EXPLORING THE ROLE OF AUXIN IN PHENOTYPIC PLASTICITY IN *ARABIDOPSIS THALIANA* ROOT DEVELOPMENT

BY

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> That though the radiance which was once so bright be now forever taken from my sight. Though nothing can bring back the hour of splendor in the grass, glory in the flower. We will grieve not, rather find strength in what remains behind.
>
> William Wordsworth

> Open up your mind and see like me; open up your plans and damn you're free. Look into your heart and you'll find that the sky is yours.
>
> Jason Mraz

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ABSTRACT

Lovin, Mary Elizabeth

EXPLORING THE ROLE OF AUXIN IN PHENOTYPIC PLASTICITY IN *ARABIDOPSIS THALIANA* ROOT DEVELOPMENT

Dissertation under the direction of Gloria Muday, Ph.D., Professor of Biology

Auxin is a plant hormone that positively regulates the initiation and emergence of lateral roots. In this study we used several approaches to reduce auxin transport moving either from the shoot toward the root apex or from the root tip toward the base and examined the effect of these treatments on lateral root initiation and emergence. Our results are consistent with shoot derived auxin driving lateral root initiation, while we could detect no role for root tip auxin in lateral root initiation or elongation. We examined how environmental variables regulate root formation with a focus on day length and growth temperature and found that elevated temperature and increased day length positively regulate root formation. We explored several mechanisms by which root initiation is enhanced under these conditions including elevated auxin synthesis, transport, and signaling. Both auxin accumulation and auxin dependent gene expression increase during these treatments, suggesting a mechanistic basis for regulation of root branching. We also asked whether populations isolated from different latitudes might exhibit differences in root architecture. Environmental variables across a latitudinal gradient have been shown to both transiently affect growth, development, and physiological responses, as well drive the evolution of ecotypes with unique genotypes that increase fitness. We hypothesized that early root architecture as well as resource allocation would differ for populations across latitude based on the latitudinal

provenance. We observed differences in lateral root initiation, elongation, and developmental patterns under higher UV that followed a latitudinal cline. In populations from higher latitude, there were fewer lateral root primordia and emerged lateral roots, as well as less shoot biomass. We observed that in mature plants grown at lower UV levels, the photosynthetic rate and shoot biomass were lower in populations from higher latitudes leading to a different carbon balance. Root respiration and root biomass did not change between these populations isolated from different latitudes. Together these results provide insight into the hormonal, environmental, and genetic controls of root developmental patterning.

CHAPTER I

INTRODUCTION

Roots are one mystery of the plant world. They are often overlooked because they are difficult to study *in situ* and even when they are retrieved from their growth substrate, root structure and tissue are lost. Roots anchor the plant; they also extract, transport, and store resources (Abrahamson and Caswell, 1982; Barley, 1970; Ennos, 1990). The underground nature of roots provides a refuge from the high and low extremes of temperature, wind, evaporation, light, and dryness to which the above ground tissues must adapt. The plant must maintain a balance between the shoot surface and the absorptive root surface; this balance depends on life cycle stage and environmental conditions that affect both above and below ground plant surfaces (Abrahamson and Caswell, 1982).

Root Morphology

Primary roots are formed within the embryo and are usually the first tissue to emerge during germination. The primary root elongates and the lateral roots initiate from what was previously the non dividing tissue of the pericycle. There is branching of lateral roots with secondary and tertiary branches forming along primary lateral roots (Rose, 1983; Charlton, 1983). The branching can be simple or complex depending on the species and growing conditions. Adventitious roots grow from the stem above the ground and increase stability (Bray, 1954). The extensive branching allows exploration of much more soil volume resulting in greater nutrient and moisture uptake. Root hairs extend from epidermal cells along primary and lateral roots and greatly increase the absorptive surface of the root.

Model plant—*Arabidopsis thaliana*

The process of root development has been best studied in the well characterized genetic system, *Arabidopsis thaliana*. *A. thaliana* is a small annual member of the Brassicaceae or mustard family. It was first used as a model system in plant genetics in the 1950s (Goodman et al., 1995). There are many advantages for using this plant; it has a small genome and a small number of chromosomes with a complete genomic DNA sequence. It is easy to transform, easy to grow at high density, and it has a fast life cycle (Pigliucci, 1998). Additionally, a diversity of natural populations have been isolated with variation in a number of interesting traits, including root branching (Andalo et al., 1999; Banta et al., 2007; Stratton and Bennington, 1996; Dorn et al., 2000; Stinchcombe et al., 2004).

The anatomy of *A. thaliana* is well characterized with a filamentous root system, a basal rosette of leaves, a main flowering stem with lateral branches, and often additional flowering stems established at the base of the plant (Pigliucci, 1998; Napp-Zinn, 1985). The rosette grows a certain number of leaves depending on genotype and environmental factors before receiving a signal to bolt (which is the term for the flowering stem to elongate). Flowers open and fruit or siliques develop. Once the seeds are fully matured in the fruit, the plant begins to senesce; the siliques open and the seeds are released.

Lateral root development

The developmental biology of lateral root formation in *A. thaliana* has been extensively studied (Malamy, 2008). The mature primary root has a simple structure. The four outer tissues consist of one cellular layer each, the epidermis, cortex,

endodermis, and pericycle. Lateral root initiation occurs in pericycle cells that are in specific alignment with protoxylem poles (Malamy and Benfey, 1997; Dubrovsky et al., 2001). A schematic diagram of this division and subsequent divisions that give rise to lateral roots is shown in Figure I-1. A subset of these pericycle founder cells develops into lateral roots, with precise signals that are regulated to initiate division in some cells and restrict division in others (Malamy and Benfey, 1997). The first morphological step in initiation is when two pericycle founder cells within the same cell file, adjacent to one of the xylem poles, undergo almost simultaneous polarized asymmetric transverse divisions; this creates two short cells flanked by two longer cells (Casimiro et al., 2001; Laskowski et al., 1995). These daughter cells divide continuously (Casimiro et al., 2001; Malamy and Benfey, 1997). Following a period of radial expansion, the central short daughter cells divide periclinally; this will become the basic composition of the primordia with inner and outer cell layers (Malamy and Benfey, 1997). As the primordium continues to expand, it will break through the layers of the primary root (Casimiro et al., 2003). As lateral roots develop they take on the developmental structure of a primary root tip quickly with cell layers including the epidermis, cortex, endodermis, and pericycle. Once lateral roots emerge, then further expansion occurs through division originating in the root meristem (Malamy and Benfey, 1997).

Auxin and its role in root development

Root development is regulated by plant hormones, with a clear role for auxin. Indole-3-acetic acid (IAA) is the most abundant naturally occurring auxin. Auxin is critical for many developmental processes including lateral root initiation and elongation (Casimiro et al., 2003; Teale et al., 2005; Malamy, 2008). Appropriate distribution of

auxin has been shown to be necessary for phototropism, gravitropsim, root elongation, and lateral root development (Leyser, 2006; Malamy, 2005; Muday and Rahman, 2008). Application of exogenous auxin stimulates lateral root development in a variety of plant species (Muday and Haworth, 1994). Similarly, plants with either mutations or transgenes that cause elevated endogenous auxin concentration have proliferation of lateral roots (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995). Auxin acts at the earliest stages of initiation of lateral root primordia during the activation of the previously quiescent pericycle cells to begin division (Himanen et al., 2002), but also has been shown to enhance elongation of lateral roots (Wu et al., 2007).

Auxin moves via a unique polar transport mechanism that is tied to growth and developmental processes. In shoots, polar auxin transport occurs by cell-to-cell movement from the shoot toward the root in a basipetal direction (Muday and DeLong, 2001; Leyser, 2006). In roots, two auxin transport streams have been detected in different tissues. Acropetal transport occurs from the shoot toward the root apex through the central cylinder, while basipetal transport occurs from the root apex toward the base through the outer root layers (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978; Rashotte et al., 2000, Muday and DeLong, 2001). Auxin delivery to the cells via polar auxin transport is essential for lateral root development, as demonstrated by treatment with inhibitors of auxin transport that reduce the number of lateral roots (Muday and Haworth, 1994; Reed et al., 1998; Casimiro et al., 2001). Direct application of NPA to root tissue arrests lateral root development by blocking the first transverse divisions of xylem pole pericycle cells, suggesting that a threshold concentration of auxin might be

required before cells could respond to lateral root-patterning signals (Casimiro et al., 2001).

IAA transport is mediated by 2 classes of auxin transport proteins, IAA influx and IAA efflux carriers. Influx is mediated by AUX1 and other LAX proteins (Leyser, 2006). Efflux has been tied to 2 classes of proteins, PIN proteins and ABCB/MDR/PGP proteins (Leyser, 2006; Geisler et al., 2005). In roots, AUX1 protein participates in both basipetal and acropetal IAA transport (Negi et al., 2008). In contrast PIN and ABCB proteins are tied to distinct polarities of auxin transport. ABCB4 and PIN2 mediate basipetal transport (Rashotte et al., 2000; Lewis et al., 2007) and ABCB19 and PIN1 mediate acropetal transport (Titapiwatanakun et al., 2009; Lewis et al., 2007; D.R. Lewis, personal communication). The transport of auxin by PIN3, PIN4, and PIN7 has not been reported, but their localization patterns suggests that they may function to mediate acropetal IAA transport. The asymmetrical positioning of the PIN proteins are essential for the polarity of auxin transport (Friml, 2003; Kramer and Bennett, 2006), while ABCB and AUX/LAX proteins are found on all sides of the membrane (Titapiwatanakun et al., 2009).

Chapter 2: Source of auxin during lateral root formation

Although the role of auxin in root development is well established, the auxin source that drives root formation is still debated (Casimiro et al., 2001, Bhalerao et al., 2002, Reed et al., 1998). There are two sources of auxin in the developing seedling, the first true leaves and the primary root tip (Leyser and Day, 2003). Most agree that the development of lateral root primordia and the emergence of lateral roots are two separate processes and may be controlled by separate sources of auxin (Casimiro et al., 2001;

Bhalerao et al., 2002). Some results suggest the initiation and elongation of lateral root primordia is driven by primary root tip derived auxin (Casimiro et al., 2001, Benkova et al., 2003), while other studies suggest that shoot derived auxin is important for lateral root elongation (Reed et al., 1998; Bhalerao et al., 2002). In Chapter 2, the hypothesis that shoot derived auxin drives lateral root initiation and emergence of lateral roots was tested by using chemical, physical, and genetic techniques to block the movement of auxin; the results directly supported the hypothesis that shoot-derived auxin drives lateral root formation. If shoot derived auxin drives lateral root development; it is logical to hypothesize that the environmental cues that are perceived by the shoot may be translated to the root to modify development.

Phenotypic plasticity

Root development exhibits a shifting phenotype depending on environmental conditions (Malamy and Ryan, 2001). In a population of immobile organisms living in variable and in some cases unpredictable environments the ability of a single genotype to produce phenotypes that vary in response to the environment is a valuable adaptation that is generally termed phenotypic plasticity (Schlichting, 1986). The extent and importance of phenotypic plasticity in a given species depend on how much of a generalist it is, since this determines the range of conditions over which it must thrive; a given genotype with the capacity for phenotypic plasticity has greater potential for survival and successful reproduction (Zhivotovsky et al., 1996; Bachmann, 1983). All plants have constraints and thus limited plasticity due to either lack of genetic variation (Via and Lande, 1985), architectural constraints (Watson and Casper, 1984), and/or resource allocation (Adams, 1967; Antonovics, 1976; Thomas et al., 1971).

Plants respond differently to environmental variables and thus can be categorized by their plastic response (Miner et al., 2005). A plant with a very plastic growth system, a generalist, could adapt well in many different environments. A plant could also be a specialist for a particular ecotype (alpine) and thus not need a variable system; it would not be considered plastic especially for something like root architecture (Van Tienderen, 1991). Finally there are plants that are not plastic but can live in a variety of environments. They survive but not well. Their "average" phenotype is suboptimal in all environments but not bad enough to cause it to go extinct (Pigliucci, 2001). It is often assumed that the plasticity of a plant trait is adaptive, but a response has to be appropriate to the environment to be adaptive (DeWitt et al., 1998).

A. thaliana has been shown to exhibit phenotypic plasticity; with flowering time being the best characterized plastic phenotype (Pigliucci, 1998). Groups of natural populations have been identified by their flowering phenology (Napp-Zinn, 1985). There is a very early flowering plant or spring ephemeral which bolts quickly and does not respond to vernalization, unless grown under short days; these are most ecotypes used in laboratory conditions. There is also an early summer annual that flowers later and responds only slightly to vernalization and a late summer annual, which bolts even later with no need for vernalization. Finally there is the winter annual that germinates in late fall and overwinters. Many argue that these characterizations are based on physiological responses to a given environment and that there is a lack of field studies to confirm that all of these differing types of growth histories indeed exist (Le Corre et al., 2002; Johanson et al., 2000; Lawrence, 1976; Koornneef et al., 2004; Wilczek et al., 2009). Much of the variation in flowering time in *A. thaliana* can be attributed to molecular

variation in genes that are responsible for sensing light and temperature which would indicate seasonal changes. These genes include *FRIGIDA* (*FRI)* (Stinchcombe et al., 2004), *FLOWERING LOCUS C* (*FLC)* (Wollenberg et al., 2008, Schmitz and Amasino, 2007), *GIGANTEA (GI)* (Sawa et al., 2007), and *CONSTANS* (*CO)* (Suarez-Lopez et al., 2001; Roden et al., 2002)

Environmental control of root development: a plastic phenotype

Lateral root formation is a plant phenotype that is sensitive to external cues (Malamy and Ryan, 2001). The development of an optimal root system is a key factor in a plant's ability to survive adverse conditions. Plants monitor both local cues like nutrients and soil structure as well as long-distance cues like ambient temperature, day length, and light intensity. Based on these environmental cues, the plant alters development.

Root structure is plastic and it is sensitive to both soil structure and nutrients and the environmental factors that affect soil (Bray, 1954). If you examine the soil horizons of different ecosystems, you will see that they differ greatly. This is caused by many factors. The relative abundance of clay, sand, and humus in the soil leads to different root structure. Soil pH is also important; a soil's acid or alkaline measurement is related to the availability of inorganic nutrients for plant growth. If roots are unable to obtain nutrients, all but a few species of plants may be forced out. It is an interactive system; this in turn leads to the evolution of different root systems.

Nutritional Control of Root Development

Patch size, quality and duration of nutrient availability are all important to a root system when determining whether or not to invest energy. There are temporal pulses of

nutrients, which are followed by temporal root responses. The primary root, lateral roots, and root hairs all change in different ways in response to different nutrients. Two well studied examples that provide insight on nutrient regulated root development are the effects of nitrogen and phosphate (Chevalier et al., 2003; Malamy and Ryan, 2001; Al-Ghazi et al., 2003; Lopez-Bucio et al., 2003; Lopez-Bucio et al., 2005). For example, if phosphate is limited, lateral root growth increases over primary root growth, and this effect is local so that roots grow toward patches of high P (Al-Ghazi et al., 2003). Using the mutant *root hair defective2* (*rhd2*), the importance of root hairs for P uptake in limiting conditions was demonstrated (Bates and Lynch, 2000). A temporal pattern of *A. thaliana* root response to P starvation has also been observed (Al-Ghazi et al., 2003). There is an interaction found between levels of P and N (Zhang et al., 1999). Lateral root density remains the same over a range of nitrate concentrations but decreases dramatically when P is increased. If there is a large amount of N or P in the soil, there is a uniform suppression of lateral roots (Linkohr et al., 2002). Auxin-resistant *A. thaliana* mutant, *axr4*, may be important in elucidating the coordination between auxin signaling and the adaptive response to nitrate starvation (Zhang et al., 1999; Fitter et al., 2002).

Water as a signal to modulate root development

Water availability can also be a limiting factor in root development. In moist fertile regions, roots grow extensively until the resources become limited (Shaver and Billings, 1975). When water is depleted, roots grow into new soil regions by formation of more root branches. If water is available deeper, then the roots grow down in a process called hydrotropism (Takahashi, 1997). Plant roots in desert regions may use this strategy or they may have a shallow root system to take advantage of brief rain events or

they may do both (Hunt et al., 1987). In terms of water availability, the individual plant or to a greater extent the plant species in a specific region must do a cost/benefit analysis; it maintains the root only until efficiency of resource acquisition is maximized. Here root replacement may be modulated because of its life history or because of its ecosystem (Fitter et al., 1988).

Presumably because of these different life histories, individual species might have different strategies for maximizing foraging for water. Annual grasses possess a fibrous and highly branched root system near the soil surface while perennial grasses extend far deeper. By growing a deeper root system, perennials invest additional sources of energy because they are there for a longer period of time and they will deplete the shallower resources. This strategy may provide long-term protection against drought or other extreme weather issues (Fransen et al., 1999; Fitter, 1986).

Light regulated root development

Photoperiods and light intensity are long-distance signals that modulate plant developmental responses including root development. Plants use light as an energy source via photosynthesis and a signal to activate and modify endogenous developmental processes such as auxin synthesis, transport, and signaling. Day length can limit the amount of photosynthate a plant can produce; it can also limit auxin processes. This might in turn change the response of seedlings as it pertains to lateral root initiation and emerged lateral roots.

Plants sense light via three known classes of photoreceptors: cryptochromes, phytochromes, and phototropins (Casal, 2002; Chen et al., 2004; Casal, 2000). Cryptochromes mediate light responses in the plant including, but not limited to de-

etiolation; they have not been implicated in root development (Chen et al., 2004; Lin, 2002; Liscum et al., 2003). Phytochromes play a large role in light signaling pathways in the shoot. Phytochromes are encoded by small gene family composed of *PHYA-PHYE.* There is evidence for phytochrome activity within the root system controlling phototropism (Correll et al., 2003; Kiss et al., 2003; Ruppel et al., 2001). The *phyD* mutant had slightly more lateral root production than wild type while *phyA, B, E* have reduced lateral root outgrowth, suggesting that phytochromes play a role in controlling root development (Salisbury et al., 2007). Phototropins also mediate physiological responses to light. Phototropism is the directional growth of the plant toward a unilateral light source, which is a blue light response mediated by phototropins (Chen et al., 2004).

Auxin has been shown to alter expression of a range of light-regulated genes confirming the close association of light and auxin signaling (Nagpal et al., 2000). HY5 protein is potentially a signal integration point in light and hormone signaling networks. The *hy5* mutant, defective in phytochrome signaling, has altered lateral root production (Cluis et al., 2004; Sibout et al., 2006). Aux/IAA proteins are involved in auxin response; studies of *aux/iaa* mutants suggest a strong link between auxin signaling and photomorphogenesis (Nagpal et al., 2000).

PHOT1 localization and the localization of members of the PIN family of auxin efflux carriers, particularly PIN3 overlap, suggesting that phototropin activation may direct asymmetric auxin distribution and growth orientation (Friml, 2003; Sakamoto and Briggs, 2002). PIN3 is important in establishing auxin gradients in response to changes in phototropism (Friml et al., 2002).

Temperature regulated root development

Plants are sessile and can experience significant temporal variation in temperature (Atkin et al., 2006; Larcher, 2004). Variations in temperature affect metabolic processes that contribute to biosynthesis of auxin (Gray et al., 1998) and cellular maintenance and photosynthesis. Biomass allocation is temperature sensitive (Loveys et al., 2002). During long term exposure to low temperatures, contrasting plant species exhibit reduced investment in shoots and increased root biomass, as compared to plants grown at higher temperatures (Loveys et al., 2002). These results are important in a real world context as global warming increases.

Temperature is key to organ growth and IAA synthesis in *A. thaliana* (Gray et al., 1998). Gray et al. (1998) find that elevated temperature (28ºC versus the normal 22ºC) increases hypocotyl elongation. The higher temperature causes increased IAA synthesis in this tissue and this response requires intact auxin signaling pathways. The authors suggest that temperature regulates auxin synthesis that in turn regulates developmental responses (Gray et al., 1998). It would be reasonable to predict that if temperature affects auxin synthesis and growth in the hypocotyl, it may also affect root development.

Chapter 3: Environmental variation

Chapter 3 of this thesis tests the hypothesis that light and temperature regulate root development. External cues that affect the shoot response such as day length or growth temperature also mediate auxin synthesis, transport, and signaling. When studying any plant model, there are multiple factors that can alter root development but I chose to look at two long distance signals (day length and growth temperature) that affect the shoot and may alter root development via long distance auxin signaling. Chapter 3 reports evidence that these two environmental variables regulate root development and

explores the role of auxin in the transduction of these environmental inputs into root growth and developmental patterns.

Genetic differences in natural populations

Another important question is whether distinct lateral root developmental patterns have been observed in natural populations. Day length and growth temperatures vary along latitudinal gradients; they are representative of potential genetic variation and accompanying metabolic responses found in natural populations. The evolution of this genetic variation may be due to the environmental variables in their specific ecosystem. Large changes in these variables are evident over latitudinal gradients. *A. thaliana*'s geographic range is amazingly expansive. This is surprising because as a weed, it does not compete well and it is rarely locally abundant; instead it is found in small clumps scattered far from other local populations. *A. thaliana* is therefore an ideal model for examining whether genetic variation leads to unique root architectural patterns.

In one study, seeds were collected from European populations across a north-south latitudinal gradient from northern Spain to southern Sweden to see if photoperiod was at least partially responsible for local adaptation of the growth measures such as bolting time, number of leaves, rosette size, plant height, branches, ripening time, and number of fruit (Banta et al., 2007). They found that plants were not locally adapted to photoperiods using different growth chambers to simulate natural conditions for the three latitudes (Banta et al., 2007). The results indicate that populations native to these conditions did not have higher fitness than any of the other non-native populations in reciprocal conditions. Genes are often thought to have a pleiotropic effect on suites of environmental variables that affect a species in a specific ecosystem. However,

populations may be locally adapted only to some but not other suites of environmental variables, even if those variables co-vary across the landscape. Since *A. thaliana* populations can be genetically distinct even 30 kilometers away from one another (personal correspondence, M. Pigliucci), it is important to examine variation, both within and across populations in a given latitude.

Chapter 4: Variation in root development in natural populations

The goal of chapter 4 of this thesis is to ask whether *A. thaliana* populations that have evolved at different latitudes, with the resulting light and temperature differences, result in different root developmental patterns. Latitudinal studies are invaluable for examining genetic variation in growth characteristics, although they have not examined variation in root development on a population level. We found plasticity in root development in the laboratory lines (that were not selected for any specific latitude) under various day lengths and growth temperatures. I hypothesized that natural populations would have genetic variation based on these environmental variables that may define unique developmental patterns of lateral root development. I found that lateral root development in populations across latitude does significantly vary.

Conclusion

Root development in *A. thaliana* is a fascinating process that is amenable to study because of the many tools to elucidate growth and development such as mutants, transgenic reporter lines, physiological and genetic assays, and natural populations. In the laboratory, the conditions that evoke a specific developmental program can be held relatively constant. We found that lateral root development was driven by shoot derived auxin. This led us to environmental variables such as day length and growth temperature

that affect the shoot and thus long-distance signaling affecting auxin synthesis, transport, and signaling. We discovered that root systems exhibit plastic responses to these environmental variables. Examining natural populations over latitudinal gradients allowed us to identify genetic variation that may have evolved in response to day length and growth temperatures. We found that populations from different latitudes show variation in early root development. Together these results provide insight into the hormonal, environmental, and genetic controls of root developmental patterning.

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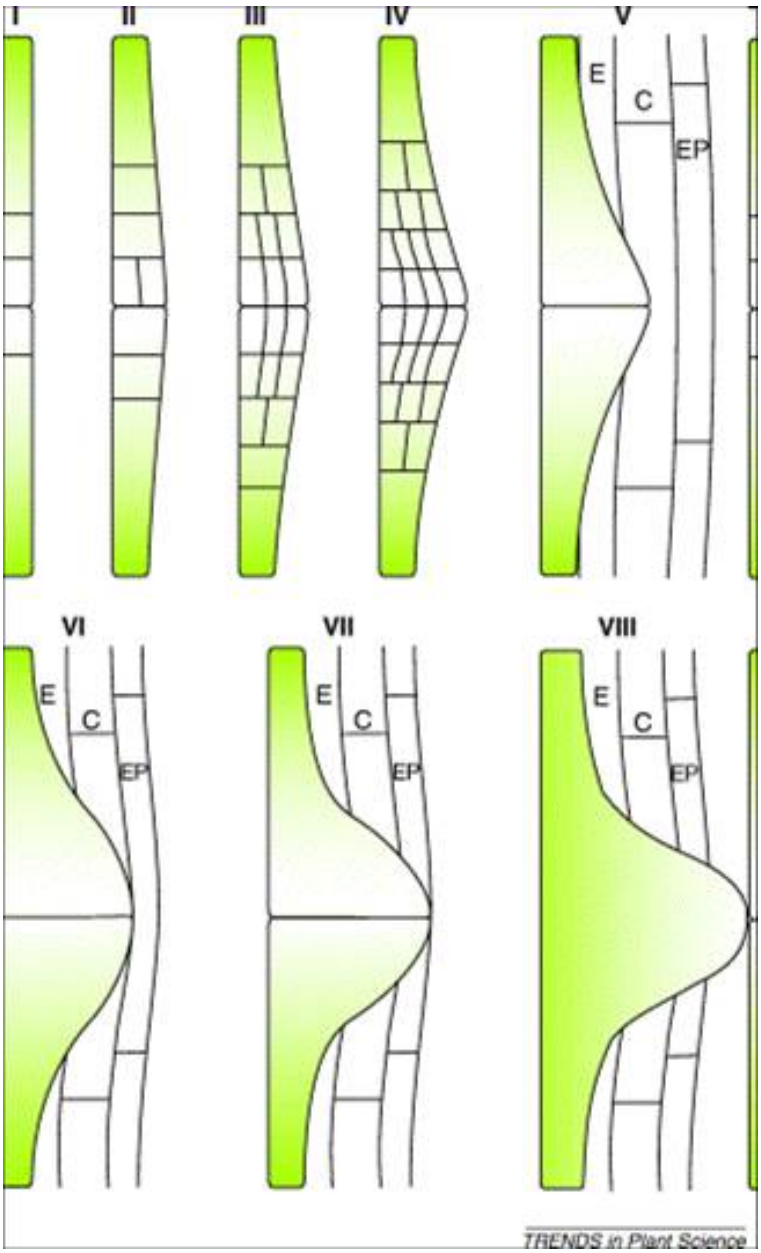

Figure I-1. Developmental stages during lateral root (LR) formation in *Arabidopsis.*

The figure shows a series of longitudinal sections through LR primordia at specific developmental stages as identified as stages I-VIII. The stage-I LR primordia contains a pair of short pericycle cells lying end to end and flanked by two longer cells. At stage II,

cells undergo transverse asymmetric divisions, forming an inner layer (IL) and outer layer (OL). In stage-III LR primordium, OL cells undergo periclinal divisions to create a three-layered LR primordium. At stage IV, the LR primordium forms four layers because of periclinal divisions in the IL. By stage V, LR primordia are midway through the parent cortex, finally emerging at stage VIII. Abbreviations: C, cortex; E, endodermis; EP, epidermis.

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CHAPTER II

SHOOT DERIVED AUXIN IS REQUIRED FOR LATERAL ROOT PRIMORDIA INITIATION AND EMERGENCE

Abstract

Auxin positively regulates the initiation and emergence of lateral roots. We used several approaches to selectively disrupt the two polar transport streams in roots and examined the effect of these treatments on lateral root initiation and emergence. Localized application of the auxin transport inhibitor, *N*-1-naphthylphthalamic acid (NPA), to the root/shoot junction blocked acropetal transport from the shoot to the root and reduced lateral root initiation and emergence. In contrast, NPA application to the root tip, to block basipetal IAA transport, had limited effect. Application of IAA below the site of NPA application at the root/shoot junction was able to restore lateral root initiation. Mutants with defects in acropetal IAA transport due to defects in influx and efflux carrier proteins, developed few lateral root primordia. Mechanical removal of the shoot, which eliminates shoot derived auxin, blocked lateral root initiation and emergence, while removal of the root tip did little to alter either root initiation or elongation. Since shoot excision, also reduced photosynthate availability, we used the herbicide, Norflurazon (NFZ), to inhibit photosynthesis and found that there were no lateral root primordia or emerged lateral roots. NFZ treatment reduces free IAA accumulation in the root and shoot and was reversed by application of IAA at the root/shoot junction, consistent with photosynthate needed for auxin synthesis. Our results are consistent with root tip derived auxin playing no role in root formation; while shoot derived auxin is required for lateral root initiation and elongation.

Keywords: Arabidopsis thaliana, lateral root development, auxin, auxin transport

Introduction

Appropriate root development is essential for a plant's success. A primary root forms in the embryo and emerges at germination; from this primary root, secondary roots emerge laterally. These lateral roots are important because they offer anchorage, provide access to below ground water sources, and harvest micro and macronutrients that are vital to plant growth. It is beneficial to the plant to have a root network that is flexible and readily adapts to changing availability of nutrients and water. As conditions change, signals can be sent to initiate more lateral roots or elongate the lateral root primordia that are already formed (Malamy and Ryan, 2001; Nibau et al., 2008). Understanding the basic underlying mechanisms that control lateral root development is important before one can understand how root phenotypes change in an ecological context.

The mature primary root has a simple structure consisting of four outer tissues, each of one cell layer, surrounding the central vasculature: the epidermis, cortex, endodermis, and pericycle (Malamy and Benfey, 1997). Division occurs in the quiescent center of the root tip and newly divided cells move toward the root base and eventually begin to elongate. Lateral root initiation occurs in pericycle cells that are in specific alignment with protoxylem poles, which are termed pericycle founder cells (Malamy and Benfey, 1997; Casimiro et al., 2003). Once activated, these founder cells begin to divide to form the lateral root primordia (Malamy and Benfey, 1997). Although these founder cells have the capacity to become lateral roots, not all founder cells begin this division process. A precise series of divisions occurs during lateral root primordia formation with the early stages of lateral root development giving rise to a four layered dome as a result of sequential divisions of the pericycle-derived tissue. Malamy and Benfey (1997) define

seven developmental stages that precede emergence of lateral roots. The images in Figure II-1A-D show lateral root primordia at several stages. Once emerged, division originates in the lateral root meristem followed by elongation of cells further back from the root tip. The developing lateral roots mirror the primary root anatomy through similar patterns of cell division and elongation (Malamy and Benfey, 1997).

There appears to be a narrow developmental window in which lateral root initiations may occur. There are environmental and/or hormonal regulatory mechanisms that determine competence of pericycle cells to produce founder cells and operate during this narrow developmental window (Dubrovsky et al., 2006; Dubrovsky et al., 2008). Consistent with this suggestion, lateral root development can be arrested at several stages. These stages seem to be independently controlled and provide flexibility during root development (Dubrovsky et al., 2006). This evidence also suggests that initiation and emergence of lateral roots are fundamentally distinct processes; signals may regulate initiation and/or elongation separately.

Auxin is critical for many developmental processes including lateral root initiation and elongation (Casimiro et al., 2003; Teale et al., 2005; Malamy, 2008). Appropriate distribution of auxin has been shown to be necessary for phototropism, gravitropsim, root elongation, and lateral root development (Leyser, 2006; Malamy, 2005; Muday and Rahman, 2008). Application of exogenous auxin stimulates lateral root development in a variety of plant species (Muday and Haworth, 1994). Similarly, plants with either mutations or transgenes that cause elevated endogenous auxin concentration have proliferation of lateral roots (Sibout et al., 2006; Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995). Auxin acts at the earliest stages of initiation of lateral root

primordia during the activation of the previously quiescent pericycle cells to begin division (Himanen et al., 2002), but also has been shown to enhance elongation of lateral roots (Wu et al., 2007).

Auxin moves through plant tissues via a unique polar transport mechanism that is tied to growth and developmental processes. In shoots, polar auxin transport occurs by cell-to-cell movement from the shoot toward the root (Muday and DeLong, 2001; Leyser, 2006). In roots, two auxin transport streams have been detected in different tissues. Acropetal transport occurs from the shoot toward the root apex, through the central cylinder, while basipetal transport occurs from the root apex toward the base through the outer root layers (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978; Rashotte et al., 2000, Muday and DeLong, 2001). Auxin delivery to the cells via polar auxin transport is essential for lateral root development, as demonstrated by treatment with inhibitors of auxin transport that reduce the number of lateral roots (Muday and Haworth, 1994; Reed et al., 1998; Casimiro et al., 2001). Casimiro et al. (2001) found that direct application of NPA to root tissue arrests lateral root development by blocking the first transverse divisions of xylem pole pericycle cells, suggesting that a threshold concentration of auxin might be required before cells could respond to lateral root-patterning signals.

IAA transport is mediated by 2 classes of auxin transport proteins, IAA influx and IAA efflux carriers. Influx is mediated by AUX1 and other LAX proteins (Leyser, 2006). Efflux has been tied to 2 classes of proteins, PIN proteins and ABCB/MDR/PGP proteins (Leyser, 2006; Geisler et al., 2005). In roots, AUX1 protein participates in both basipetal and acropetal IAA transport (Negi et al., 2008). In contrast PIN and ABCB proteins are tied to distinct polarities of auxin transport. ABCB4 and PIN2 mediate

basipetal transport (Rashotte et al., 2000; Lewis et al., 2007) and ABCB19 and PIN1 mediate acropetal transport (Titapiwatanakun et al., 2009; Lewis et al., 2007; D.R. Lewis, personal communication). The transport of auxin by PIN3, PIN4, PIN7 has not been reported, but their localization patterns suggests that they may function to mediate acropetal IAA transport. The asymmetrical positioning of the PIN proteins are essential for the polarity of auxin transport (Friml, 2003; Kramer and Bennett, 2006), while ABCB and AUX/LAX proteins are found on all sides of the membrane (Titapiwatanakun et al., 2009).

Inhibition of influx or efflux carriers by transport inhibitors and mutations in auxin transport proteins block lateral root formation. The influx carrier mutant, *aux1* shows a reduction of lateral roots (Marchant et al., 2002) and has reduced acropetal and basipetal transport (Rashotte et al., 2000; Negi et al., 2008). The *lax3* mutant and the *aux1lax3* double mutant show reduced number of primordia (Bainbridge et al., 2008). Treatment with IAA influx inhibitors also blocks lateral root formation (Negi et al., 2008). Mutants in genes encoding efflux carrier proteins, *pin2* and *mdr4/abcb4*, are defective in basipetal auxin transport and show no effect on root development (Rashotte et al., 2001; Lewis et al., 2007; Benkova et al., 2003; Laskowski et al., 2008). *pin1*, *pin3, pin4*, and *pin7* mutants defective in auxin efflux carrier, also show substantial reduction in the density of lateral root primordia (Laskowski et al., 2008). Benkova (2003) found that *PIN* genes are expressed in developing lateral root primordia, changing positions during development, representing the molecular basis for a polar transport system that can mediate auxin redistribution during primordium development (Benkova et al., 2003).

These mutant analyses suggest that acropetal IAA transport may be directly tied to root formation, as mutations that affect basipetal IAA transport do not affect root formation.

Several additional lines of evidence suggest causative role of acropetal auxin transport in root development. Acropetal transport of shoot derived IAA was reduced by placing localized NPA at the root/shoot junction to inhibit the flow of auxin (Reed et al., 1998). The number and density of elongated lateral roots were significantly reduced to near zero by NPA treatment. Excision of the shoot to block auxin flow from the shoot also had profound effects; no lateral roots emerged (Reed et al., 1998). Bhalerao et al. (2002) measured the concentration of free auxin in shoot and root tissues over several days after germination. They identified a spike in auxin in young leaves followed by a sharp decline that was then followed by an increase of auxin in the root, which was correlated with the time of emergence of lateral roots. These results suggest that a pulse of auxin from the shoot may drive lateral root elongation. Together, these experiments indicate that shoot derived auxin drives emergence of lateral roots.

The role of basipetal auxin transport in root formation was also investigated by Reed et al. (1998). They placed NPA on the root tip or excised the root tip, in order to inhibit basipetal transport. Neither treatment had an inhibitory effect on the number of emerged lateral roots. This experiment suggests that auxin originating from the root tip was not essential in lateral root elongation. In contrast, Rashotte et al. (2000) using experimental techniques to block basipetal IAA transport, found that basipetal auxin flow was tightly linked to primary root growth and gravitropsim.

Mutants were also used to examine the role of basipetal auxin flow in primordia development. The mutant, *aux1*, which accumulates IAA at the root apex, has 50% fewer

lateral root primordia than wild-type, suggesting that AUX1 facilitates an important auxin flux reaching pericycle cells. Casimiro et al. (2001) examined lateral root formation in the *stm1* mutant, which has no shoot apical meristem and no true leaves. This mutant was predicted to have less shoot derived IAA and less acropetal IAA transport, although IAA levels and transport were not measured. The mutant still forms normal levels of lateral roots. The authors conclude that the important source of auxin for lateral root development was root tip derived, due to the phenotype of *stm1* and *aux1* mutants. Yet the defects in both basipetal and acropetal IAA transport in *aux1* (Rashotte et al., 2000; Negi et al., 2008) and the absence of information on free IAA and IAA transport in *stm1* weaken this conclusion.

Three groups have examined the role of auxin in lateral root development when root tip growth directionality is environmentally regulated. De Smet et al. (2007) induced formation of root waves by growth of seedlings on hard agar held at a 45º angle. They found that root formation correlates with waving pattern, with initiations occurring on the outer sides of most bends. Ditengou et al. (2008) also reports that lateral root initiation occurs at the position of root bending in response to gravity response. Laskowski et al. (2008) and Ditengou et al. (2008) both show mechanical bending of roots leads to lateral root formation at the outside of the bent root. De Smet et al. (2007) detected an oscillation in auxin induced DR5-GUS expression in the basal meristem of waving roots, and interprets this result to suggest that this oscillation may prime pericycle cells for lateral root initiation. This supports the earlier hypothesis put forth by Casimiro et al. (2003) and Bhalerao et al. (2002) that the auxin pool in the root tip drives the initial stages of lateral root primordia formation. Yet Ditengou et al. (2008) showed that

excision of the root tip in mechanically bent roots had no effect on lateral root initiation and elongation. Therefore, these experiments do not fully clarify the source of auxin that drives lateral root formation.

This study tested the hypothesis that shoot-derived IAA drives lateral root initiation and emergence. We characterized the pattern and position of lateral root formation in the earliest stages of division to develop a spatial context for this process. Acropetal and basipetal auxin transport were manipulated using several chemical and mechanical means. These experiments find no evidence for a role of root tip derived auxin in lateral root formation and suggest that acropetal IAA transport provides auxin needed for both initiation and elongation of lateral roots. These results are consistent with shoot derived auxin being vital to lateral root initiation and emergence and suggest a mechanisms by which shoot growth environment can control root development through long distance communication through auxin transport.

Results

Early development of primordia does not occur near the root tip

We examined the distribution of lateral root formation along a primary root using a reporter expressed in the earliest stages of root formation. The *cycl1-At*-GUS transgene is expressed in rapidly dividing cells, lateral root primordia and cells at the apex of emerged lateral roots and primary roots. This reporter is expressed at all stages of lateral root development, as shown for primordia at stages 1,3, 5 and 7 in Figure II-1A-D.

The number of lateral root primordia and emerged lateral roots were quantified between six and eleven days after sowing (DAS) in *cycl1-At*-GUS seedlings, as shown in Figure II-2A. Early stage primordia (stages 1-3) were observed beginning at day 6 while primordia of stages 4-7 are not detected until one day later, and lateral roots emergence begins after one more day of growth. The number of primordia stays relatively constant over this time frame as they form at a constant rate, but shortly after initiation they progress to emerged lateral roots. Consistent with this progression, emerged lateral roots increase linearly between 7-11 days after sowing.

The position of primordia and emerged lateral roots were determined along a primary root over this time course providing spatial and temporal information about primordia development at lateral root emergence, as shown in Figure II-2B. The position of primordia formation is highest in the root 5-10 mm from the tip, in most of these samples. The number of primordia in the later stages (Stage 4-7) is fewer than in the early stage and the location where these are found is also most abundant in the region 5-10 mm from the root tip, but these later stage primordia have a broader distribution and are found further from the root tip. Finally, the emerged lateral roots are most distant

from the root tip and spread along the root, consistent with the increasing abundance at later time points. What this data clearly shows is that lateral root initiation rarely occurs at the tip of the root. Rather, it occurs well into the elongation region of the root. It is important to note that this region is largely outside the zone of basipetal IAA transport, which extends about 7 mm from the root tip (Rashotte et al., 2001). Although the earliest stages of root development occur closer to the tip, it is conceivable that signals from the shoot act on these tissues, as they have a unique developmental competence to form primordia. On Day 9 and 10 most primordia and emerged lateral roots are in the 20mm closest to the base as compared to the 5mm closest to the tip. By day 11, there is more of an even distribution of lateral root development with less than one primordia in stages 1-5 and less than one emerged lateral root in the 5mm closest to the tip.

NPA blocks root initiation and elongation when applied to the root shoot junction

To understand the source of auxin that drives lateral root initiation and elongation, the two polarities of root auxin transport were blocked by local application of the IAA efflux inhibitor, NPA, to the root/shoot junction or the root tip. NPA at 10 μM was added to growth media and poured into one half of Petri dishes with central dividers. The plates were prepared with control agar in both halves or with half control and half NPA containing media. Seedlings were transferred to experimental media conditions at six days after sowing, which is, approximately three days after germination, and after three additional days, images of root growth were captured and are shown in Figure II-3A. Additionally, the number of lateral root primordia and emerged lateral roots were quantified for each treatment and are reported in Figure II-3B. When roots were grown on control agar, either placed with only the tip (data not shown) or the entire root on the

lower agar, there were large numbers of lateral roots initiated, with smaller numbers of elongated lateral roots (Figure II-3A-B). When seedlings were grown with shoots positioned on control media and root tips positioned on NPA media, there was a slight but not statistically significant increase in lateral root primordia relative to seedlings positioned similarly onto uniform control media ($P<0.05$). There was a small, but significant, 1.4-fold reduction in number of emerged lateral roots compared to control ($P<0.05$). If the shoot (including the root/shoot junction) was positioned on NPA media and the root was positioned on control media, there was a significant 2.7-fold decrease in both initiation of lateral roots and 18-fold decrease in number of elongated roots relative to roots positioned similarly on control media ($P<0.0001$). These results suggest that when auxin transport from the shoot to the root was inhibited, lateral root initiation and elongation were both severely affected, even though the majority of the root was not in direct contact with NPA containing media. In contrast, the effects of NPA treatment at the root tip were small and only occurred in regions of root in contact with NPA.

The effect of localized NPA treatment on primary root growth and the density of lateral roots, defined by the number of primordia or emerged lateral roots divided by root length, are reported in Table II-1. Treatment with NPA at the tip actually increased the number of lateral roots by 1.2-fold, but reduced root elongation. The decrease in emerged lateral roots closely parallels the root length changes, resulting in similar density of elongated lateral roots by this treatment, as compared to controls. Control/Control and NPA/Control treatment had similar root lengths, but the density of both lateral root initiation and emerged lateral roots in the NPA/Control was significantly reduced by 2- and 10-fold, respectively.

Localized IAA treatment can reverse NPA inhibition of root initiation

We also asked whether IAA could reverse the inhibition of root formation by localized NPA treatment. Seedlings were transferred to plates with NPA and/or control media, as described above, at five days after sowing. An agar line consisting of either control agar or agar containing 10 μM IAA was placed directly below the thin partition or the root/shoot junction and after four additional days of growth under yellow light, images of root growth were captured and are shown in Figure II-4A-D. Additionally, the number of lateral root primordia, emerged lateral roots, and densities were quantified for each treatment and are reported in Figure II-4E-F. When NPA was in the top half of the Petri dish, both the number of primordia and emerged lateral roots were dramatically reduced, 7.5-fold and complete reductions, respectively, similar to the half plate experiment described above. When a 10μM IAA agar line was placed on seedlings directly below the thin partition, the emerged number of lateral roots increased over control by 1.4-fold and over NPA treated by almost 12-fold. The application of IAA had an inhibitory effect on primary root growth. This result suggests that IAA was able to partially reverse the inhibition of the initiation of primordia and fully reverse the emergence of lateral roots, as shown in Figure II-4E. When the densities of initiation, emergence, and totals of initiation and emergence were examined, an interesting trend emerged. In the experimental conditions where 10μM IAA agar lines were applied, the root length decreased by about 2-fold. If one examines the total density, one can see that the application of these IAA lines actually increases the total number of lateral roots per root length as compared to control. These results suggest that when IAA is applied to the root/shoot junction, it can completely reverse the effects of NPA; in both the control and

NPA conditions, the IAA flow from the shoot is increased and lateral root development is increased; thus providing another piece of evidence that shoot derived auxin is vital for lateral root development.

Transport mutants, defective in both auxin influx and efflux carriers, profoundly reduce the initiation and emergence of lateral roots.

To understand acropetal flow of auxin from the shoot, *pin3* and *pin7* mutants, defective in auxin efflux carriers and *aux1*, *lax3*, and *aux1lax3* mutants, defective in auxin influx carriers, were grown and lateral root primordia and emerged lateral roots were examined. Mutants were placed on control agar for nine days after sowing and lateral root primordia and emerged lateral roots were quantified, as shown in Figure II-8. By using mutants defective in acropetal transport from the shoot, we see that there is a profound reduction in lateral root primordia and emerged lateral roots. *aux1* and *pin3* mutants have the greatest reduction compared to control, with a 5- and 6-fold decrease in the initiation of primordia. Emerged lateral roots were not affected by these mutations with the exception of *aux1lax3* with an approximate 2-fold decrease. The proteins associated with these mutants are important in acropetal auxin transport; when they are null or defective, the initiation of primordia and emerged lateral roots decrease suggesting that shoot derived auxin is driving lateral root development. In contrast, previous reports have indicated that *pin2* (Laskowski et al., 2008) and *abcb4/mdr4/pgp4* mutants (Lewis et al., 2007), which are only defective in basipetal IAA transport, have wild-type levels of lateral root formation.

Shoot excision blocks lateral root initiation and elongation

A second approach was used to separately block the two polarities of auxin transport in roots. We physically removed the shoot and/or the root tip source of auxin at six days after sowing, and seedlings were placed on uniform control agar. After three additional days of growth, images were captured that illustrated the treatment and its effect on root growth and lateral root formation, as shown in Figure II-6A. Additionally, the number of lateral root primordia and emerged lateral roots were quantified for each treatment and are reported in Figure II-6B. When roots were grown on control agar, with neither the root tip nor shoot removed, there were large numbers of lateral roots initiated, with smaller numbers of elongated lateral roots (Figure II-6B). When the shoot source of auxin was removed, there were virtually no formation of lateral root primordia or emerged lateral roots. However, when the root tip source of auxin was removed, lateral root primordia still developed at a decreased capacity while emerged lateral roots significantly increased.

The effects of the excision treatment on primary root growth, and density of lateral roots, are reported in Table II-3. We found that when root tips were excised, there were 2-fold fewer lateral root initiations but 1.5-fold more emerged lateral roots ($P=0.005$). There was a 2.9-fold decrease in the length of the primary root as compared to the control seedlings, as the primary root stops growing once the root tip is excised. Presumably the number of primordia decreases because there is now limited space on the primary root to initiate them. Consistent with this prediction, the density of lateral root primordia increases 1.2-fold and the density of emerged lateral roots increases 4-fold, as compared to control. The advantage of enhanced emergence of lateral roots when the

root tip is damaged or removed is obvious, so that root development can compensate for the loss.

When the shoot was removed the number of initiations and emerged lateral roots were reduced 28-fold and 43-fold when compared to control ($P<0.0005$). This treatment also reduced root elongation by 1.6-fold, so this reduced the effect on density of initiations and elongated roots to 15- and 25-fold, respectively. These results suggest that when auxin transport from the shoot to the root was inhibited, lateral root initiation and elongation were both severely affected, suggesting an important role of shoot derived compounds in both stages of lateral root development.

Excision of shoot in a root waving experiment abolishes root initiations

Several reports have recently indicated that lateral root formation can be induced in *Arabidopsis* roots that are bent in response to gravitropic stimulation, root waving, or manual bending (Laskowski et al., 2008; Ditengou et al., 2008). To ask if lateral root initiation in response to altered root orientation is fueled by either shoot or root derived auxin, we induced root waving. Seedlings were grown on standard agar and then switched to 1.5% agar at five days after sowing and placed at a 45º angle. As roots of these seedlings can not penetrate into the agar, they will form a waving pattern along the agar. Additionally, lateral roots emerge from the outer region of a wave. The seedlings were allowed to grow and their shoots were excised at the root/shoot junction on 5, 6, 7, or 8 days after sowing. The number of primordia and emerged lateral roots were quantified, as shown in Figure II-7A. Intact seedlings show few primordia in the straight root region, but have extensive primordia forming on the outside of the root waves. Seedlings with their shoots excised at five days after sowing did not have any emerged

lateral roots and fewer than one initiation per root (Figure II-7A). If shoots are excised one day earlier at 4 days after sowing, lateral root initiation is almost completely shut down. Seedlings with shoot excision at 6, 7, or 8 days after sowing form lateral root primordia in the straight region and the waving region. The longer the shoot is present (6, 7, 8 DAS), there are more primordia and emerged lateral roots each day as shown in figure II-7A. Throughout this time course root tip auxin should be constant, but availability of shoot derived auxin will vary. These results indicate that in both straight and curvy regions, shoot derived auxin is critical for lateral root formation.

To ask if IAA may be limiting for root development in these excised seedlings, IAA droplets of 10, 25, 50, 75, and 100 μM concentrations were added to the root/shoot junction at five DAS (the same day the shoot was removed). After four additional days of growth, the number of primordia and emerged lateral roots were quantified and the results are shown in Figure II-7B. Roots of the seedlings treated with IAA did not grow after transfer to experimental treatment. Since IAA acts as a negative regulator for primary root growth, these seedlings did not exhibit root waving. The dose response curve from 25μM-100μM all showed a significant increase of lateral root primordia and emerged lateral roots in the straight primary root section as compared to the 0μM dose. The IAA treatment did, however, partially rescue lateral root primordia and emerged lateral roots over the dose response curve, as compared to control and to shoot excised seedlings that did not receive any IAA droplets.

Norflurazon reduces lateral root initiation and emergence

Reduction of available auxin from the shoot by local NPA application or shoot excision indicate that auxin from the shoot is important for both initiation of primordia

and emergence of lateral roots. However, shoot excision reduces availability of other shoot derived resources, including photosynthate. Although the seedlings in these experiments are grown on sucrose containing media, carbohydrates may be limited by shoot excision. We therefore asked about the contribution of photosynthate in lateral root formation. One approach to limit the amount of photosynthate production is to treat the seedlings with the herbicide, Norflurazon (NFZ), which blocks chlorophyll production (Tkalec et al., 2003). Seedlings were germinated and grown on 0.1 μM NFZ control plates with sucrose under 24 hours of light until nine days after sowing (Figure II-8A). In Figure II-8B, seedlings grown on NFZ formed less than one primordia per seedling and no detectable emerged lateral roots compared to controls ($P < 0.0001$). Seedlings grown an additional three to six days on NFZ, also had minimal primordia and no emerged lateral roots (data not shown). This result is consistent with a role for photosynthate in root formation, either directly or indirectly through photosynthate dependent IAA synthesis.

We tested the possibility that the effect of NFZ on photosynthate may reduce free IAA, rather than limit energy available for lateral root initiation. In Table II-4 free IAA accumulates at lower levels in both the shoots and roots of NFZ treated plants; there is a 1.8-fold decrease in shoot and 1.1-fold decrease in roots of NFZ treated seedlings as compared to control samples; neither NFZ treated roots or shoots are significantly different from control. This is a different pattern, though, than dark grown plants, where there is almost no detectable IAA accumulation, indicating that there is another light dependent signal, that is independent of photosynthate that is also important in IAA synthesis and transport.

We asked whether the NFZ effect was reversible by treatment with exogenous IAA. IAA droplets containing 10, 50, and 100 μM concentrations of IAA were added to the root/shoot junction of the NFZ treated seedlings at 6 DAS. After three additional days of growth, the number of primordia and emerged lateral roots were quantified and the results are shown in Figure II-7. Lateral root formation was partially restored at nine days after sowing. The number of primordia in all IAA treatments showed an approximate 7.5- to 8.5-fold increase over NFZ treated seedlings. Treatments were compared to the NFZ without IAA; all were statistically significant ($P<0.0001$). Emerged lateral roots at 10μM showed a 3-fold increase as compared to NFZ treated seedlings and a 3-fold decrease as compared to control. All other concentrations of IAA showed a complete rescue of emerged lateral roots as compared to controls.

Discussion

Auxin positively regulates the initiation and emergence of lateral roots (Casimiro et al., 20003; Teale et al., 2005; Malamy, 2008). An important question is how auxin reaches the site where lateral roots form. A number of experiments indicate that shoot derived auxin is required for lateral root emergence (Reed et al., 1998; Bhalerao et al., 2002; Ditengou et al., 2008). However experimental results have provided contradictory information on the source of auxin that converts quiescent pericycle cells into actively dividing primordia during the initiation of lateral roots. In this study we used several approaches to block either basipetal transport from the root tip toward the base or acropetal auxin transport from the shoot toward the root apex and examined the effect of these treatments on lateral root initiation and emergence. Our results are consistent with shoot derived auxin driving both lateral root initiation and elongation.

Blocking the basipetal source of auxin in the root tip does not affect the development of lateral root primordia or emergence of lateral roots. We find that when NPA was used to block basipetal IAA transport there was a slight increase of lateral root primordia as compared to controls. When the root tip was excised, there was a increase in the density of lateral root primordia and emerged lateral roots. Additionally, mutants that have defects in only basipetal IAA transport, such as *pin2* (Laskowski et al., 2008) and *abcb4* (Lewis et al., 2007), form wild-type numbers of lateral roots. Reed et al. (1998) placed an NPA line mid-root and found that there were no changes in the number of emerged lateral roots above the NPA line, although there were significant decreases below that point. These experiments together indicate the lack of influence of basipetal auxin flow on the development of lateral root primordia and emerged lateral roots.

Our results indicate that blocking the acropetal transport of auxin from the shoot profoundly reduces the development of lateral root primordia and emergence of lateral roots. The localized application of NPA to block acropetal IAA transport significantly reduced initiation of primordia and blocked all emergence of lateral roots. Shoot excision also virtually blocked all lateral root initiation and elongation at either five or six days after sowing, in both straight roots and roots grown under conditions designed to induce waves, which define the position of lateral root formation. Finally, mutants with reduced acropetal IAA transport show reduced numbers of initiated and elongated lateral root, consistent with shoot derived IAA driving root formation.

We have shown that blocking acropetal auxin transport from the shoot drastically, and in some cases, completely abolishes the initiation and emergence of lateral roots. We asked whether we could restore lateral root development by applying IAA to the root/shoot junction in shoot excised or NPA treated plants. In the root waving experiment, IAA application to the root/shoot junction partially rescued both primordia and emerged lateral roots. When IAA agar lines were applied directly below the NPA treated tissues, the lateral root phenotype was partially restored with additional lateral root growth.

In several experiments where acropetal auxin was blocked, some lateral root primordia did form. With NPA treatment, we were unable to completely block root formation by blocking acropetal IAA transport. When the shoot was placed on NPA agar six days after sowing, there were approximately six primordia as seen in Figure II-3D. When seedlings were placed on the NPA/Control plates earlier at five days after sowing in Figure II-4, we only found two primordia as compared to 16 in the control; less than

two primordia were found in the root waving experiment when the shoot was excised at five days after sowing (Figure II-7). This data is consistent with the data presented in Figure II-2A where primordia have already formed by six days after sowing. Whether observing lateral root development in vivo or using various experimental methods to inhibit acropetal auxin flow, which severely effected lateral root initiation and elongation, one can conclude that this acropetal transport of auxin from the shoot is vital for early lateral root development.

Ditengou et al. (2008) and Laskowski et al. (2008) found that when one mechanically bent the root, initiation of primordia occurred at the outer bends. Dintengou et al. (2008) removed the shoot apex at six days after sowing, a single lateral root primordia formed and no lateral roots elongated. We suggest that like our NPA treatments, this excision occurred after a previously observed auxin pulse had already been released from the shoot causing the initiation of these primordia (Bhalerao et al., 2002). This evidence is strengthened by our data in Figure II-2 where we can follow the natural progression of lateral root development.

Excision of the shoot suggests that shoot derived auxin is essential for the development of lateral root initiation and emergence. When shoots are removed as exhibited in experiments shown in Figure II-6 and II-7, both auxin and other important compounds, such as photosynthate are not transported from the shoot to the root. Even though these seedlings were grown on sucrose containing agar, preventing transport of photosynthate reduces available energy. Photosynthate is also severely reduced by growing seedlings in the dark, which results in no lateral roots emergence (Reed et al, 1998). Bhalerao et al. (2002) found that in dark grown seedlings there was almost no

detectable IAA accumulation. This suggests that when there is little photosynthate, there is little, if any, auxin synthesis.

To test the role of photosynthate in root development, we applied the herbicide Norflurazon (NFZ) to seedlings as they germinated and developed. NFZ causes a carotenoid deficiency which leads to photooxidation of chlorophyll and reduced photosynthesis and photosynthetic pigment accumulation (Tkalec et al., 2003; Sauret-Gueto et al., 2006). As with dark grown seedlings (Bhalerao, 2002; Reed et al., 1998), we see no primordia or emerged lateral roots in the NFZ treated seedlings. The seedlings were small and chlorotic. When accumulation of free IAA was measured in NFZ treated seedlings, there was a trend that represented a reduction of IAA in both the roots and shoots, as compared to control. The magnitude of these effects is substantially less than in dark grown seedlings where almost no free IAA was detected. Even though there was IAA accumulation detected in the NFZ treated seedlings, no lateral root primordia or emerged lateral roots formed. This suggests that NFZ grown seedlings, exposed to 24 hours light, were receiving a light signal that allowed auxin synthesis so that the limitation in root formation is not at the level of free IAA. Alternatively, there may be localized changes in free IAA that are not detected by this method. In NFZ treated seedlings, auxin was applied to the root/shoot junction and there was a significant increase in primordia as well as full restoration of emerged lateral roots. This result parallels the treatment of dark grown seedlings; IAA application to the root/shoot junction also partially restored the emergence of lateral roots (Reed et al., 1998). In this experiment photosynthate was limited, but auxin was able to overcome this limitation and

drive lateral root development, suggesting that auxin transport might be limiting in NFZ treated seedlings.

Consistent with the hypothesis that light signaling is important for lateral root development, Salisbury et al. (2007) found that phytochromes, the light receptors that control many plant light responses (Chen et al., 2004) participate in the regulation of lateral root production. The *phyA and phyB* mutants, have reduced lateral root production relative to wild-type which suggests that the proteins, PHYA and PHYB, play a significant role in the regulation of root architecture and communicate light signals from the shoot to the root. They also measured auxin transport in the mutants, *phyB* and *phyAphyB*; they found that the mutants had significantly less transport as compared to wild type (Salisbury et al., 2007). We found that mutants defective in the proteins PIN3 and PIN7 showed profoundly decreased numbers of primordia and emerged lateral roots. Therefore, it may be NFZ alters the expression of *PIN3* and *PIN7*, to reduce root formation. Both the NFZ experiment and the results presented by Salisbury et al. (2007) suggest that light signals play a significant role in both auxin synthesis and transport.

IAA is essential for lateral root formation and to develop models for the regulation of this process it is necessary to understand the source of auxin that drives root formation. We tested the possibility that shoot derived auxin was essential to lateral root initiation and that root tip derived auxin is not critical for this process. We provide insight into the position of root initiation in the developing primary root. Through both chemical and mechanical means, we manipulated the flow of shoot derived auxin at the earliest stages of lateral root development and found dramatic if not complete decline of initiation of lateral root primordia. We also saw no emergence of lateral roots further

confirming the findings of Reed et al. (1998), who examined similar treatments on lateral root elongation. Finally by using the transport mutants, *pin7*, *pin3*, *aux1*, *aux1lax3*, and *lax3*, defective in both auxin influx and efflux carriers, we found a dramatic reduction in lateral root primordia. These results supported our hypothesis that shoot derived auxin drives both lateral root initiation and elongation.

Understanding the source of auxin that drives lateral root formation has important physiological and ecological implications. If shoot derived auxin modulates root formation, then this could act as a long distance signal to control root architecture when the shoot environment changes. The shoot is exposed to many unpredictable environmental variations and it must respond in a way that protects and benefits the entire plant. In contrast, root growth occurs in a more constant environment, with total darkness and more constant temperatures. Modulation of light signaling to control root architecture is one clear area where long distance shoot signal modulates root architecture (Reed, et al., 1998; Bhalerao et al., 2002; Salisbury et al., 2007).

Materials and Methods

Chemicals

Naphthylphthalamic acid (NPA) was purchased from Chemical Services (West Chester PA). Murashige and Skoog (MS) salts were purchased from Caisson Labs. Norflurazon was purchased from Chem Service. All other chemicals were acquired from Sigma (St. Louis, MO).

Seed Germination and Plant Growth

Nossen transformed with *cyc1-At-GUS* were used. Seeds were soaked in distilled water for 30 min and surface sterilized with 95% (v/v) ethanol for five minutes, 20% (v/v) bleach with 0.01% (v/v) Triton X-100 for 5 minutes, and then washed with distilled water for five minutes. After three additional washes in sterile distilled water, seed were transferred to Petri dishes containing sterile control medium (0.8% (w/v) agar [Sigma Type M, plant tissue culture], 1 x Murashige and Skoog salts, 0.05% (w/v) Mes, pH 6.0, 1.5% (w/v) sucrose, 1 $\mu g \cdot mL^{-1}$ thiamine, 1 $\mu g \cdot mL^{-1}$ pyridoxine HCl, and 0.5 $\mu g \cdot mL^{-1}$ nicotinic acid). Seeds were germinated and grown on vertically oriented Petri dishes with 24 hours of continuous fluorescent light (90-100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) at room temperature (24°C). The seeds were exposed to 24 hrs of light throughout germination and the experimental treatments.

Initiation and elongation measurement

After treatments, activity from the *cycl1At*-GUS reporter was detected histochemically. Seedlings were transferred from agar to GUS staining solution, which is 2 mM X-glucuronide dissolved in 0.5 mM K ferricyanide, 0.5 mM K ferrocyanide, 0.5% Triton X-100 and 100 mM sodium phosphate buffer, pH 7.0 (Craig, 1992). They were

stained overnight at 37^{o}. After staining was complete, tissue was washed in 100 mM sodium phosphate buffer three times followed by 70% ethanol. Seedlings were stored in ethanol until analysis under a compound microscope.

The length of each seedling's root was determined with a ruler to the nearest mm. The roots were placed on a microscope slide with cover glass and examined under a dissecting microscope. The number of primordia at stages I-VII (as defined by Malamy and Benfey, 1997) were quantified, as were emerged lateral roots.

NPA Treatment and reversal by IAA

Media containing NPA was prepared from a 10^{-2} M stock dissolved in DMSO and made fresh for each experiment. NPA was added to agar cooled to 50 ºC to a final concentration of 10 μM. Seeds were germinated on control agar. At six days after sowing (DAS), seedlings were transferred to divided Petri dishes with ten seedlings per dish. Petri dishes with plastic dividers yielded two sections that were filled with either control agar or NPA, so that seedlings could be in contact with different agar mixtures. The *cycl1At*-GUS seedlings were placed on plates with either NPA in contact with shoot with control media on bottom, NPA in contact with the root tip with control media on top or control on both top and bottom. The root/shoot junction was placed just above the partition when the top was NPA or with the control plates. The root tips were placed below the partition when the bottom was NPA or with control plates. There were two controls for each experimental treatment. There was no statistical difference between the controls so only the first control was shown. The number of primordia and emerged lateral roots were quantified after three additional days of growth as described above.

For experiments involving IAA and NPA application, there were four experimental treatments including the control. Divided plates as mentioned in above section were used. The control condition contained control agar in the top and bottom of the Petri dish. The NPA condition was identical to the above experiment with a control line of agar placed directly below the thin partition. The IAA condition contained control agar in the top and bottom of the Petri dish with a line of 10µM concentration of IAA placed directly below the partition. Finally there was a plate with NPA on top and control on the bottom of the Petri dish with a line of 10µM concentration of IAA below the partition. The conditions containing IAA were placed under yellow light (Stasinopoulos and Hangarter, 1989).

Transport Mutant Analysis

For experiments involving transport mutants, *pin3*, *pin7*, *aux1*, *lax3*, and *aux1lax3* were used. All mutants were grown on Petri dishes containing control agar under 24 hours of light for nine days after sowing. Roots were cleared (Dubrovsky et al., 2009) and lateral root primordia and emerged lateral roots were quantified.

Excision Treatment

For experiments involving excision, at six DAS, seedlings were transferred to new media with ten seedlings per Petri dish. The seedlings were placed in one of four experimental groups. One group had the root tip excised; one group had the shoot excised at the root/shoot junction; one group had both the shoot and root tip excised and one group was intact (control). Using spring loaded scissors, the shoot was excised at the root/shoot junction. The root tip was excised approximately 1 mm from the tip. The length of the root at transfer was noted. After transfer to fresh media, seedlings were

exposed to a 24 hour light cycle at constant temperature for three additional days. At nine DAS, root length was measured and seedlings were GUS stained and the number of primordia and emerged lateral roots was counted.

Root Waving Treatment

For experiments involving root waving, five day old seedlings (from the time of sowing) were transferred to 1.5% agar media, and the Petri dishes were placed at an angle of 45^0 under yellow light. The shoots of seedlings were removed on various days after transfer and the number of primordia and emerged lateral roots were quantified after four additional days of growth.

For experiments involving IAA application, five day old seedlings (from the time of sowing) were transferred to 1.5% agar plates with or without shoot removed. Agar droplets were applied with concentrations of 10, 25, 50, 75 or 100 μM IAA to seedlings with their shoots removed and were placed under yellow light (Stasinopoulos and Hangarter, 1989). The number of lateral root initiation and emerged lateral roots were quantified and were compared to control intact seedlings after 4 additional days of growth.

Norflurazon Treatment

For experiments involving Norflurazon (NFZ) application, NFZ media was made from a 2mM stock dissolved in ethanol and used for no longer than two weeks. NFZ was added to agar cooled to 50 ºC to a final concentration of 0.1μM. Seeds were germinated on NFZ agar and exposed to a 24 hr light cycle at constant temperature. At six days after sowing (DAS), seedlings were transferred to plates with freshly made NFZ agar with ten seedlings per Petri dish and then grown for 3, 6 or 9 additional days. At each time point,

seedlings were GUS stained and the number of primordia and emerged lateral roots was counted.

For experiments involving Norflurazon (NFZ) application with the addition of IAA droplets, seeds were germinated on NFZ agar. At 6 DAS, seedlings were transferred to new agar containing NFZ with ten seedlings per Petri dish. A droplet of 1% agar containing a 10, 50, or 100 μM concentration of IAA was administered to the root/shoot junction. Plates were placed under yellow light to prevent the degradation of the IAA (Stasinopoulos and Hangarter, 1989). IAA droplets were left on the root/shoot junction for 3, 6 or 9 additional days respectively. At each time point, seedlings were GUS stained and the number of primordia and emerged lateral roots was counted.

Statistics

Student *t*-tests were employed to identify statistical difference between controls and treatment groups. Individual t-tests were performed on independent experiments (plates containing 10 seedlings) to ensure that all data from individual plates could be pooled. Once pooled, unpaired *t*-tests were used to examine the range for the actual mean of each sample. A statistical program from the website http://www.physics.csbsju.edu/stats/t-test.html was used to calculate whether statistical differences exist between experimental treatments and controls using a Student's *t*-test. Averages and SE were calculated using excel.

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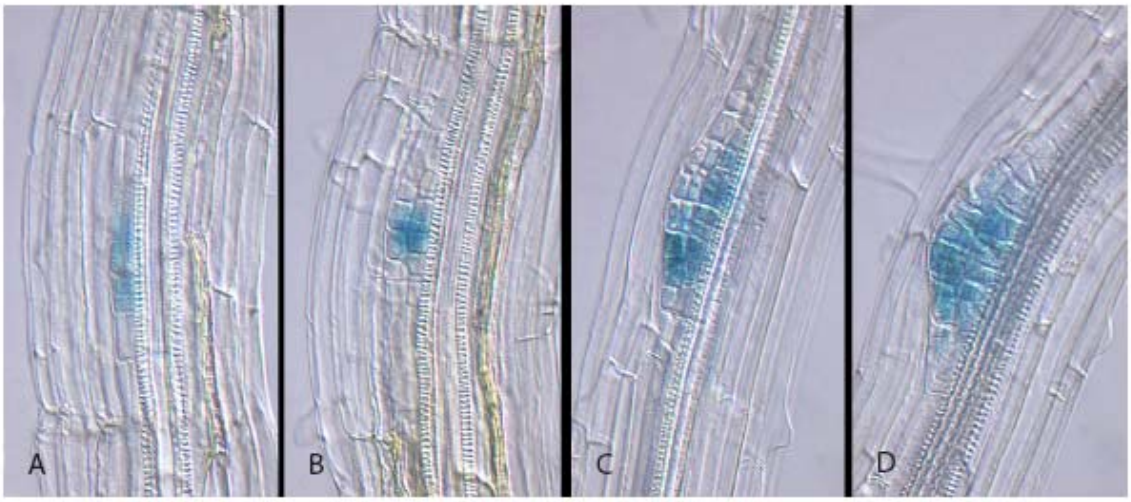


Figure II-1. Early developmental stages for lateral root primordia were visualized using *cycl1-At*-GUS, which is expressed in rapidly dividing cells. A-D, Stages 1, 3, 5, and 7 of lateral root primordia development as defined by Malamy and Benfey, (1997).

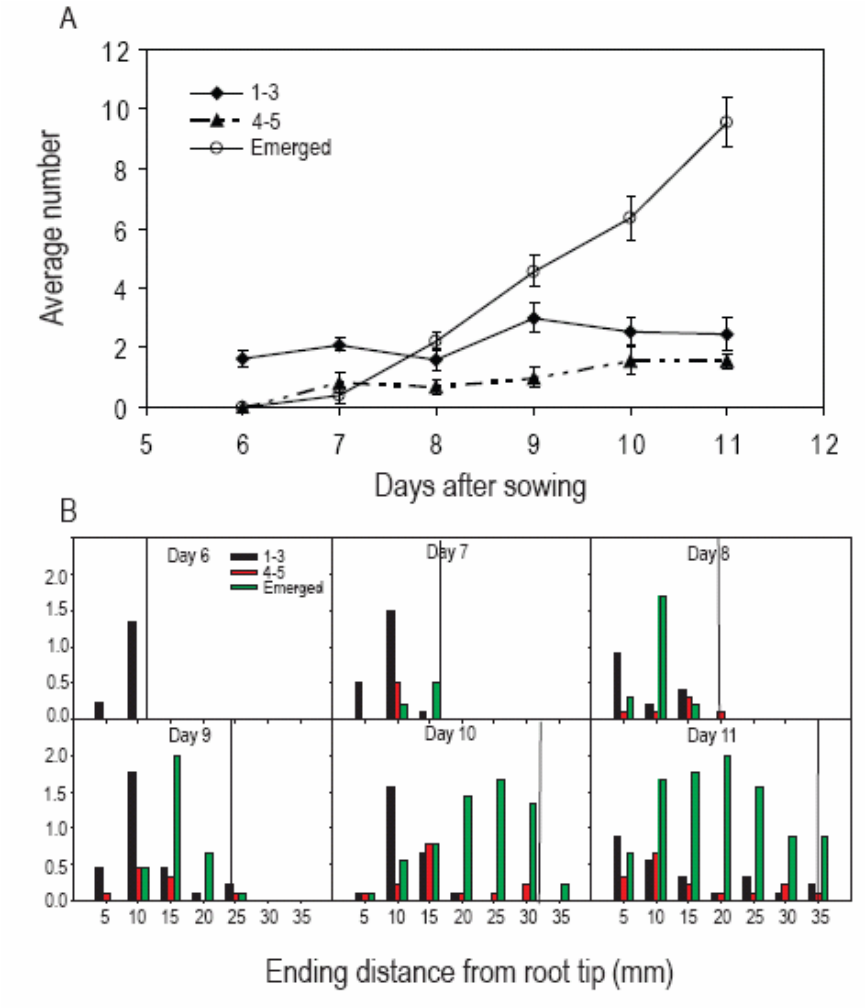


Figure II-2. The number of lateral root primordia begin to form at 6 days after sowing and lateral roots emerge from 7-8 days after sowing. A, The number of lateral root primordia at early and later stages (stage 1-3 and 4-7, respectively) and emerged lateral roots were quantified at the indicated times after sowing. The average and SE of nine seedlings in one experiment are reported. B, The position of formation of lateral root primordia and emerged lateral roots was determined along primary roots at 6 to 11 days after sowing. The average root length is indicated by the vertical line in each square of this figure. The average and SE of nine seedlings from one experiment are reported.

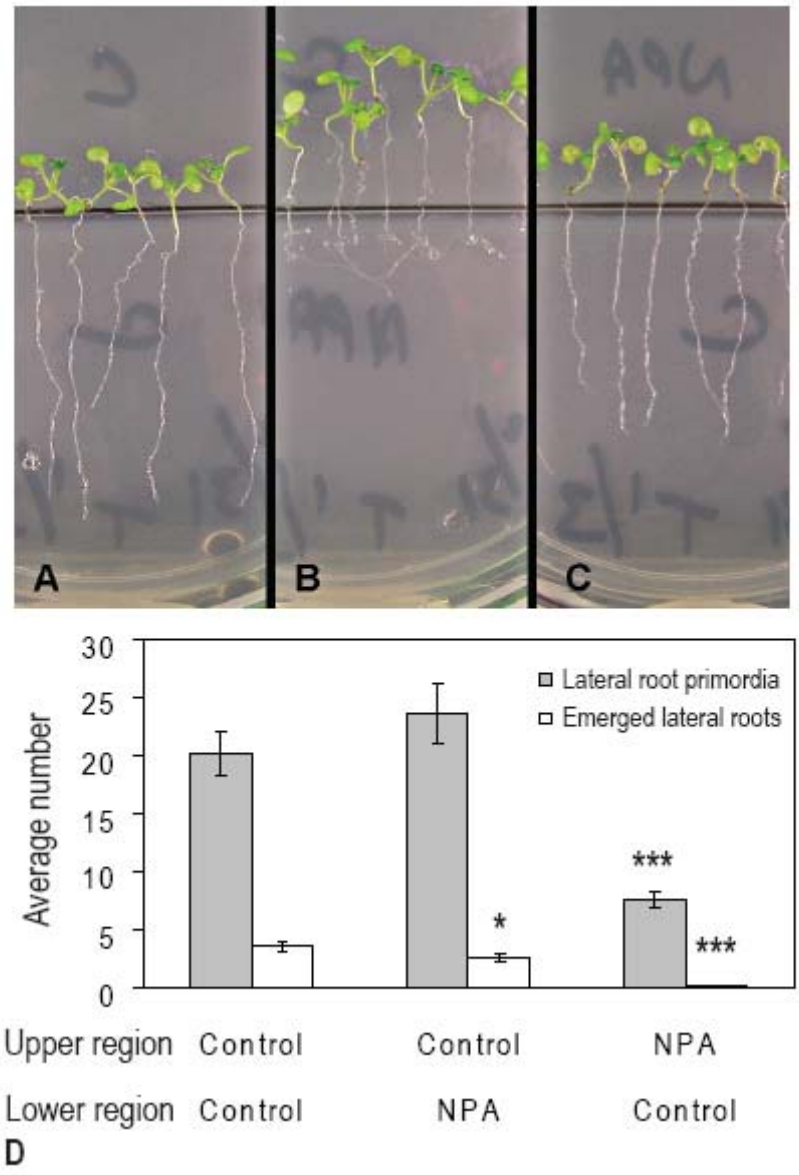


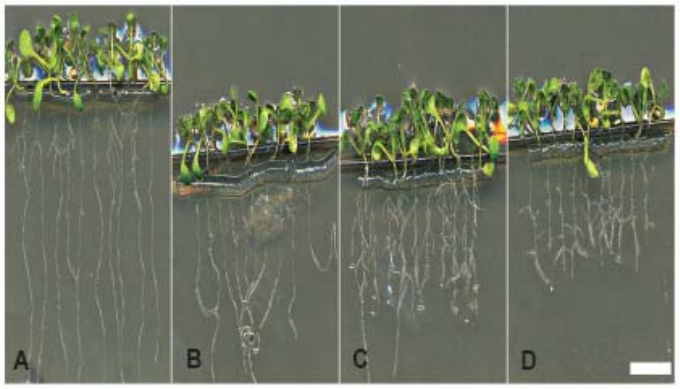
Figure II-3. Chemical reduction of auxin transport from the shoot and the root tip. Seedlings were germinated on control media and after six days transferred to divided Petri dishes containing control media or NPA containing media in each half and allowed to grow for 3 additional days. A, Seedlings were grown with upper region and lower regions of roots in contact with agar containing control agar. B, Seedlings were grown with upper region in contact with control agar and the lower region in contact with NPA agar. C, Seedlings were grown with the upper region on NPA agar and the root tip in

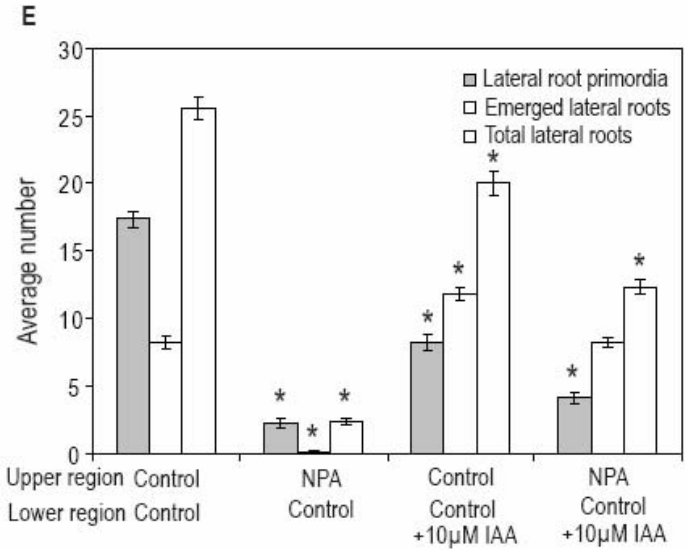
contact with control agar. D, The average and standard error of 40 seedlings in 4 separate experiments are reported. The number of primordia and emerged lateral roots found in each treatment are compared to controls by Student's t-test * $P < 0.05$, *** $P<0.0001$.

Table II-1. Effect of localized NPA treatment on primary root growth, lateral root initiation, emergence, and density in *Arabidopsis*. Six day old seedlings were treated on Petri dishes divided with a thin plastic partition allowing control or NPA agar poured on the top or bottom. After three additional days of growth, the number of initiated lateral roots, emerged lateral roots, and primary root length were determined. The reported values are averages ± SE of 40 seedlings from 4 separate experiments.

Top Treatment	Bottom Treatment	Lateral root primordia (LRP), (number)	Lateral root emerged (LRE), (number)	Length of primary root (mm)	LRP density (number/ mm)	LRE density (number/mm)
Control	Control	20.2 ± 1.90	3.6 ± 0.40	28.2 ± 0.90	0.6 ± 0.10	0.10 ± 0.01
Control	NPA	23.7 ± 2.60	2.5 ± 0.30[a]	20.4 ± 1.10[a]	1.27 ± 0.17[a]	0.13 ± 0.02
NPA	Control	7.6 ± 0.68[a]	0.2 ± 0.06[a]	27.6 ± 0.87	0.28 ± 0.02[a]	0.01 ± 0.002[a]

[a] Values that are statistically different from controls, as judged by Student's t-test are indicated, $P<0.05$


A
B
C
D


E
Lateral root primordia
Emerged lateral roots
Total lateral roots
Average number
30
25
20
15
10
5
0
Upper region Control
Lower region Control
NPA
Control
Control
Control
+10μM IAA
NPA
Control
+10μM IAA

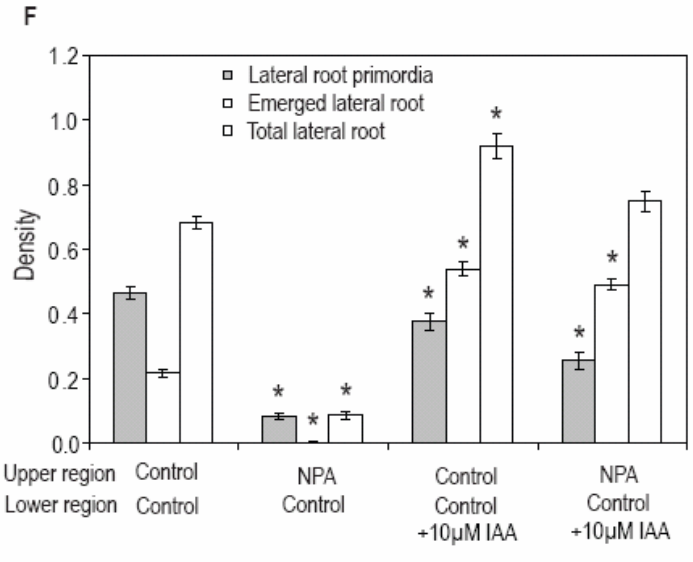

F
Lateral root primordia
Emerged lateral root
Total lateral root
Density
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Upper region Control
Lower region Control
NPA
Control
Control
Control
+10μM IAA
NPA
Control
+10μM IAA

Figure II-4. Chemical reduction of auxin transport using NPA, an auxin transport inhibitor. Five day old seedlings were treated on Petri dishes divided with a thin plastic partition allowing NPA agar poured on the top and control agar on the bottom or control agar to be poured on both top and bottom. The seedlings were allowed to grow for four additional days of growth. The scale bar represents 1 cm. A, Seedlings were grown with upper and lower regions of roots in contact with agar with a control line of agar placed directly below the thin plastic partition. B, Seedlings were grown with the upper region on NPA agar and the root tip in contact with control agar with a control line of agar placed directly below the thin plastic partition. C, Seedlings were grown with upper and lower regions of roots in contact with control agar with an agar line containing 10μM IAA applied directly below the thin plastic partition. D, Seedlings were grown with the upper region on NPA agar and the root tip in contact with an agar line containing 10μM IAA applied directly below the thin plastic partition. E, The average and standard error of 30-35 seedlings from 4 separate experiments are reported. F, The average and standard error for the densities of primordia, emerged lateral roots, and total lateral roots of 30-35 seedlings from 4 separate experiments are reported. The number of primordia, emerged, and total lateral roots and densities for each found in each treatment as compared to controls by Student's t-test * $P < 0.01$.

Table II-2. Effect of localized NPA treatment with the addition of localized IAA treatment on primary root growth, lateral root initiation, emergence, and density in *Arabidopsis*. Six day old seedlings were treated on Petri dishes divided with a thin plastic partition allowing NPA agar poured on the top or control agar to be poured on the top and bottom. After three additional days of growth, the number of initiated lateral roots, emerged lateral roots, primary root length, and densities were determined. The reported values are averages ± SE of approximately 30 seedlings from 4 separate experiments.

	Initiated (I) lateral root	Emerged (E) lateral root	Total (T) lateral roots	root length	I density	E density	T density
Control/Control	17.3 ± 0.60	8.2 ± 0.48	25.6 ± 0.83	37.5 ± 0.68	0.47 ± 0.02	0.22 ± 0.01	0.68 ± 0.02
NPA/Control	2.3 ± 0.30*	0.1 ± 0.06*	2.4 ± 0.31*	28.8 ± 0.92*	0.08 ± 0.01*	0.003 ± 0.002*	0.08 ± 0.01*
Control/Control +10μM IAA	8.2 ± 0.69*	11.8 ± 0.43*	20.0 ± 0.85*	21.9 ± 0.48*	0.38 ± 0.03*	0.54 ± 0.02*	0.92 ± 0.04*
NPA/Control +10μM IAA	4.1 ± 0.39*	8.2 ± 0.35	12.4 ± 0.54*	16.7 ± 0.40*	0.26 ± 0.03*	0.49 ± 0.02*	0.75 ± 0.03

* Values that are statistically different from controls, as judged by Student's t-test are indicated, $P<0.01$.

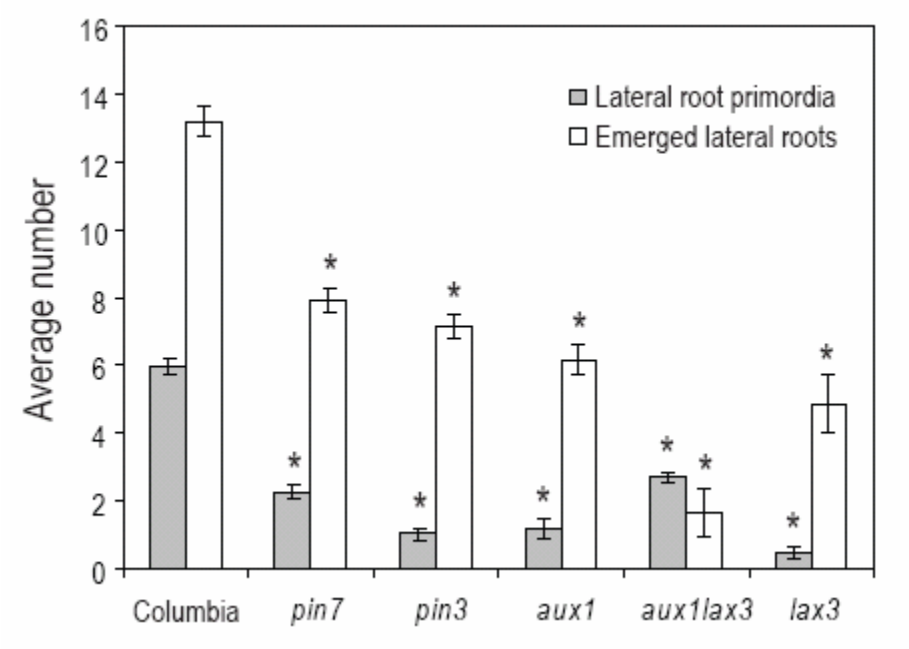


Figure II-5. IAA transport mutants have fewer lateral root primordia. *pin3* and *pin7* are mutants defective in auxin efflux carriers while *aux1*, *lax3*, and *aux1lax3* are mutants defective in auxin influx carriers. The average and standard error of 30-35 seedlings in 4 separate experiments are reported. * Significant differences for lateral root primordia and emerged lateral roots between mutants and wild type as determined by Student's t-test are indicated ($P<0.0001$).

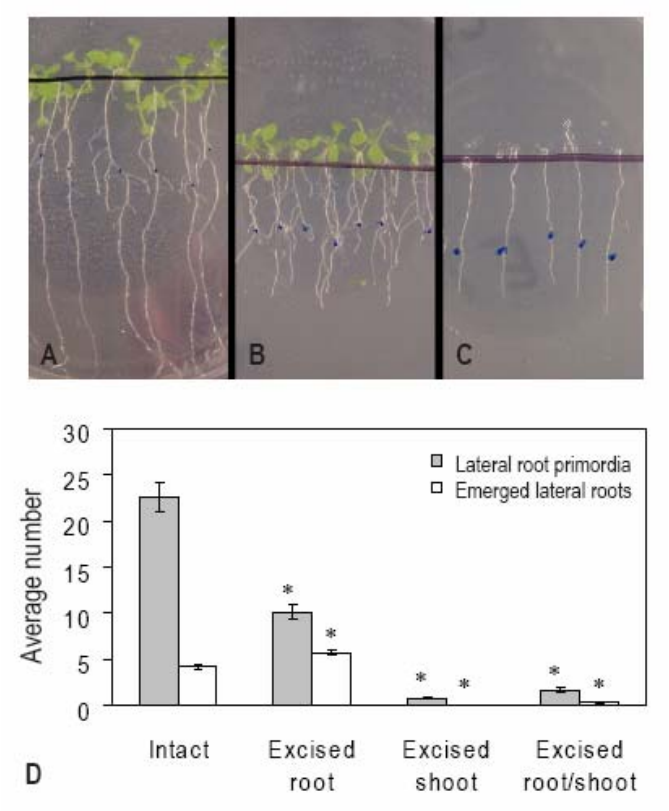


Figure II-6. Mechanical reduction of auxin flow from the shoot reduces root initiation. At six days after sowing plants were treated as indicated and after 3 additional days images were captures or the number of primordia or emerged lateral roots quantified. A, Seedlings were left intact. B, Root tips were excised 3mm above the root tip. C, Shoots were removed by excision at the root/shoot junction,. The blue dots represent the position of the root tip when seedlings were transferred to experimental condition. The roots extending beyond this mark seen in Panel B, after the root tip excision are elongated lateral roots. D, The average and standard error of 38 seedlings from 3 separate experiments are reported. Significant differences between intact controls and indicated treatments were determined by Student's t-test as indicated, * $P < 0.005$

Table II-3. Effect of excision on primary root growth, lateral root initiation and emergence, and lateral root density in *Arabidopsis*. Six day old seedlings were either subjected to root excision, shoot excision, root and shoot excision, or left intact. After three additional days of growth, the primary root was measured, the seedlings were GUS stained and primordia and emerged lateral roots were quantified. The reported values are averages and SE from three combined independent experiments (n=38).

Treatment	Lateral root primordia (LRP)	Lateral root emerged (LRE)	Length of primary root	LRP density	LRE density
Control	22.5 ± 1.60	4.3 ± 0.30	36.7 ± 1.00	0.6 ± 0.10	0.1 ± 0.01
Excised Root	9.4 ± 0.90[a]	5.5 ± 0.30[a]	12.7 ± 0.50[a]	0.7 ± 0.10	0.4 ± 0.03[a]
Excised Shoot	0.8 ± 0.20[a]	0.1 ± 0.10[a]	22.9 ± 0.50[a]	0.04 ± 0.01[a]	0.004 ± 0.003[a]
Excised R/S	1.7 ± 0.20[a]	0.3 ± 0.10[a]	12.4 ± 0.80[a]	0.1 ± 0.02[a]	0.03 ± 0.01[a]

[a] indicates which values are statistically different from controls as judged by Student's t-test ($P<0.005$).

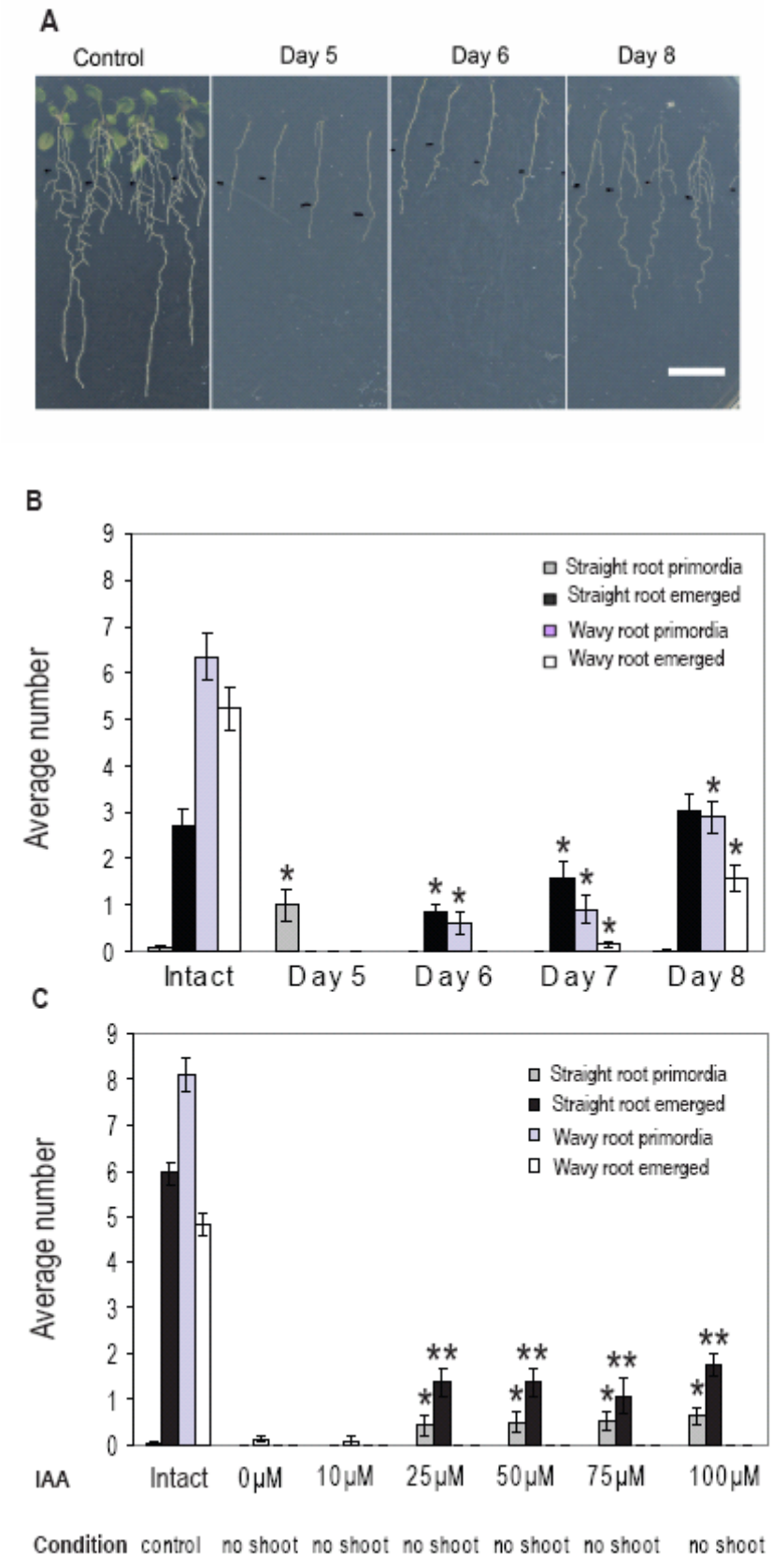

A
Control
Day 5
Day 6
Day 8
B
Average number
Straight root primordia
Straight root emerged
Wavy root primordia
Wavy root emerged
Intact
Day 5
Day 6
Day 7
Day 8
C
Average number
Straight root primordia
Straight root emerged
Wavy root primordia
Wavy root emerged
IAA
Intact
0μM
10μM
25μM
50μM
75μM
100μM
Condition
control
no shoot
no shoot
no shoot
no shoot
no shoot
no shoot

Figure II-7. Lateral root formation on wavy roots is sensitive to shoot derived auxin. Five days after sowing the seedlings were transferred from 0.8 % agar to 1.5% agar and placed at a 45º angle which causes the root to wave. The shoot was removed when plants reached the indicate days after sowing and seedlings were allowed to grow to day 8 after sowing when the number of lateral root primordia and emergence were quantified. A, Images of seedlings were captured at 8 days after sowing (3 days after treatment) as indicated for controls (intact seedlings) and seedlings with the shoot excised at 5, 6, and 8 days after sowing. B, The number of lateral root primordia and emerged lateral roots were determined in the straight region formed prior to transfer to conditions that induce wave formation and the wavy region after transfer to conditions that induce waves. The average and standard error of approximately 30 seedlings were measured from three independent experiments. * The effect of excision of time course was compared to intact seedlings by Student's t-test and significant differences are indicated, ($P<0.0001$). C, The seedlings were grown as described in Figure II-7A. At five days after sowing agar droplets containing IAA at the indicated concentration was applied to the root/shoot junction. After three additional days of growth, the number of primordia and emerged lateral roots was quantified. The average and standard error of approximately 30 seedlings from three independent experiments are reported. * the effect of IAA concentration on lateral root primordia in the straight section of primary root was compared to 0μM seedlings and significant differences are indicated ($P<0.02$). ** the effect of IAA concentration on emerged lateral roots in the straight section of primary root was compared to 0μM seedlings and significant differences are indicated ($P<0.003$).

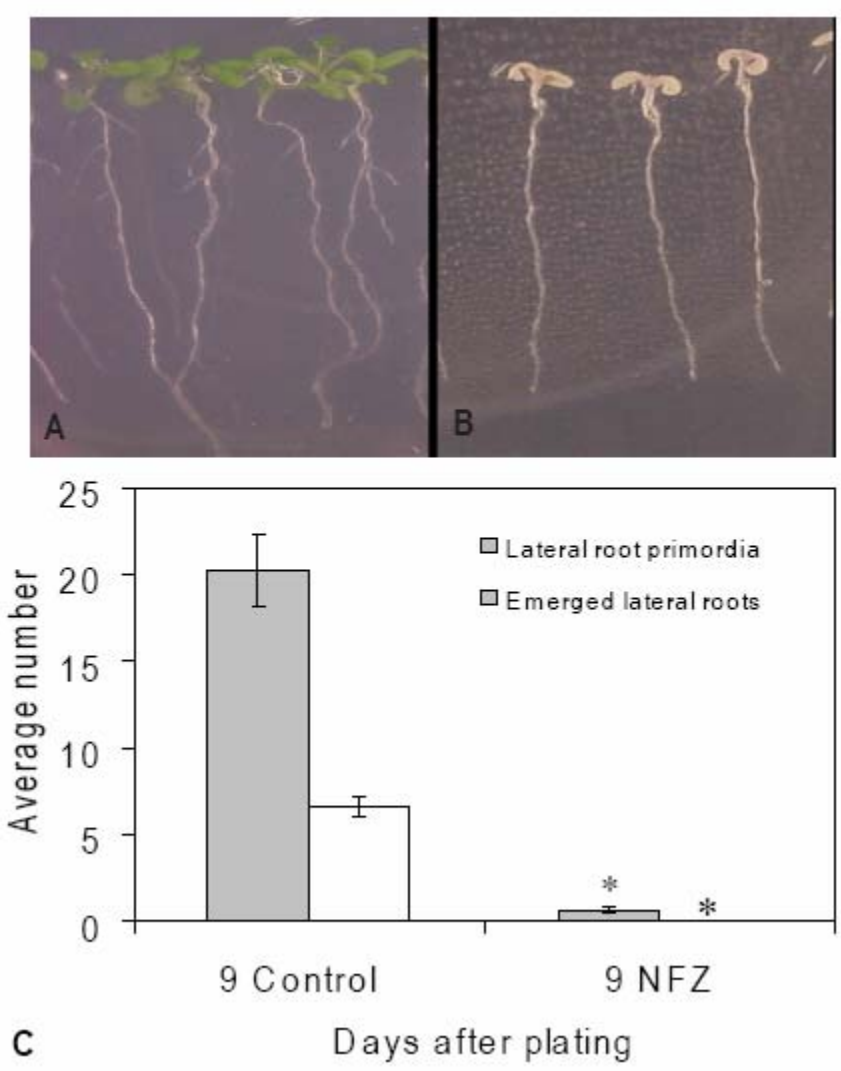


Figure II-8. Seedlings grown on 0.1µM of Norflurazon (NFZ) for 9 days after sowing. The seedlings were germinated on agar with NFZ and grown for 9 days after sowing. A, Control seedlings at 9 days after sowing are shown. B, NFZ grown seedlings at 9 days after sowing are shown. C, The average and standard error of 30 seedlings are reported. * The effect of NFZ on the primordia and emerged lateral roots was compared to controls by Student's t-test and significant differences are indicated ($P<0.0001$).

Table II-4. The effect of norflorazon on free IAA levels on number of lateral root primordia and emerged lateral roots. Seedlings were grown at 24 hours of light for 9 days on either control or NFZ treated agar. Root or shoot tissue was harvested separately from seedlings and frozen for determination of free IAA concentration. The number of replicates for root samples was 5-6 for control and 3 for NFZ and 3 for shoot samples for both control and NFZ; each root sample contained approximately 100-200 roots while the shoot samples contained approximately 20-30 shoots. The average and standard error of 50 seedlings from five independent experiments are reported for the average number of primordia and emerged lateral roots.

Condition	Shoot Auxin Content (ng/g fw)	Root Auxin Content (ng/g fw)	Number of primordia	Number of emerged LR
Control	8.37 ±1.60	11.53 ± 0.62	19.3 ± 0.58	5.7 ±0.29
NFZ	4.63 ± 0.12	10.71 ± 1.93	0.6 ± 0.17	0.0 ± 0.00

There were no significant differences in either the shoot or root free IAA accumulation as compared to control as determined by Student's t-test ($P>0.05$). There were no significant differences between NFZ treated and control roots as judged by Student's t-test.

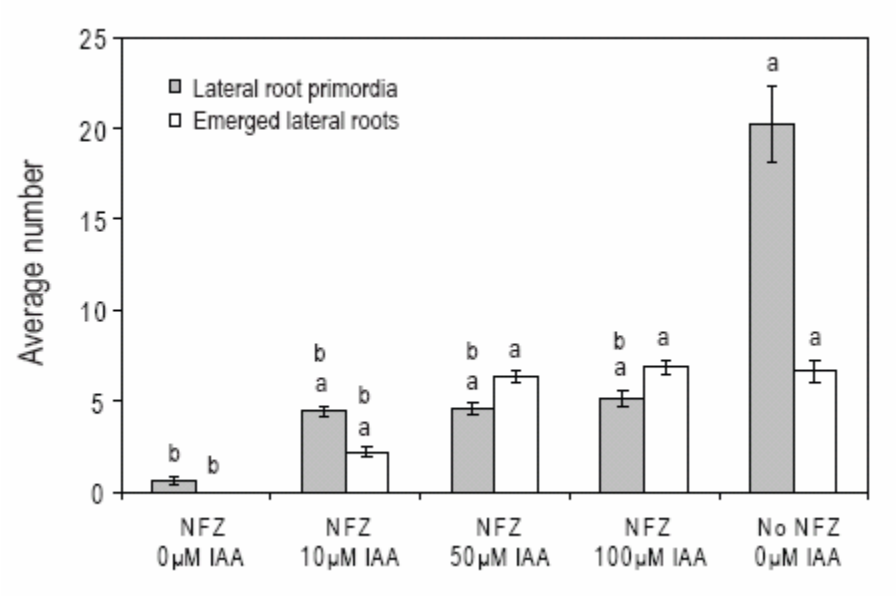


Figure II-9. IAA treatment rescues the effect of NFZ on lateral root elongation. The seedlings were grown on media containing NFZ. At 6 days after sowing droplets containing IAA at the indicated concentrations were applied to the root/shoot junction. After three additional days of growth, the number of primordia and emerged lateral roots was quantified. The average and standard error of 50 seedlings from 5 independent experiments are reported. "a" Indicates the number of primordia and emerged lateral roots formed after IAA treatment were compared to the NFZ treated roots without IAA and significant differences are indicated as determined by Student's t-test ($P<0.0001$). "b" Indicates the number of initiated and emerged lateral roots was also compared to the untreated control and those treatments with significant differences are indicated ($P<0.0001$). For most IAA treatments there was no difference in the number of emerged lateral roots, indicating that emerged lateral root numbers for 50 and 100µM IAA were fully rescued these treatments.

CHAPTER III

LATERAL ROOT INITIATION AND EMERGENCE ARE REGULATED BY LIGHT AND TEMPERATURE

Abstract

Developmental plasticity of roots is vital for successful exploitation of growth conditions; the environment is unpredictable and the plant sessile. Primary and lateral roots must respond to a host of environmental cues that are both local, i.e. nutrients, soil, and water availability, and global such as day length and growth temperature. Environmental parameters perceived by the shoot may influence synthesis and movement of long distance shoot signals, such as auxin, to optimize root system architecture. This study tested the hypothesis that growth temperature and day length, which are perceived by the shoot, may control lateral root development. Lateral root initiation and elongation were positively regulated by increasing day length and showed more complex temperature dependence. Auxin is a plant hormone that positively regulates the initiation and emergence of lateral roots and could serve as the long distance signal to modulate root architecture. We examined plants with mutations in auxin transport proteins and found that the protein, PIN3, is important in this light dependent pattern. We examined possible mechanisms driving this developmental plasticity and find that changes in auxin transport and signaling are strongly correlated with light and temperature dependent root formation, while auxin synthesis is weakly correlated.

Key words: Arabidopsis thaliana, lateral root development, auxin, auxin transport, auxin synthesis, auxin signaling, day length, temperature

Introduction

Physiological plasticity of plant roots is vital for successful exploitation of heterogeneous underground environments. Primary and lateral roots must respond to a host of cues defined by where they initiate. Environmental cues including nutrient distribution, density and compaction of soil, type of soil, salinity, competition among and between species, and interactions with micro-organisms are local determinants of root growth (Lynch, 1995). Root architecture also responds to dynamic environmental factors, which include changes in moisture, temperature, light intensity, and day length (Hodge, 2004; Malamy, 2005; Lynch, 1995). Many of these transient controls are perceived by the shoot and potentially communicate signals from the shoot to the roots to influence development. The ability of a plant to have an active developmental response to both local and long distance environmental cues enables the plant to be adaptive to the environment and be able to develop an optimal root system (Dorn et al., 2000; Malamy and Ryan, 2001). The plasticity of root architecture has a profound impact on plants' productivity as well (Lynch, 1995). Malamy and Ryan (2001) argue that the formation of lateral roots is a good model for how plant development is coordinated with environmental conditions.

Auxin is critical for many developmental processes including lateral root initiation and elongation (Casimiro et al., 2003; Teale et al., 2005; Malamy, 2008). Exogenous application of auxin has been shown to stimulate lateral root development (Torrey, 1976; Reed et al., 1998). Mutants that overproduce IAA, superroot (*sur*) and rooty (*rty*), have significantly increased levels of endogenous IAA compared to wild type plants and increased numbers of lateral roots (Boerjan et al., 1995; King et al., 1995).

Auxin resistant mutants, *axr1* and *axr4 aux1* have reduced lateral root numbers (Hobbie and Estelle, 1995; Timpte et al., 1995). Auxin acts at the earliest stages of initiation of lateral root primordia during the activation of the previously quiescent pericycle cells to begin division (Himanen et al., 2002), but also has been shown to enhance elongation of lateral roots (Wu et al., 2007). These data suggest that auxin plays a significant role in lateral root development.

Auxin moves through plant tissues via a unique cell to cell polar transport mechanism that is tied to growth and developmental processes including lateral root development. In shoots, polar auxin transport occurs from the shoot toward the root in a basipetal direction (Muday and DeLong, 2001; Leyser, 2006). In roots, transport occurs from the shoot toward the root apex through the central cylinder (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978; Rashotte et al., 2000; Muday and DeLong, 2001). Auxin delivery to the cells via polar auxin transport is essential for lateral root development, as demonstrated by treatment with inhibitors of auxin transport that prevent formation of lateral roots (Muday and Haworth, 1994; Reed et al., 1998; Casimiro et al., 2001).

Additional evidence for the role of transport in root development comes from studies of plants with mutations in auxin transport proteins. IAA transport is mediated by two classes of auxin transport proteins, IAA influx and IAA efflux carriers. Inhibition of influx or efflux carriers by mutations in these auxin transport proteins (Marchant et al., 2002; Laskowski et al., 2008) or by treatment with inhibitors that act on these proteins (Reed et al., 1998; Casimiro et al., 2001; Negi et al., 2008) both reduce lateral root formation. The influx carrier mutant, *aux1* shows a reduction in lateral roots (Marchant

et al., 2002) and has reduced acropetal and basipetal transport (Rashotte et al., 2000; Negi et al., 2008). The second influx carrier mutant, *lax3,* and the *aux1lax3* double mutant also show reduced number of primordia (Bainbridge et al., 2008). Mutants in genes encoding efflux carrier proteins, *pin2* and *mdr4/abcb4*, are defective in basipetal auxin transport (Rashotte et al., 2001) and show no effect on root development (Wu et al., 2007). The *pin1*, *pin2*, *pin3, pin4*, and *pin7* mutants have defects in auxin efflux carriers; Laskowski et al. (2008) showed that mutant, *pin2*, which is defective in basipetal IAA transport (Rashotte et al., 2001) exhibited significantly increased lateral root density over wild type. These results suggest that basipetal auxin transport is not important in lateral root development. In contrast, transport proteins tied to acropetal IAA transport have a more complex set of effects. *pin3* showed a significant reduction in formation of lateral roots. *pin7* and *pin1* mutants did not exhibit significantly altered lateral root density as compared to wild type. It would be useful to resolve the discrepancy by comparing the number of initiations of primordia and emerged lateral roots in these mutants, rather than just focusing on the density of root formation.

Root development is both genetically defined and environmentally responsive. There is a basic plant genetic makeup that defines root architecture, which has been called the intrinsic pathway (Malamy, 2005). This intrinsic pathway is modulated by the response pathway which co-ordinates environmental cues with development by modulating intrinsic pathways. Although auxin is essential in the intrinsic pathway, it is also an excellent candidate to examine as a major contributing factor in the response pathway that is important in perception and subsequent response to environmental cues (Malamy, 2005).

Auxin signaling is important in the development of lateral roots. Genome-wide studies indicate transcriptional response to auxin rapidly impact expression of a diverse set of genes (Goda et al., 2004; Tian et al., 2002; Mockaitis and Estelle, 2008). The AUX/IAA genes are transcriptionally regulated by auxin and modulate the activities of Auxin Response Factors (ARF), which are a series of transcription factors that act as repressors of gene expression. The TIR1 protein has recently been shown to act as the auxin receptor (Dharmasiri et al., 2005). The *tir1* mutant is deficient in a variety of auxin related growth processes including lateral root formation. Additionally mutants in ARF proteins such as *monopteros* (*mp*) and *bodenlos* (*bdl*) do not develop roots due to the impaired development of the founder cell of the basal meristem (Mockaitis and Estelle, 2008; Berleth and Jurgens, 1993; Hardtke and Berleth, 1998; Weijers and Jurgens, 2005; Hamann et al., 1999; Hamann et al., 2002). Similarly Fukaki et al. (2002) isolated the solitary root (*slr-1*) mutant, which forms no lateral roots due to the absence of divisions of pericycle cells and have shown that this mutant has a defect in *IAA14* which acts as a transcriptional repressor.

Auxin synthesized in the shoot has been shown to be necessary for both lateral root initiation and emergence (chapter 2*;* Bhalerao et al., 2002; Casimiro et al., 2003; Reed et al., 1998). The environmental variables, day length and growth temperature, are two cues that have a profound effect on shoot architecture and growth (Gray et al., 1998; Morelli and Ruberti, 2002; Casal et al., 2003; Bláha and Hnilička, 2004). In this study we will examine these early long distance environmental cues on lateral root architecture.

Plants use light as an energy source via photosynthesis and a signal to activate and modify endogenous developmental processes. Plants sense light via three known classes

of photoreceptors: cryptochromes, phytochromes, and phototropins (Casal, 2002; Chen et al., 2004; Casal, 2000). Cryptochromes mediate light responses in the plant including but not limited to de-etiolation; this developmental transition includes an inhibiton of hypocotyl growth, promotion of cotyledon expansion, and synthesis of pigments including chlorophyll and anthocyanins upon transfer of seedlings from light to dark (Chen et al., 2004; Lin, 2002; Liscum et al., 2003).

Phytochromes play a large role in light signaling pathways in the shoot, but few studies have examined their role in root development. Phytochromes are encoded by small gene family designated *PHYA-PHYE*. There is evidence for phytochrome activity within the root system controlling phototropism (Correll et al., 2003; Kiss et al., 2003; Ruppel et al., 2001). Salisbury et al. (2007) found the *phyD* mutant had slightly more lateral root production than wild type while *phyA, B, E* reduced lateral root outgrowth, suggesting that phytochromes play a role in controlling root development. Exogenous application of IAA to *phyB* and wild type showed opposite effects; there was increased DR5::GUS expression in wild type but no increased DR5::GUS expression in *phyB* suggesting an involvement of PHYB in auxin response. PHYB may regulate auxin transport into the root system and/or reduce the response of auxin-inducible genes.

Phototropins also mediate physiological responses to light. Phototropism, which is bending toward a unilateral light source, is a blue light response mediated by phototropins (Chen et al, 2004). Auxin and light signaling are tightly bound. The mutant, *phot1*, was identified for its inability of the hypocotyl to bend toward unilateral blue light (Liscum and Briggs, 1995). The protein, PHOT1, is evenly distributed at the plasma membrane; this distribution might be relevant for asymmetric growth response

initiation by unilateral light. PHOT1 localization and the localization of members of the PIN family of auxin efflux carriers, particularly PIN3, overlap suggesting that phototropin activation may direct asymmetric auxin distribution and growth orientation (Friml, 2003; Sakamoto and Briggs, 2002). PIN3 is important in establishing auxin gradients in response to changes in phototropism (Friml et al., 2002).

Light signals have also been shown to exhibit cross talk with auxin signals. Auxin has been shown to alter expression of a range of light-regulated genes confirming the close association of light and auxin signaling (Nagpal et al., 2000). HY5 protein is potentially a signal integration point in light and hormone signaling networks. The *hy5* mutant, defective in phytochrome signaling, has altered lateral root production (Cluis et al., 2004; Sibout et al., 2006). Links between light and auxin have been defined using primary auxin response genes; 3 major gene classes are *Aux/IAA, GH3*-lik*e* and *SAUR* gene families (Guilfoyle, 1999). Aux/IAA proteins are involved in auxin response; studies of *aux/iaa* mutants suggest a strong link between auxin signaling and photomorphogenesis (Nagpal et al., 2000).

Plants are sessile and can experience vast temporal variation in temperature. (Atkin et al., 2006; Larcher, 2004). Variations in temperature affect metabolic processes that contribute to biosynthesis of auxin and other compounds and cellular maintenance and photosynthesis (Gray et al., 1998). Biomass allocation is temperature sensitive. During long term exposure to low temperatures, plants exhibit reduced investment in shoots and increase root biomass, as compared to plants grown at higher temperatures (Loveys et al., 2002). These results are important in a real world context as global warming is occurring. Examining the role of temperature plasticity could elucidate a

plant's response to changing environments and provide useful information to applied plant researchers trying to prepare for this inevitable event.

Temperature is important to organ growth and IAA synthesis in *Arabidopsis* which is examined by Gray et al. (1998). They find that elevated temperature (28ºC versus the normal 22ºC) increases hypocotyl elongation. This higher temperature causes increased IAA synthesis in this tissue and this response requires intact auxin signaling pathways. Based on their results, Gray et al., (1998) suggested that temperature regulates auxin synthesis, which in turn regulates developmental responses. Although they do not discuss the effect of temperature on root development, images in their paper suggest that there is enhanced root branching at the higher temperature.

Both changes in growth temperature and day length can alter growth variables such as flowering time, rosette size, leaf number, seed count and size, germination, and vernalization (Westerman, 1970; El-Assal et al., 2001; Blazquez et al., 2003; Engelmann and Purugganan, 2006; Ungerer et al., 2003). Photoperiod length and temperature are important; both contain valuable information that impacts directly on the plant's developmental program. Photoperiod length is a powerful flowering signal that ensures reproductive development is synchronized with day length. Small changes in photoperiods or a fall in temperature may signal unfavorable conditions or early onset of winter. Little, however, has been studied on the effect of growth temperature and day length on early development as it pertains to lateral root development.

This study tested the hypothesis that the environmental variables, growth temperature and day length, are important in the plastic response of lateral root development. As shown in Chapter 2, we find that shoot derived auxin positively

regulates lateral root initiation and emergence. Day length and growth temperature affect the shoot directly. In this study we characterized the pattern of lateral root formation under different growth temperatures and day lengths and we found a plastic response to both, suggesting the importance of the shoot and the auxin synthesized there. We also examined possible mechanisms driving this physiological plasticity such as auxin transport, auxin signaling and auxin synthesis; all appear to play a role in the plastic response.

Results

With increasing day length, the number of primordia and emerged lateral roots increase

We tested whether the number of lateral root primordia and emerged lateral roots vary with increasing day length. Seeds were plated on control agar and grown vertically in 24 hour light to synchronize germination rates. Three days after sowing (DAS) the newly germinated seedlings were transferred to 8, 12, 16, 20, and 24 hour days; examples of seedlings grown at 8, 16, and 24 hours are exhibited in Figure III-1 (A-C). After six additional days, the number of lateral root primordia and emerged lateral roots were quantified for each treatment and are reported in Figure III-1(D) and Figure III-3. We found that with increasing day length (between 8 and 20 hours) the number of lateral root primordia increase. Days lasting 24 hours were slightly less optimal for root initiation than 20 hour days. For the measurement of lateral root primordia, all day lengths were significantly different from 8 hours as analyzed by ANOVA followed by Tukey–Kramer multiple test, ($P<0.01$). For emerged lateral roots, 8, 12, and 16 hours were significantly different from 20 and 24 hours as analyzed by ANOVA followed by Tukey–Kramer multiple test, ($P<0.0001$).

Growing seedlings on vertical Petri dishes places the roots in constant light and with asymmetric nutrient and moisture supply. Several additional growth conditions were used to ask if light effects on root formation were due to exposure of the shoot or root to light. Seedlings were germinated and grown in magenta boxes containing agar with no effort to block out light or in magenta boxes containing agar with a thin layer of agar/charcoal mixture on the surface of the agar and with foil around the sides of the box

to block light transmission to the root. Magenta boxes that simulated the dark grown root condition best replicate seedlings in soil conditions. Seedlings were grown in magenta boxes under 24 hour light for three days after sowing to unify germination then transferred to light banks with 8, 12, 16, 20, or 24 hour day length. After six additional days of growth, seedlings were gently extracted from the agar, stained for cyclin-GUS activity and the number of lateral root primordia and emerged lateral roots were quantified for each treatment. The average and SE of approximately 45 seedlings in 3-6 separate experiments is reported in Figure III-2.

Lateral root primordia formation and emergence changes as day length increases when roots are shaded, but lateral root primordia and emerged lateral roots exhibit different responses. Lateral root primordia formation exhibits a bell shaped curve with 12, 16, and 20 hour days leading to the highest number of lateral root primordia. For the measurement of lateral root primordia, all day lengths were significantly different from 8 hours as analyzed by one-way ANOVA, ($P<0.05$). In contrast, there was little difference in the number of emerged of lateral roots over 8-16 hour day lengths; 20 and 24 hour day lengths seemed to be the most optimal.

To better understand the relationship between light and dark grown plants and their day length dependence, Figure III-3 shows a comparison of lateral root primordia and emerged lateral roots in these two conditions. This graph illustrates that there is a similar relationship between day length and number of emerged lateral roots whether or not the roots are shaded or grown in light, although the shaded roots form more laterals at all day lengths. For 8, 12, and 16 hours, we saw 6-, 8-, and 5-fold increases, respectively, when emerged lateral roots in dark grown roots were compared to roots grown in the

light. At 20 and 24 hours, the differences between plates and magenta boxes was less profound with only 1.8- and 2-fold increases

In contrast, the relationship between number of lateral root primordia and day length is very different for plants grown under these two conditions. Light grown roots displayed a near linear relationship between number of primordia and increasing day length, while in shaded roots, there is a bell shaped curve with a plateau between 12 and 20 hour days. Like emerged lateral roots, the shaded and agar embedded roots form more primordia.

The effect of day length on primary root growth and the resulting density of lateral roots for both plates and magenta boxes are reported in Table III-1. As day length increases in seedlings grown in Petri dishes, there is a statistically significant increase in root length with the exception of 16 hours in which the root elongation decreases, as analyzed by a one-way ANOVA, ($P<0.01$). In magenta grown seedlings, there is no statistical difference in root length over the day length gradient as analyzed by ANOVA followed by Tukey-Kramer multiple test, ($P>0.05$).

Potentially important day length sensitive transitions in both the average number of primordia and emerged lateral roots and their corresponding densities in both Petri dishes and magenta box grown seedlings are seen in Table III-1. In plate grown seedlings, initiation of primordia are day length limited at 8 hours. There is a significant increase in the number of primordia at 12-16 hours followed by another jump in average number of primordia at 20-24 hours as analyzed in one-way ANOVA, ($P<0.05$). The density of primordia follows a similar pattern as the number of primordia. In magenta box grown seedlings, lateral root initiation is also day length limited at 8 hours of light.

The numbers of primordia significantly increase from 12-20 hours with a sharp decrease at 24 hours suggesting a limiting day length as analyzed in a one-way ANOVA, ($P<0.05$). Densities of primordia for magenta grown seedlings follow the same pattern.

When examining emerged lateral roots there is only one day length sensitive transition for both Petri dish and magenta grown seedlings. For Petri dish grown seedlings, 8-16 hours of day length seems to limit numbers of primordia with a significant increase in primordia numbers at 20 and 24 hours of light. This day length dependent transition is mimicked in emerged root densities; these growth dependent transitional day lengths are statistically different as analyzed by a one-way ANOVA, ($P<0.05$). In magenta grown seedlings, there is a similar pattern although not as pronounced; 20 and 24 hour day lengths increase over 8, 12, and 16 hour day lengths with the same density pattern. These day length dependent transitional periods are not statistically different as analyzed in a one-way ANOVA, ($P>0.05$) but a trend is evident. These transitional patterns possibly signal developmental divisions of growth corresponding with given day lengths. For plate and magenta grown seedlings, they receive considerably less light signaling and photosynthate at 8 hours of light; the development of primordia and emerged lateral roots lag. The shift to 12-16 hour day lengths appears to be a significant developmental shift and the seedlings produce more primordia although the emergence of lateral roots still seems arrested. Finally 20-24 hours of light appear to be ideal for both primordia and emerged lateral root development. With magenta grown seedlings, the ideal day lengths for primordia development are 12-20 hours and for emerged lateral roots, 20-24 hour day lengths seem to be ideal in magenta grown seedlings. In magenta box grown seedlings, root growth is

more stable over all day lengths. This might suggest that the roots in the magenta boxes are buffered from the increasing day length.

Increasing growth temperature is correlated with the number of primordia and emerged lateral roots

We tested if there was a different response of lateral root development over different growth temperatures. Seeds were plated on control agar and grown vertically in 24 hours of light in growth chambers set at 18, 21, 24, 26, 28, or 30 ºC. Examples of seedlings grown at 22 and 27 ºC are exhibited in Figure III-4 (A-B) At three days after sowing light cycles were changed to 16 hr day/ 8 hr night, but temperature was maintained as constant. Seedlings of all temperatures germinated on the same day; there was no developmental lag due to temperature differences. After six additional days of growth, the number of lateral root primordia and emerged lateral roots were quantified for each treatment and are reported in Figure III-4 (C).

The number of lateral root primordia exhibited a bell shaped curve; the largest numbers of lateral root primordia formed between the temperatures of 21 and 26 ºC. Temperature extremes lead to reduced number of primordia at the lower and upper ends of the temperature spectrum. Emerged lateral roots exhibited a skewed bell shaped curve, with increasing numbers from 18 to 28 ºC with a 2.3-fold statistically significant decrease at 30 ºC. Differences in length of primary roots were evident with a trend of longer lateral roots in the optimal temperature range between 21 and 26 ºC with a sharp decline in length at 30 ºC, leading to relatively consistent lateral root density (data not shown). These results suggest that there is an optimal temperature for growth and

development of lateral roots as well as limiting temperatures at both ends of the temperature spectrum used.

Auxin transport capacity is not changed with increasing day length or increasing growth temperatures

We tested whether increasing growth temperatures would result in increased auxin transport capacity in the roots. Seeds were plated on control agar and grown in 24 hours of light in growth chambers set at 18, 24, or 28 ºC. At 3 days after sowing the growth chambers were set to 16 hr day/ 8 hr night cycle. After 6 additional days of growth, acropetal IAA transport was measured and is shown in Figure III-5B. There were no statistical differences between any of the treatments using a Student t-test, ($P>0.05$) consistent with the absence of an effect of growth temperature on capacity to transport IAA.

We tested whether increased day length would result in increased auxin transport capacity in the roots. Seeds were plated on control agar and grown in 24 hour light. Three days after sowing the seedlings were placed under conditions with 8, 16, and 24 hour day length. After six additional days, the amount of acropetal auxin transport was determined. A droplet containing 1μM ^{3}H-IAA was placed on the root/shoot junction for two hours and transport into a 3mm segment, 5 mm below the application site was quantified, as shown in Figure III-5A. There were no statistical differences between any of the treatments, ($P>0.05$) consistent with the absence of an effect of day length on capacity to transport IAA.

Auxin transport proteins are required for lateral root formation and for day length dependent changes

We tested whether mutants defective in auxin transport proteins exhibit altered lateral root initiation and elongation upon variable day length. *aux1*, *lax3*, and the double mutant, *aux1lax3,* are defective in influx carriers while *pin3* and *pin7* are defective in efflux carriers. Seeds were plated on control agar and grown in 24 hour light. Three days after sowing the newly germinated seedlings were transferred to 8, 16, and 24 hour days. After six additional days, the seedlings were cleared and the number of lateral root primordia and emerged lateral roots were quantified for each treatment and are reported in Figure III-6.

In all auxin transport mutants tested, the number of primordia and emerged lateral roots are significantly less than wild type in seedlings grown under 24 hour days , as judged by a Student's t-test, ($P<0.0001$). Wild type Col seedlings exhibited an increase of lateral root primordia and emerged lateral roots over increasing day length, like the NO ecotype, used in previous experiments. If a specific auxin transport protein is needed for this light dependent change in root formation, we would expect that the number of primordia and lateral roots would be constant with variable day length. The *pin3* mutant exhibited this behavior, as the number of primordia is not significantly different when 8 and 24 hour days are compared. When examining the emerging lateral roots, one sees a similar pattern in the *pin3* mutant. There is a statistically significant difference between 8-16 and 24 hours ($P<0.04$) but the difference is smaller than 2-fold; in contrast to the 17-fold increase in Col. This result suggests that the PIN3 protein is also important in the day length dependent pattern exhibited in wild type. For all the other mutants, means become significantly less, so they are implicated. However, they appear not to be implicated in the plasticity of response to day length.

The effect of day length on primary root growth and the resulting density of lateral roots for these auxin transport mutants are reported in Table III-2. Wild type seedlings exhibited an increase in primary root length with increasing day length. In contrast the mutants exhibited relatively constant root length with different day length. Under 16 and 24 hour light conditions, these transport mutants have significantly less root elongation as compared to wild type; at 8 hours most transport mutants either equaled root length or surpassed the wild type. *lax3* had one of the longest primary root over all day lengths, yet had virtually no primordia.

Day length and temperature affect free IAA in roots

We measured free IAA levels in roots and shoots grown under three day lengths, 8, 16, and 24 hours. Seedlings were grown at 24 hours of light for three days and then grown for six additional days at indicated day length. For these seedlings root or shoot were separately harvested and tissue was frozen with liquid nitrogen. Free IAA concentration was measured for root and shoot tissues separately. Levels of free IAA in the shoot do not exhibit a pattern of increasing concentrations with increasing day length; the concentration increases 1.5-fold from 8 to 16 hours, but then exhibits a 1.4-fold decrease from 16 to 24 hours. However, levels of free IAA in the root remain stable at 8 and 16 hours with no significant difference, ($P>0.05$) but exhibit a 1.3-fold increase at 24 hours judged by a Student's t-test, ($P<0.02$). This data suggests that free IAA accumulation in the roots with increasing day lengths is correlated with increase in the number of emerged lateral roots, although the magnitude of the increase in root numbers is greater. The pattern of increasing auxin concentration in the roots is correlated with

the pattern of increasing number of emerged lateral roots over increasing day lengths, as seen in Table II-2.

Similarly, we tested whether a pattern of IAA accumulation would emerge with increasing growth temperature. Seedlings were germinated at the indicated growth temperature with 24 hours of light. At three days after sowing, the light cycle was changed to 16 hr day / 8 hr night. Root and shoot tissues were harvested and frozen separately. Free IAA was measured for separate root and shoot samples. In the shoot, free IAA concentration decreased as the temperature increased. In the root, free IAA concentration increased as temperature increased with a significant difference between 18 and 28 ºC, ($P<0.003$). There was a correlation between the increasing concentration of free IAA in roots and the number of emerged lateral roots as temperatures increased as seen in Table III-4. In contrast, there was no apparent corresponding trend between changes in free IAA and frequency of lateral root primordia formation. There was no apparent relationship between IAA accumulation from the shoot and the number of lateral root primordia or emerged lateral roots as temperature increased.

GH3-GUS expression increased with increasing day length and growth temperatures.

We tested the possibility that increasing day length leads to increased root formation via enhanced auxin signaling. At three days after sowing, GH3-GUS transgenic seedlings were placed with the auxin responsive GH3 promoter driving GUS synthesis. to the indicated day lengths. Expression of the GH3 promoter/GUS reporter gene can be specifically induced by auxin in all cell types throughout the plant; expression of the GH3-GUS gene correlates with physiological events which are

presumably mediated by auxin. After six additional days of growth, the ß-glucuronidase activity in roots was determined using fluorescent MUG substrate. As day length increases, the amount of GUS activity increases; all time points are significantly different from one another, ($P<0.01$) as seen in Figure III-7. These results suggest that there is enhanced auxin signaling and more lateral root development in the seedling as day length increases.

We tested whether auxin induced gene expression changed with increasing growth temperature. At three days after sowing, seedlings were transferred to a light cycle of 16 hr day / 8 hr night at the indicated temperature. After six additional days of growth, the ß-glucuronidase activity in GH3-GUS roots was determined using fluorescent MUG substrate. The bell shaped pattern of expression of GUS activity is similar to the number of lateral root primordia and emerged lateral roots found with increasing temperatures. There is a significant difference between 18 and 24ºC, ($P<0.05$) as seen in Figure III-8, but no difference between 18 and 28ºC, ($P<0.05$). The bell shaped pattern of auxin signaling is similar to that of lateral root emergence, which exhibits the same peak at 24ºC as GH3-GUS expression.

***axr1-3* day length dependent increase in number of lateral root primordia and emerged lateral roots.**

We asked whether the auxin signaling mutant, *axr1-3*, showed day length dependent changes in lateral root initiation and elongation. At three days after sowing, under 24 hours of light, seedlings were transferred to the indicated day lengths. After six additional days of growth, roots were cleared and the number of lateral root primordia and emerged lateral roots were quantified. The *axr1-3* mutation has a small effect on

lateral root primordia formation, but with increases at low light and decreases at high light. The number of emerged lateral roots is significantly reduced in *axr1-3* as compared to Col controls at all day lengths except 8 hour days. Both wild type and *axr1-3* show a day length dependent pattern with wild type having the more pronounced pattern. If the protein, AXR1-3, were important in the day length dependent root formation, we would have expected to see constant numbers of roots forming with differing day lengths. The evidence provided here does not support this hypothesis.

Discussion

Long distance signals such as day length, light intensity, and growth temperature affect the shoot; in turn these long distance signals may change patterns of root growth (Dorn et al., 2000; Malamy and Ryan, 2001). Physiological plasticity of roots is necessary for the plant to take advantage of environmental resources. Auxin positively regulates the initiation and emergence of lateral roots (Casimiro et al., 20003; Teale et al., 2005; Malamy, 2008); it is a strong candidate for long distant signaling from the shoot to the root. We examined the physiological plasticity of lateral root growth under different day lengths and growth temperatures; we also examined possible mechanisms such as auxin transport, signaling, and synthesis that might change to induce different root growth phenotypes. Our results demonstrate that light and temperature regulate the amount of lateral root formation; we also explore the role of auxin signaling and transport in this environmentally sensitive root development.

We find that the number of primordia and emerged lateral roots positively correlated with increasing day length in roots grown in the light and dark. The number of primordia and emerged lateral roots correlated with growth temperatures over a naturally occurring range. The pattern across the range of temperatures exhibited a bell shaped curve with optimal growth temperatures in the middle and limiting temperatures on either side of the range.

Auxin transport was examined as a possible mechanism for this physiological plasticity seen in both day length and growth temperature. There was no significant difference in acropetal IAA transport when 8, 16, and 24 hour days or 18, 24 and 28 ºC temperatures were compared. We used IAA transport mutants to further test the

possibility that auxin transport might be a factor in the day length dependent plasticity. These mutants affect acropetal auxin flow; at 24 hours, all mutants formed significantly fewer lateral root primordia and emerged lateral roots than wild type suggesting that the proteins associated with these mutants are important in auxin transport from the shoot required for lateral root development. The *pin3* mutant specifically showed no light dependent pattern in the initiation of primordia and a weak but significant light enhanced lateral root emergence. These results taken together suggest that although there were no detected changes in acropetal IAA transport, auxin transport proteins are required for lateral root formation and that PIN3 is required for day length dependent root development.

When we examined whether auxin synthesis might be a possible mechanism in both day length and growth temperature, we discovered a positive correlation between root free IAA levels and number of emerged lateral roots. With increasing day length or elevated temperatures, we found an increasing amount of auxin accumulation in the roots that paralled changes in the number of emerged lateral roots. The differences in the amount of auxin accumulation establishing this pattern was of a much smaller magnitude than the change in lateral root emergence across day lengths or temperature changes. As these measurements were done with whole roots, localized changes in free IAA would not be evident in these analyses. The positive, albeit small, changes in free IAA levels suggest that these may play a role in lateral root elongation.

We tested a third potential regulatory mechanism to see if changes in auxin signaling play a role in day length and growth temperature dependent root formation. With increasing day length, we found increasing expression of the auxin response GH3-

GUS reporter. We also found that with increasing temperature, expression of the auxin response GH3-GUS reporter increases in parallel to increases in the number of primordia and emerged lateral roots. These results suggest that auxin signaling correlates with increasing day length and growth temperature. We also used the signaling mutant, *axr1-3*, to examine the role of the gene, *AXR1-3*, in the day length dependence response. At 24 hours there were significantly fewer primordia and emerged lateral roots in the mutant as compared to the wild type. This suggests that the protein, AXR1-3, has a small role in lateral root formation, but we find no evidence that it is required for the day length dependent changes in root formation.

Light and temperature have profound effects on lateral root developmental plasticity. Previous experiments have also examined the relationship between auxin synthesis and light. Dark grown *Arabidopsis* seedlings do not form lateral roots (Reed et al., 1998), and there is almost no detectable free IAA present (Bhalerao et al., 2002). When exogenous IAA is applied to the root/shoot junction of dark grown seedlings, lateral root emergence is partially rescued (Reed et al., 1998), consistent with the absence of IAA synthesis. These two experiments show the extremes of day length, which are plants in either 24 hours of dark or light. In our experiments we examined the data across a day length spectrum. In terms of auxin synthesis, we saw more subtle changes until we got to the extreme of 24 hours where there was a distinct jump in auxin synthesis.

In dark grown seedlings, photosynthate is limited, but exogenously applied auxin was able to overcome this limitation and rescue lateral root development suggesting that auxin was the driving force behind lateral root emergence. Bhalerao et al. (2002) detected a pulse of auxin moving from the shoot to the root. If seedlings were placed in

darkness at three days after germination, prior to this auxin pulse (Bhalerao et al., 2002), no lateral root development was observed (Salisbury et al., 2007); this suggests that this pulse is essential for root formation and that light is required for this pulse of auxin; it also reinforces the importance of the role of the shoot derived auxin for lateral root development (Salisbury et al., 2007).

Temperature also has a profound effect on root development. This study is the first time that the effect of growth temperature across a natural range has been examined on *Arabidopsis* root development. Gray et al. (1998) examined the effect of high versus low temperature on hypocotyl elongation in *Arabidopsis*. When grown at high temperatures, *Arabidopsis* seedlings have a marked increase in elongation growth of the hypocotyl accompanied by elevated free auxin concentrations. When auxin response or transport pathway mutants were grown at these two temperatures, the dramatic growth seen in the higher temperature was greatly reduced. Based on their results, Gray et al. (1998) suggested that temperature regulates auxin synthesis that in turn regulates hypocotyl elongation. It is interesting that when we used similar temperatures, we did not detect the large differences in free IAA detected in their studies. It is worth noting that our light intensity was significantly higher which might explain the difference as IAA might have been less limiting under our conditions.

In the following studies the authors have found profound effects in IAA synthesis; their use of other species, which have potentially different metabolic processes might explain why we do not see this large difference across temperature points. Rapparini et al. (2002) have shown that IAA biosynthesis and turnover also change in response to changing temperatures in a monocot species. There is a correlation between measured

IAA levels and growth rate with increasing temperature. Musatenko et al. (2003) saw that under short heat shock conditions, IAA accumulation controls the development of plant protective responses at earlier stages of the shock. In roots, free IAA increases 1.3-fold in one maize hybrid and 2.4-fold in another hybrid. IAA is important for a plant's entrance into a stress state after heating (Veselov et al., 1998). Gladish and Rost (1993) examined primary root growth in Pea (*Pisum sativum* L. cv. Alaska) at two different temperatures. At the higher temperature, development proceeds as normal until 4 days after germination when growth quickly slows; fewer lateral root primordia form, emergence of lateral roots is almost totally inhibited and primary root growth is arrested. These results taken together strongly suggest that temperature is a long distance signal that affects auxin synthesis, transport, signaling and thereby modulates lateral root development.

Understanding the signals that drive plasticity in root development has important physiological and ecological implications. The shoot environment changes which may affect the concentration or transport of auxin; these changes may act as a long distance signal controlling root architecture. The shoot is exposed to potentially harsh and unpredictable environmental variation and the plant as a whole must respond with long distance signals that protect and benefit and ultimately assure survival. Light signals through phytochromes provide plants with circadian, seasonal, and positional information important for control of germination, seedling development, shade avoidance, reproduction, and dormancy (Mathews, 2006). These response pathways are important in perceiving environmental cues in varying environments and set in motion signaling cascades that ultimately influence the plant's physiology (Fankhauser and Staiger, 2002).

Bláha and Hnilička (2004) found growth temperatures can have short- and long-term affects on root morphology. Increased growth temperature of a single generation can change these variables of the root system in the next generation especially in root traits; the root length, surface, weight, nutrient uptake, number of root tips, hairs and lateral roots, density, and water uptake can be affected by this abiotic stressor. We studied the early developmental effects of changes in the shoot environment and the importance of auxin as a part of the long distance signaling pathway; obviously these changes can also have long term effects that are beyond the scope of this study.

Modulation of light signaling and growth temperature to control root architecture are clear areas where long distance shoot signals modulate root architecture (Reed, et al., 1998; Bhalerao et al., 2002; Salisbury et al., 2007; Bláha and Hnilička, 2004; Gray, 1998). In this study we found that as day length increased, lateral root development changed quantitatively. We also found that auxin signaling and that PIN3, an auxin transport protein, plays a significant role in light dependent root formation. Changes in free IAA accumulation also appear to correlate with changing root emergence. We also discovered that with increasing growth temperatures, there were corresponding trends in lateral root development. Auxin signaling and synthesis, in a more minor role, appear to be important in these differences in lateral root architecture. We were not able to examine the role of transport proteins for a possible role in this temperature dependent pattern.

We found that day length and growth temperatures were important in a laboratory ecotype of *A. thaliana*. In natural populations, day length and growth temperatures vary significantly for plants depending on their ecological range. *Arabidopsis thaliana* has a substantial latitudinal range both in Europe, Asia and North America. In natural conditions, it would be important to this species' survival to be physiologically plastic. Environmental factors change quickly and it is vital to the plant to be able to change with them.

Materials and Methods

Chemicals

For the MUG assay, MUG was purchased from Gold Biotechnology; DDT was purchased from Fisher Biotech; Na_2CO_3, Na_2EDTA, sodium lauroyl sarcosine were purchased from Fisher. Bradford assay reagent was purchased from BioRad. ^{3}H-IAA was purchased from Amersham. Formaldehyde, DMSO, sodium iodine, and glycerol were purchased from Sigma; $Na_2S_2O_3$ was purchased from JT Baker chemical Co. Murashige and Skoog (MS) salts were purchased from Caisson Labs. All other chemicals were acquired from Sigma (St. Louis, MO).

Seed Germination and Plant Growth

Wild-type *Arabidopsis thaliana*, ecotype Nossen (No), was used in the IAA accumulation experiment. No transformed with *cyc1-At-GUS* and Col transformed with GH3-GUS were generously provided by John Celenza,, and Gretchen Hagen, respectively. The transport mutants, *aux1*, *aux1lax3*, *lax3*, *pin3*, and *pin7* (Benkova et al., 2003; Laskowski et al., 2008; Salisbury et al., 2007; Casimiro et al., 2003; Swarup et al., 2008) were in the Columbia background and were provided by Malcolm Bennett and Marta Laskowski. *axr1-3* provided by ABRC (Tiryaki and Staswick, 2002), a signaling mutant in the Columbia background, was used in the mutant signaling experiment. Seeds were soaked in distilled water for 30 minutes and surface sterilized with 95% (v/v) ethanol for five minutes and 20% (v/v) bleach with 0.01% (v/v) Triton X-100 for five minutes and then washed with distilled water for five minutes. After three additional washes in sterile distilled water, seed were transferred to Petri dishes containing sterile control medium (0.8% (w/v) agar [Sigma Type M, plant tissue culture], 1 x Murashige

and Skoog salts, 0.05% (w/v) Mes, pH 6.0, 1.5% (w/v) sucrose, 1 $\mu g \cdot mL^{-1}$ thiamine, 1 $\mu g \cdot mL^{-1}$ pyridoxine HCl, and 0.5 $\mu g \cdot mL^{-1}$ nicotinic acid).

Day length and Growth Temperature

For experiments in which day length was varied, seedlings were grown on Petri dishes containing control media. All seeds were allowed to grow at 24 hours of light (approximately 100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) for three days to allow for uniform germination. At three days after sowing seedlings were transferred to light banks set for 8, 12, 16, 20, or 24 hour days. After three additional days of growth, seedlings were transferred to new control media with 10 plants per Petri dish. At nine days after sowing, seedlings were GUS stained and the number of primordia and emerged lateral roots were quantified.

For experiments involving magenta boxes (9.5 X 6.5 X6.5 mm), boxes were filled halfway with control agar. The magenta box has a vented top to allow gas exchange. A thin layer of control agar and charcoal was poured on top of control agar. Foil was tightly wrapped around the outside of box to the height of the agar and taped in place. Seeds were germinated on top of the charcoal/agar layer. At three days after sowing, seedlings were transferred to light banks of 8, 12, 16, 20, or 24 hours. After six additional days of growth, seedlings were carefully pulled from agar, GUS stained and the number of primordia and emerged lateral roots were quantified.

For experiments involving growth temperatures, seedlings were grown on Petri dishes containing control media in 24 hours of light in growth chambers set at 18, 21, 24, 26, 28, or 30 ºC. At three days after sowing a light cycle of 16 hour day and 8 hour night was implemented for all growth chamber conditions. After six additional days of growth,

seedlings were GUS stained and the number of primordia and emerged lateral roots was quantified.

Initiation and elongation measurement

After treatments, activity from the *cycl1At*-GUS reporter was detected histochemically. Seedlings were transferred from agar to GUS staining solution. They were stained overnight at 37^{o} in 2 mM X-glucuronide dissolved in 0.5 mM K ferricyanide, 0.5 mM K ferrocyanide, 0.5% Triton X-100 and 100 mM sodium phosphate buffer, pH 7.0 (Craig, 1992). After staining was complete, tissue was washed in 100 mM sodium phosphate buffer three times followed by 70% ethanol. Seedlings were stored in ethanol until analysis under a compound microscope. Mutants were cleared using the following method found in (Dubrovsky et al., 2009). Roots were fixed in 4% formaldehyde solution buffered to pH 7.2 for 4 hour to overnight. The fixative was replaced with 30% glycerol containing 2% of DMSO and left for at least 30 minutes at ambient temperature. The roots were mounted into clearing solution.

The length of each seedling's root was determined with a ruler. The roots were placed on a microscope slide with cover and examined. The number of primordia at stages I-VII (as defined by Malamy and Benfey, 1997) were quantified as were emerged lateral roots.

Auxin transport

For the ^{3}H-IAA experiment involving day lengths and growth temperatures, seedlings are grown as described above. For growth temperature experiments, the plants were transported from the location of growth (UNC-Greensboro) to the location of the transport assay (Wake Forest University) which took approximately 45 minutes. The

plants were placed in growth chambers at WFU for approximately two hours to acclimate to the growth temperatures. A concentration of 1µM ^{3}H-IAA was added to warm 1.0% agar media. 5µl agar droplets containing ^{3}H-IAA were allowed to harden in a Petri dish. One droplet was placed on the root/shoot junction. After the ^{3}H-IAA droplet was placed on the root/shoot junction, seedlings were placed at the indicated temperature for two hours. At the designated time, a 3mm section was cut 5mm below the root/shoot junction. This section was put individually in scintillation vials and placed in a scintillation counter, the Beckman LS6500 and radioactivity was determined.

Mutant experiments

For experiments involving day lengths and mutants, seedlings of different mutants were grown on Petri dishes containing control media. At three days after sowing seedlings were transferred to light banks of 8, 16, or 24 hours. At six days after sowing seedlings were transferred to new control media with 10 plants per Petri dish. After three additional days of growth, seedlings were cleared and the number of primordia and emerged lateral roots was quantified. Transport mutants, *pin3*, *aux1*, *aux1lax3*, *lax3*, and *pin7,* and *axr1-3*, a signaling mutant, were used.

Auxin accumulation

For experiments involving auxin accumulations for day length and growth temperature, seeds were densely sown in a straight line for easy harvesting. Seedlings were grown for nine days after sowing in their respective conditions. At nine days after sowing, the shoot was excised from the root. Approximately 50-80 mg of root or shoot tissue was frozen separately using liquid N_2. Frozen tissue was homogenized with a bead beater in 150µl homogenization buffer (35% of 0.2M imidazole, 65% isopropanol, pH 7),

containing 4ng of ^{13}C-IAA as an internal standard IAA. The samples were incubated on ice for an hour, and were subjected to centrifugation at 10,000g for 8min. The homogenate was transferred to deep 96 well plates and samples were purified over two successive columns (amino anion exchange column and SPE columns with polymethymethacrylate epoxide resin) using an automated robot system. The extract collected was then treated with diazomethane, dried using N_2 and redissolved in 20-25 µl of ethyl acetate. The samples were then analyzed using gas chromatography mass-spectroscopy. The free IAA was calculated using a ^{13}C-IAA internal standard (Barkawi et al., 2008).

Auxin induced gene expression

For experiments involving determination of auxin induced gene expression as a function of day length and growth temperature, seedlings were grown as described above. The roots of 10-15 GH3-GUS seedlings (approximately 1-2mg of tissue) were excised and frozen in liquid nitrogen. Tissue was ground in 100µL extraction buffer. Extraction buffer consists of 50mM sodium phosphate buffer, pH 7.0; 10µM DDT, 1mM Na_2EDTA, 0.1% sodium lauroyl sacrsosine; and 0.1% Triton X-100. The tissue was subjected to centrifugation at 13,000 rpm for five minutes in a 4ºC microcentrifuge. 50µL aliquot of the supernatant was added to 500µL of prewarmed extraction buffer containing 0.22mg of MUG. MUG fluorometric assays are used for quantitative analysis of ß-glucuronidase (GUS) activity. The reaction was incubated at 37ºC. A 100µL aliquot of the reaction was added to 900µL of the MUG stop buffer at 2, 4, 6, and 8 hour time points. The stop buffer consists of 0.2M Na_2CO_3. Fluorescence was detected by a Corning 96 well plate reader with CW-lamp filter F485. Details of the MUG assay were described in (Lewis et

al., 2007). Data was normalized relative to protein concentrations as detected by a Bradford protein assay using manufacturer's specification.

Statistics

Student *t*-tests were employed to identify statistical difference between controls and treatment groups. Individual t-tests were performed on independent experiments (plates containing 10 seedlings) to ensure that all data from individual plates could be pooled. Once pooled, unpaired *t*-tests were used to examine the range for the actual mean of each sample. A statistical program from the website http://www.physics.csbsju.edu/stats/t-test.html was used to calculate whether statistical differences exist between experimental treatments and controls using a Student's *t*-test. Averages and SE were calculated using excel. One-way ANOVA were used for the day length and temperature experiments followed by Tukey–Kramer multiple test for some analyzes.

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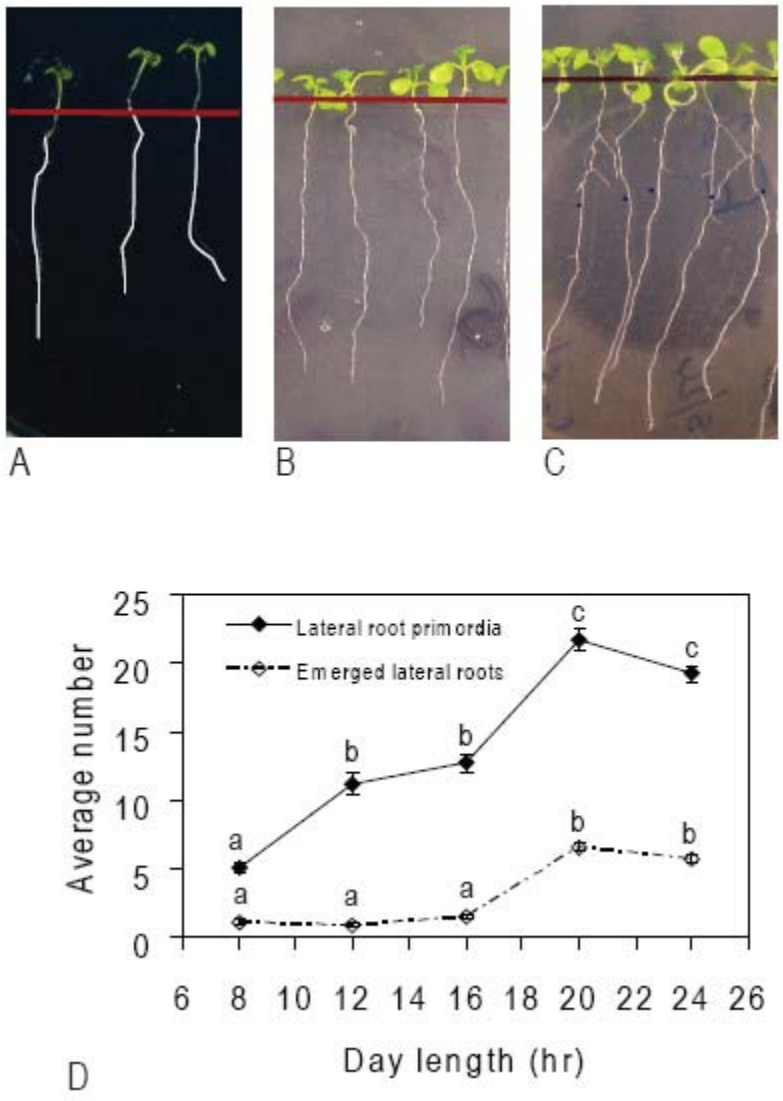

Figure III-1. Lateral root formation and emergence are positively regulated by increasing day length. A, example of seedling grown at 8 hour day length; B, example of seedling grown at 16 hour day length; C, example of seedling grown at 24 hour day length; D, Seedlings were grown on Petri dishes at indicated day lengths until 9 DAS and the number of lateral root primordia and emerged lateral roots were determined for approximately 65 seedlings in 5-7 separate experiments for each day length. "a", "b", and "c" indicate differences from each other. The effect of day length

on average number of primordia and emerged lateral roots was examined by ANOVA followed by Tukey-Kramer multiple test, ($P<0.01$).

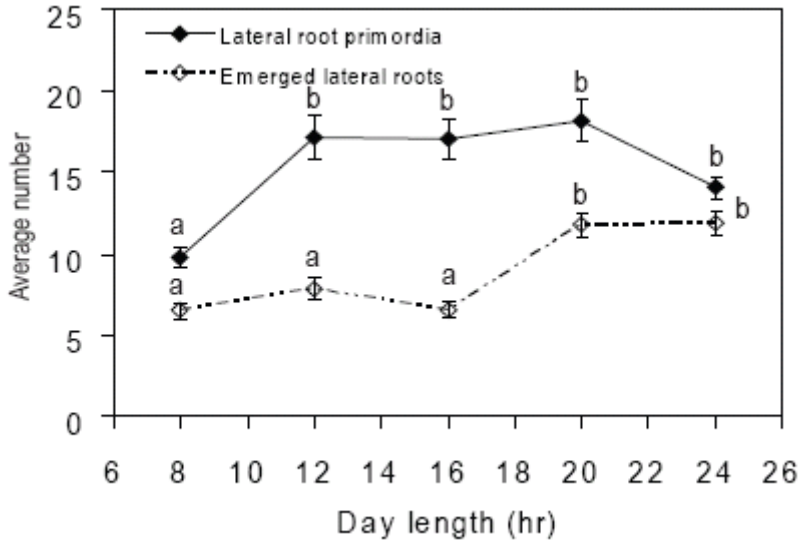

Figure III-2. Shaded roots exhibited day length dependent root formation. Seedlings were grown at indicated day lengths until 9 DAS and the number of lateral root primordia and emerged lateral roots were determined for approximately 50 plants in 3-5 separate experiments. The effect of day length on average number of primordia and emerged lateral roots was determined by one-way ANOVA, ($P<0.05$). "a" indicates values that are significantly different from "b".

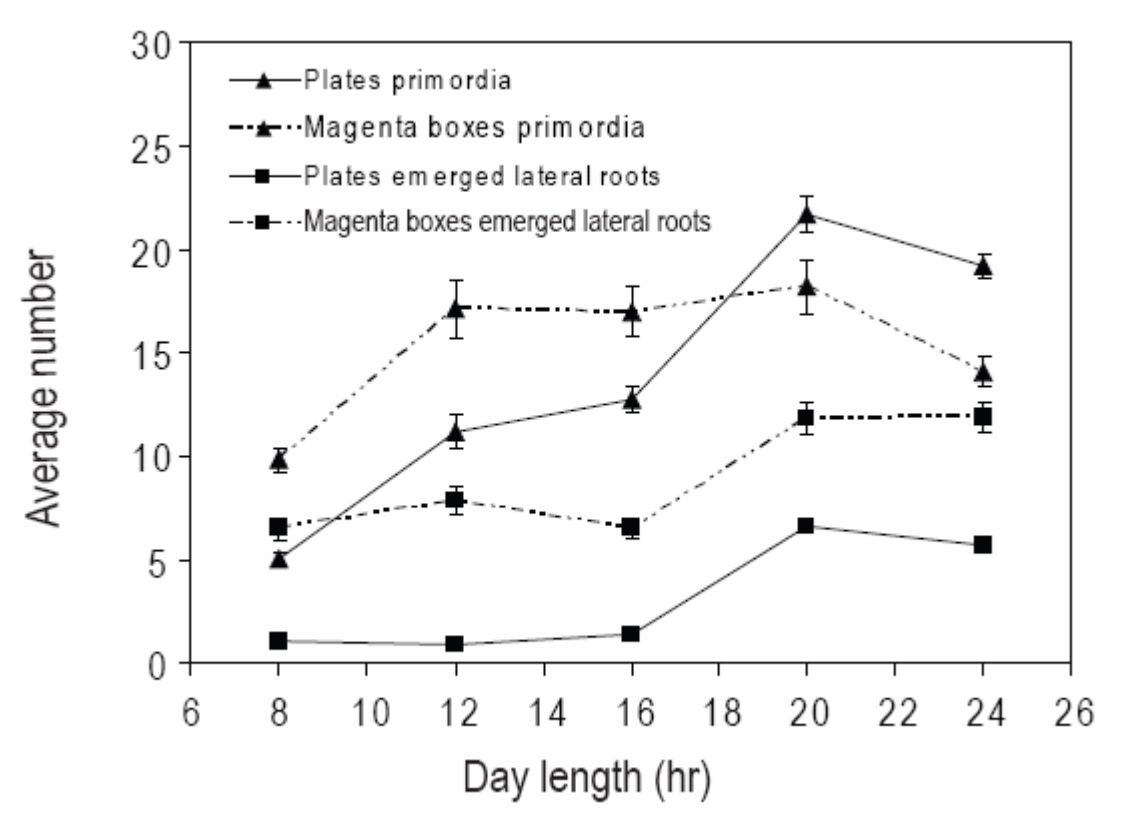

Figure III-3. A comparison of lateral root primordia and emerged lateral roots from plate grown and magenta grown seedlings exhibits similar patterns of growth. The graph is the combined data from Figures III-1 and III-2, to allow direct comparisons of seedlings grown on Petri dishes versus seedlings grown in magenta boxes.

Table III-1. Effect of vertical plates (top) and magenta boxes (bottom) on primary root growth, lateral root initiation, emergence, and density in *Arabidopsis*. Nine day old seedlings were either germinated and grown on vertical Petri dishes (control) or in magenta boxes with foil covering the outside of the box (up to the level of agar) and a thin layer of charcoal and agar on top of the existing agar. After nine days of growth, the number of initiated lateral roots, emerged lateral roots, and primary root lengths were determined. The reported values are averages ± SE of 65 seedlings from 5-7 separate experiments and 50 seedlings from 3-5 separate experiments respectively.

Day length (hr)	Initiated (I) lateral root	Emerged (E) lateral root	Length of primary root	I density	E density
8[a]	5.0±0.38	1.1±0.15	23.6±0.60	0.22±0.02	0.05±0.01
12[a]	11.2±0.81	0.9±0.15	29.0±0.59	0.39±0.03	0.03±0.01
16[a]	12.7±0.65	1.4±0.19	26.3±0.68	0.49±0.02	0.05±0.01
20[a]	21.8±0.85	6.6±0.24	35.3±0.42	0.62±0.03	0.19±0.01
24[a]	19.3±0.58	6.0±0.29	37.3±0.44	0.52±0.02	0.15±0.01
8[b]	9.8±0.61	6.5±0.53	25.6±0.81	0.39±0.02	0.26±0.03
12[b]	17.2±1.40	7.9±0.67	29.1±0.66	0.59±0.04	0.27±0.02
16[b]	17.0±1.20	6.6±0.52	26.1±0.93	0.66±0.04	0.25±0.02
20[b]	18.2±1.30	11.8±0.73	30.9±0.87	0.60±0.04	0.39±0.02
24[b]	14.1±0.69	12.0±0.70	28.8±0.93	0.51±0.02	0.43±0.03

[a] represents seedlings grown on Petri dishes. [b] represents seedlings grown in magenta boxes

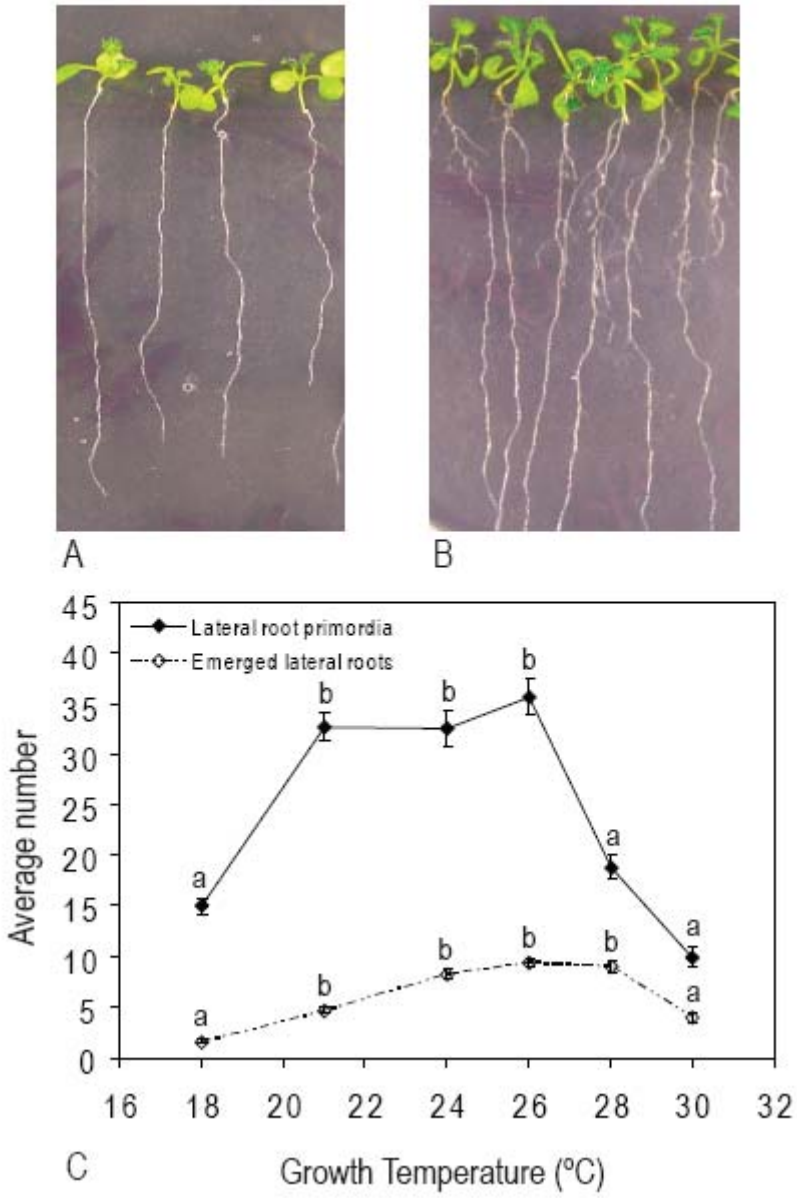

Figure III-4. Lateral root primordia and emergence exhibit temperature dependence. A, example of seedling grown at 22ºC; B, example of seedling grown at 27ºC; C, At 3 days after sowing under uniform light (24 hours) on Petri dishes at indicated growth temperatures, seedlings were transferred to a light cycle 16 hr day / 8 hr night. After 6 additional days of growth, the average and SE of the number of lateral root primordia and emerged lateral root for 45 plants per treatment were determined. The effect of growth temperature on average number of primordia and emerged lateral roots

was determined by a one-way ANOVA, ($P<0.02$). "b Indicates values that are significantly different from "a".

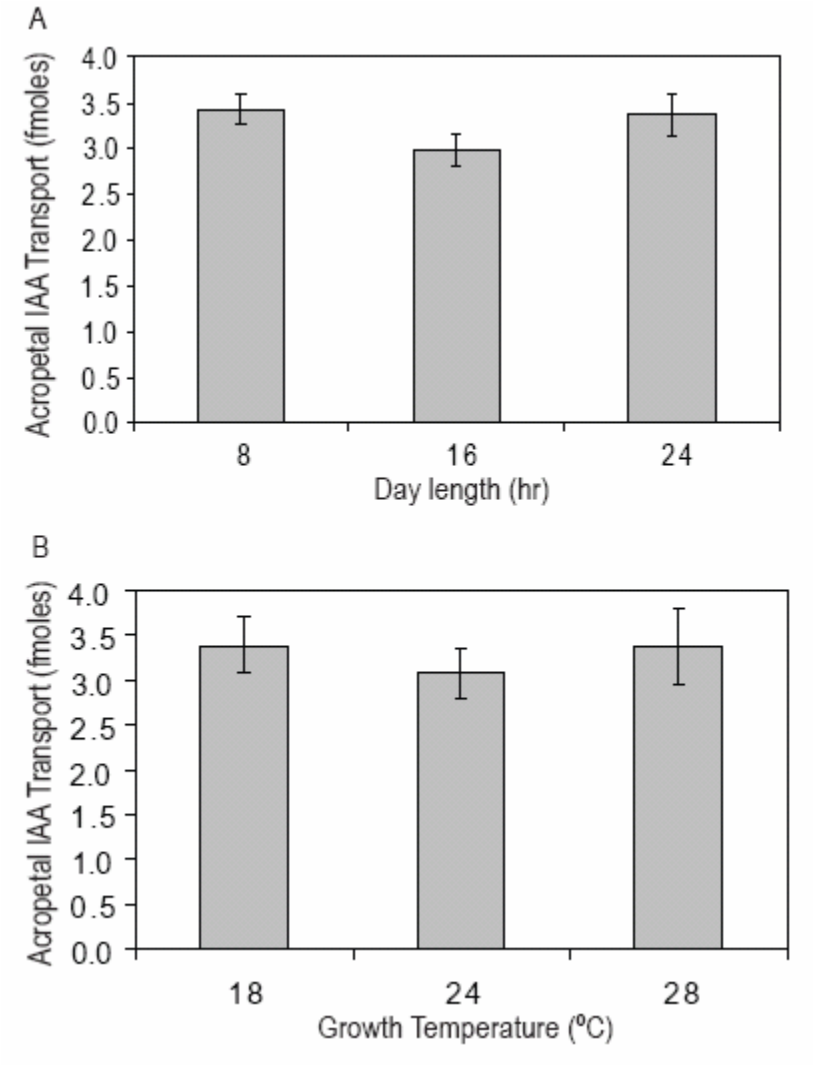

Figure III-5. Auxin transport capacity is not changed with increasing day length or growth temperature. A, Seedlings were grown at the indicated day length and transport was measured in plants that were nine days after sowing. The average and standard error of 65 plants per treatment are reported. The effect of day length on acropetal IAA transport was examined by Student's t-test and there was no statistical difference between all three samples, ($P>0.05$); B, Plants were grown on Petri dishes at 16 hour days at the indicated temperature. The average and standard error of 45 plants per treatment are reported. The effect of transport and growth temperature was examined by Student's t-test and there was no statistical difference, ($P>0.05$).

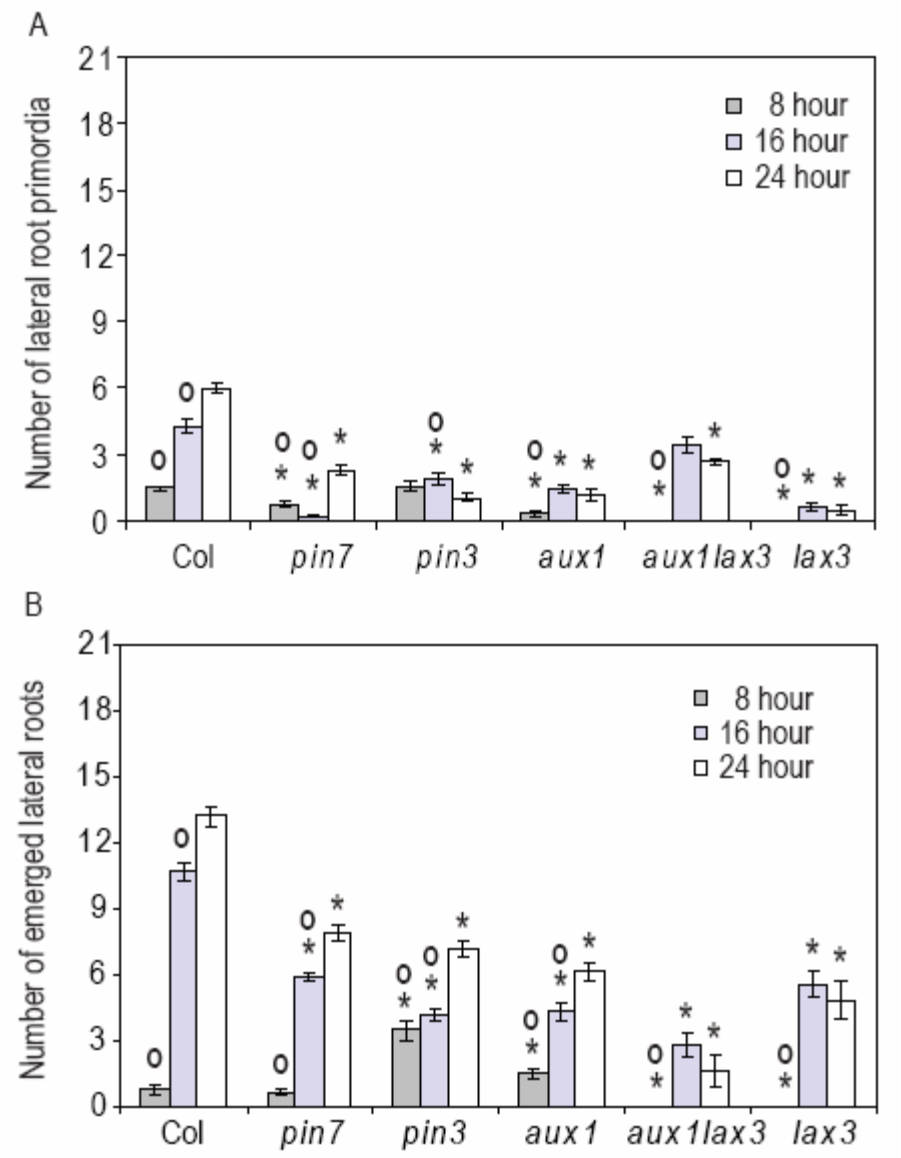

Figure III-6. Auxin transport mutants exhibit a significantly different pattern in lateral root primordia, but not emerged lateral roots. Seedlings were grown at indicated day lengths until nine DAS and the average and SE of lateral root primordia and emerged lateral roots were determined for approximately 35 seedlings in 3-4 separate experiments for each day length. A, The number of lateral root primordia of transport mutants grown at different day lengths are reported. B, The number of emerged lateral roots of transport mutants grown at different day lengths are reported. The effect of day length on lateral root primordia and emerged lateral roots of transport mutants were

examined by Student's t-test, ; * Indicates all significant differences as compared to wild type, ($P<0.03$); º Indicates significant differences as compared to 24 hour day length within genotype, ($P<0.04$).

Table III-2. Effect of transport mutants with defects in influx and efflux carriers on primary root growth, lateral root initiation, emergence, and density in *A. thaliana*. Seedlings germinated and grew for nine days when they were cleared. Number of initiated lateral roots, emerged lateral roots, and primary root length were determined. The reported values are averages ± SE of approximately 35 seedlings from 3-4 separate experiments.

Day length (hr)	Genotype	Initiated (I) lateral roots	Emerged (E) lateral roots	Length of primary root	I density	E density
8	Columbia	1.5 ± 0.13	0.8 ± 0.24	20.6 ± 0.63	0.07 ± 0.01	0.04 ± 0.01
8	*pin7*	0.7 ± 0.15	0.7 ± 0.14	13.9 ± 0.30	0.05 ± 0.01	0.05 ± 0.01
8	*pin3*	1.6 ± 0.21	3.5 ± 0.42	21.5 ± 0.73	0.07 ± 0.01	0.16 ± 0.02
8	*aux1*	0.3 ± 0.11	1.5 ± 0.20	20.8 ± 0.58	0.02 ± 0.01	0.07 ± 0.01
8	*aux1lax3*	0.0 ± 0.00	0.0 ± 0.00	19.0 ± 0.45	0.00 ± 0.00	0.00 ± 0.00
8	*lax3*	0.0 ± 0.00	0.0 ± 0.00	30.1 ± 0.83	0.00 ± 0.00	0.00 ± 0.00
16	Columbia	4.3 ± 0.30	10.7 ± 0.40	40.8 ± 0.54	0.10 ± 0.01	0.26 ± 0.01
16	*pin7*	0.2 ± 0.06	5.9 ± 0.20	11.4 ± 0.26	0.02 ± 0.01	0.53 ± 0.02
16	*pin3*	1.9 ± 0.27	4.2 ± 0.26	20.4 ± 0.46	0.09 ± 0.01	0.21 ± 0.01
16	*aux1*	1.4 ± 0.21	4.4 ± 0.42	19.8 ± 0.73	0.07 ± 0.01	0.23 ± 0.02
16	*aux1lax3*	3.5 ± 0.32	2.8 ± 0.54	13.3 ± 0.36	0.27 ± 0.03	0.22 ± 0.04
16	*lax3*	0.6 ± 0.21	5.6 ± 0.61	29.2 ± 0.97	0.02 ± 0.01	0.21 ± 0.03
24	Columbia	6.0 ± 0.24	13.2 ± 0.42	42.4 ± 0.84	0.14 ± 0.004	0.32 ± 0.01
24	*pin7*	2.3 ± 0.24	7.9 ± 0.33	21.8 ± 0.68	0.10 ± 0.01	0.38 ± 0.02
24	*pin3*	1.0 ± 0.17	7.2 ± 0.34	22.7 ± 0.70	0.04 ± 0.01	0.32 ± 0.02
24	*aux1*	1.2 ± 0.28	6.2 ± 0.43	19.5 ± 0.85	0.06 ± 0.02	0.33 ± 0.02
24	*aux1lax3*	2.7 ± 0.31	1.7 ± 0.37	12.9± 0.39	0.21 ± 0.02	0.13 ± 0.03
24	*lax3*	0.5 ± 0.14	4.9 ± 0.71	29.4 ± 0.90	0.02 ± 0.01	0.17 ± 0.03

Table III-3. Pattern of emerged lateral roots under increasing day length is consistent with pattern of increasing IAA accumulation in roots but not in shoots. Seedlings were grown at 24 hours of light for three days and then transferred for six additional days of growth in indicated day length. Seedlings were harvested separately as root or shoot tissue and frozen. IAA accumulation was measured for root and shoot. The number of replicates for root samples was 5-6 and 3 for shoot samples; each root sample contained approximately 100-200 roots while the shoot samples contained approximately 20-30 shoots.

Day length (hr)	8	16	24
Shoot free auxin (ng/gfw)	7.75 ± 0.61	11.76 ± 0.59*	8.37 ± 1.60
Root free auxin (ng/gfw)	9.13±0.56	9.11±0.56	11.53±0.62*
Number of primordia	5 0±.38	12.7±0.65*	19.3±0.58*
Number of emerged LR	1.1±0.15	1.4±0.19	5.7±0.29*

* Indicates which value are statistically different from 8 hr day length as judged by Student's t-test, ($P<0.009$).

Table III-4. Pattern of emerged lateral roots under increasing temperature is consistent with pattern of root IAA accumulation.

Seedlings were grown in indicated growth temperature with 24 hours of light. At 3 days after sowing, the light cycle was changed to 16 hr day / 8 hr night. Seedlings were harvested separately as root or shoot tissue and frozen. IAA accumulation was measured for root and shoot. The number of replicates was 5-7 for root samples and 3 for shoot samples; each root sample contained approximately 100-200 roots while the shoot samples contained approximately 20-30 shoots.

Temperature (ºC)	18	24	28
Shoot free auxin (ng/gfw)	7.80 ± 0.37	7.10 ± 0.34	6.37 ± 0.17*
Root free auxin (ng/gfw)	7.66 ± 0.30	9.54 ± 0.33*	10.0 ± 1.12*
Number of primordia	15.0 ± 0.86	32.5 ± 1.78*	18.8 ± 1.22
Number of emerged LR	1.6 ± 0.22	7.5 ± 0.41*	9.0 ± 0.51

* Indicates which value are statistically different from 18ºC growth temperature as judged by Student's t-test, ($P<0.04$).

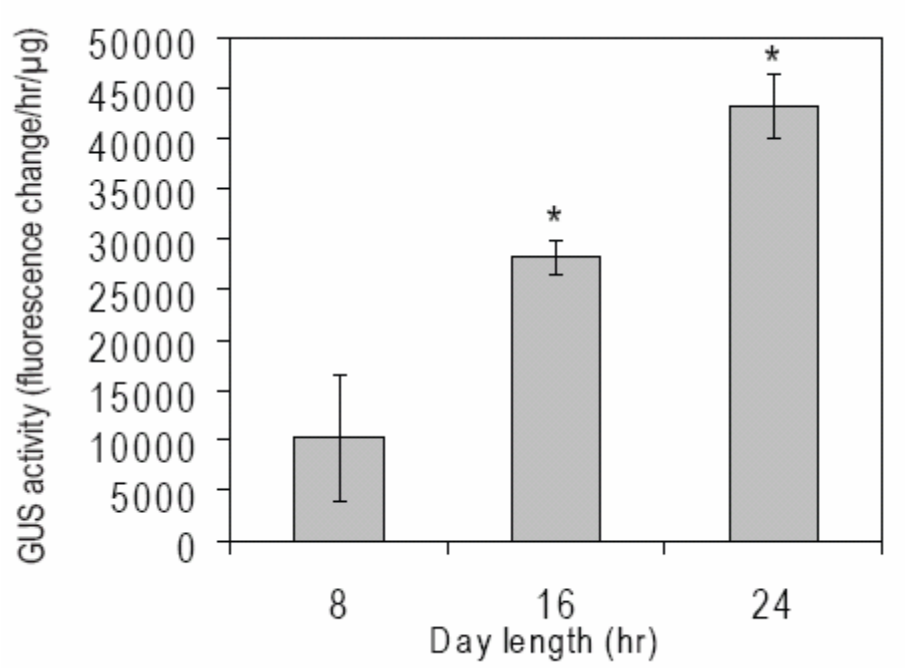

Figure III-7. Expression of the auxin response GH3-GUS reporter increases with increasing day length. At 3 days after sowing, under 24 hours of light, seedlings were transferred to the indicated day lengths. After 6 additional days of growth, the ß-glucuronidase activity was determined using a MUG substrate. The average and standard error are reported. The effect of day length on auxin induced gene expression was examined by Student's t-test as compared to the 8 hour day length, (* $P < 0.05$).

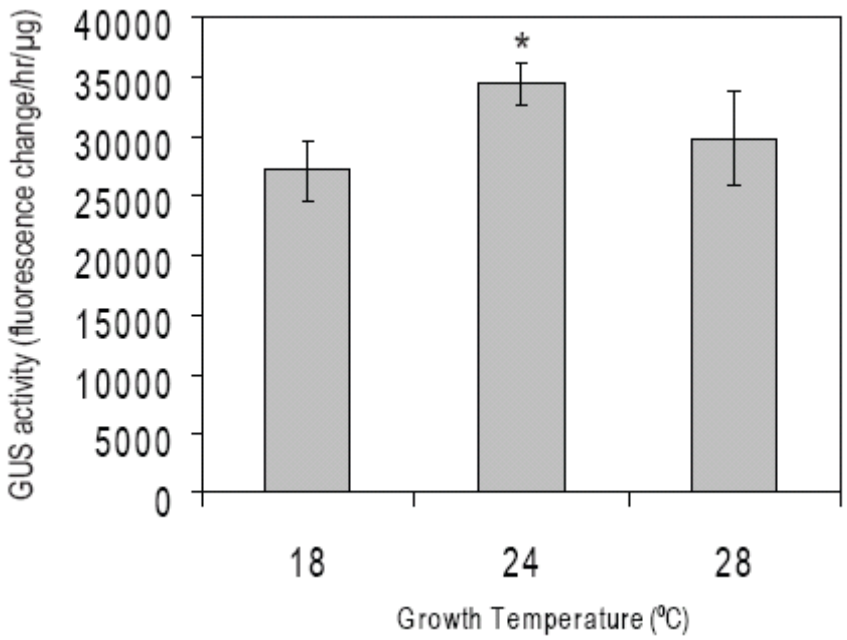

Figure III-8. Expression of the auxin response GH3-GUS reporter is consistent with pattern of lateral root phenotype with increasing growth temperatures. At 3 days after sowing under uniform light (24 hours) at the indicated growth temperature, seedlings were transferred to a light cycle of 16 hr day / 8 hr night. After 6 additional days of growth, the ß-glucuronidase activity was determined using MUG substrate. The average and standard error per treatment are reported. The effect of growth temperature on auxin induced gene expression was examined by Student t-test as compared to 18ºC, (* $P < 0.05$).

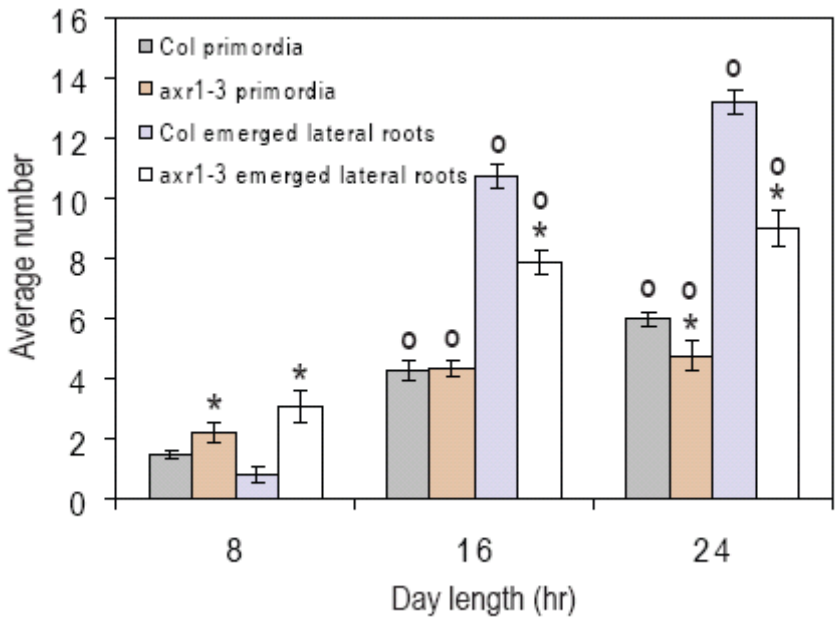

Figure III-9. *axr1-3*, a signaling mutant, increases number of lateral root primordia and emerged lateral root with increasing day length. At 3 days after sowing, under 24 hours of light, seedlings were transferred to the indicated day lengths. After 6 additional days of growth, roots were cleared and the number of lateral root primordia and emerged lateral roots were quantified. Averages and standard errors were calculated for approximately 30-35 seedlings for 4 separate experiments are reported. The effect of day length on *axr1-3* was examined by a Student t-test; * indicates a statistically significant difference as compared to 8 hour day length between axr1-3 and wild type, ($P<0.04$); º indicates a statistically significant difference as compared to 8 hour day length within a genotype, ($P<0.0001$). The number of primordia and emerged lateral roots for *axr1-3* were profoundly different from control at all day lengths.

CHAPTER IV

ARABIDOPSIS THALIANA ROOT DEVELOPMENT AND RESOURCE ALLOCATION IN POPULATIONS ISOLATED ACROSS A LATITUDINAL GRADIENT

Abstract

This study focused on the possibility that populations isolated from different latitudes may have genetic changes that alter root development and architecture. Environmental variables across a latitudinal gradient have been shown to affect root growth, development, and physiological responses. We hypothesized that early root architecture, as well as resource allocation, would differ between populations isolated from different latitudes. We also hypothesized that in mature plants, biomass allocation and carbon exchange rate would vary among populations across latitude.

Nine European *Arabidopsis thaliana* populations from three different latitudes (40ºN-60ºN) were used to test these hypotheses through a common garden experiment. We found a chamber effect potentially linked to UV differences. In early lateral root development in higher UV, there was a latitudinal cline; in populations from higher latitude, there were fewer lateral root primordia and emerged lateral roots. In developing seedlings, there was less shoot biomass in populations isolated from higher latitude; root biomass was similar in all populations. In lower levels of UV, the photosynthetic rate was lower in populations from higher latitudes, leading to a different carbon balance. In this experiment, root respiration and root biomass was the same in populations across latitude. In the absence of UV, there was more shoot biomass in populations from higher latitude. Together these experiments indicate that root development, shoot development,

and resource allocation vary between populations, with complex relationships influenced by light quality.

Keywords: Arabidopsis thaliana, carbon exchange, lateral root architecture, biomass allocation, genetic variation

Introduction

Root development is a highly plastic process that is modulated by environmental and genetic factors. The root senses local cues like water and nutrient availability and the shoot senses environmental cues such as day length, light intensity, light quality, and ambient temperature. Complex signaling pathways exist between the roots and the shoot to coordinate growth and development to maximize growth and fitness. Lateral roots, in particular, are influenced by external cues that influence the shoot (Malamy and Ryan, 2001). One third of the earth's land surface is arid and the entire surface is subject to periodic drought long enough to inhibit plant growth. Nutrient limitations such as N or P are nearly universal and the imbalance of pH and salinity in native soils often leads to the plant's inability to access soil nutrients (Lynch, 1995). Thus the development of an optimal root system is a key factor in a plant's ability to survive adverse conditions. *Arabidopsis thaliana* is a well studied plant model. Natural populations of this species are especially interesting because there are potential selective pressures that might give rise to genetic variation due to the latitude of the source population.

Environment and genes act upon a basic program of root development. Primary roots are formed within the embryo and are usually the first tissue to emerge during germination. The primary root elongates and lateral roots initiate from what was previously the non-dividing tissue of the pericycle. There is branching of lateral roots with secondary and tertiary branching (Rose, 1983; Charlton, 1983). Adventitious roots grow from the stem above ground and increase stability (Bray, 1954). The extensive branching allows exploration of a substantial soil volume resulting in efficient nutrient

and moisture uptake. Root hairs extend from epidermal cells along primary and lateral roots and greatly increase the absorptive surface of the root (Fitter, 1987).

In chapters two and three of this thesis, we found that auxin synthesized in the shoot controls lateral root development and that the environmental variables, day length and growth temperature, have profound effects on root architecture and growth. Auxin is involved in the mechanistic underpinnings of this plasticity. Day length and temperature, which affect optimal growth and reproduction of a species, vary across a latitudinal gradient; this likely affects nutrient and water availability. This suite of environmental cues may have led to the evolution of genetically different populations with root architecture best suited to a specific ecosystem.

Plants use sunlight as an energy source via photosynthesis and as a signal to activate and modify endogenous developmental processes. In turn, developmental processes depend partly on the energy available for growth. Plants sense light via three known classes of photoreceptors (phytochromes and phototropins), and crypochromes, with the former two affecting the root system (Casal, 2002; Chen et al., 2004; Casal, 2000). Although phytochromes play a large role in the light signaling pathways in the shoot, there is evidence for phytochrome activity within the root system controlling phototropism (Correll et al., 2003; Kiss et al., 2003; Ruppel et al., 2001). Salisbury et al. (2007) found the *phyD* mutant, which has a defect in a gene encoding one of four phytochromes, had slightly more lateral root production than wild type while mutations in the other members of the gene family, *phyA, B, E* showed reduced lateral root outgrowth, suggesting that phytochromes play a role in controlling root development.

Phototropins mediate a blue light dependent directional growth of the plant stem which leads to bending toward a unilateral light source (Chen et al, 2004). The mutant, *phot1*, was identified for the hypocotyl's inability to bend toward unilateral blue light (Liscum and Briggs, 1995). Galen (2007) found that the presence of PHOT1 increases fitness in *A. thaliana* in the field. The protein functions to inform growing roots of their position relative to the soil surface and potentially promotes drought tolerance by enhancing the efficiency of root growth away from the soil surface (Galen, 2007).

Analysis of the growth of plant tissues, such as the hypocotyl, leaves, and flowering stems, can be used to examine potential genetic variation in a latitudinal gradient that result in different responses to light quality. Light quality, intensity, and day length vary across latitude which may result in genetic changes that allow plants to thrive und these variable conditions. Stenøien et al. (2002) found a latitudinal cline in hypocotyl response to red and far-red treatments across Norway (59ºN to 67ºN). Northern populations had more de-etiolated hypocotyls than southern populations after red and far-red treatments; Stenøien et al. (2002) suggested that the northern populations are more sensitive to these stimuli. Clapham et al. (1998) observed increasing requirement for far-red light to maintain growth and prevent budset in *Picea abies* seedlings as latitude of origin increased. Using natural populations of *A. thaliana*, Maloof et al. (2001) suggested that light intensity is an important environmental factor driving latitudinal shifts in hypocotyl length. They speculated that plants at lower latitudes compensate for higher light intensity by being less sensitive to light while northern strains are on average more light sensitive, thereby apparently compensating for lower ambient light intensity at these latitudes.

Day length, which changes with latitude, regulates a wide range of developmental processes including bud formation and dormancy, stem elongation, formation of storage organs, vegetative reproduction, lateral root production, and flowering (Chazdon and Pearcy 1986). Banta et al. (2007) found that when populations were grown in the environmental conditions, including photoperiod, consistent with the latitudinal provenance from which they were isolated, time to bolting increased from south to north; number of rosette leaves increased from south to north; and rosette diameter decreased from south to north. In a common garden experiment, Li et al. (1998) examined 40 populations of *A. thaliana* from a latitudinal gradient (16-63ºN) which exhibited genetic variation in plant size and relative growth rate. Populations from higher latitudes had smaller seeds, cotyledon width, rosette size, number of rosette leaves, size of largest leaves, total leaf area, and total dry weight per plant. By growing plants in a common garden, they removed the environmental variation in day length and examined the possible effects of genetic variation that have evolved in populations from these distinct lattitudes.

Seasonal changes include differences in day length, which control flowering time in *A. thaliana* (Engelmann and Purugganan, 2006). Treatments that mimic changes relative to the environment lead to abortion of flowers and very young seeds, but also allow for the formation of new flowers and the production of normal siliques. The ability to adjust patterns of assimilation, storage, and utilization of carbon in response to changes in the environment may determine fitness in terms of reproductive success (Engelmann and Purugganan, 2006). In *A. thaliana*, one might expect different patterns of carbon acquisition, storage, and utilization in populations isolated across latitudinal gradient.

For example, if a plant is genetically programmed to avoid heat and water stress by growing and flowering early, then carbon exchange might be different than in a plant which is not under the same constraints.

Schlichting and Smith (2002) found there are plastic responses that originate at a cellular level which receive and process signals from the environment. For example, plants can adjust their carbon assimilation and storage to exactly serve the anticipated demand during the following night. This implies that the rate of starch synthesis in the day is set by mechanisms that anticipate the amount of carbon required in the night (Chatterton and Silvius 1979, 1980, 1981; Jablonski and Geiger 1987; Lorenzen and Ewing 1992; Matt et al. 1998; Gibon et al. 2004). *A. thaliana* plants can adjust in this way to an extensive range of day lengths including days as short as 4 hours. Adjustment is also seen in response to changes in light level (Smith and Stitt, 2007).

Growth temperature is also a driving force behind variation in resource acquisition and storage across a latitudinal gradient. Zhen and Ungerer (2008) found a steep latitudinal cline in freezing tolerance among natural populations of *A. thaliana*. Potvin (1986) used *Echinochloa crus-galli* grown in controlled environments under different temperatures. The northern plants exhibited the highest rates of photosynthesis (Potvin and Strain 1985). This high photosynthetic activity is coupled with a more efficient biomass allocation (Potvin, Goeschl and Strain, 1984). These data indicate that southern and northern populations have different physiological tolerance to low temperature as well as different growth patterns. This grass is a perennial, however, and can not be directly compared to an annual in resource allocation.

Temperature and precipitation along a latitudinal gradient affect how a plant might acquire and store resources. Temperature cues affect moisture content of the plant. Photosynthesis is greater in northern populations for ericaceous shrubs (Llorens et al., 2004) and for shrub land species, (Peñuelas et al., 2004), the authors found that plants from cold/wet northern sites were more sensitive to warming and that plants from warm/dry southern sites were more sensitive to drought. Water availability and temperature also play a role in root system architecture. In general, in moist fertile regions, roots grow extensively until the water resources become limited (Shaver and Billings, 1975). If water is available deeper then the roots grow down in a process called hydrotropism (Takahashi, 1997). Grainer (2006) found that for nine populations of *A. thaliana*, different populations required different amounts of soil water content for maximum efficiency. One population was more successful at low soil water content indicating genetic adaptation to a specific ecosystem. Bray (2004) found that cellular water deficit stress triggers many changes in gene expression which can be used to define the response of a plant to an environmental condition. Roots are often less affected by drought stress than the shoot. In severe drought, the shoot may stop growing completely while the root continues to explore the soil for water (Bray, 2004). van der Weele et al. (2000) found changes in water deficit changed root architecture. With moderate water stress, lateral roots were unaffected but with severe water stress lateral root development and elongation were arrested. Severe stress partially reduced the growth of the primary root (van der Weele et al., 2000).

A. thaliana is often used as a plant model because its genetics are well characterized and a diversity of mutants, transgenics, and natural populations have been

identified. It is also useful in ecological and evolutionary studies because it is a small annual weed distributed throughout Europe, spanning a latitudinal range from northern Africa to the Arctic Circle. This diversity of natural populations offers variation in a number of interesting traits, including root branching (Andalo, et al., 1999; Banta et al., 2007; Stratton and Bennington, 1996; Dorn et al., 2000; Stinchcombe, et al., 2004).

Using this expansive range, scientists can potentially identify genetic variation in a specific trait and identify the function of individual genes (Koornneef et al., 2004; Hoffman,2002). Christophe et al. (2008) used *Arabidopsis thaliana* as a model plant to simulate growth at the whole-plant scale. They describe the first model of *Arabidopsis* growth which combines organogenesis, morphogenesis, and carbon-partitioning processes for above ground and below ground plant tissue throughout its development. Most of the latitudinal gradient studies have not examined the potential genetic variation and corresponding physiological responses in root development in populations across latitude when growth temperature, nutrients, day length, and water availability are held constant. This study examines nine different natural populations of *Arabidopsis thaliana* within and across latitude during early and mature vegetative development to understand the variation in root development and whole-plant carbon allocation. Specifically, we tested the following hypotheses:

1. Given that root development is affected by environmental variables such as moisture, temperature, and light, early root development including lateral root primordia and emerged lateral roots, and biomass allocation will vary among populations from different latitudes.

2. Given the plastic response of roots to environmental variables in early development, biomass allocation will vary among populations of various latitudes in a similar pattern in the mature plant.

3. Given that carbon uptake determines the energy available for growth and maintenance respiration to all organs, trade-offs exist among different populations adapted to different environments.

Results

Overall treatment effect

For most measurements there was a chamber effect so chambers were considered separately, as determined by ANOVA analysis followed by Tukey-Kramer multiple test. Each chamber had identical environmental conditions except for UV levels. We will refer to the chambers as "lower UV levels" and "higher UV levels" even though there is no definitive proof that the differences we observe here are due to UV levels.

Lateral root primordia and emerged lateral roots were lower in populations from higher latitudes in the low UV chamber.

To assess the potential for genetic variation in early root development in *Arabidopsis* populations of different latitudinal provenance, we asked whether the number of lateral root primordia and emerged lateral roots vary among populations isolated from different latitudes. The pattern of emerged lateral roots for all populations are shown in photographs of young seedlings from all these populations at 21 day after sowing for plants grown in the higher UV chamber, as shown in Figure IV-1. Qualitatively, in northern populations, most lateral roots are near the root/shoot junction in a tight cluster. In the middle latitude populations, one sees the same clustering near the root/shoot junction but the lateral roots are longer and more intricate (more secondary and tertiary lateral roots). In the southern populations, there are more lateral roots but they are shorter and they are evenly dispersed along the primary root. .

After 21 days of growth on agar plates, the number of lateral root primordia and emerged lateral roots were counted for each population and an ANOVA was performed to determine if there were significant differences of latitude and population on early

development and biomass, as shown in Table IV-1. There was a significant effect of chamber for lateral root primordia and emerged lateral roots but not for shoot or root weight. There was an effect of latitude on shoot weight in both chambers but no effect for root weight in either chamber. The numbers of lateral root primordia and emerged lateral roots are shown in Figure IV-2. With higher UV levels, there were fewer primordia and emerged lateral roots in populations from higher latitudes. In contrast, at lower UV levels, the average number of primordia or emerged lateral roots from populations across latitudes was not significantly different.

Length of primary roots and density of lateral roots are also important measures of root architecture. The variation among populations from different latitudes in primary root growth and the resulting density of lateral roots are reported in Table IV-2. At higher UV levels, root length was not statistically different for populations across latitude, yet there were fewer primordia and emerged lateral roots in populations from higher latitudes. Consequently the density of both primordia and emerged lateral roots were significantly less in populations from higher latitudes. With lower UV levels, primary root length almost doubled as compared to the primary root length exhibited in higher UV conditions. In contrast, when the populations from different latitudes were analyzed at low UV levels, root length and numbers of primordia and emerged lateral roots, and density of primordia and emerged roots were not statistically different.

Dry shoot weight of seedlings is lower in populations from higher latitudes at low UV.

We tested whether shoot and/or root weight during early development would differ in populations isolated within and across latitudes. The shoot and root weight of

the five seedlings in each plate were averaged; the five replicates for each population were then averaged and are reported in Figure IV-2; there was a significant difference between shoot weight for populations between 40ºN and 60ºN in both chambers, ($P<0.05$); the There was lower shoot weight in populations from higher latitudes. Interestingly, even though there is no measurable difference in root weight from populations across latitudes; both the number of lateral root primordia and lateral roots and the pattern of root primordia formation change, at least under higher UV levels.

Photosynthetic rates and shoot respiration are lower in populations from higher latitudes at low UV.

We tested whether photosynthetic rate, shoot respiration, root respiration, and the carbon budget would differ in populations isolated from three different latitudes. Representative images of plants are shown in Figure IV-5 and the smaller rosette diameter and leaf size in populations from higher latitudes is evident. The values from these measurements are also shown in Figure IV-5 and and the ANOVA demonstrating that the effect of population and latitude on the carbon balance of mature plants is shown in Table IV-3. There was a chamber effect for all measurements except for root respiration. Root respiration was not significantly different within or across latitudes. The chamber with lower UV level showed an effect of all other measurements. With higher UV levels, there was no significant variation in any of these measures among populations across latitude. With decreased UV, a pattern emerged across latitude. The photosynthetic rate and root respiration rates were higher in populations from lower latitudes, ($P<0.05$). In contrast, root respiration did not significantly differ between any populations.

Dry shoot weight, leaf number, and rosette size are lower in mature plants from higher latitudes at low UV.

In plants that have not yet bolted, we examined whether germination, rosette diameter, number of leaves per rosette, and dry shoot and/or root weight (mature rosette prior to reproduction) differ across populations. There was no significant difference in days until germination in either chamber. There was no significant difference in any growth processes for populations across latitude with higher UV levels. With lower UV, populations from higher latitudes had lower shoot weight, leaf number, and rosette diameter, ($P<0.05$). The southern populations have bigger shoots, both in weight and size; this in turn, leads to greater photosynthetic capacity. In this common garden study, the growth substrate, water regime, and nutrient supply were all the same over populations. The effect of population on growth measures of mature plants are shown in Table IV-4 which showed a chamber effect in all measurements except root biomass; there was no effect of populations within or across latitude in either chamber for root biomass. In the chamber with lower UV levels there was a significant effect of latitude on photosynthetic rate and shoot respiration with the lower latitude having higher rates.

Time to bolting is prolonged in populations from higher latitudes

A plant species' phenology shifts across a latitudinal gradient; shorter time to bolt might indicate a population's need to avoid harsh environmental conditions. We observed and quantified time to bolting for populations within and across latitudes. In terms of bolting, there was no chamber effect, so the data from both chambers were considered together. The southern latitude required the shortest time to bolt. In southern latitudes, these populations grow and reproduce in April to early May; the average

monthly temperature rises quickly and there are water constraints as the spring progresses into summer (Pigliucci, 1998). One potential explanation is that the southern populations may grow and seed quickly to avoid the harsh summer environment and competition from hardier species.

Discussion

The goal of this study was to examine potential genetic variation resulting from selective pressures due to environmental changes associated with different latitudes and to ask if this variation modulates early root development, allocation of carbon between roots and shoots, and respiration in mature plants. The latitudinal gradient we explored in this study (40ºN-60ºN) coincided with a climatic gradient in environmental variables such as day length and growth temperature. For the nine populations used in this study, one potential limiting factor was photoperiod. Banta et al. (2007) stated that plants were not locally adapted to photoperiods across a latitudinal gradient in *A. thaliana*, meaning that populations from a given latitude did not do better in growth or fitness measures in their own latitude than other populations subjected to the same environmental situation. Therefore in this study, we did not vary the photoperiod and examine its effect on root development, but rather we performed a common garden experiment to test for potential genetic variation in root development. For this analysis we examined nine European populations of *Arabidopsis thaliana* from three different. We found differences in early lateral root architecture in this range of populations, which were not accompanied by differences in root allocation and respiration across this latitudinal gradient.

This study was performed with two chamber replicates but we were surprised to observe qualitative chamber difference in growth parameters late in development and to demonstrate that there was a significant chamber effect through ANOVA analysis. Upon examination of all potential environmental factors like CO_2 levels, light quality and intensity, and humidity, the only difference detected was the levels of UV light. Most of the UV light in this chamber was UV A with low contributions of UV B and UV C (both

of which have been determined to be the most detrimental to plants and humans alike). However, the potential UV effect was enough to induce substantial differences between the chambers in many variables measured. It is important to remember that the assumption of this study is that any latitudinal differences among populations describe relevant patterns, no matter in which chamber they occurred. Whether the trends are significant in one chamber versus the other, significant in both, or significant in neither, we did not see contradictory significant trends in the two chambers, although most differences in populations were observed in only one of the chambers.

Our main hypothesis for early root development was that due to differing environmental variables seen across a latitudinal gradient, there was potential genetic variation which altered root development. In the higher UV chamber in populations from higher latitudes, there were fewer lateral root primordia and emerged lateral roots and shorter roots, as shown in Table IV-2. Yet, because the number of roots that form and the length of the primary root decrease in parallel, there were no significant differences in density of root formation between population groups in this chamber. Additionally, differences in the length and placement of emerged lateral roots across latitude in the chamber with higher UV levels are evident in Figure IV-1. Populations from northern and middle latitudes have lateral roots clustered near the root/shoot junction; however in the populations from middle latitudes, the lateral roots are longer and more complex; southern populations have short lateral roots that are placed along the length of primary root. In contrast, in the lower UV chamber, there no significant difference in number or density of primordia and emerged lateral roots across latitude.

In both chambers the dry shoot biomass is lower in populations from higher latitude, but there is not a significant difference in dry root biomass between any populations. These results suggest that while UV has no effect on early growth biomass of the root, UV does have a profound effect on root architecture. There is very little root biomass in early development, so while we see a pattern of less shoot biomass, which is easier to measure, as latitude increases, we may not be able to detect a difference in root biomass. Architectural measurements are easier with the use of software programs which would give a finer measure of the lateral root architecture for populations within and across latitude.

In this study, our results for early development root formation were not focused on the role of auxin, but there is much evidence that auxin does play a substantial role in mediating external signals such as UV. Molinier et al. (2008) tested whether *A. thaliana* genes are involved in the repair of UV-induced DNA lesions affected root growth. In mutants lacking genes that might protect from UV-C exposure, there was a loss of root mass; in plants where there was an over-expression of these proteins, tolerance to UV was enhanced. In our study on early development, we did not see any significant effect of UV in root biomass but we did see drastic changes in lateral root architecture. Rakitina et at. (2001) suggests that high-penetrating UV radiation changes the content of IAA, ABA, and ethylene in plants (Brederode et al., 1991). This suggests that phytohormones may play an important role in plant response to UV stress. Other studies show alterations in gene expression and resource allocation including changes in root and shoot biomass (Hectors et al., 2007) that can be attributed to changes in auxin metabolism and transport (Ros and Tevini, 1995; Meijkamp et al., 2001; Huang et al., 1997; Jansen,

2002; Jansen et al., 1998). In chapters two and three of this thesis, we found that auxin mediates changes in lateral root development under differing environmental conditions. It is an intriguing possibility that auxin may be involved in lateral root development in response to the higher UV levels observed in one of the chambers in this study.

We also asked whether there was genetic variation in root allocation and respiration that was associated with the latitudinal provenance of these natural populations. We found a highly significant chamber or potential UV effect; populations from the southern latitude differed from the other two sets of populations. With lower UV levels, populations from higher latitudes exhibited a lower photosynthetic rate, shoot respiration, and carbon balance, which was accompanied by more shoot biomass, leaf number, and greater rosette diameter but no significant difference in root biomass or respiration. It is not surprising to see more shoot biomass accompanied with increased photosynthetic rate. In southern populations where we see more biomass and increased photosynthetic rate, we hypothesize that *A. thaliana* would allocate more resources to the shoot because it would be beneficial to those populations to have a quicker life cycle to avoid heat and drought stress.

We hypothesized that root biomass would differ in populations across latitude; while we found that shoot biomass does vary across latitude, root biomass differences were not detected. Root architecture, however, does vary across latitude when plants were grown under certain UV levels. We did not intend to introduce UV light as an environmental variable, UV; however it yielded interesting results, if not another layer of complexity. Our second hypothesis was that in mature plants, root biomass and respiration would differ in populations across latitude. We were unable to detect

significant differences in these parameters between populations in either UV condition. Photosynthetic rate and shoot respiration rate as well as shoot biomass, leaf number, and rosette diameter did vary in populations across latitude with more in the southern populations. Southern populations need to adapt to their harsh environment; *A. thaliana* is not a strong competitor and it does not fare well in extreme growth temperatures. It turns hot more quickly in southern latitudes, thus the plants develop in early spring; they use more of their acquired energy from higher photosynthetic rates for development of the shoot. Populations from southern latitude flower and senesce more quickly than middle and northern latitudes.

From our results in chapters 2 and 3, we found a plastic response in root development to day length and growth temperature; we tentatively hypothesized that we might find a pattern of growth differences with natural populations across a latitudinal gradient with natural variation in day length and growth temperature. Although we did not observe such differences universally, we did find that the early stages of root development were accelerated in populations from southern latitudes and that roots in these populations exhibited unique root architectural patterns, suggesting a specific growth strategy to adapt in the natural environments of these populations.

Materials and Methods

Plant material and handling

We used nine natural populations of *A. thaliana* from the Arabidopsis Biological Resource Center at http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm. The populations were chosen to represent three clusters of latitude (40, 50, 60ºN) as well as corresponding environmental variation, and all were from low altitude (maximum 170 m). The populations were Solomennoye (N4, Russia, 61ºN); Espoo (Es-O, Finland, 60ºN); Oystese (Oy-0, Norway, 60ºN); Limburg (Li-2, Germany, 50ºN); Anholt (ANH1, Germany, 52ºN); Bulhary (Blh-1, Czechoslovakia, 49ºN); Blanes/Gerona (Bla-1, Spain, 41ºN); Tossa de Mar (Ts-6, Spain, 41ºN); Playa de Aro (Pla-1, Spain, 41ºN). All populations were labeled as "late" flowering. The northern populations normally flowered in June at an average day time temperature of 15ºC and day length of 18 hours per day. The mid-latitude populations normally flowered in May at an average day time temperature of 15ºC and a day length of 16 hours per day. The southern populations normally flowered in April at an average day time temperature of 14ºC and a day length of 14 hours per day. (Approximate flowering times provided by Joshua Banta; personal communication).

All populations were grown under uniform conditions while the seeds were bulked. They were grown under continuous light (90 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) and an average ambient temperature of 23ºC. At rosette stage, the plants received a brief cold treatment to induce flowering. The F1 seeds were used in experiments after the following sterilization. Seeds were soaked in distilled water for 30 minutes and surface sterilized with 95% (v/v) ethanol for five minutes, 20% (v/v) bleach with 0.01% (v/v) Triton X-100

for 5 minutes, and then washed with distilled water for five minutes. Seeds were washed three additional times in sterile distilled water.

Lateral root development and biomass allocation in young seedlings

In the experiments that examined lateral root development in young seedlings, seeds were transferred to Petri dishes containing sterile control medium (0.8% (w/v) agar [Sigma Type M, plant tissue culture], 1 x Murashige and Skoog salts, 0.05% (w/v) MES, pH 6.0, 1.5% (w/v) sucrose, 1 $\mu g \cdot mL^{-1}$ thiamine, 1 $\mu g \cdot mL^{-1}$ pyridoxine HCl, and 0.5 $\mu g \cdot mL^{-1}$ nicotinic acid). Seeds were germinated and grown on vertically oriented Petri dishes with 16 hour day / 8 hour night and 15ºC day / 10ºC night under a combination of fluorescent and incandescent light at 135-140 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Five plates of each population were placed randomly in each of two controlled environmental growth chambers (EGC, model M-13) at the Duke University Phytotron. The UV level for chamber 1 was measured at 6.3 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ and at 1.8 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ for chamber 2. This was not an intentional environmental variable but after the experiment was complete, growth differences were observed. The intended study had two chambers with identical environmental conditions.

After six days of growth, five seedlings of similar length of the same population were transferred to new sterile Petri dishes. There were five replicate plates per population in each chamber. After another 15 days of growth, pictures were taken of each plate. The length of each seedling's root was determined with a ruler to the nearest mm. The roots were placed on a microscope slide with cover glass and examined under a Meiji dissecting microscope under 10X magnification. The number of lateral root primordia and emerged lateral roots for each seedling were counted. For each of the five

plants, the biomass was harvested, root and shoot tissue were separated, then dried at 55˚C for several days, and weighed.

Mature plant experiments

For mature plant experiments, ten seeds were placed in 10 cm pots containing Turface growth substrate. For each of the nine populations, seven replicates were placed in each of two controlled growth chambers. The day length, growth temperature, and light intensity were the same as above and UV levels varied between chambers.

Plants were measured for germination. It took approximately ten days for all populations in all pots to germinate. Once they matured into small 2-4 leaf seedlings, they were thinned to one plant per pot. Time until thinning took about 3-4 more weeks; growth rate varied. At 7 ½ weeks after sowing seeds, four mature replicates from each population in each growth chamber were measured for (1) rosette diameter; (2) number of leaves; (3) dry shoot and root weight; and (4) carbon exchange rate which included photosynthetic rate, shoot and root respiration rate, and total carbon balance. These plants were sacrificed for biomass measurements. The three remaining plants (replicates) were allowed to progress to bolting. Days to bolting was recorded.

The rosette photosynthetic rate, and shoot and root respiration were measured using the LI-6400 portable gas exchange system (Licor, Lincoln, NB) using a custom-made cuvette to include the whole plant and the above ground rosette separately. The rosette photosynthetic rate was measured first, sealed from root and soil gas exchange. The whole apparatus was then covered in thick black felt to simulate dark conditions for approximately five minutes. The rosette respiration rate was measured, sealed from root

and soil gas exchange. Finally the barrier was removed between the rosette and soil, and still in darkness, the whole plant respiration rate was measured.

Data analyses

All statistical analyses were performed using Proc Mixed from SAS version 9.1 (SAS, Inc., Cary, NC, USA). The early development data were first considered as a linear mixed model with chamber (two levels), latitudinal groups (three levels), and population (nine levels) in a factorial arrangement and considered to be in a completely randomized design. The average number of primordia, emerged lateral roots, length of primary root, primordial density, emerged lateral root density, dry shoot biomass, and dry root biomass were each modeled separately. Tests for the assumption of homogeneity of samples were run and showed no need for transformations. Linear mixed models were also used to determine if chamber had an effect on population and latitudinal group; chamber did have an effect and we dealt with chambers separately. All pair-wise comparisons were tested using the Tukey–Kramer procedure. The same procedures were repeated for the mature variables: dry shoot biomass, dry root biomass, number of leaves, rosette diameter, time to bolt, photosynthetic rate, shoot respiration rate, root respiration rate, and carbon balance.

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Table IV-1. General ANOVA for the effects of latitude and population on the early development and biomass.

Factor	Lateral root primordia			Lateral root emergence			Shoot weight			Root weight		
	df	F	P	*df*	F	P	*df*	F	P	*df*	F	P
Lat	2	0.8	0.4592	2	23.0	0.0001	2	14.1	0.0001	2	0.2	0.8454
Pop(Lat)	6	8.2	0.0001	6	25.5	0.0001	6	4.3	0.0009	6	2.0	0.0735
Chmbr	1	123.8	0.0001	1	162.1	0.0001	1	2.8	0.1015	1	1.0	0.3269
Lat * chmbr	2	10.8	0.0001	2	3.1	0.0514	2	1.1	0.3404	2	0.9	0.4220
Pop (Lat) * Chmbr	2	13.9	0.0001	6	7.6	0.0001	6	2.3	0.0435	6	0.7	0.6579

Abbrevations for this table: Lat-latitude; Pop(Lat)-population nested in latitude; Chmbr-chamber.

The F-Test is a *parametric* test: F=variance of the group means / mean of the within group variances.

The P-value is the probability that the variation between conditions may have occurred by chance.

df = degrees of freedom

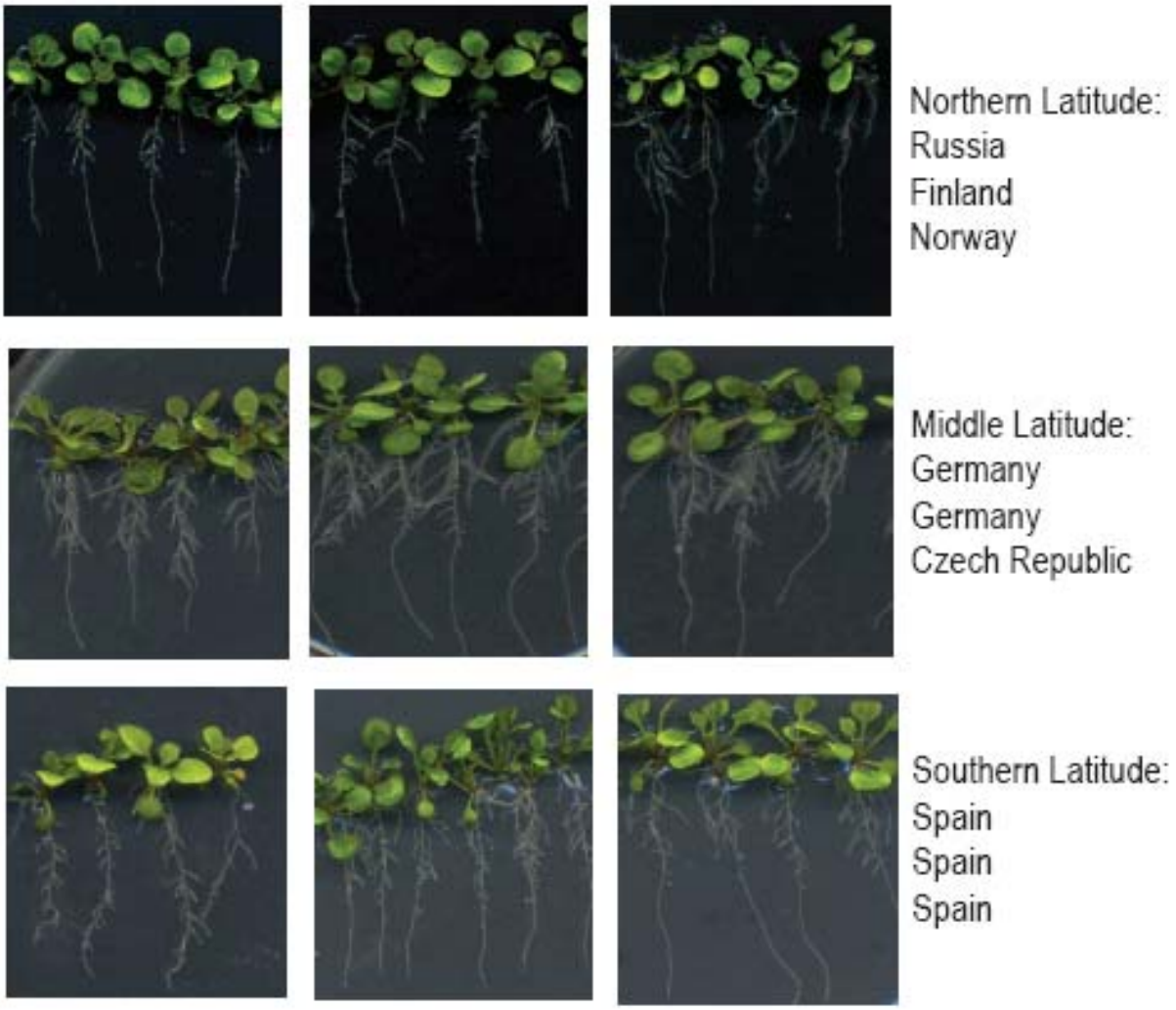


Figure IV-1. Variation in root architecture in populations isolated across latitude. Seedlings were grown in the higher UV level chamber and were 21 days past sowing at the time images were captured.

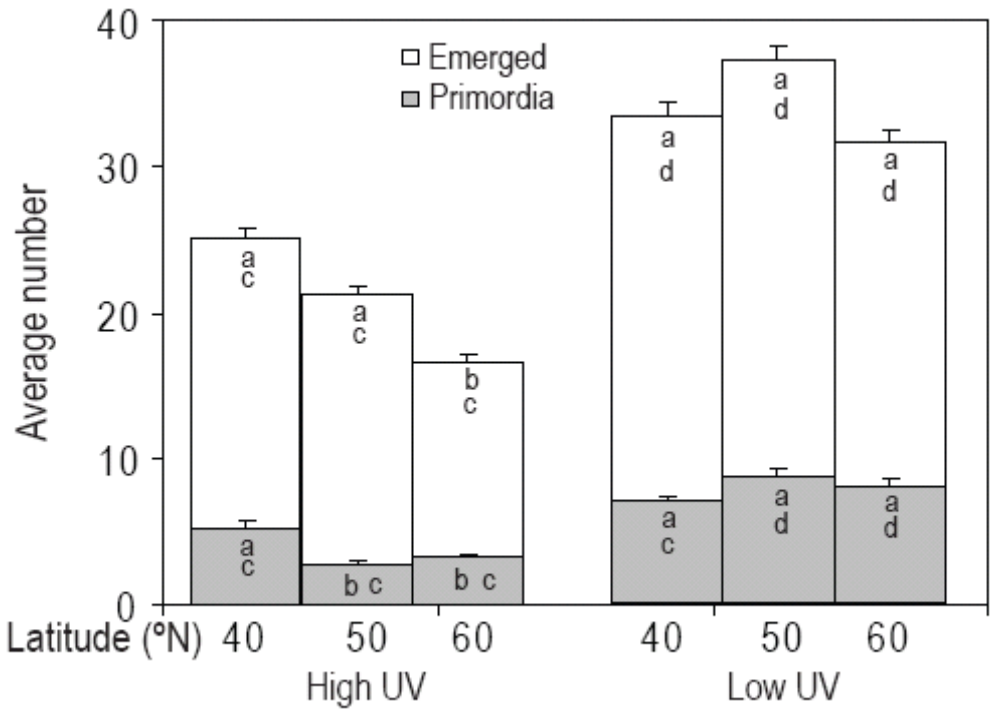


Figure IV-2. Lateral root formation and emergence are reduced in populations from higher latitudes. Within chamber comparisons, **"**b" indicates a significant latitudinal difference from "a", ($P<0.05$). For comparisons of chamber effect, "d" indicates a significant difference from "c" at a given latitude, ($P<0.05$). All comparisons were examined by ANOVA followed by Tukey-Kramer multiple test.

Table IV-2. Primary root growth, lateral root primordia, emerged lateral roots and their densities in wild populations of *Arabidopsis thaliana* isolated at several latitudes. The number of lateral root primordia, emerged lateral roots, and primary root lengths were determined. The reported values are averages ± SE of approximately 70 plants for each population in each chamber.

		Latitude (ºN)		
	Chamber	40	50	60
Lateral root primordia (P)	1	5.2 ± 0.46^{ac}	2.7 ± 0.21^{bc}	3.2 ± 0.22^{bc}
	2	6.9 ± 0.35^{ac}	8.6 ± 0.48^{ad}	8.0 ± 0.43^{ad}
Emerged (E) lateral roots	1	19.8 ± 0.70^{ac}	18.6 ± 0.54^{ac}	13.4 ± 0.52^{bc}
	2	26.3 ± 0.91^{ad}	28.5 ± 0.97^{ad}	23.5 ± 0.84^{bd}
Length of primary root	1	26.8 ± 0.71^{ac}	27.3 ± 1.07^{ac}	24.5 ± 0.41^{ac}
	2	47.0 ± 1.02^{bd}	51.7 ± 1.31^{ad}	46.2 ± 1.09^{bd}
P density	1	0.19 ± 0.01^{ac}	0.09 ± 0.01^{bc}	0.13 ± 0.01^{bc}
	2	0.14 ± 0.01^{ad}	0.16 ± 0.01^{ad}	0.17 ± 0.01^{ac}
E density	1	0.75 ± 0.02^{ac}	0.72 ± 0.02^{ac}	0.54 ± 0.02^{bc}
	2	0.55 ± 0.01^{ad}	0.54 ± 0.01^{ad}	0.50 ± 0.01^{ac}

In this table, **"**b" indicates a significant latitudinal effect from "a" within a chamber, ($P<0.05$). For chamber effect comparisons, "d" indicates a significant difference from "c" at a given latitude, ($P<0.05$). Chamber 1 had higher UV levels than Chamber 2. All comparisons were examined by ANOVA followed by Tukey-Kramer multiple test.

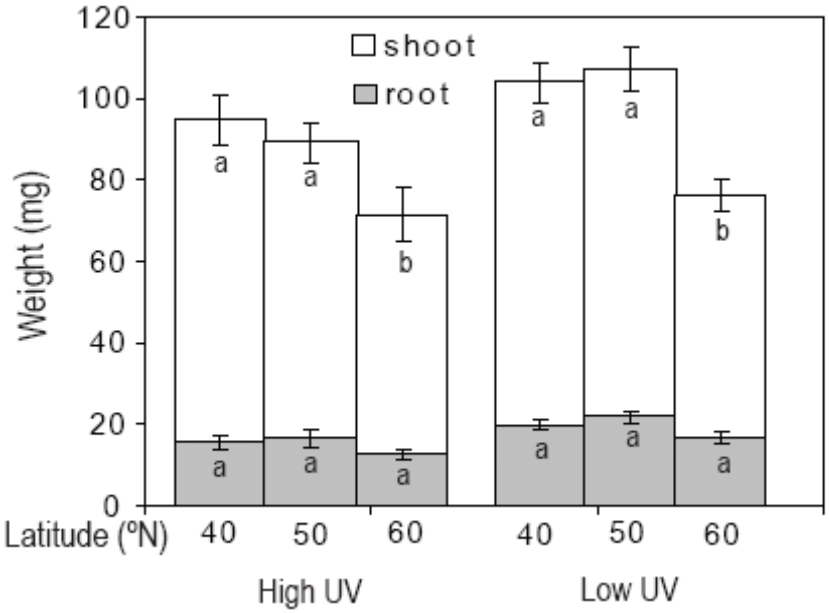


Figure IV-3. Seedling shoot weight is lower in populations with decreased UV levels in early development. Within chamber comparisons, **"**b" indicates a significant latitudinal difference from "a", ($P<0.05$). There is no significant difference between chambers. All comparisons were examined by ANOVA followed by Tukey-Kramer multiple test.

Table IV-3. General ANOVA for the effect of population on carbon balance of mature plants.

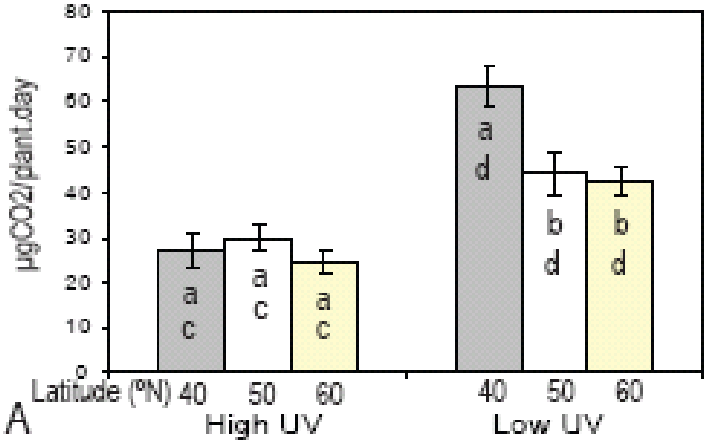
Factor	Photosynthetic rate			Shoot respiration			Root respiration			Carbon balance		
	df	F	P	*df*	F	P	*df*	F	P	*df*	F	P
Lat	2	8.7	0.0006	2	3.0	0.597	2	2.0	0.1615	2	8.5	0.0007
Pop(Lat)	6	5.7	0.0001	6	2.3	0.0501	6	1.3	0.2764	6	6.2	0.0001
Chmbr	1	84.0	0.0001	1	10.8	0.0018	1	1.0	0.3180	1	10 3.5	0.0001
Lat * chmbr	2	8.3	0.0007	2	3.6	0.0329	2	2.7	0.0762	2	7.8	0.0011
Pop (Lat) * Chmbr	2	1.8	0.0118	6	1.0	0.4189	6	0.9	0.5380	6	2.1	0.695

Abbrevations for this table: Lat-latitude; Pop(Lat)-population nested within latitude; Chmbr-chamber.

The F-Test is a *parametric* test: F=variance of the group means / mean of the within group variances.

The P-value is the probability that the variation between conditions may have occurred by chance.

df = degrees of freedom


µgCO2/plant.day
80
70
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Latitude (°N) 40 50 60 40 50 60
High UV
Low UV

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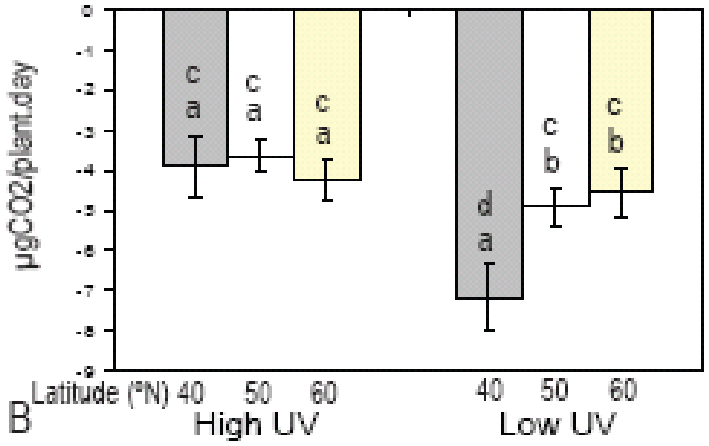
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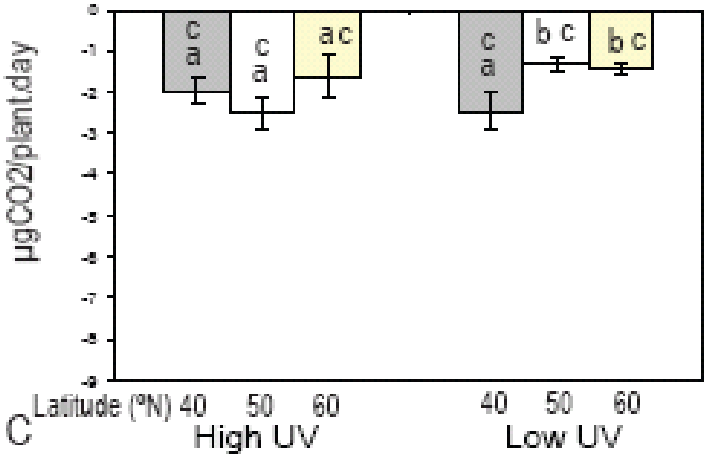
B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Latitude (°N) 40 50 60 40 50 60
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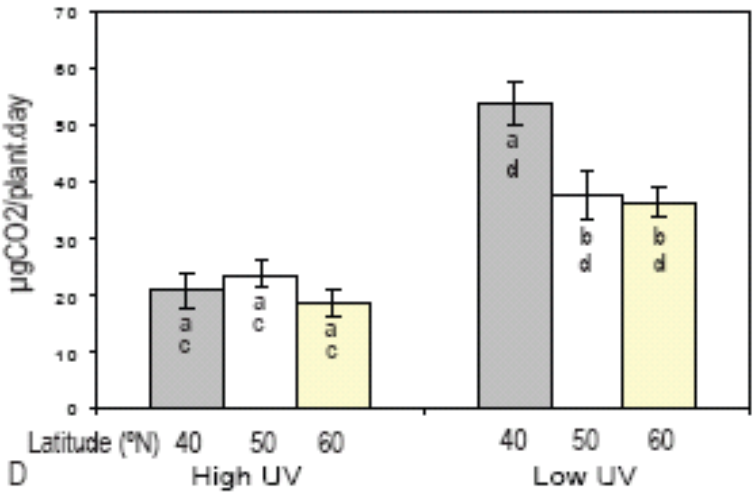


Figure IV-4. Carbon budget components differ in populations from low latitude.

A, Photosynthetic rate; B, Shoot respiration; C, Root respiration; D, Carbon balance. Within chamber comparisons, **"**b" indicates a significant latitudinal difference from "a", ($P<0.05$). For comparisons of chamber effect, "d" indicates a significant difference from "c" at a given latitude, ($P<0.05$). All comparisons were examined by ANOVA followed by Tukey-Kramer multiple test.

Table IV-4. General ANOVA for the effect of population on growth measures of mature plants.

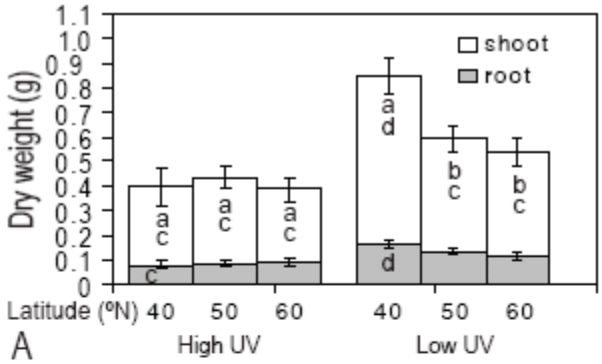
Factor	Leaf number			Rosette diameter			Shoot weight			Root weight		
	df	F	P	*df*	F	P	*df*	F	P	*df*	F	P
Lat	2	24.4	0.0001	2	15.5	0.0001	2	3.8	0.0286	2	8.4	0.0007
Pop(Lat)	6	2.6	0.0303	6	2.8	0.0213	6	2.1	0.0654	6	6.3	0.0001
Chmbr	1	18.2	0.0001	1	34.2	0.0001	1	18.2	0.0001	1	0.9	0.3554
Lat * chmbr	2	9.2	0.0004	2	5.7	0.0058	2	3.7	0.0323	2	8.9	0.0005
Pop (Lat) * Chmbr	2	1.1	0.3752	6	0.7	0.6285	6	1.1	0.3961	6	3.2	0.0095

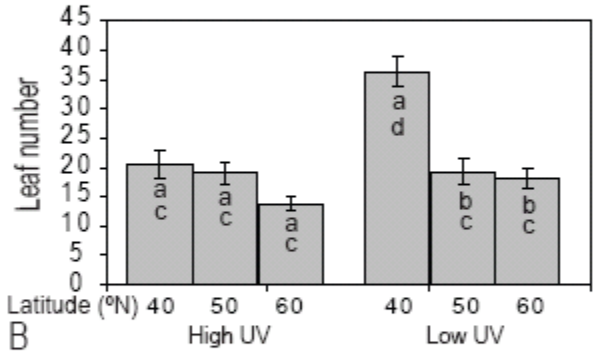
Abbrevations for this table: Lat-latitude; Pop(Lat)-population nested within latitude; Chmbr-chamber.

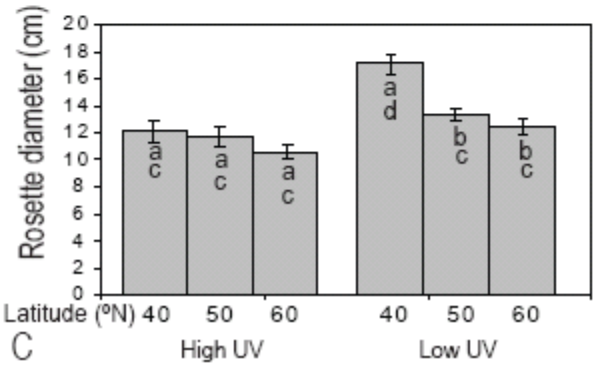
The F-Test is a *parametric* test: F=variance of the group means / mean of the within group variances.

The P-value is the probability that the variation between conditions may have occurred by chance.

df = degrees of freedom







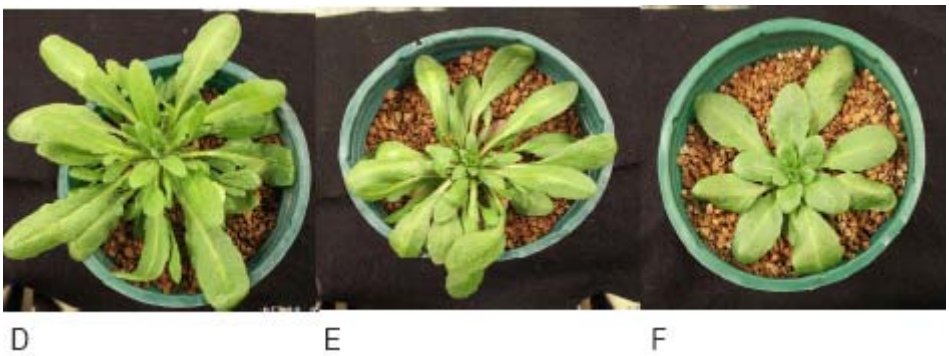


Figure IV-5. Mature plant size is smaller in low UV plants from high latitude. A, Dry shoot weight of shoot and root; B, Leaf number; C, Rosette diameter; D-F, Mature

rosettes from southern, middle, and northern latitudes respectively from lower UV level chamber. Within chamber comparisons, **"**b" indicates a significant latitudinal difference from "a", ($P<0.05$). For comparisons of chamber effect, "d" indicates a significant difference from "c" at a given latitude, ($P<0.05$). All comparisons were examined by ANOVA followed by Tukey-Kramer multiple test.

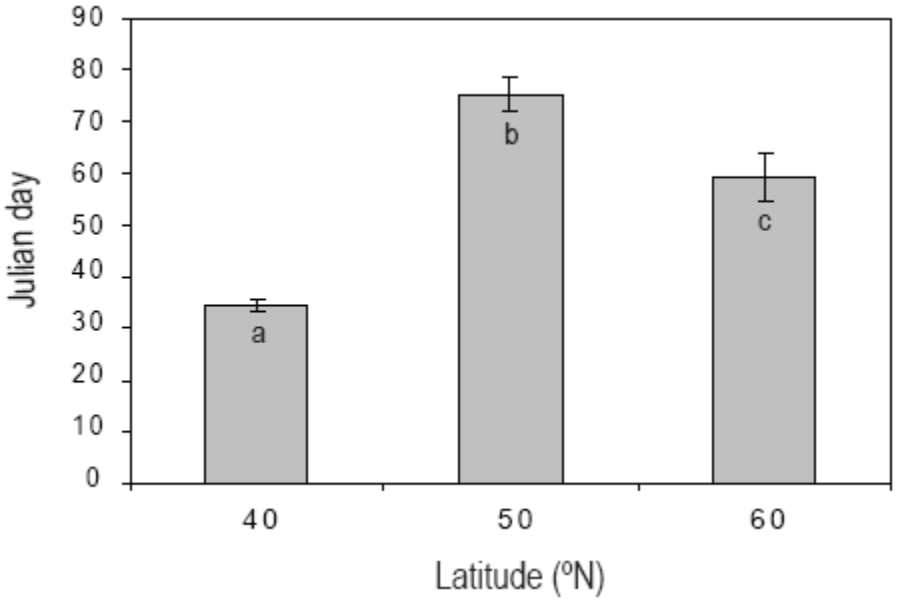


Figure IV-6. Time to bolting varies between populations from different latitudes. All latitudes were significantly different from one another, "a" indicated a difference difference from the middle and northern latitudes; "b" indicates a significant different from the southern and northern; and "c" indicates a significant difference from the southern and middle latitudes, ($P<0.05$). There was no chamber effect. All comparisons were examined by ANOVA followed by Tukey-Kramer multiple test.

CHAPTER V

CONCLUSION

This dissertation addresses important physiological and ecological questions about lateral root development and the mechanisms that drive this developmental process. Most scientists agree that auxin, a phytohormone, is the driving force behind lateral root development. There are two sources of early synthesis in developing seedlings (shoot tip derived auxin and primary root tip derived auxin) but the source of auxin which is responsible for the development of lateral root primordia and emerged lateral roots has been debated. Understanding the source of auxin involved in lateral root development has important physiological and ecological implications. If shoot-derived auxin modulates root formation, then this could act as a long distance signal to control root architecture when the shoot environment changes. The shoot is exposed to many unpredictable environmental variables and it must respond in a way that protects and benefits the entire plant.

In agreement with Reed et al. (1998), we found that shoot-derived auxin drives emergence of lateral roots. In chapter II, we used chemical, physical, and genetic techniques to examine which source of auxin modulates lateral root primordia. We found no role for root tip derived auxin in lateral root formation; we also found that shoot-derived auxin is required for the initiation of lateral root primordia.

Many environmental variables such as day length, light intensity and quality, and temperature affect shoot growth and development. In chapter two,we asked whether signals from these environmental variables then affect lateral root development. The roots are vital for carbon storage but they also forage the soil for nutrients and moisture.

Complex signaling between the shoot and root maximize resource acquisition and minimize the cost to the overall plant. In chapter three we examine the role of day length and growth temperature on the lateral root phenotype. We found that as day length increased over the range of 8-24 hours, lateral root primordia and emerged lateral roots also increased with optimal development at 20 hour days. We also examined growth temperatures that were ecologically relevant. We saw a bell shaped curve with optimal temperatures for lateral root primordia and emerged lateral root development between 21ºC-26ºC and 24ºC- 28ºC, respectively.

We established that root development is plastic when presented with varying day length and growth temperature. What role does auxin play in this plastic response? We examined auxin transport, synthesis, and expression as potential mechanisms for this plastic root phenotype. Auxin transport, using traditional transport assays, showed no significant differences in day length or temperature changes. Using transport mutants at different day lengths (8, 16, 24), we found that the protein, PIN3, is important in this light dependent pattern; this suggests certain transport proteins do play a role in auxin transport that determine the plastic response to day length. Changes in auxin responsive gene expression appear to play the most significant role in light and temperature dependent root formation, while changes in auxin accumulation may play a minor role.

We observed a plastic response to the environmental variables, day length and growth temperature in a laboratory strain of *A. thaliana*. Day length and temperature, which affect optimal growth and reproduction of a species, vary across a latitudinal gradient; these likely affect nutrient and water availability, and in turn, root development.

These environmental cues may have led to the evolution of genetically distinct populations with root architecture best suited to a specific latitudinal driven ecosystem. *A. thaliana* is also useful in ecological and evolutionary studies because it is a small annual weed distributed throughout Europe, spanning a latitudinal range from northern Africa to the Arctic Circle.

We examined how genetic variation, as a result of adaptation to different environments at a range of latitudinal, modulates early root development and allocation and root allocation and respiration in mature plants. We chose nine European populations from a latitudinal gradient (40ºN-60ºN) which coincided with a climatic gradient in environmental variables such as day length and growth temperature. We found differences in early lateral root architecture in this range of populations under specific UV levels; we also found that root allocation and respiration did not differ in these populations isolated across a latitudinal gradient. Auxin synthesis and transport have been linked to a plant's response to environmental variables including UV and drought stress. It is an intriguing idea but beyond the scope of this thesis, that these different root patterns in these natural populations may result from changes in auxin signaling, synthesis or transport. Together these results provide insight into the hormonal, environmental, and genetic controls of root developmental patterning.

CURRICULUM VITAE

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RESEARCH INTERESTS

Plant Biology, Plant Community Ecology, Plant Physiology, Plant Physiological Ecology, Plant Genetics, Plant Evolutionary Ecology, Medicinal Plant Biology

EDUCATION

Ph.D. Candidate, Wake Forest University, August 2009
Major: Ph.D. program in plant physiology/biochemistry.
Dissertation: Exploring the role of auxin in phenotypic plasticity in *Arabidopsis* root development

University of North Carolina at Greensboro, NC. MA 1996.
Major: Psychology – Concentration in Developmental Psychobiology
Thesis: Development of Peer Recognition in Ducklings

Davidson College, NC. BA 1992.
Major: Psychology

GRANTS AND AWARDS

WFU: Science Research Fund (2003) with Dr. Gloria Muday.

WFU: Research and Publication Fund (2007) with Dr. Gloria Muday.

PUBLICATIONS

Lacey, E.P., Lovin, M.E., Richter, S.J., and D.A. Herrington. *in review*. Floral Reflectance and reproductive thermoregulation in a common perennial herb: A test of adaptive plasticity in temporally variable environment. American Naturalist.

Lovin, M. E., Sukumar, P., Buer, C., and G. Muday. *in preparation*. Shoot derived auxin is required for lateral root primordia initiation and emergence.

Lovin, M.E., Sukumar, P., and G. Muday. *in preparation*. Lateral root initiation and emergence are regulated by light and temperature.

Lovin, M.E., Muday, G., Reed, C., and E.P. Lacey. *in preparation*. *Arabidopsis thaliana* root development and resource allocation in populations isolated across a latitudinal gradient.

Lovin, M.E. 1996. Development of peer recognition in ducklings: how long does it take, what sensory modalities are used, and what are the indications for neural development. Masters Thesis, University of North Carolina at Greensboro, Greensboro, N.C.

PRESENTATIONS

2008 Anderson, E.R., M.E. Lovin, and E.P. Lacey. A comparison of floral reflectance in *Plantago* species. Botanical Society of America annual meeting, Vancouver, Canada.

2008 Lacey, E.P., M.E. Lovin, S.J. Richter, and D.A. Herington. Adaptive thermoregulation in plants: A comparative study of floral reflectance patterns in European populations of *Plantago lanceolata*. Botanical Society of America annual meeting, Vancouver, Canada.

2008 Lovin, M. E. Exploring the role of auxin in phenotypic plasticity in *Arabidopsis* root development. Wake Forest University. Winston-Salem, NC.

2009 Lovin, M.E. Exploring the role of roots in early development and mature natural *Arabidopsis* populations over a latitudinal gradient in a daily carbon budget. Invited seminar. UNCG. Greensboro, NC

WORK EXPERIENCE

Research Associate: UNC-Greensboro, Greensboro, NC. 2004-2008.

- Supervised a plant ecology/evolution research lab. The NSF funded project integrates studies of ecology, population biology, genetics, functional morphology, phylogenetics, physiology, and biochemistry to examine aspects of the evolution of floral design, phenotypic plasticity, and reproductive thermoregulation in plants. In this project we have taken the plant model, *Plantago lanceolata*, and examined all aspects of a single reproductive process. The project is unique because we look at this process on so many different levels. It has prepared me to logically examine any plant model on many different levels; one level of a plant level is not detached from another.
- Assisted in researching and writing scientific presentations, grants and papers.
- Designed and implemented daily and long-term research programs. I assisted in the planning of new experiments and how logically they might fit in the overall project. In general, this experience has strengthened my ability to think on my own.

- Performed statistical analysis on and interpretation of experimental data using the statistical program, SAS.
- In charge of all undergraduate workers.

Teaching Assistant: Wake Forest University, Department of Biology, Winston-Salem, NC. 2001-2003.

- Supervised the WFU greenhouse and its care.
- Developed new growing regimes for various ecosystems.
- Supervised work study students.

Teaching Assistant: Wake Forest University, Department of Biology, Winston-Salem, NC. 2001.

- Instructed Biology 111 lab (introductory biology for non-majors) for undergraduates.
- Prepared and presented weekly lectures and supervised lab experiments for undergraduates.

Research Assistant: Wake Forest University, Department of Biology, Winston-Salem, NC. 2000-2001.

- Assisted in grant preparations and submissions.
- Assisted in plant ecophysiology field research: North Carolina, Wyoming.

Research Technician: Department of Physiology/Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC. 1996-1997.

- Researched nonhuman primate models of alcohol abuse.
- Assisted in programming equipment programs for the experiment.
- Obtained scheduled blood samples from wild, awake primates.
- Performed statistical analysis on and interpretation of experimental data.

Lab Manager: Department of Psychology, UNCG, Greensboro, NC. 1992-1995.

- Researched duckling models of development.
- Performed delicate operating techniques on newly hatched ducklings.
- Supervised weekly lab schedules and experiments.

Research Assistant: Department of Psychology, UNCG, Greensboro, NC. 1992-1993.

- Observed the mating habits of mocking birds in their natural habitat.

- Tagged and collected blood samples from wild, awake mocking birds for study.
