varying gravity conditions.

The regulation of TE production in seedlings may be influenced by additional factors. It is interesting to note that DGT positively regulates TE production in seedling tissue. This may be due to the effect of a range of other light- or developmentally-regulated genes. Testing the effect of the *dgt* mutation on the expression of genes involved in TE development in seedling tissue, possibly under varying gravity conditions, could shed more light on the interactions between DGT and gravity also in this developmental process.

In summary, we have demonstrated that the auxin-resistant *dgt* mutant can respond to treatment with 2,4-D with respect to callus growth and TE development, however, only *dgt* callus can produce TEs in the absence of exogenous auxin. Further, our data suggest that TE differentiation is regulated by gravity in wild-type callus. TE development is freed from negative regulation by gravity in the *dgt* mutant, suggesting that gravity-dependent cell differentiation during early vascularization is mediated by the DGT gene - perhaps via an auxin-regulated pathway.

Fig. 3.6.: A model for possible regulation of TE development in seedlings (A) vs. callus (B). Seedlings mutated in the DGT gene show a reduction in the development of TEs (A). In callus cells, TE production is negatively regulated by the DGT gene. This negative regulation appears to be reduced in wild-type callus under increased gravity forces. The effect of gravity on the *dgt* mutant is reduced if DGT cannot exert its negative effect on TE production.

Figure 3.6.

A

DGT (and other developmental factors?)

(age dependent ?)

auxin ───────────────────────► seedling TE production

B

gravity ────┤ DGT

auxin ───────────────────────► callus TE production

3.6. Acknowledgements

4. ETHYLENE AND AUXIN INTERACT DURING ADVENTITIOUS ROOTING, HYPOCOTYL ELONGATION, THE GRAVITROPIC RESPONSE, AND FLOWER DEVELOPMENT IN TOMATO

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4.1. Abstract

A double mutant between the auxin-resistant *diageotropica* (*dgt*) and ethylene-insensitive *Never-ripe* mutants of tomato (*Lycopersicon esculentum*, Mill.) was constructed to test auxin-ethylene interactions during plant growth and development. Flowers of the *Nr;dgt* double mutant exhibited increased variation in the number of flower organs, unusual fusion patterns of anthers and style, and a reduction in postgenital carpel fusion. Further, the double mutant displays inhibition of adventitious root formation and an increase in anthocyanin levels as compared to wild-type and single mutant plants. Adventitious rooting in response to auxin is strongly inhibited in the *dgt* mutant, somewhat reduced in the *Nr* mutant, and completely inhibited in the *Nr;dgt* double mutant, indicating an additive effect of the two hormone responses. Hypocotyl elongation in response to auxin is also strongly inhibited in the *dgt* mutant and somewhat reduced in the *Nr* mutant. For both rooting and elongation the shift in auxin responsiveness noticed for the *Nr* mutant suggested increased sensitivity to inhibitory concentrations of auxin. The *Nr;dgt* double mutant, however, displayed no response to auxin application. The *dgt* mutant exhibits elevated anthocyanin levels and although the *Nr* mutation alone has no effect on anthocyanin production, the double mutant displays even further increased anthocyanin levels than observed in the *dgt* mutant alone. In contrast, the *Nr* mutation antagonizes the effect of the *dgt* mutation on chlorophyll production in the double mutant resulting in an intermediate

phenotype between the chlorophyll-overproducing *dgt* mutant and the *Nr* mutant. Surprisingly, while the *dgt* graviresponse is delayed, the graviresponse of the double mutant was restored to wild-type levels. These results demonstrate that both additive or synergistic and antagonistic interactions exist between the phytohormones auxin and ethylene in different developmental pathways and suggest multiple interaction mechanisms.

4.2. Introduction

Plant growth and development are regulated by the interplay of phytohormones. The interaction of two of these hormones, auxin and ethylene, plays an important role in a wide array of processes from germination to seed development. Distinct interactions between the two hormones include regulation of shoot and root elongation growth, asymmetric growth during gravitropism and phototropism, auxin transport, flower and fruit development, and abscission (Abeles et al., 1992). Auxin promotes the synthesis of ethylene (Abeles et al., 1992) and, through feedback regulation, ethylene can inhibit the polar transport of auxin (Abeles et al., 1992; Schwark and Schierle, 1992). Auxin is involved in numerous aspects of plant development ranging from cell division, cell elongation and cell differentiation to fruit- and flower development (Metzger, 1995), lateral root initiation, and repression of anthocyanin production in post-harvest fruit (Ludford, 1995). Auxin also plays a pivotal role in the orientation of roots and stems in response to gravity or light (Went and Thimann, 1937).

Ethylene inhibits growth in etiolated *Arabidopsis* and pea plants, a phenomenon referred to as the triple response because exposure to ethylene results in stunted growth, radial expansion and exaggerated hook curvature (Neljubov, 1901; Ecker 1995). Conversely, it has also been reported that ethylene can promote cell growth and elongation in light-grown *Arabidopsis* (Smalle et al., 1997) and rice (Kende et al., 1998) as

well as in a number of other species (Abeles, 1992; Satler and Kende, 1985). Ethylene is implicated in maintaining the seedling apical hook (Peck et al., 1998), and delaying flower formation in most plants (Metzger, 1995). In contrast, ethylene is well known to induce flowering in the Bromeliaceae (Reid, 1995). In addition, ethylene has been reported to be involved in sex expression where it may induce femaleness (Reid, 1995).

Most recently, interactions between ethylene and auxin were reported for the function of *EIR1* from *Arabidopsis*. A mutation in *EIR1* (allelic to *agr1, wav6-52* and *pin2*) confers reduced ethylene sensitivity and root agravitropism, and there is evidence that the *EIR1* gene encodes an auxin efflux carrier, involved in polar auxin transport (Luschnig et al., 1998; Gälweiler et al., 1998; Utsuno et al., 1998; Chen et al., 1998). Many other plant mutants that are altered in their gravitropic response also exhibit reduced sensitivity to both auxin and ethylene (for review see Lomax, 1997).

Many of the aspects described above appear to be tightly regulated by the ratio of auxin to ethylene within the tissue. Interactions between these two important phytohormones have been studied mostly on the whole organism level. Due to the multitude of interactions between not only ethylene and auxin but also the other plant hormones, studies teasing apart specific pathways in which interactions between ethylene and auxin occur have so far been limited. In an attempt to further our understanding of the interactions between auxin and ethylene in plant development, we have taken a genetic approach using the auxin-resistant *diageotropica* (*dgt,*

Zobel, 1973, 1974; Kelly and Bradford, 1986) and the ethylene-insensitive *Never ripe* (*Nr,* Wilkinson et al., 1995) mutants of tomato. The *dgt* mutant is a single gene, recessive mutant (semi-dominant only with respect to leaf shape; Ursin and Bradford, 1989) with a pleiotropic phenotype that exhibits, among other things, a reduced gravitropic response, decreased auxin-induced elongation growth, reduced vasculature and an overproduction of anthocyanins (Zobel, 1973,1974; Kelly and Bradford, 1986; Lomax et al., 1993, Madlung et al., 1999). Further, *dgt* is unable to produce ethylene in response to auxin, while it can produce basal levels of ethylene and synthesize ethylene in response to other stimuli such as fusicoccin and anaerobiosis (Kelly and Bradford, 1986). The *Nr* mutant (Wilkinson et al., 1995; Yen et al., 1995; Lashbrook et al., 1998) is a single-gene dominant mutant which is the result of a mutation in an ethylene receptor gene. This gene is a member of the ETR gene family of ethylene receptors. The mutation results in a dominant negative effect and renders the plant insensitive to applied or endogenous ethylene (Wilkinson et al.,1995; Bleeker, 1999; Chang and Shockey, 1999) which is manifested by the absence of the triple response in high ethylene and greatly delayed fruit ripening (Wilkenson et al., 1995).

A double mutant was constructed for both of these genes, in order to test the importance of the interaction between auxin and ethylene during normal development. We report here evidence for multiple levels of interaction between auxin and ethylene throughout the lifecycle of the plant, indicating that responsiveness to these hormones must be regulated

by multiple perception or signal transduction mechanisms. Further, we demonstrate that ethylene insensitivity compromises auxin responsiveness with respect to adventitious rooting and hypocotyl elongation.

4.3. Materials and Methods

4.3.1. Plant Material

Seeds of the wild-type tomato (*Lycopersicon esculentum* Mill.) varieties Chatham (C), and Pearson (P) as well as the two single mutants *dgt* (*diageotropica,* allele *droopy;* Jones and Jones, 1996) and *Nr* (*Never-ripe*) were used. The dgt^{dp} mutant (in this paper simply referred to as *dgt*) was maintained in the isogenic C background while *Nr* was maintained in the isogenic P background . Seeds of *dgt* and C were originally obtained from C.M. Rick (University of California, Davis). The phenotype of the dgt^{dp} allele is essentially identical to the phenotype of the dgt^{1-1} allele in the isogenic VFN8 background (data not shown). Seeds of *Nr* and P were a gift of Dr. Harry Klee (University of Florida, Gainesville). All lines were propagated by selfing on the Oregon State University Botany Farm (Corvallis, OR).

4.3.2. Genetic Crosses and Screening Assays

To select double mutants, all F1 progeny of a cross between homozygous *dgt* and *Nr* plants were grown to maturity in the greenhouse and allowed to self-pollinate. F2 seed collected from these populations was screened for the double mutant phenotypes as follows: F2 seeds of the *Nrxdgt* cross were sown in potting soil (Bi Mart, Eugene, OR) in 6 cm x 6 cm styrofoam pots and scored for the horizontal growth habit, hyponastic leaves, and overall wilty phenotype typical for *dgt* (Zobel, 1973; 1974). Only plants exhibiting *dgt*-like growth were transplanted into 4-liter-pots when they were 4-6 weeks old and subsequently screened for the lack of complete fruit ripening typical of *Nr*. Plants bearing only green and orange fruit at the age of 6 months were scored as heterozygous or homozygous *Nr* mutants. Plants showing one or more mature red fruit were scored as homozygous for the *nr+* gene and discarded. The ratio of *Nr* to *nr+* was, as expected, 3:1 (data not shown).

To verify homozygosity at both loci, putative double mutants were backcrossed to the *dgt* single mutant parent. The offspring were scored for *dgt* phenotypic traits. To verify homozygosity of the dominant *Nr* gene, seeds of the putative double mutant, wt P, and *Nr* seeds were planted in scintillation vials filled with vermiculite and germinated for 4-5 d in the dark at 29°C. The vials were transferred into 1L Mason jars and sealed airtight. They were injected with 0.5 to 1mL L^{-1} ethylene and scored for hypocotyl

elongation after 48 hours in darkness. Only seed from plants whose F3 seedlings showed no triple response symptoms in 100% of the offspring (n=approximately 50) were considered homozygous and used in further experiments. Three individual double mutant lines (#7, 37, 56) were isolated and one of them (#37) was used for all experiments, with the exception of the flower analysis for which all lines were used. The F2 *Nrxdgt* sibling generation was included in all experiments to account for possible background effects due to the different single mutant parent varieties. F2 plants used in the experiments were, where possible, selected for wild-type appearance (=F2 select population). Due to the dominance of the *Nr* mutation, roughly 56 % of the wild-type appearing seedlings were expected to carry the *Nr* lesion, however, there is no morphological *Nr* phenotype at this developmental stage.

4.3.3. Rooting assays

To test the effect of auxin and ethylene responsiveness on adventitious root formation, 7-week-old wild-type, single, and double mutant plants as well as the F2 select population were cut at their base and dipped approximately 1.5 cm into talcum powder (Sigma, St. Louis, MO) containing 0 - 75 μM of the auxin indole-3-butyric acid (IBA; Sigma). Plants were grown under greenhouse lighting (16 h light/8 h dark) in pre-moistened Perlite™ in 6 cm x 6 cm plastic pots at 23°C/28°C (night/day) for an additional 3 weeks. The plants where then taken from their pots and the

newly-formed adventitious roots were visually inspected and counted. This experiment was repeated twice and the data pooled. An average of 10 plants was used for each data point.

4.3.4. Hypocotyl elongation assays

Wild-type (C and P), *dgt*, *Nr*, and *Nr;dgt* double mutant seedlings, as well as seedlings of the F2 select population were grown in darkness for 6 d at 29°C in plastic boxes lined with one layer of Kimtowels (Kimberly Clark, Roswell, GA) and covered with wet filter paper. 10 mm hypocotyl sections were excised immediately below the hook, placed in small Petri dishes (5.5 cm diameter) and incubated in 0.01 M KPi buffer (pH 6.0) containing 0 - 10^{-3} M of indole-3-acetic acid (IAA; Sigma). The sections were incubated for 12 h in dim light with gentle shaking and their increase in length subsequently measured with a caliper. The experiment was repeated 4 times with essentially the same results. A representative experiment is shown. Error bars reflect SE of the means. An average of 17 individuals was used per data point. Sample sizes varied from n = 11 - 28 (mean = 19).

4.3.5. Pigment Analysis

Anthocyanin and chlorophyll were extracted from the cotyledons and the first true leaf of 4-5 week-old wild-type (P and C), *Nr*, *dgt*, and the

Nr;dgt double mutant, as well as the F2 select population were grown in potting soil (Bi Mart) in the greenhouse at 25°C under 16h light/8 h dark cycles in 6 cm x 6 cm plastic pots. Leaf tissue was cut into fine pieces and extracted in 1% HCl v/v in methanol at a ratio of 30 mL g $^{-1}$ FW for 20 h on ice. The absorbance of the extract was measured using a DU 64 spectrophotometer (Beckmann, Fullerton, CA) at 530 nm (anthocyanin) and 645 nm (chlorophyll). The experiment was repeated twice and the data pooled. Sample sizes varied from n = 35-53 (mean = 49).

4.3.6. Curvature assays

Seedlings of both wild-type cultivars, *Nr, dgt,* and the *Nr;dgt* double mutant, as well as seedlings of the F2 select population, were grown in darkness for 5 d at 29°C as described above for hypocotyl elongation assays. Seedlings of uniform size (1-2 cm in length) were aligned on 1% agar in Petri dishes (15 cm diameter) and the Petri dishes were set on edge in red light (2.51 μmol m^{-2}s^{-1}, measured from 640-680 nm) at approximately 25°C, such that the seedlings were oriented horizontally for gravistimulation. Dishes were photocopied at the indicated times and the angles were measured from the photocopies using a protractor. The experiment was repeated 3-4 times with similar results and the data pooled. Each data point reflects approximately 30-40 individual measurements.

4.3.7. Statistical analysis

To determine statistical significance between means, analyses of varience (ANOVA) were computed using SAS software (SAS Institute, Cary, NC) version 8.0. Statistical significance, where reported, was determined from 95% confidence intervals.

4.4. Results

4.4.1. Reduced auxin and/or ethylene responsiveness inhibits the formation of adventitious roots

Both ethylene and auxin have been implicated in the formation of adventitious roots (Visser et al., 1996; Clark et al. 1999). To test the interaction of auxin and ethylene in regulating the formation of adventitious roots, cuttings of the parental lines, single mutants, the double mutant, and the F2 sibling population were subjected to increasing concentrations of the auxin IBA (Fig. 4.1.). Application of IBA to both wild-type parent lines resulted in a concentration-dependent increase in adventitious root growth, which reached its optimum at approximately 50 μM. The Pearson wild-type line exhibited a more dramatic peak response at the 50 μM concentration than the Chatham wild-type line. In contrast to a previous report (Clark et al., 1999) the *Nr* mutant also responded to applied IBA in a concentration-dependent manner, however, the response was reduced to Chatham-like levels at the optimum peak at 50 μM when compared to the parent line

Pearson. The *dgt* mutant had a greatly reduced but statistically significant response to auxin, with the number of adventitious roots increased two-fold at 50 μM IBA. Interestingly, the F2 select population displayed slightly less auxin responsiveness than the expected wild-type-like response. While the F2 select population response was lower than the wild-type responses, it was, however, not statistically different from the *Nr* or Chatham wild-type response . There was no statistically significant change in adventitious root formation in the *Nr;dgt* double mutant in response to any concentration of IBA tested.

4.4.2. Shoot elongation in response to applied auxin

A classical test of auxin responsiveness is the elongation of hypocotyl sections in response to applied IAA. To test the effect of the *Nr* mutation on auxin responsiveness, wild-type plants (background P and C), the *Nr* and *dgt* single and *Nr;dgt* double mutant hypocotyls as well as wild-type-appearing F2 hypocotyls were incubated in various concentrations of IAA. The data presented in Fig. 4.2. indicate that both wild-type varieties elongate similarly in response to auxin. Interestingly, the *Nr* mutant displays a concentration-dependent increase in elongation in response to IAA application, however, the magnitude of the response is reduced compared to its parental line (P) and the optimal auxin concentration is shifted lower by an order of magnitude.

Figure 4.1.: Ethylene-insensitivity and auxin-resistance reduce the plant's ability to form adventitious roots. 7-week-old wild-type plants (Pearson, closed square (red); Chatham, closed circles (blue); *Nr*, closed triangles (green); *dgt*, closed diamonds (black); *Nr;dgt* double mutant, open squares (light blue); F2 select population, open circles, (orange)) were cut and dipped in talcum powder containing IBA and planted in individual pots. Adventitious root formation was measured after 3 weeks. Experiment was performed three times and data were pooled. Sample sizes were n=8-15 per data point (mean sample size 10). Error bars shown where SE is greater than symbol.

Figure 4.1.
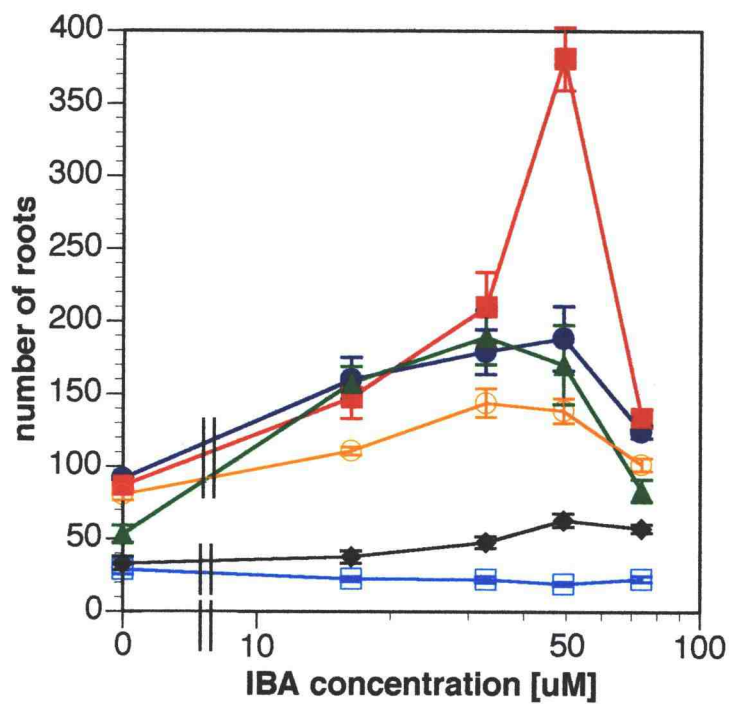
Fig. 4.2.: Auxin-resistant *dgt* and the ethylene-insensitive *Nr* hypocotyls show decreased elongation responsiveness to IAA when compared to the wild-type parents. The double mutant shows no response to applied IAA. 5-6 day-old, dark-grown wild type hypocotyls (Pearson, closed square (red); Chatham, closed circles (blue); *Nr*, closed triangles (green); dgt, closed diamonds (black); *Nr;dgt* double mutant, open squares (light blue); F2 select population, open circles, (orange)) were cut into 1 cm sections and incubated in IAA for 20h. Length increase was measured using a digital caliper. Error bars are shown where SE is larger than the symbol. (n = 15-28 per data point, pooled from 3 - 4 independent experiments.)

Figure 4.2.
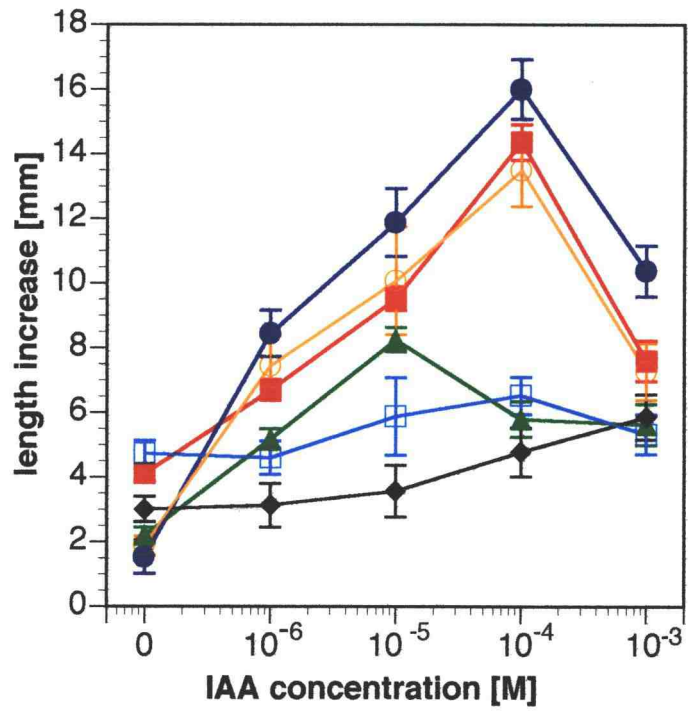
This shift appears to be due to increased sensitivity of *Nr* hypocotyls to auxin with respect to inhibition of elongation. Application of IAA to *dgt* stem segments results in only a minor increase in stem elongation at the highest concentrations tested, confirming the resistance of *dgt* to auxin with respect to shoot elongation. Elongation in response to IAA was also completely reduced in the *Nr;dgt* double mutant. The response of the wild-type-looking seedlings of the F2 select population. Their response was expected to be influenced in roughly 56% of the seedlings by the dominant *Nr* mutation. However, the observed response was almost identical to the response seen in the P wild-type line.

4.4.3. Auxin and ethylene interact in opposite manners to regulate anthocyanin and chlorophyll levels.

Ethylene has been reported to cause the breakdown of chlorophyll in ripening fruits and leafy vegetables and that response can be retarded or even reversed by applied gibberellic acid (Abeles, 1992; Woolhouse, 1984; Ludford, 1995). The influence ethylene has on pigment production in seedlings is, however, not well understood, although the auxin-insensitive *dgt* mutant has been reported to have enhanced levels of both chlorophyll and anthocyanins (Lomax et al., 1993). We measured chlorophyll and anthocyanin production in the *Nr* and *dgt* single mutants as well as the *Nr;dgt* double mutant (Fig. 4.3.). As seen previously, young *dgt* plants produce increased amounts of anthocyanins whereas the *Nr* mutant showed no statistically significant difference in its production of

anthocyanins when compared to the isogenic parent plants. However, in the *Nr;dgt* double mutant, anthocyanins are produced at an even greater level than seen with *dgt* alone. In contrast, while chlorophyll production is strongly enhanced in the *dgt* mutant, that enhancement is reduced in the double mutant, although it is still elevated compared to the parent lines. The *Nr* single mutant exhibited only a slight and statistically insignificant increase in chlorophyll over Pearson wild-type levels. Anthocyanin levels in the F2 select population appeared to be suppressed compared to either parent line or *Nr*, while chlorophyll levels in the F2 select population were similar to those observed in the wild-type or *Nr* plants.

4.4.4. Ethylene resistance can restore the graviresponsiveness of *dgt*

In previous studies, it has been shown that ethylene plays multiple, non-primary roles in modulating the gravitropic response in tomato (Wheeler and Salisbury, 1980, 1981; Philisoph-Hadas et al., 1996; Madlung et al., 1999). Also, the *dgt* lesion results in a reduction of the gravitropic response which can vary in its severity from a delayed to an incomplete response depending on environmental and experimental conditions (Madlung et al., 1999; Rice and Lomax, 2000), or the age of the seedling (Lomax et al, 1993). Ethylene can restore graviresponsiveness to the *dgt* mutant when applied at very low levels (Zobel, 1973, Madlung et al. 1999), although not with wild-type kinetics (Madlung et al. 1999).

Figure 4.3.: Auxin-resistance leads to an increase in anthocyanin (A) and chlorophyll (B) production, while ethylene-insensitivity does not alter anthocyanin production and raises chlorophyll levels only marginally. The double mutant shows increased levels of both pigments. Pigments were extracted from cotyledons and the first true leaf of 3-week-old plants and measured using spectrophotometry. Data were pooled from two experiments, n = 30-40. Error bars represent SE. Statistical difference on the 95% confidence level is indicated by different letter combinations. Same letters indicate no statistical difference between genotype responses.

Figure 4.3.
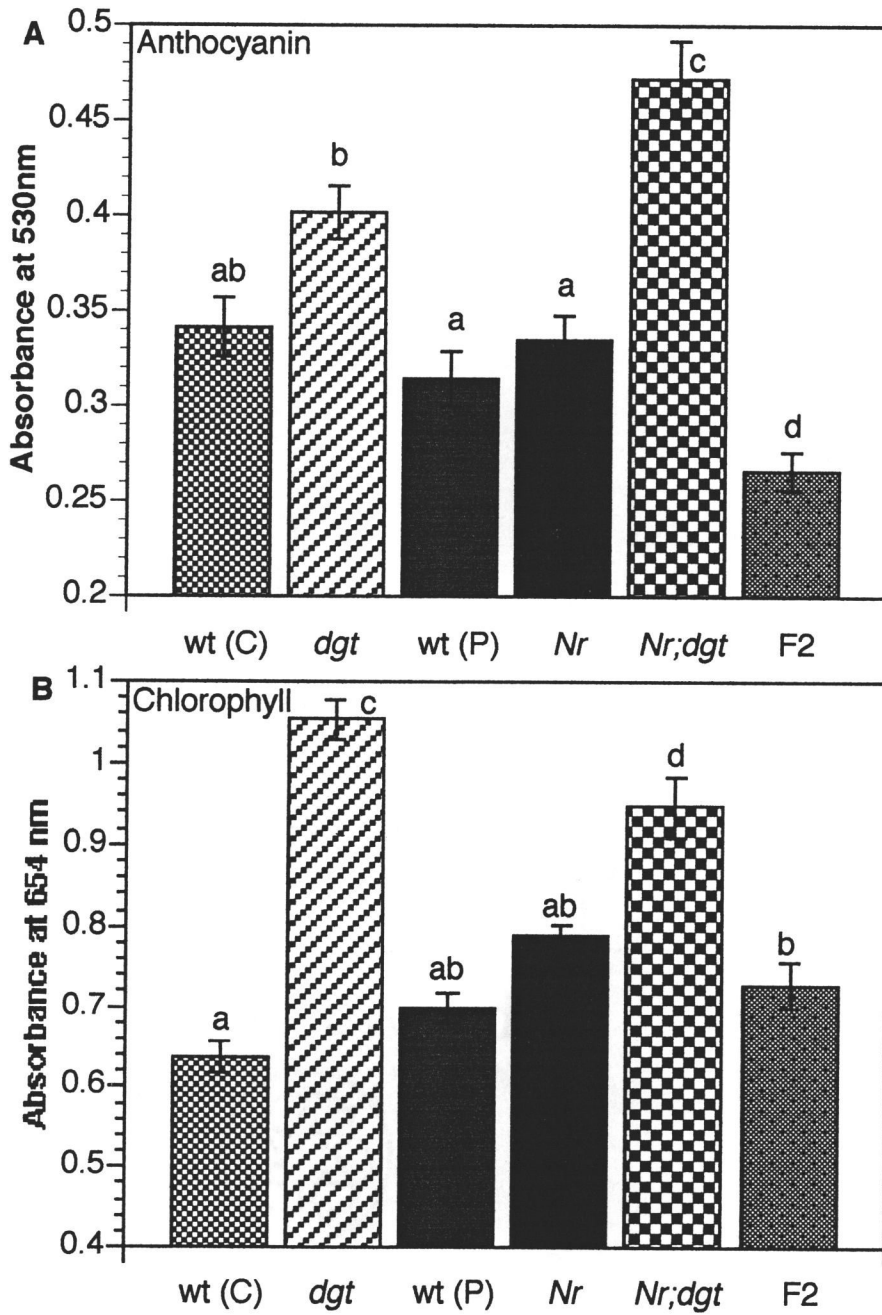
In addition, the concentration of ethylene is crucial for the nature of the response as both the absence of or overexposure to ethylene result in reduction of graviresponsiveness. The ethylene-resistant *Nr* mutant showed only a slight retardation in its graviresponse in previous experiments (Madlung et al. 1999). It was therefore our goal to examine the graviresponse of a mutant altered in both the auxin and ethylene responsiveness. We confirmed the delayed response of the *dgt* mutant (statistically significant differences were seen between the *dgt* response and the responses of the other genotypes from 2h on after gravistimulation) and the lack of the effect of the *Nr* mutation alone on the graviresponsiveness (Fig. 4.4.). Interestingly, the kinetics of the *Nr;dgt* double mutant response exhibited wild-type kinetics when compared both to the wild type parents C and P and the *Nr* single mutant (Fig. 4.4.). No statistically significant differences were found in the response between the double mutant and the wild-type lines at 8 and 12 hours after gravistimulation. The response of the F2 select population was very similar to wild-type and *Nr* levels.

4.4.5. Double mutants of *Nr;dgt* display an abnormal flower structure

The *Nr;dgt* double mutant displays the drastic delay in ripening typical of *Nr* and the wilty appearance, horizontal orientation and hyponastic leaves (Figure 4.5.A), and darker pigmentation typical of *dgt*. In addition, double mutant flowers showed varying degrees of abnormality.

The tomato cultivars used here as parents normally have five to seven petals, sepals and anthers (Fig. 4.5.C), and five carpels that are postgenitally fused bearing one common style (Fig. 4.5.E). Flowers of the *Nr* and the *dgt* single mutants display normal organ numbers. The *dgt* flowers tend to be slightly smaller in size (data not shown) and the carpel more oblong in shape (Fig. 4.5.F) when compared to wild-type plants. Occasional double mutant flowers displayed as many as 12 sepals and 12 petals while others had a normal number of sepals and petals but an increased number of anthers (up to 10, Fig.4.5.). All flowers considered as abnormal exhibited what appeared to be a lack of postgenital carpel fusion resulting in multiple, separate carpels (Fig. 4.5.B, 4.5.D, 4.5.G, 4.5.H, 4.5.O).The percentage of abnormal flowers with any degree of severity in the double mutants ranged from 5% - 50% on individual plants with an estimated mean of 10% (n > 40; data not shown). Occasional floral abnormalities in any other variety were unusual and never exceeded one per plant. While the normal count of sepals and petals can vary slightly in tomato depending on the cultivar (P and C usually have 6 petals and rarely as many as 8), abnormal flowers with up to 12 sepals and 12 petals were never seen in single mutants of *dgt* or *Nr* nor in any of the wild-type parent lines used here (data not shown). Even rarer was the occurance of twin flowers in the *Nr;dgt* double mutant. In twin flowers, two separate whorls of anthers and carpels were surrounded by one whorl of petals and sepals with greatly increased organ numbers (Fig. 4.5.P-T). Figure 4.5.T shows fusion of anthers and carpels (arrow) in the twin flowers. Abnormal flower

development was noticed in three independently generated double mutant lines and was a heritable trait in the two subsequent generations. While carpels harvested 2d post anthesis show complete fusion in the wild type (Fig. 4.5. L) and the *dgt* mutant (Fig. 4.5. M, *Nr* mutant and *Nrxdgt* F2 siblings data not shown), incomplete carpel fusion can be seen in carpels at the same developmental stage in the abnormal double mutant flowers (Fig. 4.5. N, O). These separate carpels developed into mature ovaries, often producing individual styles which were in some cases fused to each other to varying degrees (Figure 4.5.B, J, K) or completely separate from each other (Fig. 4.5. G, H). Occasionally, fusion was also noticed between filaments and style(s) (Figure 4.5.J, 4.5.K, 4.5.T see arrow). Pollination normally occurs by self-fertilization in cultivated tomato. We noticed a reduced rate of self-fertilization (data not shown) but hand-pollinated flowers developed normally. Fruits developing from multicarpel flowers often gave rise to fruits with multiple lobes (Fig. 4.5.I).

Figure 4.4.: The gravitropic response is delayed in *dgt* , but normal in the *Nr;dgt* double mutant. Plants were germinated in the dark at 29°C for 5 days, placed on agar plates and put on edge to induce gravistimulation. Angles were measured from photocopies at the given times with a protractor. Pearson, closed square (red); Chatham, closed circles (blue); *Nr*, closed triangles (green); *dgt*, closed diamonds (black); *Nr;dgt* double mutant, open squares (light blue); F2 select population, open circles (orange). Error bars reflect SE where larger than symbol, n = 20-48 per data point, data were pooled from 3-4 independent experiments.

Figure 4.4.
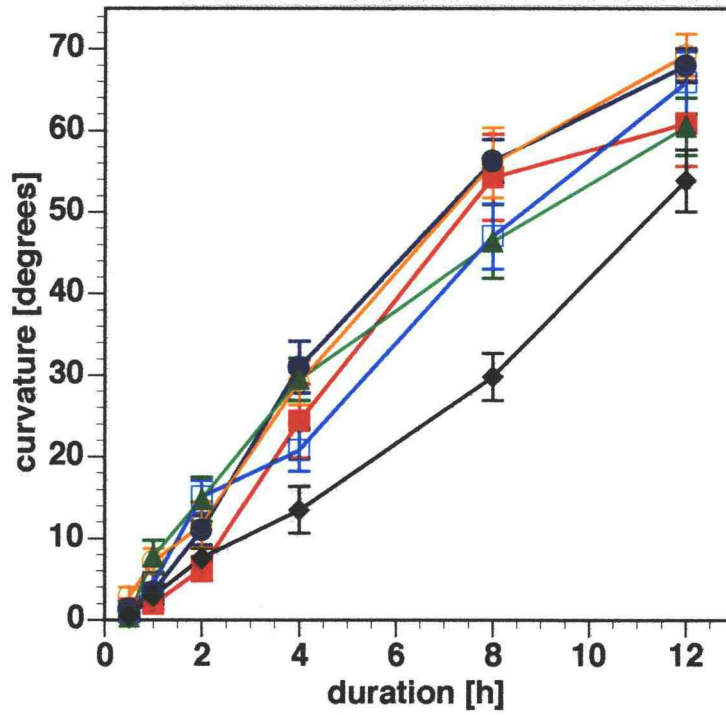
Fig. 4.5.: Flower morphology is altered in *Nr;dgt* mutants: growth habit of a mature *Nr;dgt* plant(A); flower of *Nr;dgt* mutant with the anthers removed (B); flower of wild-type Chatham with the anthers removed (C); gyneoeceum of *Nr;dgt* mutant, carpels are separate, anthers are reduced to style-like structure (D); wild-type Chatham pistil 2d post-anthesis (E); *dgt* pistil 2d post-anthesis (F); carpel of *Nr;dgt* mutant with all petals, sepals and anthers removed, each carpel produces a separate style (G); scanning electron micrograph of *Nr;dgt* mutant carpel (H); fruit resulting from multiple-carpel *Nr;dgt* flowers (I); fusion between styloid anther (arrow) and multiple styles (J, K); transverse light micrograph (100x magnification) of wild-type Chatham carpel. The pericarp tissue is completely fused (L); transverse light micrograph (100x magnification) of *dgt* carpel. The pericarp tissue is completely fused (M); transverse light micrograph (100x magnification) of *Nr;dgt* mutant carpel. The pericarp tissue is partially fused but the epidermis extends into the pericarp tissue (N); transverse light micrograph (100x magnification) of *Nr;dgt* mutant carpel. The pericarp tissue is unfused leaving a cleft (arrow) between carpels (O); twin flower, arrows point to separate whorls of anthers (P); transverse light micrograph (25x magnification) of *Nr;dgt* mutant twin flower carpel (Q) arrows in Q point to two separate carpels; transverse light micrograph (40x magnification) of *Nr;dgt* mutant twin flower carpel (R, S, T), arrow in T points to fusion between anther and carpel.

Figure 4.5.
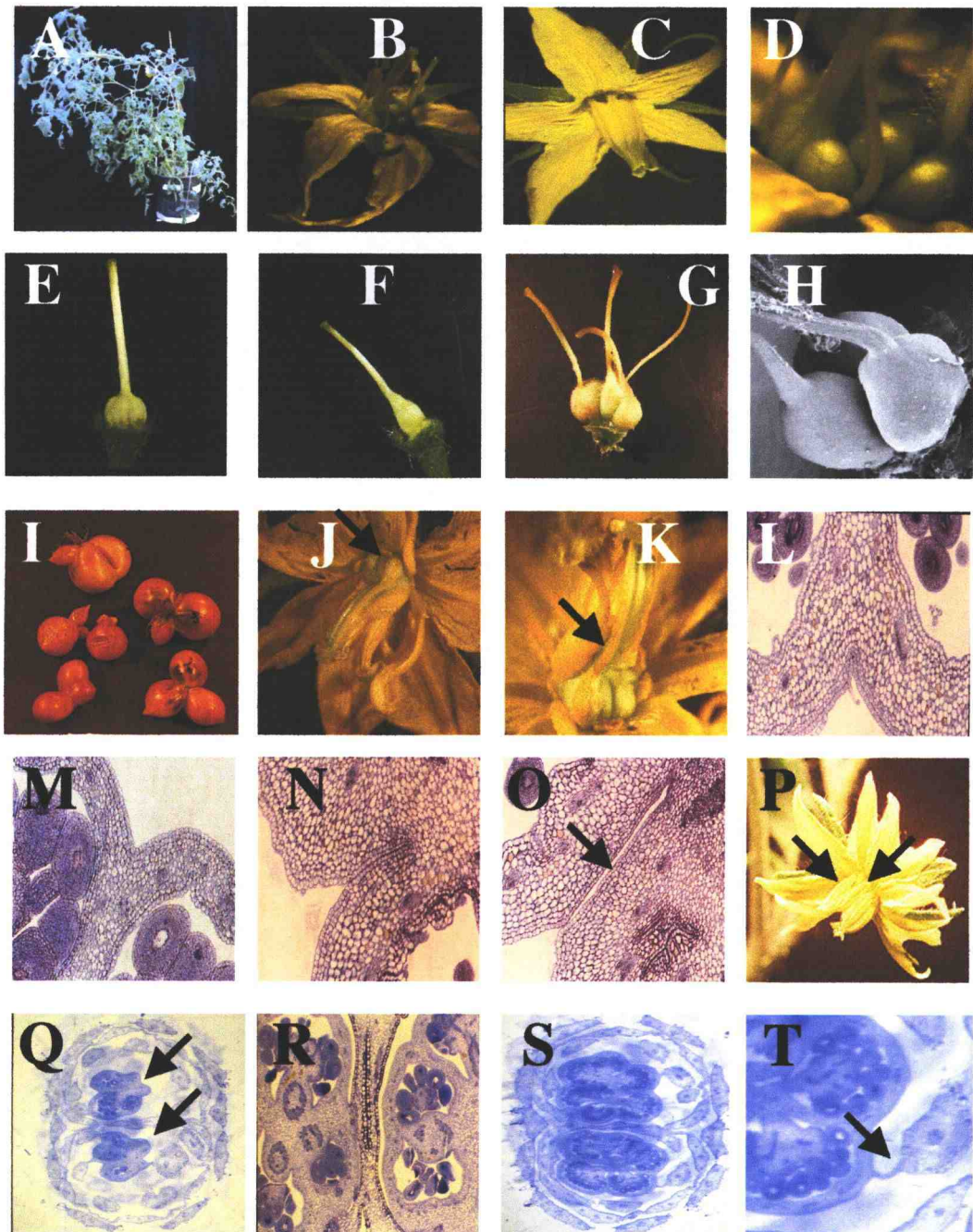
4.5. Discussion

Auxin and ethylene interact at multiple steps in the development of plants. Many auxin-resistant mutants, such as *aux1* (Roman et al. 1995), *axr1* (Lincoln et al., 1990), *axr2* (Wilson et al., 1990), and *axr3* (Leyser et al., 1996), have been shown to have reduced sensitivity to ethylene. We show here that an ethylene-insensitive mutant, *Nr*, displays reduced responsiveness to auxin in assays for adventitious root formation and hypocotyl elongation (Fig. 4.1., Fig. 4.2.). *Nr* encodes a member of the ethylene receptor gene family. Mutation in this gene results in ethylene insensitivity in a dominant negative fashion (Wilkinson et al., 1995), likely as a result of "poisoned" heteromeric receptor complexes. The *dgt* mutant is defective in its auxin-induced ethylene production (Kelly and Bradford, 1986), and ethylene sensitivity in *dgt* is altered in roots (Muday et al., 1995) and shoots (Shi and Cline, 1992, Madlung et al., 1999). Since single gene mutations often affect both auxin and ethylene responses, we predicted major alterations in a mutant that carried *bona fide* mutations for both auxin and ethylene responsiveness. Interestingly, the overall phenotype of the double mutant appeared to stem mostly from the additive effects of the *dgt* mutation (hyponastic leaves, wilty appearance, lack of lateral roots) and the *Never-ripe* mutation (lack of triple response, delay in fruit ripening) but otherwise seemed to be largely normal. Physiological experiments were conducted to distinguish between additive and non-additive effects in the plant. These experiments revealed that combining the two mutations

affected pigment production and graviresponsiveness, as well as auxin-sensitivity during adventitious root initiation and hypocotyl elongation. Surprisingly, normal flower development also appears to be disrupted by the double mutation.

4.5.1. Lack of ethylene and auxin sensitivity leads to abolition of adventitious root formation and hypocotyl elongation growth

Previous studies have concluded that ethylene is necessary for the formation of adventitious roots (Reid, 1995; Clark et al., 1999). Our studies show that ethylene insensitivity reduces the ability of the plant to form adventitious roots, but does not abolish it. Auxin sensitivity appears to play a more significant role in adventitious rooting, as the *dgt* mutant is severely compromised in its ability to form adventitious roots (Fig. 4.1.). The elimination of both auxin and ethylene responsiveness completely eliminates the plant's ability to form adventitious roots. The F2 select population displayed a slightly greater reduction in auxin responsiveness than expected from the wild-type and the *Nr* single mutant responses which may indicate a background effect of the PearsonxChatham parental lines. This reduction was found to be statistically significantly different from the wild-type Chatham parent, but not from the *Nr* single mutant at intermediate auxin concentrations. A parental background effect could be responsible for the suppression of the double mutant response as compared to the *dgt* single mutant.

In contrast to a previous report in which 4-week-old *Nr* seedlings were reported to be insensitive to auxin with respect to adventitious root formation (Clark et al., 1999), we report here that 7-week-old *Nr* mutant plants responded to applied IBA by forming adventitious roots in a concentration-dependent manner. However, the response was reduced when compared to its parent wild-type line Pearson. Higher sensitivity to inhibitory concentrations of auxin or reduced sensitivity to optimal concentrations of auxin may suggest that ethylene modulates the plant's sensitivity to auxin concentrations in the tissue. The older age of the seedlings as well as an overall greater rooting response in this experiment may explain the differences seen in this report compared to Clark's observations (Clark et al., 1999). The *Nr;dgt* double mutant was completely inhibited on its ability to produce adventitious roots in response to auxin. These results may suggest an interaction between ethylene and auxin via *DGT* and *NR* which may be necessary for any response with respect to auxin-induced adventitious rooting.

The *Nr* mutant had a similarly reduced response to applied IAA (Fig. 4.2.) in the hypocotyl elongation assay. The *Nr* mutant again appeared to be more sensitive to inhibitory concentrations of IAA, displaying a reduction in hypocotyl elongation at a ten-fold lower concentration as compared with its parent line. This suggests that, as for adventitious root formation, ethylene insensitivity leads to a reduction in auxin responsiveness at inhibitory levels with respect to elongation growth. Given the presumed presence of at least one *Nr* allele in roughly 56% of the F2 select

population, the similarity of the F2 response to that of both parental types was surprising. As in the rooting response, this may be due to background interactions between the parental lines which compensate for the effect of the *Nr* mutation.

As in the rooting assay, the hypocotyl elongation response is most reduced by the *dgt* mutation. Interestingly, in the hypocotyl assay ethylene insensitivity, when combined with partial auxin insensitivity in the double mutant, produces a response that is intermediate to either of the two responses alone. While it appears that this change in auxin responsiveness is caused by the mutation of the *dgt* and *Nr* genes, it cannot be ruled out that some of the effects seen are due to background effects resulting form genetic contribution from the parent lines Chatham and Pearson.

The responses of both *dgt* and *Nr* to auxin was to IAA was, however, statistically significantly different from the response in buffer or low auxin concentrations.

In both the rooting assay (Fig. 4.1.) and the hypocotyl elongation assay (Fig. 4.2.) auxin sensitivity is reduced most in the double mutant, arguing for an additive effect of the *Nr* and *dgt* mutation on auxin responsiveness. These experiments do not provide evidence for a direct interaction of the *dgt* and *Nr* signal transduction pathways. They do, however, show that auxin sensitivity can be modulated via the *Nr*-mediated ethylene response.

4.5.2. Auxin-resistance leads to increase in pigment production

Several studies have shown that ethylene can increase as well as decrease chlorophyll levels in different plant species (Alscher and Castelfranco, 1972, Buhler et al., 1978). It is also known that IAA can halt or decrease chlorophyll catabolism, even though IAA has also been demonstrated to increase ethylene levels (Abeles, 1992). Interestingly, ethylene inhibitors have failed to reduce chlorophyll catabolism (Abeles, 1992) suggesting an intricate network of interactions in the processes of anabolism and catabolism of chlorophyll. Chlorophyll degradation may, in fact, be the result of ethylene-induced aging, not ethylene itself (Abeles, 1992).

Visual inspection of the *dgt* mutant shows that the mutant has a darker green color than the wild-type parent and increased chlorophyll levels in the mutant have been demonstrated (Lomax et al., 1993). The results of our studies show a near doubling of chlorophyll content in *dgt* but no significant increase in chlorophyll levels in the *Nr* mutant. The double mutant shows an intermediate phenotype with respect to chlorophyll production. The significance of this is as of yet unclear but it appears that auxin negatively regulates chlorophyll production, and the *Nr* lesion in the double mutant appears to ameliorate the effect that the *dgt* lesion has on chlorophyll levels. One possible way to explain the relationships between NR and DGT in the regulation of chlorophyll is a model in which NR acts upstream of DGT and in which DGT acts negatively on chlorophyll

production. This would explain the increase in chlorophyll levels in the *dgt* mutant and the absence of a response in the *Nr* mutant. The intermediate double mutant response can not be explained by this simplistic model alone. The results of the double mutant suggest that the amelioration of the *dgt* response by the *Nr* mutation may be caused by different or indirectly *Nr*-mediated pathways.

Anthocyanins are pigments mainly involved in the coloration of flowers, leaves, and fruit. Their color is influenced by the acidity of the plant cell's vacuole. Anthocyanin synthesis genes are regulated by at least six different transcription factors in maize (Westhoff, 1998). Anthocyanin production can be affected by environmental influences such as light, temperature, nutrients and stress (Fosket, 1994; Murphy and Thompson, 1988) and can be artificially enhanced in apple and cherry by daminozide, which inhibits ethylene production by interfering with its biosynthetic enzymes (Gianfagna, 1995). It is therefore evident that anthocyanin levels can be affected by a vast number of biotic and abiotic influences on the plant. Interestingly, in tomato the ethylene-insensitive *Nr* single mutant did not show an alteration in anthocyanin levels (Fig. 4.3. A). However, we did observe increased amounts of anthocyanin in the *dgt* mutant and further increase in anthocyanin levels in the *Nr;dgt* double mutant. One hypothesis is that auxin may via DGT directly negatively regulate anthocyanin production and that NR-mediated ethylene perception plays a role in the regulation of anthocyanin productionb y interaction with the DGT-mediated response. From our experiments it cannot, however, be

concluded that the described effects are directly linked to auxin and ethylene action but may rather be caused by multiple stresses on the double mutant. Since anthocyanin levels are influenced by stress, the accumulation of anthocyanins could also be the result of increased stress in the double mutant.

4.5.3. Ethylene-auxin ratios may determine gravicurvature kinetics

The gravitropic response is governed by the transport of auxin in response to the gravity vector and the resulting unequal redistribution of auxin causes differential growth and upward curvature (Went 1937). The role that ethylene plays in this process has been the focus of many studies (Wheeler and Salisbury, 1980, 1981; Philisoph-Hadas et al., 1996; Madlung et al., 1999). Recently, we showed that the *Nr* mutant displays an essentially normal response during gravistimulation and from this and other evidence concluded that this indicates that ethylene plays a secondary, not primary role during the gravitropic process is secondary, not primary (Madlung et al. 1999). The *Nr;dgt* double mutation does, however, appear to offset the delay in the gravitropic response caused by the lesion in the *dgt* gene (Fig. 4.4.). The concentration of ethylene perceived by the plant is crucial full response to a gravity stimulus and altering this amount either by adding ethylene or reducing the active ethylene concentration with ethylene inhibitors can change the plant's gravitropic responsiveness drastically (Madlung et al., 1999). It is interesting to note that while auxin-resistance

leads to a decrease in graviresponsiveness (Fig. 4.4.; Bradford and Kelly, 1987; Lomax et al., 1993; Madlung et al., 1999) and ethylene resistance has little (Madlung et al. 1999) or no (Fig. 4.4.) effect on the gravitropic response, the combination of both auxin and ethylene resistance appears to offset the *dgt* phenotype in the double mutant and results in normal gravitropism (Fig. 4.4). This experiment supports an earlier hypothesis that a very exact balance of ethylene governs graviresponse kinetics (Madlung et al., 1999).

The double mutant analysis also allows us to put forth a more detailed working model (Fig. 4.6.) for the action of ethylene and auxin in the gravitropic response. In this model DGT acts as a negative regulator of protein X which negatively regulates protein Z. Z has a positive effect on the ability to respond to gravistimulation. NR, in this model, also has a negative effect on X but at a lower level of activity than DGT. Protein Y is negatively regulated by NR and DGT but regulates X in a negative fashion when active. In the case of the *dgt* mutant, negative regulation of X is eliminated leaving X at higher levels than normal. This leads to repression of Z and therefore decreases the ability of the *dgt* mutant to respond to gravistimulation. The *Nr* mutant results in derepression of X and Y. This, however, has no or only slight effects on the levels of X when DGT is active. In the double mutant, repression of Y by NR is eliminated, leading to high levels of Y. The absence of repression of X by DGT in the double mutant could in this case be compensated for by repression of X by protein Y (Fig. 4.6.).

This model also takes the effects of applied ethylene on graviresponsiveness into account: Low levels of ethylene could increase repression of X by NR leading to the repair of the *dgt* mutation to normal gravitropic response levels, however, with altered kinetics (Madlung et al., 1999). Higher levels of ethylene could lead to the initiation of unrelated pathways that may result in the in the inhibition of Z and/or the triple response. The first case could account for the reduction in graviresponsiveness at levels that inhibit gravicurvature but do not elicit the triple response (Madlung et al., 1999).

4.5.4. Mutation of both *Nr* and *dgt* may lead to flower organ abnormalities and alterations in organ fusion patterns

The most interesting example of the combined effects of reduced auxin- and ethylene responsiveness in the *Nr;dgt* double mutant may be the alteration of flower structure in a subset of the double mutant flowers (Fig. 4.5.). In the common cultivated tomato, the pistil is formed when central meristem cells form a column, which postgenitally fuses with the adaxial part of each of the five carpels forming the tomato ovary (Chandrar Sekhar and Sawheny, 1984). The style of the cultivated tomato is single and also arises from fusion of the carpels (for review see Gasser and Robinson-Beers, 1993). Genes involved in gynoecium development in *Arabidopsis* include *MONOPTEROUS* (*MP*), *ETTIN* (*ETT*), and *TOUSELED* (*TSL*).

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Figure 4.6.: Double mutant analysis of the graviresponse suggests that the ability to respond to gravistimulation may be affected by DGT, NR and several other factors. B, Y, X, and Z symbolize proteins not yet identified. Several scenarios of single and double mutations in this proposed pathway are discussed in the text.

Figure 4.6.
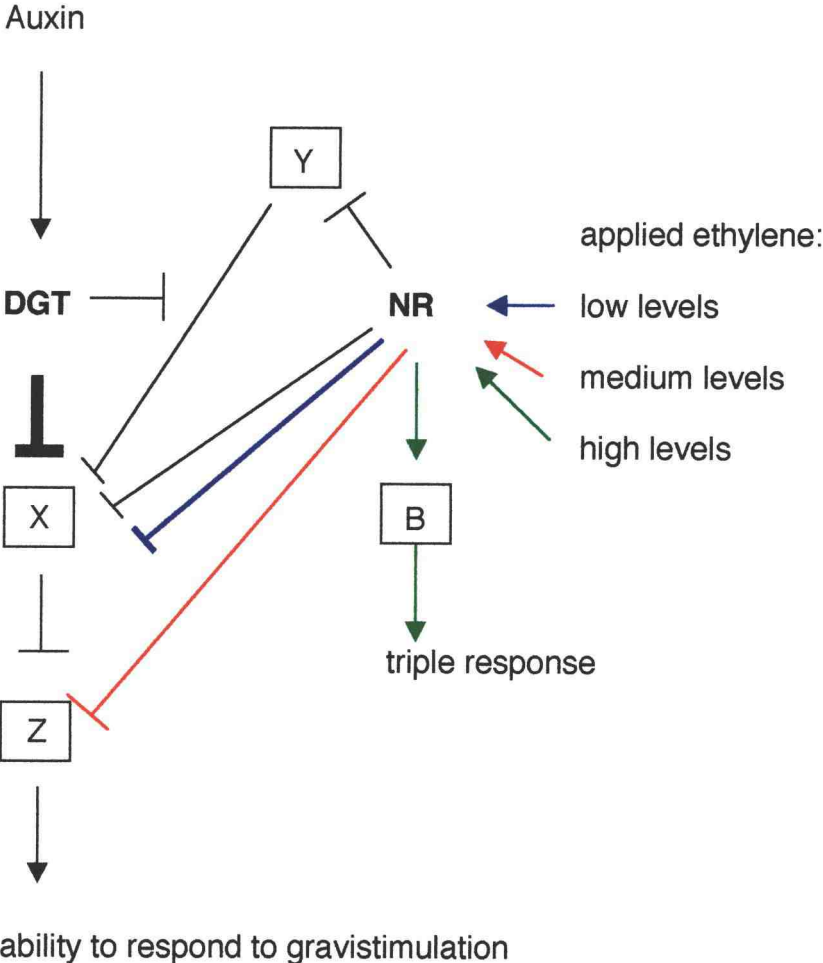
While *mp* mutants display an extremely pleiotropic phenotype throughout development, *ETT* and *TSL* are chiefly involved in flower development. *ETT*, also known as *ARF3*, has been implicated in the establishment of boundaries in the floral meristem for normal patterning (Nemhauser et al., 1998). Mutations in the protein kinase *TSL* lead to reduced tissue differentiation in the flower and eliminate fusion of style and septum in *Arabidopsis* (Roe et al., 1997). As with *ETTIN* and *TSL*, mutations in the *Arabidopsis* genes *LEUNIG* (*LUG*) and *PERIANTHIA* (*PAN*) cause abnormal floral organ numbers (Liu and Meyerowitz, 1995; Running and Meyerowitz, 1995). Some of these genes are known to be auxin regulated transcription factors, such as *MP* (also known as *ARF5* or *Aux/IAA 24*) and *ETT/ARF3*. It is therefore a formal possibility that the lack of carpel fusion and the increased number of floral organs in the *Nr;dgt* double mutant (Fig. 4.5.) may be caused by improperly relaying the auxin signal required in floral tissue patterning and carpel fusion.

The tomato mutant *solanifolia* is characterized by a general lack of fusion of floral organs (Chandrar Sekhar and Sawhney, 1984). The lack of fusion in *solanifolia* was suggested to be due to the significantly larger diameter of the floral apex resulting in a greater distance between fusing organ primordia (Verbeke, 1992). With respect to the *Nr;dgt* double mutant, increased size of primordial carpel tissue could give rise to the separation of the tissue and partitioning into distinct carpels or, as in the case of the twin flowers, distinct gynoecia. This could be the result of dilution effects of morphogen-like substances expressed on opposite sides of the floral

primordia. Greater than normal dilution of such a signal might trigger the primordium to divide and result in twin flowers.

The higher than normal number of petals observed in some flowers of the double mutant also raises the possibility that patterning by floral meristem identity genes (Okamuro, et al., 1993) may be altered by combining the two mutations. Hormonal control of *Arabidopsis* floral meristem identity genes such as *AGAMOUS, APETELA* and *LEAFY* has been shown for gibberellic acid under short-day conditions (Okamuro, et al., 1993). Lozano et al. (1998) reported flower abnormalities in tomato, which had been grown under low temperature conditions (17°C/7°C), which resulted in increased numbers of organs as well as increased fusion between anthers and carpels as well as increased levels of tomato MADS-box gene expression and an alteration in stage-specific expression of the MADS-box gene *TM4* in these flowers. It remains to be tested if transcription of these genes is also altered in the *Nr;dgt* double mutant causing the abnormal flower phenotype.

In the *Nr;dgt* double mutant, we also noticed a higher than normal number of anthers that were fused to the pistil (Fig. 4.5. J,K) and often slightly shorter and not as straight as normal anthers. Ethylene can change the ratio of male to female flowers in cucurbits (Reid, 1995). How such changes are achieved on the molecular level, however, is not well understood. Whether or not the lack of ethylene signaling in the *Nr;dgt* double mutant leads to "feminization" of anthers remains to be shown. Hamdi (1987) reported auxin to cause a higher degree of "maleness" in

Mercuralis annua (for review, also see Dellaporta and Caldera-Urrea, 1993). It is evident from the literature that different species have evolved different mechanisms of employing hormones in sex development. In many double mutant flowers we also noticed a decreased rate of self-fertilization. This appears to be due to a smaller than normal amount of pollen in the anthers of the double mutant (data not shown), but could also be caused by a lower percentage of pollen germination or a lesser degree of anther dehiscence.

4.6. Conclusions

Interactions between phytohormones are responsible for a large number of plant adaptations to environmental conditions and morphological changes during plant growth and development. It has been known for a while that auxin induces ethylene production and that ethylene can inhibit polar auxin transport (Abeles, 1992, Schwark and Schierle, 1992). We have shown here that ethylene-resistance can also result in an inhibition of responses normally attributed to auxin action, such as the initiation of adventitious rooting and hypocotyl elongation growth. We have shown that while blocking the response to either one, auxin (mediated by DGT) or ethylene (mediated by NR), does not result in a complete abolition of the tested responses, mutations in both *Nr* and *dgt* genes result in severe insensitivity to auxin-induced responses. Specifically, the *Nr;dgt* double mutant acts in an additive manner during adventitious rooting. The *Nr;dgt*

double mutation produces an additive phenotype in the elongation assay and during anthocyanin production. In the gravitropic response, *Nr* acts antagonistically to *dgt* or negatively on a third, unknown signaling pathway.

Our experiments suggest that auxin-sensitivity may be in part regulated by ethylene with respect to rooting and elongation and possibly other processes in the plant. For example, it appears that auxin resistance leads to the lifting of a repressor that negatively affects pigment production. When auxin exerts a negative effect, ethylene insensitivity alone does not seem to affect the level of pigment production. However, the combination of both auxin and ethylene resistance reveals enhanced effects (in the case of anthocyanin levels) in these response patterns as well. In this instance, ethylene and auxin interactions seem to be on a different level of interaction than in the responses where ethylene-insensitivity alone results in a reduced response. In order to make firm conclusions, it will be of great importance to elucidate the function of the DGT gene product. With respect to carpel fusion and flower development, the effect of the double mutation in auxin and ethylene sensitivity may influence transcription events downstream of *Nr* and *dgt* that could affect genes involved in flower identity and/or carpel development genes. The complete elucidation of these interactions will help reveal the level of involvement of phytohormones in flower development but will depend on the further identification and characterization of molecular transmitters of hormone signals.

4.7. Acknowledgements

5. CONCLUSIONS

Interactions between plant hormones, light, and gravity are ubiquitous throughout the life of the plant. The goal of the work presented in this thesis was to analyze specific developmental and physiological responses to auxin, ethylene, and gravity during plant growth and development. A specific set of developmental processes were selected and analyzed with respect to hormonal interactions by using hormone-response mutants and by constructing a double mutant affected in perception or signal transduction of both auxin and ethylene.

In the last five years, tremendous progress has been made on the molecular level in discovering molecules involved in the signal transduction pathways of all the major phytohormones. While molecular technology has helped to break ground in the identification of major components of the hormonal perception pathways, much work needs to be done to understand the implications of genetic control on the whole plant level.

I have utilized physiological and classical genetic tools to elucidate interactions of multiple hormones during important physiological processes. Figure 5.1 attempts to synthesize information gained from this work and existing knowledge from work in *Arabidopsis* and tomato into one coherent model of hormone interactions as they pertain to the gravitropic response,

elongation growth, radial expansion, root and callus initiation and growth, and tracheary element differentiation.

One of the most intriguing questions in the field of plant hormone action is the question of how response specificity is achieved when very few different molecules are available to orchestrate the multitude of processes regulated by hormones. Recently, research on mutants altered in auxin transport resulted in the cloning and molecular characterization of two similar auxin efflux carriers, *eir1* and *pin1* (Luschnig et al, 1998; Gälweiler et al., 1998). Both efflux carriers are similar in their protein structure but the tissue localization of their expression is quite different. *PIN1* is localized in the basal membrane of shoot starch parenchyma cells, allowing for basipetal auxin transport through the stem. *EIR1* localizes to the apical membrane in root cortex cells and facilitates acropetal transport through the root (Jones, 1998). Directed efflux (see step 1 in Fig.5.1) allows the distribution of auxin to specific tissues in the plant and thus may control spatial and temporal auxin-induced gene regulation. Efflux can be blocked by treatment with chemicals, such as TIBA and NPA. During gravistimulation, the first cell organelles responding to the change in the gravity vector are the amyloplasts (step 2, Fig. 5.1) which sink towards the gravity vector (Fukaki et al., 1998) and start a signaling cascade, most likely activated by changes in stress on cytoskeletal fibers (step 3, Fig. 5.1). There is evidence that actin filaments are linked to the auxin efflux carrier via the NPA-binding protein, an auxin efflux carrier-associated protein (Muday et al., 1999).

The graviresponse occurs when auxin is redistributed to the lower side of the gravistimulated stem (Salisbury, 1993). Efflux carriers specifically involved in lateral auxin transport have to date not been identified. There is preliminary evidence that the *lazy-2* gene may be involved in such lateral auxin transport (Kim, 1996) and could presumably encode a protein either directing a lateral auxin transporter to the distal (outward facing) side of a graviresponding cell or representing a transporter itself (step 4, Fig. 5.1).

Subsequent to targeted transport, auxin is imported into the cell via an uptake carrier (step 5, Fig. 5.1, Lomax et al., 1995). Further evidence for such an uptake carrier comes from the cloning of the *aux 1* gene of *Arabidopsis* which was found to have high similarity to a permease with affinity for tryptophan, an amino acid closely resembling the chemical composition of auxin (Bennett et al., 1996).

During the graviresponse, auxin is transported towards the epidermal cells which are the primary target tissue for auxin in gravitropism. Here, auxin is perceived by an as yet unknown auxin receptor (step 6, Fig. 5.1). This auxin receptor could be either inside the cell or on the cell surface. The AXR1 gene of *Arabidopsis* has similarity to E1 (Leyser et al., 1993), a protein involved in ubiquitination. In mammals, ubiquitination targets the plasma membrane-associated IgE receptor for degradation. In analogy, plant cells may use *AXR1* to control abundance and turn-over of an auxin receptor (step 7, Fig. 5.1) and/or auxin-regulated transcription factors.

Figure 5.1. Model for signal transduction events during the gravitropic response in tomato. CS = cytoskeleton, SG = starch granule, RL = red light, *HLS* = *HOOKLESS*. Numbers are explained in the text.

Figure 5.1.

starch sheeth

amount and direction of gravity

IAA

efflux carrier

putative lateral auxin transporter

uptake carrier
or permease (aux1?)

cortex

epidermis
(for clarity shown as
2 cell layers)

elongation
-graviresponse
-hypocotyl
-root hair growth?
root elongation
microfibrills

149
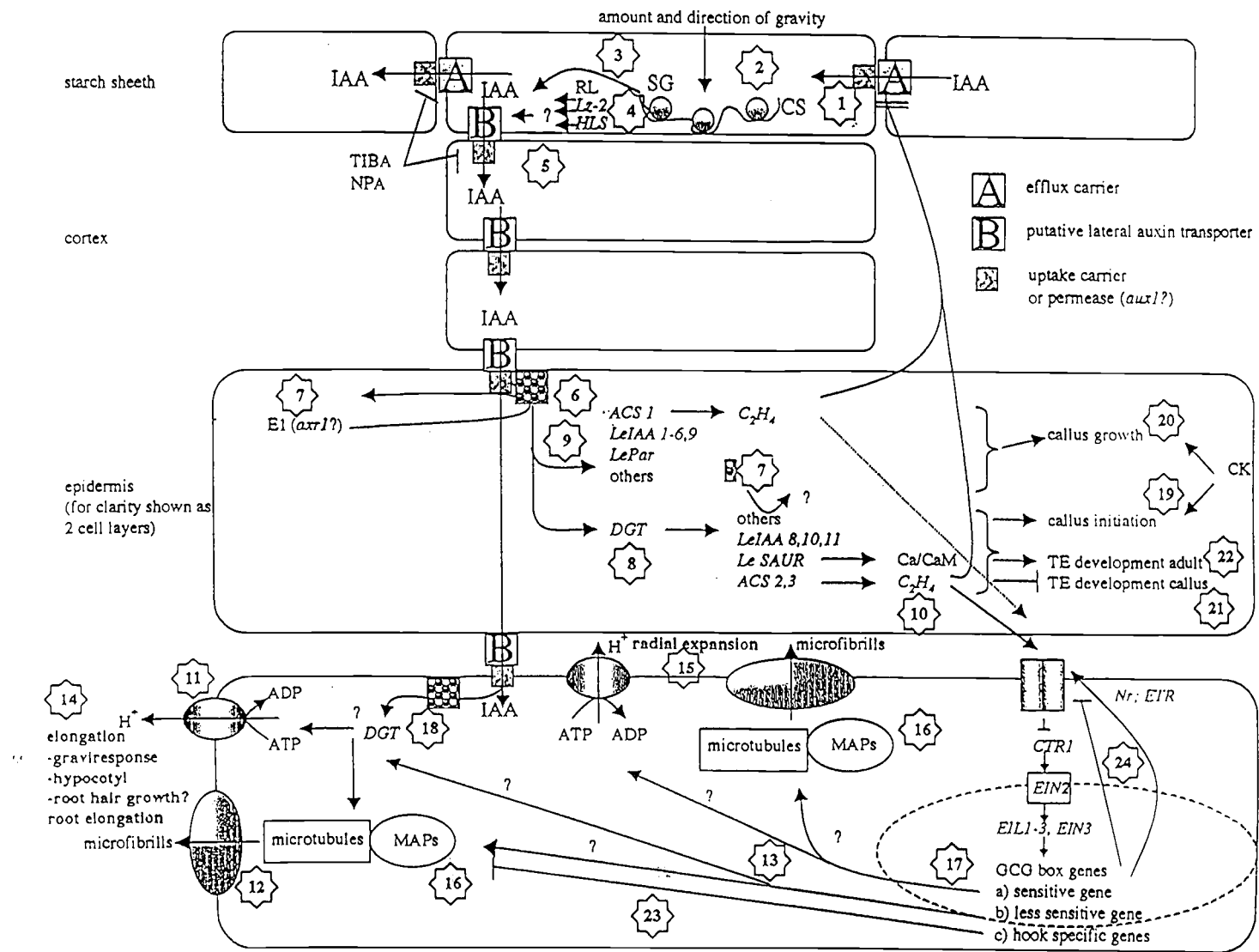
Downstream from the elusive auxin receptor, auxin affects the

transcription of a multitude of genes, such as ACC synthase genes (*ACS*1-3),

LePar, and*LeSAUR*, as well as members of auxin-regulated gene families,

such as *Aux/IAA,* and *ARF*. Previous work has shown that only some specific

members of these gene families are *DGT*-dependent in their transcriptional

activation (step 8, Fig. 5.1), such as *LeIAA 8, 10* and *11* (Nebenführ and

Lomax, 2000) and *ACS2* and *ACS3* (Coenen and Lomax, 1998). Dependence

of only a subset of auxin-regulated genes on the *DGT* gene product explains

the reduced but not in all instances abolished sensitivity of the *dgt* mutant to

auxin (steps 8 vs 9, Fig. 5.1).

The dependence of transcription of two of the known ACC synthase

genes (*ACS2* and *ACS3*) on a functional DGT gene opens a possible

explanation for the *DGT*-dependency of the gravitropic response. Reduction in

the ethylene levels normally induced by gravitropic stimulation within the cell

or tissue may downregulate the transcription of ethylene-responsive genes

which could affect signal transduction pathways necessary for cell elongation

(step 10, Fig. 5.1). This process takes place in two steps: First, to initiate short

term elongation growth, an ATPase excretes hydrogen ions into the apoplastic

space, resulting in cell wall loosening and protoplast expansion (step 11, Fig.

5.1). This mechanism is known as the acid growth theory and has been shown

to be auxin-dependent (Cleland, 1995). Second, sustained, rather than short-

term growth, may be achieved by reorientation or new deposition of

microfibrils in the cell wall, which allows the cell to grow and assume a different shape. Such changes can result in elongated cells, radially-expanded cells, or even the formation of protrusions from the cell, resulting in root hairs or adventitious roots. Microfibril orientation is dependent on cellulose synthase located in the membrane which in turn is guided by microtubules from within the cell (step 12, Fig. 5.1).

In wild-type plants, ethylene has been reported to affect microtubule alignment and to alter their orientation from horizontal to vertical. This would allow the plant to expand radially, as is the case in the triple response. In this model (Fig. 5.1), ethylene regulates or affects microfibril arrangements through differential sensitivity to ethylene concentrations (step 13 or 17, Fig. 5.1). It is formally possible that such microfibril-orienting enzymes are localized in different areas of the plasma membrane guided by the cytoskeleton and resulting in differential activity, much like the auxin efflux carriers now being elucidated from root and shoot tissues (Luschnig et al., 1998; Gälweiler et al., 1998). This could explain why small quantities of auxin-induced ethylene result in stimulation of elongation growth (step 14, Fig. 5.1) as reported in chapter 2 (Madlung et al., 1999) for gravistimulated *dgt* seedlings and dark-grown *Arabidopsis* seedlings (Smalle et al., 1997), while higher doses of ethylene lead to the triple response (step 15, Fig. 5.1). Auxin, on the other hand, may change microtubule orientation to horizontal placement allowing for longitudinal expansion and elongation (Lloyd et al., 1999). Rearrangement of

microtubules may be controlled by a number of microtubule-associated proteins (MAPs; step 16, Fig. 5.1). Using antibodies against MAPs, Lloyd et al. (1999) have shown that microtubules are aggregated or cross-linked by different MAPs possibly linking them to the plasma membrane where they have been proposed to guide cellulase situated in the membrane to produce new cellulose strands during the formation of new cell walls. Differential sensitivity to ethylene may cause activation of different ethylene-inducible genes regulating the activity of differentially-localized MAPs or intermediates which would account for either radial or longitudinal growth (step 13 or 17, Fig. 5.1). This would allow the plant to utilize ethylene as a signal for radial expansion at high concentrations and elongation - as in the gravitropic response - at low concentrations.

Initiation of root hairs (Pitts et al., 1998) and adventitious roots (Clark et al., 1999; chapter 3) could be caused by the same mechanism. Protrusions from cells in the epidermis may be caused by microfibril rearrangement of roughly 90° prior to outgrowth of root hairs and possibly in a similar fashion from the pericycle in the case of adventitious and lateral roots. Auxin is, however, suggested to be the major cause for elongation growth (step 18, Fig. 5.1). Microfibril rearrangement would therefore have to be primarily regulated by auxin in the basal and/or apical cell walls with only secondary regulation by ethylene. Experimental evidence presented here (chapter 3) supports this theory: the *Nr* mutant displays reduced production of adventitious roots in

response to ethylene while resistance to auxin in the *dgt* mutant leads to a more severe reduction. Combination of both mutations finally leads to an almost complete reduction of adventitious root initiation.

Supporting an alternative explanation to the direct regulation of cell growth by ethylene, experimental evidence has shown that auxin-induced ethylene can block auxin transport. This mechanism essentially results in a feedback control of auxin-induced ethylene production (Schwark and Schierle, 1992). Ethylene is well known to both upregulate and under certain conditions, downregulate its own production (step 24 Fig. 5.1; Abeles, 1992). Upregulation is probably caused by increased concentrations and activity of *ACS* as well as *ACO* (ACC oxidase), formerly known as ethylene-forming enzyme (Abeles, 1992). Multiple mechanisms of feedback regulation of ethylene production suggest multiple genes involved in this process which may either be differentially sensitive to ethylene or their expression is spatially or temporally regulated. One such example is hook maintenance where auxin-induced ethylene is responsible for growth retardation on the lower side of the hook (step 23, Fig. 5.1; Schwark and Schierle, 1992). Differential auxin transport has been suggested to be guided by the N-acetyltransferase *HLS* (*HOOKLESS*; Lehman et al. 1996) via an unknown mechanism.

Development of callus and tracheary elements is regulated by auxin, cytokinin and gravity (chapter 4). While callus initiation is *DGT*-dependent (step 19, Fig. 5.1; Coenen and Lomax, 1998), callus growth is *DGT*-

independent in more mature callus (step 20, Fig. 5.1; chapter 4). This suggests that several auxin-regulated genes are involved in callus initiation and growth. Development of tracheary elements is regulated both by gravity and *DGT*-dependent auxin signaling. A repressive function appears to be lifted in *dgt* mutant callus (step 21, Fig. 5.1, Chapter 4, Fig. 4.1), while normal TE development is positively regulated by *DGT* in adult plants (step 22, Fig. 5.1). Chapter 4 provides further evidence that TE development is regulated by gravity dosage in the wild-type plant and suggests partial dependency on *DGT*-mediated auxin signaling in this process.

In summary, this work has analyzed the simultaneous contributions of auxin, ethylene, and gravity in several well-studied plant developmental processes and attempted to further our understanding of hormone-hormone interactions on the tissue and cellular level. Molecular plant biologists have only recently begun to elucidate major components of the signal transduction pathways of hormones. Any attempt at presenting a comprehensive model for signal transduction events during complex physiological responses has to be at this point fairly speculative. The studies described here have demonstrated that ethylene plays a non-primary role in the gravitropic response, with ethylene decreasing the magnitude of the gravitropic response without interfering with overall elongation. Absence of ethylene leads to a reduction in gravitropic responsiveness and the addition of ethylene to the auxin-resistant *dgt* mutant can restore the magnitude but not the kinetics of the gravitropic

response in the *dgt* mutant. Further, these studies have shown that auxin-ethylene interactions play a crucial role in giving the plant full responsiveness to auxin treatment with respect to adventitious root formation, the gravitropic response, and hypocotyl elongation. The results of the *Nr;dgt* double mutant studies also demonstrate the necessity of functional *dgt* and *Nr* genes during flower development. These studies suggest that *dgt* and *Nr* in combination play a role in the regulation of proper carpel fusion and floral organ identity. Finally, the data presented here provide supportive data for the hypothesis that gravity in combination with auxin exerts a direct influence on the development of tracheary elements. These results contribute several pieces to the puzzle of hormone response pathways which, when completed, will help us better understand and possibly manipulate plant growth and development.

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APPENDIX

APPENDIX

Appendix 1:Gravicurvature is inhibted in 5d-old etiolated wild-type tomato seedlings after addition of the ethylene synthesis inhibitor AVG. The experiment was performed the same way as decribed in Fig. 2.4. Overall elongation was measured to ensure that possible growth inhibition was not the cause of the decrease in curvature.

[AVG]	angle	SE	length	SE	inhibition [%]
0μl AVG	88.30	2.40	1.11	0.02	0.00
10 μl	78.20	5.20	1.20	0.02	12.00
100 μl	71.80	7.40	1.27	0.05	19.00