DELAYED MAIZE LATERAL ROOT DETERMINACY INDUCED BY MILD WATER DEFICIT

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by

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And hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

This dissertation is dedicated to my mother Linda Dowd for all her unwavering support, love and enthusiasm for everything I do. This would not have been possible without her guidance and good grace. Thank you.
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ABSTRACT

The capability of roots to mine the soil for available water is essential for survival and productivity, especially under water-limited conditions. The formation and elongation of lateral roots is an important process determining the architecture of the root system, and is highly plastic in response to soil drying. In plants, organ growth is initiated, maintained, and regulated through meristematic tissues. However, in some contexts, meristems are genetically encoded to stop the production of new cells, resulting in determinate growth.

To explore the effects of water deficit on lateral root determinacy, we implemented a system allowing photosynthetically active growth in a near-stable water potential environment. The suitability of several commonly used growth media was assessed for use in controlled water potential ($\Psi_w$) experiments, and conditions allowing comparisons of maize genotypes of inherently different size, and transpiration regimes, over a narrow range of precise and reproducible media $\Psi_w$ are detailed. Two inbred maize lines (B73 and FR697) with divergent lateral root responses to water deficit were assessed. Genotypic differences specific to lateral root growth from the primary root system were observed over a series of mild water deficits, ranging from a medium $\Psi_w$ of -0.25 to -0.40 MPa, with a well-watered control of -0.10 MPa. The total lateral root length of inbred B73 plants, as well as the average length and diameter of first-order lateral roots, were unresponsive to the levels of water deficit tested. In contrast, the total lateral root length of inbred FR697 plants was 27% greater when grown at media $\Psi_w$ of -0.25 MPa, and first-order lateral roots were 30% longer. Furthermore, FR697 first-order
lateral roots were 26% wider than well-watered controls, resulting in a 96% increase in volume at a $\Psi_w$ of -0.40 MPa. Neither genotype showed a significant difference in lateral root length at -0.35 MPa compared to their respective well-watered controls. The sensitivity of genotype specific responses over this narrow range shows the need to study mild water deficit conditions using a high-resolution series of water potentials.

First-order laterals of the water-deficit tolerant maize cultivar FR697 display an ability to delay the determinacy program when grown under a mild water deficit of -0.30 MPa. Maximum root elongation rates were maintained for nearly 2.5 days longer, and were still at 44% of maximum when well-watered laterals approached their determinate length. Maintenance of lateral root elongation resulted from sustained rates of cell flux and meristem activity. In addition, kinematic (spatio-temporal growth) analysis revealed that reductions in tissue expansion rates with aging were delayed by more than two days in the longitudinal, radial and tangential planes. This study reveals that large genotypic differences exist relating to the interaction of water deficit with the developmental determinacy of maize lateral roots.
Chapter 1

Literature Review
Introduction

A wide variety of adverse conditions negatively impact the growth and development of agricultural crops, but none to the extent of water limitations (Boyer, 1982). With current climate models predicting the shift of many areas that are heavily invested in agriculture to more arid environments, coupled with the ever-increasing demands on the world water supply, it is imperative to develop new drought-tolerant crop lines to provide food security.

An excellent target to improve drought tolerance is the regulation of root adaptations to water deficit conditions. Roots are capable of continued cellular elongation and meristem function at low water potentials ($\Psi_w$) that result in the cessation of growth of other organs (Sharp and Davies, 1979; Westgate and Boyer, 1985; Sharp et al., 1988). Several large scale effects on root system architecture arise under water deficit conditions, with one of the most widely observed responses being the development of a deeper and more highly branched root system (Weaver and Bruner, 1926; Sponchiado et al., 1989).

The growth rates of many plant organs are not held constant indefinitely, but are dynamic throughout their individual developmental programs, and can be heavily affected by environmental conditions. Root growth is regulated by meristematic tissues within the small area of active growth at the root apex. There are two major processes that control the growth of a root, cellular production within the meristem, and expansion of cells as they are displaced from the root apex. Cellular production can be further broken down into the rate of new meristem
‘initial’ cell production, as well as the number and frequency of subsequent cellular divisions within the meristematic zone (Beemster and Baskin, 1998).

Through the use of root marking experiments or anatomical records, kinematic analyses can be conducted to assess the spatio-temporal patterns of cellular proliferation and expansion along the root growth zone (Erickson and Silk, 1980; Silk, 1984; Silk et al., 1989; Beemster and Baskin, 1998). Responses of the maize (*Zea mays* L.) primary axial root to water deficit conditions have been investigated in detail (Sharp et al., 1988; Sharp et al., 1990; Poroyko et al., 2007; Spollen et al., 2008). However, in depth studies of maize lateral root responses to water deficit utilizing a kinematic approach have not been undertaken. Evaluation of how water deficit influences the rates of expansion in the longitudinal, radial, and tangential planes of the lateral root growth zone could provide useful insights into the control of these vitally important organs under water deficit conditions.

**Maize root system morphology and the role of lateral roots**

Maize, like other monocots, produces a fibrous root system comprised of many separate root categories (Fig. 1-1). The first roots to emerge from a maize kernel are the primary axial root followed closely by a set of seminal axial roots. These are classified as embryonic roots and they develop from opposite sides of the scutellar node located within the embryo (Avery Jr, 1930). Additionally, there is a group of post-embryonic roots which are distinct in origin as they develop from mature plant tissue. Nodal roots, also known as crown or brace roots, originate from concentric nodes of stem tissue beginning under the soil surface. It has been
Figure 1-1: The categories of roots in *Zea mays* and their origin

Diagram of a young maize seedling depicting the multiple categories of roots, their nodes of origin, and the categorization of embryonic and postembryonic roots. Image from Orman-Ligeza et al. (2013).
shown that the first set of nodal roots to form are derived from the coleoptile node (node 1) and are genetically and physiologically distinct from nodal roots formed from shoot tissue later in development (Hochholdinger et al., 2004; Riggs and Sharp, 2016).

Lateral roots are an additional class of post-embryonic roots that develop from the mature tissue of all maize axial roots. Categorization of lateral roots is based upon branching order relative to the axial root, i.e. first-order, second-order, etc. As axial roots drive the root system deeper into the soil profile, lateral roots grow radially along the path of growth, searching for resources, sensing the environment and greatly increasing the plant’s absorptive surface area (Weaver and Bruner, 1926; Russell, 1977). Furthermore, lateral roots facilitate the majority of water uptake necessary to sustain growth and development (Ahmed et al., 2016). Maize lateral roots have the capacity to modify their growth to best suit environmental conditions, a process known as lateral root plasticity, potentially heavily impacting the architecture of the root system.

**Lateral root responses to water deficit**

As the vast majority of the maize root system is composed of lateral roots, understanding lateral root plasticity responses to water deficit is extremely important to unraveling how plants adapt to water-limited environments. However, the majority of water deficit studies that have investigated the mechanisms of altered root growth have not examined lateral roots in a detailed manner.
Most of the studies that have assessed lateral root responses have concluded that water stress leads to a reduction in lateral root growth (e.g., Malamy and Benfey, 1997) or that lateral root growth is unresponsive at mild levels of stress before becoming inhibited (van der Weele et al., 2000). However, some studies have shown a net increase in total lateral rooting induced by water stress. Promotion of the total lateral root length of a root system can be facilitated by an increase in the average length of lateral roots, an increase in the number of lateral root initiations, or a combination of both. Enhancement of the number of lateral roots occurs in some crops species such as perennial rye-grass (Lolium perenne L.), rice (Oryza sativa L.), cassava (Manihot esculenta) and soybean (Glycine max L.) when grown under water-limited conditions (Read and Bartlett, 1972; Jupp and Newman, 1987; Banoc et al., 2000; Pardales Jr and Yamauchi, 2003). Additionally, other crops including wheat (Triticum aestivum L.) and maize have been observed to increase the average length of laterals in response to water deficit (Schmidhalter et al., 1998; Ito et al., 2006). However, little attention has been paid to the detailed examination of the effects of water deficit on lateral root growth throughout development, with many studies assessing a single time point and potentially mistaking a dynamic response for a static effect.

**Root cellular plasticity responses to water deficit**

Under water deficit conditions plants often shift their carbon allocation towards root growth in an effort to maximize water uptake. As such, the energy requirements of root growth metabolism heavily impact the successfulness of
environmental adaptation to water deficit conditions (Lynch, 2007; Lynch, 2013). Mining the soil profile for resources is an extremely costly metabolic process, with needs that can surpass half of the daily photosynthate production (Lambers et al., 2002). In an effort to ameliorate the metabolic demand, many plant species have developed adaptations to assist growth under water deficit conditions. Several studies reported that roots grown under water deficit become thinner when grown in non-compacted soils (Sharp et al., 1988; Iijima et al., 2007). Additionally, internal root traits such as aerenchyma formation, reduced cortical cell file number, and increased cortical cell size can lessen the carbon cost of root respiration under water deficit conditions, leading to more vigorous and productive plants (Zhu et al., 2010; Chimungu et al., 2014a, b). Such anatomical modifications reduce metabolic costs and allow for greater amounts of resources to be allocated towards root growth.

Before describing the effects of water deficit on lateral root growth and anatomy in more detail, the processes involved in the formation and maintenance of lateral roots will be reviewed.

**Lateral root development**

Lateral roots initiate from dedifferentiated non-meristematic tissue, maintain growth through a complex series of interactions between hormones, transcription factors, signal peptides and microRNAs, and end with the loss of meristematic function, resulting in determinate growth. The initiation and development of lateral root primordia (LRP) in maize has two major phases (Laskowski et al., 1995). First,
mature pericycle and endodermal cells dedifferentiate and undergo a series of highly controlled divisions to form a LRP with an anatomical organization similar to a mature root tip. During this process, putative precursors begin to display characteristics of their mature cell type. The second stage is the development of a functional de novo lateral root meristem, which maintains the organization, growth, and development of the new lateral root.

The phytohormone auxin is required at many stages of lateral root development, including the selection of pericycle founder cells (Dubrovsky et al., 2008), organogenesis (De Smet et al., 2010), xylem cell differentiation (Jansen et al., 2012), root emergence (Swarup et al., 2008) and lateral root meristem maintenance (Jiang and Feldman, 2005). In fact, many of the lateral root mutants in arabidopsis were originally isolated as possessing other characteristics such as altered auxin transport or sensitivity (Casimiro et al., 2003).

Sites of lateral root formation are marked by a series of local auxin maxima signals in the differentiation zone of the root (Jansen et al., 2012). Before the initiation of LRP founder cells, strong shoot-derived auxin responses are restricted to the late-metaxylem precursors. At the time of protophloem development, the size of the adjacent pericycle cells increases and auxin is present in both early-metaxylem and the protoxylem precursors. Following this, an auxin maximum develops in the cells surrounding the protophloem and there is lignification of the protoxylem cell walls. The auxin in the xylem attenuates until the only auxin maximum is located at the phloem pole. This marks the site of new lateral root organogenesis, with the pericycle cells located between the lignified xylem
undertaking the first cellular divisions that produce a LRP. It has been shown through excision of aerial tissues that the auxin required early in development is not root-derived as the removal of shoot auxin sources prevents emergence of LRP (Bhalerao et al., 2002; Swarup et al., 2008). As LRP continue to develop an auxin gradient is evident, with a maximum at the LRP apex analogous to a mature root meristem. Furthermore, excision experiments have shown that when LRP have progressed a few stages into development (Stage III-V, see below) they can grow independently of parent-root derived auxin, suggesting LRP contain cell types capable of auxin production prior to completion of a fully formed meristem (Laskowski et al., 1995).

Exogenous application of the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) results in pericycle founder cells being kept in the G1 phase and unable to progress through mitosis (Casimiro et al., 2001). The presence of auxin can alleviate the arrest of the founder cells and promote the transition into the active S phase (Stals and Inzé, 2001). Furthermore, this transition is preceded by enhanced expression of *CycB1;1*, showing a direct role of auxin in the upregulation of cyclin genes (Gray et al., 1999). Additionally, *KIP-RELATED PROTEIN2 (KRP2)*, a gene encoding for cyclin-dependent kinase inhibitor 2, was highly expressed in NPA-treated roots and diminished upon exogenous auxin treatment (Casimiro et al., 2003). *KRP2* is suggested to act upon CDKA-1 to reduce the activity of the kinase and repress cell division by halting the transition from the G1 to S phase of mitosis (De Veylder et al., 2001; Himanen et al., 2002). Transcripts of *KRP2* are observed to accumulate in pericycle cells which
are not chosen to become founder cells. Moreover, transcription of \textit{KRP2} is down regulated by auxin at the time of the G1-S transition, and overexpression of \textit{KRP2} results in a large decrease in lateral rooting. Taken together, these observations suggest that \textit{KRP2} is a critical component regulating the organogenesis of lateral roots.

Pericycle cells that are not fated to become founder cells need to dissipate local auxin to prevent the G1-S phase transition. The auxin signal that promotes LRP development is in part transduced by \textit{NAC1}, a gene from the \textit{NAC} family, a large group of proteins composed of \textit{NAM}, \textit{ATAF}, and \textit{CUC} transcription factors (Xie et al., 2000). Studies utilizing over and under expression lines produced through sense and antisense \textit{NAC1} cDNA displayed a positive correlation between \textit{NAC} expression and the length and number of lateral roots. Another protein, \textit{SINA OF ARABIDOPSIS THALIANA 5} (\textit{SINAT5}), a homolog of the \textit{Drosophilla} protein \textit{SEVEN IN ABSENTIA} (\textit{SINA}), possesses ubiquitin E3 ligase function that acts to direct proteolysis of \textit{NAC1} (Xie et al., 2002). Overexpression of \textit{SINAT5} leads to a dramatic reduction in lateral roots, showing its importance in the regulation of root system branching. Decreased levels of \textit{NAC1} via ubiquitination by \textit{SINAT5} result in diminished auxin concentrations in the pericycle, maintaining the arrest of the cell cycle in the G1 state, and thereby repressing lateral root initiation.

The formation of lateral roots has been classified into eight stages of development (Fig. 1-2) characterized by a series of highly controlled cellular divisions (Malamy and Benfey, 1997). Stage I is defined by an increase in anticlinal divisions at the site of lateral root initiation. In cereal crops, lateral roots develop
Figure 1-2: Morphological stages of lateral root development

The series of highly controlled cellular divisions involved in LRP organogenesis (a) and the original images of barley roots stained with toluidine blue for clarity (b). Image modified from Orman-Ligeza et al. 2013.
from the division of pericycle cells adjacent to phloem poles, rather than near xylem poles as is seen in arabidopsis (Casero et al., 1995; Jansen et al., 2012). The newly formed cells expand radially and become noticeably wider than adjacent cells.

Stage II LRP are characterized by a periclinal division splitting the LRP into two layers, resulting in the formation of the epidermal precursor tissue. At this early stage in development, End199, an enhancer trap line with a GUS insertion upstream of the SCARECROW (SCR) gene, can be seen in a small subset of the cells in the outer layer, and is the first sign of differential expression in the LRP (Malamy and Benfey, 1997). End199 expression can be seen in the endodermal tissue of mature axial and lateral roots, with the highest activity in the meristem. Expression can be seen in the endodermal and cortical cell initials as well as the quiescent center (QC). This marker is located approximately 1 kb upstream from the SCARECROW (SCR) gene, which is critical for radial patterning in meristem formation and the separation of the endodermal and cortical cell layers (Scheres et al., 1995; Di Laurenzio et al., 1996; Sabatini et al., 2003). In situ hybridizations corroborate that the expression patterns of End199 are an accurate representation of SCR expression (Malamy and Benfey, 1997). Taken together, the evidence suggests that this small set of cells plays a critical role in LRP organization and development and may be the precursors of the QC.

Stage III begins when the outermost cell layer undergoes an additional periclinal division, resulting in a three-layer primordium. At this stage, the rounded shape becomes easily noticeable. In arabidopsis, pericycle cells are the only cell
layer to be involved in the development of LRP. However, at this stage in maize, endodermal cells undergo an anticlinal division to produce an additional cell layer that is involved in the formation of the LRP epidermis and root cap tissue (Bell and McCully, 1970).

At stage IV, the innermost layer undergoes a periclinal division, adding a fourth cell layer. Additionally, the epidermis marker line, EpiGL2, is expressed in the outermost layer to either side of the LRP apex. There is no expression in the most apical cells that underwent the periclinal division in stage III, suggesting they could be root cap progenitors.

At stage V, several centralized cells in the outermost layers divide anticlinally, forming many small central cells. Additionally, cells in the innermost layer divide and grow radially, forcing the outer layers further from the parent root axis. At this stage, marker lines specific to endodermal tissue are expressed in the second outermost layer, again excluding the few centermost cells. Interestingly, this subset of cells displays strong expression of the SCR marker End199.

Stage VI is marked by the outermost layer experiencing a periclinal division, producing a new internal cell layer. Additionally, some central internal cells divide periclinally, adding a further layer of cells that are well-placed to be the putative QC precursors. These cells are devoid of GUS activity in endodermal, cortex and epidermal marker lines, suggesting they are a distinct cell type compared to surrounding tissues. Interestingly, the SCR marker End199 is seen in these cells providing further evidence of QC and meristem development (Malamy and Benfey, 1997). Additionally, some of the cells from the inner layers begin to develop an
elongated appearance resembling vasculature. At this stage, in both arabidopsis and cereal crops such as maize and barley, the primordium possesses many of the defining characteristics of a mature root tip, including cell layers resembling endodermis, cortex, epidermis, stele and root cap precursors (Bell and McCully, 1970; Malamy and Benfey, 1997; Orman-Ligeza et al., 2013).

Stage VII is marked by continued anticlinal divisions of most putative tissue types and the increased resemblance to a fully formed root tip. In arabidopsis there are no significant changes in the number of cells present in the LRP after reaching stage VII until after emergence (Malamy and Benfey, 1997). Furthermore, the basal-most cells greatly elongate to propel the LRP through the cortex of the parent root. Together these observations suggest that if a meristem is fully formed at this stage, it is in a state of low mitotic activity and is not significantly impacting LRP elongation. Supporting evidence can be seen in the rml-1 and rml-2 as well as the alf-3 mutants, which are incapable of forming an active lateral root meristem and display the phenotype of successful LRP emergence followed quickly by the cessation of growth (Celenza et al., 1995; Cheng et al., 1995). At this stage, the cortex cell-specific marker line CorAx92 is expressed in cells in the outer layers of the LRP, and like the expression pattern of EpiGL2, is absent in the central most cells at the apex.

After emergence, basal cells in the LRP maintain expansion for a time; however, the number of cells in the outermost layers begin to drastically increase in abundance. At this point the LRP is growing by cell production and elongation via its own functional meristem and is reclassified as a lateral root (Stage VIII). It
is not until emergence that expression of the stele-specific marker Ste05 can be detected in the basal portion of the new lateral root. Furthermore, expression of the lateral root cap marker LRC244 is found in the outermost cells at the tip of the root, except for those cells expressing the epidermal marker EpiGL2. As the root continues to grow the expression of LRC224 attenuates in a small group of cells at the center of the apex. These cells later express CRC219, a marker for columella root cap specificity, indicating that the two parts of the root cap have become distinct.

During development, a LRP must push itself through the endodermis, cortical cell files, and epidermis to emerge from the parent root. In arabidopsis, there are only a few cell layers to traverse, but in monocots, such as maize, a considerable number of cortical cell files may exist. Much work has been done to understand the regulatory network required to coordinate the separation of tissues allowing for minimal damage during emergence (Fig. 1-3). Following the initial divisions of the pericycle founder cells, indole acetic acid (IAA) is exported into outlying endodermal cells to target the SHORT HYPOCOTYL 2 (SHY2) / INDOLE-3-ACETIC ACID 3 (IAA3) repressor for degradation. Reduction of SHY2/IAA3 activity leads to induction of cell wall remodeling proteins that facilitate cell separation (Swarup et al., 2008). A large group of genes related to cell wall remodeling are expressed specifically in cells overlaying the lateral root primordium, including pectate lyase, xyloglucan:xyloglucosyl transferase, subtilisin-like protease, polygalacturonase, expansins and many others.
Figure 1-3. Auxin-dependent processes facilitating cell separation and LRP emergence. (a) Pericycle (P)-derived IAA downregulates the SHY2/IAA3 repressor in the endodermis (End) to induce the expression of cell wall remodeling proteins (CWR). (b) LRP-derived IAA targets SLR/IAA14 in the adjacent cortical cell (C) for degradation, inducing expression of an IAA influx carrier LAX3. (c) The permeability of the cell to IAA influx is increased by LAX3 expression, resulting in increased IAA accumulation, further inducing CWR expression and the formation of a positive feedback loop. (d) Just before emergence, LAX3 expression is induced by auxin in the epidermal cells (Epi), increasing CWR expression, and preparing the final cell layer for separation to allow LPR emergence. The blue coloration is representative of cellular auxin concentration. Image modified from Swarup et al. 2008.
Additionally, IAA is exported from the new primordium to degrade the SOLITARY ROOT/INDOLE-3-ACETIC ACID 14 (SLR/IAA14) repressor in outlying cells, leading to induction of a high-affinity IAA uptake protein, LAX3 (Swarup et al., 2008). Increased expression of LAX3 promotes influx of auxin into the cells, resulting in a feedback loop increasing the expression of the cell wall remodeling proteins. This process continues as the lateral root grows through each cortical cell layer and emerges from the epidermis, allowing for growth and containment of the cell wall remodeling proteins in a highly controlled vector. The IAA signal that precedes the path of primordia emergence is facilitated by PIN and AUX/LAX auxin transport proteins (Marchant et al., 2002; Benková et al., 2003). Individuals with a mutation in LAX3 show a large reduction in lateral root emergence, with the majority of formed primordia being arrested at stage I (Swarup et al., 2008), demonstrating the necessity of this critical component in lateral root emergence. Recovery of the growth defects of lax3 mutants can be accomplished by treatment with the synthetic auxin 1-naphthaleneacetic acid (1-NAA), showing the process is auxin dependent.

Examination of arabidopsis roots under a scanning electron microscope revealed that cells in the path of an emerging lateral root are not destroyed, but separate along the middle lamella (Laskowski et al., 2006). Due to the significantly more complex radial anatomy of maize roots, compared to the four cell file layers in arabidopsis, simple cell separation is insufficient to facilitate LRP emergence. In
maize, cellular divisions of cortical cells occur specifically overlaying the path of LRP emergence in addition to the enhanced activity of cell wall remodeling proteins. Unlike the relatively damage free process seen in arabidopsis, a significant number of cortical cells experience hydrogen peroxide-mediated cell death preceding LRP emergence, suggesting a controlled and extensive reorganization of anatomy (Orman-Ligeza et al., 2013).

**Root meristem organization and maintenance**

Root growth is coordinated and maintained by a highly organized collection of cells at the root apex, designated the root apical meristem (RAM). Perpetual maintenance of the RAM is required for active growth and is dependent upon a balance between the production of new meristematic cells and their subsequent division and expansion. The majority of cellular divisions within the RAM are anticlinal, perpetuating cell displacement toward the differentiation zone, with less frequent periclinal divisions giving rise to additional cell files. An extremely complex interaction of several regulatory elements act together to maintain proper RAM organization and functionality.

Studies have shown that maintenance of RAM architecture, functionality and growth are dependent on the presence of a functional QC (Rodríguez-Rodríguez et al., 2003). The QC holds a central position in the RAM and has been proposed to comprise the stem cells of the plant due to their capacity for seemingly endless proliferation, self-regulating maintenance mechanisms, and aptitude for
regeneration (Barlow, 1997; Ivanov, 2004). Surrounding the QC are several tissue-specific “initial” cells, which give rise to all subsequent root cell types (Fig. 1-4).

When initial cells divide, the more basal cell continues to proliferate giving rise to a cell file, while the apical cell is repressed by the QC to remain in an undifferentiated state. The endodermal and cortical tissues, as well as the epidermis and lateral root cap, share common initial cells adjacent to the QC and require a subsequent periclinal division to form separate tissue-specific initials. The daughter cells formed from the initials undergo several divisions before exiting the meristematic zone and are then replaced by newly formed daughter cells.

Several transcription factors including *PLETHORA1 (PLT1)* and *PLT2*, *WUSCHEL-RELATED HOMEBOX 5 (WOX5)*, *SHORT ROOT (SHR)* and *SCARECROW (SCR)* have been shown to be involved in preserving QC identity and meristem maintenance (Benfey et al., 1993; Scheres et al., 1995; Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004; Jiang and Feldman, 2005). WOX5 is expressed specifically in the QC cells and plays a particularly important role in stem cell niche maintenance by maintaining the undifferentiated state of the initial cells surrounding the QC (Drisch and Stahl, 2015). Additionally, WOX5 is responsible for repression of CYCD3;3 and CYCD1;1, cyclin D genes that promote cell proliferation in the QC (Forzani et al., 2014). Mutations in these transcription factors show drastic defects in anatomy and greatly impair meristem function. Roots of the *shr* mutant are completely devoid of endodermal tissue (Lucas et al., 2011) and *scr* mutants have a fused endodermal-cortex hybrid tissue, due to improper regulation of the periclinal division that gives
Figure 1-4: Anatomical organization of the root apical meristem (RAM). (a) Tissue patterning of cell types in an arabidopsis meristem. (b) Stem cell initials surrounding the QC with the direction of daughter cell production indicated. The black line indicates the role of the QC in the repression of the apical-most daughter cell to remain in an undifferentiated state. Image from (Benfey and Scheres, 2000).
rise to the cortex and endodermis initial cells (Di Laurenzio et al., 1996). In both mutants, roots quickly lose their meristematic function after initiation and growth cannot continue. Additionally, SHR regulates the activity of SCR (Levesque et al., 2006) and several D-type cyclin genes (Sozzani et al., 2010), showing a direct role in the control of organization and rates of division of meristem cells.

A highly coordinated interaction between auxin transport and zones with sustained auxin maxima are required for full RAM functionality. The auxin efflux proteins PIN FORMED 3 (PIN3) and PIN FORMED 4 (PIN4) are expressed in the QC and surrounding initial cells and are required for RAM patterning (Friml et al., 2004). Additionally, apical transport of auxin into the RAM cells, a vital part of RAM maintenance, is facilitated via PIN FORMED 2 (PIN2). Auxin accumulation in the QC promotes an oxidized environment which represses the cells in the G1 phase of mitosis (Jiang et al., 2003; Jiang and Feldman, 2005; De Tullio et al., 2010). Finally, the regulation of several transcription factors critical for maintaining RAM organization are dependent on auxin either directly or indirectly, including SHR, SCR, PLT1, PTL2 and WOX5 (Aida et al., 2004; Drisch and Stahl, 2015).

Other potentially faster methods controlling the regulation of transcription factors can be facilitated by peptides, microRNAs (miRNAs), or the movement of transcription factors themselves between cells. Several small signal peptides serve some role in the development of Arabidopsis roots, including some involved in homeostasis of meristematic cells (Delay et al., 2013). The peptide CLAVATA3/EMBRYOSURROUNDING REGION 40 (CLE40) is produced from developed columella root cap cells and is important in regulating columella root
cap initial function. Impairment of the movement of CLE40 from the columella root cap cells back into the columella initials disrupts the correct positioning and expression of WOX5, leading to loss of meristem function (Stahl et al., 2009). Additionally, peptides in the ROOT MERISTEM GROWTH FACTOR (RGF) family have been demonstrated to regulate PLT activity at the posttranslational level in an auxin-independent manner (Matsuzaki et al., 2010).

A reported function of miRNA165/166 is the movement from their site of synthesis, the endodermis, through plasmodesmata into the stelar tissue, resulting in the specificity of xylem cell types (Miyashima et al., 2011). Prior to leaving the endodermis, miRNA165/166 are activated by a combination of SCR, which resides in the endodermis, and SHR, which is produced in the stele and enters the endodermis through plasmodesmata (Carlsbecker et al., 2010). This crosstalk between the endodermis and vasculature, utilizing multiple forms of small signaling molecules and various means of regulation, in addition to the hormonal signals discussed above, demonstrate the remarkable complexity required to maintain a properly functioning RAM.

**Determinate Root Growth**

In some plant tissues such as floral organs, leaves, thorns, spines, tendrils and root nodules, meristems can only preserve growth for a limited duration before meristematic capacity is lost and development ceases, a process called determinacy (Blaser, 1956; Tucker and Hoefert, 1968; Nap and Bisseling, 1990;
Determinate root growth occurs when the RAM initials lose their potential for proliferation, potentially due to impairment of the repressive signal produced by the QC (Dubrovsky, 1997a; Shishkova et al., 2008; Drisch and Stahl, 2015). Determinate root development is observed in a wide range of species in many groups, such as: Proteaceae (Purnell, 1960), Pteridophytes (Webster and MacLeod, 1996), Cactaceae (Dubrovsky, 1997a, b), Fabaceae (Gladish and Rost, 1993), *Lolium perenne* L. (Zobel, 2013), *Zea mays* L. (Fusseder, 1987; McCully, 1987; Varney and McCully, 1991) and many others.

Two types of determinate root growth exist. Constitutive determinacy, in which determinacy is a typical part of the developmental program, has been studied extensively in Cactaceae primary roots and is also observed in lateral root growth of many plant families. Non-constitutive or inducible determinate growth is when determinacy is activated in response to environmental cues, such as phosphorous deficiency (Sánchez-Calderón et al., 2005). During typical root development, new cells are formed via initial cells in the QC, then several cycles of cell division occur within the meristem before cells exit into the elongation zone (Ivanov, 1981, 1994; Webster and MacLeod, 1996; Barlow, 1997). Therefore, RAM functionality is reliant upon both the activity of meristematic cells as well as proper coordination with the QC. When roots are growing at a steady rate, the quantity of cells within the RAM is more or less constant (Ivanov, 1981). However, when roots approach determinacy, the number of meristematic cells begins to drop rapidly (Dubrovsky, 1997b). In arabidopsis, as the RAM ages, the density and frequency of plasmodesmata decrease, suggestive of decreased cellular communication.
when approaching determinate length (Zhu et al., 1998). Furthermore, the cells comprising the QC become less distinct and the RAM becomes disorganized (von Guttenberg, 1960; Baum et al., 2002).

Several studies have assessed the cellular and genetic basis for determinacy. Mutants of several critical transcription factors for maintaining RAM organization and functionality, *plt1* and *plt2*, *wox5*, *shr* and *scr*, display aberrant QC divisions or loss of QC cell specificity, resulting in meristem exhaustion (Benfey et al., 1993; Scheres et al., 1995; Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004; Jiang and Feldman, 2005). Additionally, auxin has been shown to be a critical factor in several processes related to meristem development and functionality, and is necessary to maintain indeterminate growth. Studies examining loss of function mutants in *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (TAA1) and *TRYPTOPHAN AMINOTRANSFERASE RELATED2* (TAR2) reported total RAM consumption (Stepanova et al., 2008). Additionally, constitutive overexpression of the serine-threonine kinase *PINOID* (*PID*) involved in auxin efflux leads to RAM exhaustion shortly after germination. However, RAM exhaustion in 35S::*PID* seedlings can be prevented by treatment with NPA (Benjamins et al., 2001; Friml et al., 2004). Auxin has additionally been known to be associated with alterations in cellular redox (Jiang et al., 2003). The QC maintains a more oxidized environment compared to surrounding meristematic cells, containing more oxidized forms of glutathione and ascorbate, which delay the progression of the cell cycle at the G1 stage (De Tullio et al., 2010). Furthermore, studies have shown
that the reduced form of ascorbate, ascorbic acid, promotes cell division in the QC of maize RAMs (Jiang and Feldman, 2005).

Recent work has focused on studying the regulation of the ‘indeterminacy-to-determinacy’ switch (IDS) in arabidopsis roots (Reyes-Hernández et al., 2014). FOLYL POLYGLUTAMATE SYNTHETASE (FPGS) is a gene associated with vitamin B9 metabolism and was identified as being responsible for the repression of IDS in a perpetual ‘off state’. Events leading to IDS being switched to an ‘on state’ through modification of AtFPGS1 result in RAM exhaustion and determinacy. Activation of the QC through the polyglutamate-dependent IDS pathway was shown to act independently of auxin gradients and RAM regulatory elements such as PLT, SCH/SCR and WOX5 (Reyes-Hernández et al., 2014). B9 vitamins are a group encompassing tetrahydrofolates (THF), folic acid derivatives which are essential for metabolism. THFs act as donors of the C1 atom required for synthesis of critical cellular compounds. Purine, thymidylate, pantothenate, amino acid, and nucleic acid synthesis are all dependent on FPGS1 (Srivastava et al., 2011). Furthermore, a study of arabidopsis plants grown on medium containing methotrexate, a powerful inhibitor of folate synthesis, resulted in determinate roots (Srivastava et al., 2011). Additionally, studies of mko2, an AtFPGS1 mutant, showed an expansion of the promoter activity domain of QC-specific transcription factors, suggesting a role of FPGS in QC quiescence (Reyes-Hernández et al., 2014). Taken together, these findings imply that FPGS functionality maintains RAM indeterminacy, both by a requirement of FPGS in meristem niche maintenance as well as its role in folate-dependent C1 metabolism.
Some studies have reported that water deficit can influence the determinate root developmental program. A review of known literature, as well as original work of this dissertation, involving the interaction of determinate root growth and plasticity to water deficit conditions are presented in Chapters 2 and 3.

Objectives

The overall objective of the presented work was to assess genotypic differences in maize lateral root plasticity to mild water deficits, and to examine the effects of sustained water deficit on the lateral root determinate growth program. Specific objectives were:

I) Development of conditions suitable for growing transpiring, light-grown maize plants in a range of sustained water potential ($\Psi_w$) environments.

II) Assess the extent of lateral root plasticity of two maize lines, FR697 and B73, over a high-resolution range of mild water deficits.

III) Use a kinematic approach to examine whether the enhanced first-order lateral root phenotype displayed by FR697 under mild water deficit conditions involves delayed root determinacy.
REFERENCES


Chapter 2

Maize lateral root developmental plasticity induced by mild water stress I: An examination of genotypic variation across a high-resolution series of water potentials
ABSTRACT

The suitability of several commonly used growth media was assessed for use in controlled water potential (Ψ_w) experiments. Conditions allowing comparisons of maize genotypes of inherently different size over a narrow range of precise and reproducible media Ψ_w are detailed. Genotypic differences specific to lateral root growth from the primary root system were observed over a series of mild water deficits, ranging from a medium Ψ_w of -0.25 to -0.40 MPa, with a well-watered control of -0.10 MPa. The total lateral root length of inbred B73 plants, as well as the average length and diameter of first-order lateral roots, were unresponsive to the levels of water deficit tested. In contrast, the total lateral root length of inbred FR697 plants was 27% greater when grown at media Ψ_w of -0.25 MPa, and first-order lateral roots were 30% longer, in comparison with well-watered control plants. Furthermore, FR697 first-order lateral roots were 26% wider than well-watered controls, resulting in a 96% increase in volume. Both genotypes showed no significant differences in lateral root length at -0.35 MPa compared to their respective well-watered controls. The sensitivity of genotype specific responses over this narrow range shows the need to study mild water deficit conditions using a high-resolution series of water potentials.
INTRODUCTION

Drought is the largest contributing factor to global limitations on crop growth and development (Boyer, 1982). In the US alone there is estimated to be up to $8 billion in damage to crops annually (Witt, 1995). With more extreme droughts being predicted regionally by climate models, net crop yields are likely to decrease (Dai et al., 2004; Trenberth et al., 2014). The shift of agricultural areas to more arid climates, combined with increased volumes of water being necessary for irrigation, leads to an imperative for the development of crop varieties capable of producing larger yields in environments with limited water. Therefore, it is essential to enhance the yield of crops through increased drought tolerance to provide adequate amounts of food for the ever-growing global population.

One of the most common adaptations that plants have evolved to maintain growth under water deficit conditions is to develop a deeper, more highly branched root system (Sponchiado et al., 1989). Some types of roots are capable of maintaining growth at low water potentials ($\Psi_w$) that completely inhibit growth of other plant organs (Sharp and Davies, 1979; Westgate and Boyer, 1985). Furthermore, there have been many observations that mild water deficit can increase the net root length of several species (Weaver and Bruner, 1926; Yeager, 1936; Jarvis and Jarvis, 1963; Hsiao and Acevedo, 1974; Sharp and Davies, 1979; Huck et al., 1983; Jupp and Newman, 1987; Nguyen and Lamant, 1989; Creelman et al., 1990; Morita and Okuda, 1994; Triboulot et al., 1995; Morita et al., 1997; Reid and Renquist, 1997; van der Weele et al., 2000; Mahajan and Singh, 2006). Maize (Zea mays L.) is a valuable candidate for efforts to increase drought
tolerance as it is the most cultivated cereal worldwide (U.S. Grains Council). Maize seedlings have been used extensively to investigate the genetic and physiological responses of the primary axial root in water-limiting environments (Sharp et al., 1988; Sharp et al., 1990; Sharp et al., 2004; Spollen et al., 2008; Yamaguchi and Sharp, 2010). However, knowledge of the mechanisms controlling the growth of lateral roots at various levels of water deficit is lacking.

The maize root system is composed of several distinct categories of roots. Upon germination, the first root to emerge is designated the primary root. The primary axial root along with its associated laterals are collectively termed the primary root system. Similarly, lateral roots also form on the seminal and nodal axial roots, which are collectively referred to as the seminal and nodal root systems. Lateral root categorization is further broken down relative to branching order from the axial root, i.e. first-order, second-order. Lateral roots are responsible for soil exploration along the vector of axis growth and are the main contributors to resource uptake (Russell, 1977). It has been shown that maize lateral roots are accountable for the majority of water acquisition and can facilitate rates of radial uptake into the vasculature up to five orders of magnitude higher than axial roots (Ahmed et al., 2016). Furthermore, lateral roots display high phenotypic plasticity, changing the shape of the root system to best suit environmental conditions. Even though lateral root growth responses to water deficits are exceptionally important to understanding how water acquisition changes under stress, the majority of drought studies have not studied them in fine detail or in a highly-controlled Ψ_w
environment. Thus, little has been ascertained regarding the mechanisms involved in growth regulation of these vitally important organs in soil drying conditions.

An increase in lateral root initiation can be seen in response to water deficit in some plants. Succulent cacti species *Ferocactus acanthodes* and *Opuntia ficus-indica* both show an approximate three-fold increase in lateral root primordia number when grown under dry down conditions (North et al., 1993; Dubrovsky et al., 1998). Stimulation of lateral root number has also been seen in crop plants such as perennial rye-grass (*Lolium perenne*), rice (*Oryza sativa*), cassava (*Manihot esculenta*) and soybean (*Glycine max*) when grown under water deficit conditions (Read and Bartlett, 1972; Jupp and Newman, 1987; Banoc et al., 2000). In addition to enhanced number, the length of laterals has also been shown to increase in some crops in response to water deficit, such as maize and wheat (*Triticum aestivum*) (Schmidhalter et al., 1998; Ito et al., 2006).

A few researchers have demonstrated that mild levels of water stress can elicit an enhancement in lateral root length that is not observed under more severe stress (Read and Bartlett, 1972; Molyneux and Davies, 1983; Jupp and Newman, 1987; Munns, 2002; Ito et al., 2006; Kano et al., 2011). Unfortunately, many stress studies implementing a stable $\Psi_w$ environment begin with treatments at relatively high levels of stress, or only use one extreme level to study survival mechanisms. An alternate common practice is to impose a dry-down by withholding water, or to limit the amount of available water by irrigating at less frequent intervals. In either of these methods the media $\Psi_w$ will likely decrease through the mild stress range quickly and any growth responses induced by mild stress could be missed entirely.
or incorrectly attributed to higher stress levels. Furthermore, these types of studies provide no detailed quantification of the level or range of stress being utilized and thus cannot be reproduced. How quickly the $\Psi_w$ of the growth media declines with drying is greatly dependent on the characteristics of the growth media itself, with some media being incapable of maintaining a stable stress of any level for a prolonged time. Furthermore, dry-down or less frequent irrigation experiments create a gradient of water deficit in pots or in the soil profile. A $\Psi_w$ gradient greatly complicates the interpretation of such studies, as the hundreds of lateral roots associated with the various root categories may be responding to numerous levels of stress simultaneously, due to differences in rooting depth. Moreover, individual roots emerging high in the soil profile grow through increasingly wetter soil the deeper they grow. These potential problems could further interact with issues inherent in pot-based plant systems, such as the dependency of aeration and soil temperature on the water content of the media (Passioura, 2006). Collectively, this shows that to study the characteristics and mechanisms driving lateral root responses to water deficit there is a need for highly controlled, reproducible conditions over a range of steady stress levels, as well as a careful selection of growth media.

In this study, I present a detailed evaluation of the suitability of various, commonly used growth media for their use in controlled $\Psi_w$ experiments. Furthermore, growth conditions capable of allowing comparisons of maize genotypes of inherently different size and transpiration regimes, over a narrow range of reproducible media $\Psi_w$ for a significant duration of growth are detailed.
First-order lateral roots from the primary root system showed genotypic differences in dynamic growth responses over a narrow range of $\Psi_w$ from -0.10 to -0.40 MPa, supporting the need for high-resolution $\Psi_w$ studies at mild stress levels.

**MATERIALS AND METHODS**

**Growth media water relations**

The media utilized in this study were: sand (QUIKRETE® Premium Play Sand®, Atlanta, GA, USA), porous ceramic particles (Greens Grade, Profile® Products, Buffalo Grove, IL, USA), vermiculite (no. 2A, Therm-O-Rock East Inc., New Eagle, PA, USA) and Pro-Mix HP potting mix (Premier Tech, Québec, Canada). To assure that adequate nutrition was available regardless of the added volume of water, the various growth media were first fully hydrated with nutrient solution to create uniform availability. The nutrient solution used was comprised of 0.50 mM KH$_2$PO$_4$, 0.50 mM MgSO$_4$ · 7 H$_2$O, 2.50 mM Ca(NO$_3$)$_2$ · 4 H$_2$O, 2.50 mM KCl, 0.31 mM EDDHA iron chelate (SPRINT ® 138), 2.30 μM H$_3$BO$_3$, 0.90 μM MnSO$_4$ · H$_2$O, 0.60 μM ZnSO$_4$ · 7 H$_2$O, 0.10 μM Na$_2$MoO$_4$ · 2 H$_2$O, 0.11 μM NiCl$_2$ · 6 H$_2$O and 0.15 μM CuSO$_4$ · 5 H$_2$O (personal communication with Dr. Dale Blevins, University of Missouri). The final solution pH was adjusted to 5.6 with 10 mM NaOH. An additional treatment of Pro-Mix HP was hydrated with deionized, distilled (DDI) water devoid of added nutrient solution to test effects of nutrients on the $\Psi_w$ of the media at various levels of moisture. Following hydration, the different media were dried in a forced air oven at 55°C for at least 48 h, until constant
weights were obtained. The dried media were then weighed and mixed with a series of increasing amounts of DDI water to produce curves of $\Psi_w$ by gravimetric water content ($\theta_g$). Growth medium $\Psi_w$ was measured by isopiestic thermocouple psychrometry (Boyer and Knipling, 1965). Pro-mix HP was deemed to have the most optimal water relations over the desired range of stress levels, and was selected as the sole medium to be used for the plant growth experiments presented in Chapters 2 and 3.

**Plant material and growth conditions**

Maize (*Zea mays* L. cvs B73 and FR697) seeds were surface sterilized with 5% bleach for 30 min and then rinsed thoroughly with tap water. Following sterilization, seeds were treated with fungicide (Spectracide Immunox® Multi-Purpose Fungicide, Spectrum Brands Holdings, Inc., Middleton, Wisconsin, U.S.A.) for an additional 30 min and then imbibed in 1 mM CaSO$_4$ for 23 h. After imbibition, seeds were transferred to moist germination paper wetted with 1 mM CaSO$_4$ and were allowed to germinate at 29°C in the dark at near saturation humidity. Seedlings with primary roots of 5-10 mm in length were selected and transplanted into 46 cm tall x 15 cm diameter Plexiglas tubes filled with Pro-Mix HP. The medium had previously been desiccated, weighed into separate 1 kg samples and thoroughly mixed with predetermined amounts of DDI water to produce media with precise $\Psi_w$ based on $\theta_g$. For the presented plant growth experiments, the $\Psi_w$ of the well-watered control medium at transplant was -0.10 MPa and levels of water deficit used were -0.25, -0.30, -0.35 and -0.40 MPa. Eight
seedlings were transplanted into each tube in a circular pattern at an equal distance from the center and the edge, at a planting depth of 5 cm.

After transplanting, tubes were weighed then moved into a controlled environment growth chamber (Conviron PGW36) kept at a constant 29°C. The light intensity was 700 μmol m$^{-2}$ s$^{-1}$ PAR at canopy height, with a 14h/10h day/night cycle and a relative humidity (RH) of 90% / near saturation, respectively. Tube bases were placed in plastic buckets and were surrounded with moist cheesecloth to minimize evaporation from tube bottoms. Each day tubes were weighed, controls were watered back to original weight, and the cheesecloth of all tubes was remoistened. Water deficit treatments did not receive any watering for the duration of experiments.

Following 9 days of growth, plants were harvested and the Ψ$_{w}$ of the medium was assessed at a tube depth of 15 cm to confirm that the levels of water deficit were maintained for the duration of the experiment. Roots were then carefully washed free of all growth medium and kept at 4°C until further analysis. Leaf area was obtained using a LI-COR LI 3000A portable leaf area meter. Following leaf area measurements, the leaves and stem of each individual were gathered together, dried in an oven at 55 °C until fully desiccated, and analyzed for shoot dry-matter accumulation.

**Root trait analysis from scans**

Clean root systems were analyzed for a variety of root traits by WinRHIZO equipment and software (Regent Instruments Inc.) using an image capture
resolution of 800 dpi. The total length of the primary and seminal root systems was assessed and the length of the axial roots were subtracted, resulting in total lateral root length for each system. The whole organ responses of first-order lateral roots growing from the top 15 cm of the primary axial root were assessed by individually analyzing 10 first-order branches, excluding all higher order laterals, to obtain an average length, diameter and volume per treatment. Following analysis, the primary, seminal, and nodal root systems from each individual were separated, dried in an oven at 55 °C until fully desiccated, and analyzed for root dry-matter accumulation.

Statistical Analysis

Student’s t-tests and ANOVA analyses were carried out at the 0.05 significance level in IBM SPSS Statistics 2017 (IBM Corporation, Armonk, New York, U.S.A.). Graph production and regression analyses were performed by Origin 2017 (OriginLab, Northampton, MA, U.S.A). Experimental sample sizes for plant growth assays were 12–16 individual plants, from two individual tubes of up to eight seedlings. Data presented for first-order lateral roots are the means generated by the longest 10 of each individual, averaged across all 12-16 plants per treatment.
RESULTS

Comparative water relations of common plant growth media

One method to attempt to maintain the water status of the soil is to reduce the water-loss by halting transpiration. However, preliminary experiments utilizing microcosms at near saturation humidity resulted in stunted plants that were more sensitive to water deficit compared to plants grown at 90% RH (Fig. A-1). Therefore, several different commonly utilized growth media, a high porosity peat-based potting mix (Pro-Mix HP), vermiculite, a ceramic clay-based medium (Greens Grade), and course sand were assessed for their suitability of use in sustained stable \( \Psi_w \) experiments supporting the growth of actively transpiring plants. The largest difference between the various growth media tested was the amount of water each could retain at maximum holding capacity. Sand held the least amount of water, with a \( \theta_g \) of 0.2 (g H\(_2\)O g\(^{-1}\) dried media) when fully saturated (Fig 2-1A). Other media tested could hold considerably more water, Greens Grade, an artificial porous ceramic medium, had a \( \theta_g \) of 1.0 g·g\(^{-1}\) (Fig 2-1B) when fully hydrated. Vermiculite, a growth medium commonly used in root research, had a maximum \( \theta_g \) of 3.0 g·g\(^{-1}\) (Fig 2-1c). Pro-Mix HP, a high-porosity peat based potting mix, held the largest amount of water, with a \( \theta_g \) up to 7.0 g·g\(^{-1}\) after drainage (Fig 2-1D).

The relationship between \( \Psi_w \) and \( \theta_g \) was found to be best modeled using hyperbolic non-linear regression. The fitted curves have two asymptote arms connected at varying angles dependent on the hyperbolic rate parameter, with a
Figure 2-1. Influence of gravimetric water content on the water potential of several common growth media

Curves showing the relationship of water potential ($\Psi_w$) to gravimetric water content ($\theta_g$) for sand (A), Greens Grade (B), vermiculite (C), and Pro-Mix HP (D) growth media. Data points are from multiple independent calibrations and are fitted by a hyperbolic regression using Origin software (Originlab 2017) with $R^2 = 0.89 - 0.99$. The effect of adding nutrients on the water relations of Pro-Mix HP was assessed by producing curves with full nutrients (black squares) as well as no added nutrients (open circles). Lines on (D) show the change in Pro-Mix HP $\Psi_w$ and $\theta_g$ for the lowest and highest stress levels tested after growing FR697 plants for 9 days. Solid lines show the changes after transplanting into media with a $\Psi_w$ of -0.25 MPa. Dotted lines show the changes after transplanting to -0.40 MPa media.
higher rate having a more acute angle and severe transition between asymptotes. Major differences were seen in the dynamics of media $\Psi_w$ in relation to $\theta_g$ between the various growth media (Fig 2-1a-d). The fully hydrated Pro-Mix HP had a $\Psi_w$ approximately -0.06 MPa lower than the other media tested and exhibited a much slower decline of $\Psi_w$ with decreasing $\theta_g$, especially for $\Psi_w$ values between -0.20 and -0.70 MPa. Sand displayed an extremely limited range of high $\Psi_w$ stability before showing dramatic declines in response to very slight changes in $\theta_g$ (Fig. 2-1a). Greens Grade similarly had a small range of high $\Psi_w$ stability before the curve transition point. While the transition occurs over a much milder angle, steep declines in $\Psi_w$ with decreasing $\theta_g$ are seen for $\Psi_w$ levels below -0.15 MPa (Fig. 2-1b). Vermiculite had the largest range of high $\Psi_w$ stability of all the media tested, as well as a relatively mild change in slope for $\Psi_w$ in the range of -0.15 to -0.60 MPa (Fig. 2-1c). The Pro-Mix HP curve has a relatively small range of high $\Psi_w$ stability. However, the transition between asymptotes of the fitted curve encompassed nearly the entire range of $\theta_g$ tested. The mild rate of change in slope exhibited over the extensive range of $\Psi_w$ from -0.10 to -0.70 MPa, together with the capacity to retain large volumes of water, demonstrate the suitability of Pro-Mix HP for sustained $\Psi_w$ studies of roots of larger, light grown and transpiring plants.

To assess the relationships between $\Psi_w$ and water-loss of the different media on a relative level, $\theta_g$ values were converted to percentages of maximum holding capacity (Fig. 2-2). The change in percentage of holding capacity of the various media over the desired range of water deficit also varied greatly, with the
Figure 2-2. Percentage of media water-holding capacity viable for the desired range of mild water deficits. Curves showing the relationship of $\Psi_w$ to % of maximum holding capacity for sand (green triangles), Greens Grade (blue triangles), vermiculite (red circles) and Pro-Mix HP (black squares) growth media. Shaded regions represent the window of percentage holding capacity viable for water deficit studies between media water potentials of -0.25 and -0.40 MPa.
amount of water loss that is tolerated over the range varying by an order of magnitude between media. With the levels of nutrients used in this study, Greens Grade was the medium which was least resistant to a change in Ψ\textsubscript{w} with a decline in percentage of holding capacity over the range of desired stresses. At the target Ψ\textsubscript{w} of -0.25 and -0.40 MPa, Greens Grade was at a holding capacity of 38.6% and 37.5% respectively. This drop of 2.9% of the total holding capacity (Fig. 2-2, blue shading) represents the workable area for examination of water deficits in the desired range. Represented as water loss of bulk media, this would equate to a loss of only 29 g H\textsubscript{2}O per 1 kg of dried media pushing the Ψ\textsubscript{w} through the entire desired stress range. The tubes used to hold media for plant growth could contain 5 kg of dried Greens Grade, allowing for a workable range of 145 g H\textsubscript{2}O for experimentation between the Ψ\textsubscript{w} values of -0.25 to -0.40 MPa.

Sand had a Ψ\textsubscript{w} of -0.25 MPa at 14.7% of its well-watered holding capacity and lost 5.5% (Fig. 2-2, green shading) before reaching a Ψ\textsubscript{w} of -0.40 MPa at 9.2% of holding capacity. With a workable range of 11 g H\textsubscript{2}O per 1 kg of dried media, and 14 kg of dried Greens Grade to fill a tube, there is a window of 154 g H\textsubscript{2}O for experimentation. Vermiculite had a Ψ\textsubscript{w} of -0.25 MPa at 15.3% of capacity and was reduced to 8.7% of capacity at a Ψ\textsubscript{w} of -0.40 MPa, a loss of 6.6% (Fig. 2-2 red shading). This represents a window of 198 g H\textsubscript{2}O out of 1 kg of dried media, and a workable range of 297 g H\textsubscript{2}O for experimentation with 1.5 kg of dried vermiculite used to fill a tube. Pro-Mix HP was the medium most resistant to changes in Ψ\textsubscript{w} in response to declines in holding capacity. Pro-Mix HP had a Ψ\textsubscript{w} of -0.25 MPa at a holding capacity of 38.7% and could sustain a drop of 14.8% (Fig. 2-2 grey
shading) to a water holding capacity of 24.6% before reaching a \( \Psi_w \) of -0.40 MPa. A change of 14.8% represents a loss of 1036 g H\(_2\)O out of 1 kg of dried media. As exactly 1 kg of Pro-Mix was used for the experiments this is the workable range for \( \Psi_w \) values between -0.25 to -0.40 MPa. Interestingly, most of the growth media had a \( \Psi_w \) of around -1.5 MPa, a level of water deficit often referred to as the permanent wilting point, at 2-9% of maximum holding capacity. In contrast, Greens Grade was still at nearly 30% holding capacity when at this level of water deficit (Fig. 2-2).

Due to the requirement of sufficiently aged plants to study lateral root growth, a medium able to maintain a stable \( \Psi_w \) environment for actively transpiring plants over a prolonged duration is needed. Pro-Mix HP was deemed ideal due to its high holding capacity, and its relative stability of \( \Psi_w \) in respect to \( \theta_g \) in the desired stress range, growth medium \( \Psi_w \) values of -0.25 through -0.40 MPa. The impact of added nutrients on the water relations of the selected medium was assessed and was shown to have a large effect (Fig. 2-1D). The addition of nutrient solution decreased the \( \Psi_w \) of the well-watered medium from approximately -0.06 to -0.11 MPa, an osmotic component of -0.05 MPa. As the media became drier, concentration effects became more pronounced. At a \( \theta_g \) of 1.0 g·g\(^{-1}\) the addition of nutrients lowered the \( \Psi_w \) from around -0.40 to -0.70 MPa, an osmotic component of -0.30 MPa. For all subsequent experiments presented in this work, Pro-Mix HP was the sole medium used, supplemented with the nutrient solution outlined above.
A near-stable water deficit environment for the comparison of root traits

Two maize genotypes, which were shown in preliminary experiments to have different lateral root phenotypes in response to water stress, were utilized to test the stability of the system over a gradient of mild water deficits. The most thoroughly DNA-sequenced maize line, B73, was compared to FR697, a line which has been previously shown to exhibit a relatively greater ability to maintain primary and nodal axial root elongation under water deficit conditions (Leach et al., 2011; Riggs and Sharp, 2016).

Table 2-1 shows the change in media $\Psi_w$ of the treatments used, with an average decrease of 0.10 MPa at a depth of 15 cm for tubes of either genotype 9 days after transplant (DAT). Preliminary experiments showed that soil samples taken at a depth of 30 cm at harvest showed no significant change in $\Psi_w$ throughout the duration of the experiment. In contrast, tubes filled with sand or Greens Grade with a transplant $\Psi_w$ of -0.40 MPa declined at least -0.30 MPa at a depth of 15 cm over 9 days of growth (Fig. A-2). Lines on Fig. 2-1d show the average change in $\Psi_w$ and $\theta_g$ for the lowest and highest stress levels tested in Pro-Mix HP. At transplant, the medium with a $\Psi_w$ of -0.25 MPa was generated by mixing 2.75 g H$_2$O g$^{-1}$ dried media. During the duration of the experiment the roots dried the upper portion of the profile to a $\Psi_w$ of -0.34 MPa, a reduction of -0.08 MPa. The drop in $\Psi_w$ was associated with a drop in $\theta_g$ of -0.36 g·g$^{-1}$. Growth medium with a $\Psi_w$ of -0.40 MPa at transplant also displayed a reduction of -0.08 MPa in the upper...
Table 2-1. Validation of stable media $\Psi_w$ across a gradient of mild water deficits

Change in media $\Psi_w$ for mild deficit treatments of FR697 or B73 seedlings for 9 days. Transplant and harvest $\Psi_w$ values are the average of two separate tubes per genotype, growing eight seedlings each. Harvest $\Psi_w$ was assessed at a tube depth of 15 cm. Data are means ± the range of the data.

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<td>Transplant</td>
<td>Harvest $\Delta \Psi_w$</td>
<td>Transplant $\Delta \Psi_w$</td>
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<td>-0.29 ± 0.02</td>
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<td>-0.36 ± 0.01</td>
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<td>-0.49 ± 0.04</td>
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profile, declining to a $\Psi_w$ of -0.48 MPa during the 9 days of growth, with a $\theta_g$ loss of -0.22 g·g$^{-1}$. No appreciable difference in the amount of media drying was observed between genotypes (Table 2-1).

**Maize axial root responses to mild water deficit conditions**

Inhibition of axial root length, as well as leaf area, was seen with increasing levels of water deficit for all root categories of both genotypes (Fig. 2-3, Fig. 2-4). Node 1 axial roots were the most sensitive to increased levels of water deficit for both genotypes, with a reduction in length of approximately 50% compared to well-watered controls at a $\Psi_w$ of -0.40 MPa. The primary and seminal axial roots were less sensitive, but an appreciable decrease in average length was seen over the range of water deficits tested compared to well-watered controls, beginning at a $\Psi_w$ of -0.35 MPa for FR697 and -0.30 MPa for B73.

**FR697 displays enhanced lateral rooting under mild water deficit conditions**

In contrast to the high similarity of axial root length, the growth response of lateral roots to the range of $\Psi_w$ tested was markedly different between FR697 and B73 (Fig. 2-5). Under all experimental treatments, B73 plants had a higher total lateral root length of the primary root system compared to FR697, a trait not reflected in lateral roots originating from the seminal axes. A gradual decrease was observed in total lateral root length of the B73 primary root system with decreasing
Figure 2-3. Effects of mild water deficit on axial root growth

Effects of deceasing $\Psi_w$ on the average length of FR697 (A) and B73 (B) primary, seminal, and node 1 axial roots. ANOVA analyses are for within a root category across multiple stress levels, for each genotype individually. For each root type, treatments with different letters are statistically different at $p < 0.05$ ($n = 12-16$; $n = 7$ for B73 -0.40 MPa treatment).
Figure 2-4. Effects of mild water deficit on leaf area

Change in leaf area with increasing water deficit of B73 (black squares) and FR697 (white circles) seedlings 9 days after transplant. Data are means ± standard error (n = 12-16; n = 7 for B73 -0.40 MPa treatment).
Figure 2-5. Effects of mild water deficit on total lateral root length

Total lateral root length responses of the primary (grey bars) and seminal (white bars) root systems of FR697 (A) and B73 (B) to water deficit conditions. ANOVA analyses are for within a root category across multiple stress levels, for each genotype individually. Treatments denoted by different letters are statistically different at p < 0.05 (n = 12-16; n = 7 for B73 -0.40 MPa treatment). Insets show total lateral root length as % of the well-watered (WW) control from four independent experiments; lines of fit are modeled with cubic ($R^2 = 0.48$) and asymptotic regressions ($R^2 = 0.77$) for FR697 and B73, respectively.
media $\Psi_w$, with no difference in length at a $\Psi_w$ of -0.25 MPa and a 20% reduction by -0.4 MPa. Conversely, FR697 plants displayed a significant increase in total lateral root length of the primary root system at a $\Psi_w$ of -0.25 MPa, with a 27% enhancement in length over well-watered controls. The increased length attenuated back to control levels at -0.35 MPa and was maintained at this length at -0.40 MPa. The insets in Figures 2-5a and 2-5b show the total lateral root length of the primary root systems from four independent experiments. Both genotypes displayed a similar reduction in total lateral root length of the seminal root system with increasing water deficit, with FR697 being more sensitive than B73 at a media $\Psi_w$ of -0.25 and -0.30 MPa (Fig. 2-5).

To further analyze the differential responses of the primary root system between genotypes, regional lateral root responses to mild water deficits were evaluated along the length of the primary axial root. As most of the total lateral root length is composed of roots from the upper portion of the axis (Fig. 2-6), the growth of lateral roots originating from the upper 15 cm (approximately the top third) of the primary axial root was analyzed separately from the remainder of the root system. Both the total lateral root length as well as the number of first-order lateral roots were determined for both sections at a medium $\Psi_w$ of -0.25 MPa, the treatment with the largest genotypic difference in lateral root response (Fig. 2-6). No change in either total lateral root length or in the number of first-order laterals was observed in B73 plants for either region of the root system. However, FR697 showed an interesting response to the imposed water deficit, with different modifications of lateral root growth characteristics between the upper and lower portion of the
Figure 2-6. Regional responses of lateral roots along the primary axis under mild water deficit conditions

Effect of mild water deficit on the regional responses of total lateral root length (A, B) and number of first-order laterals (C, D) along the primary axial root of FR697 (A, C) and B73 (B, D). Lateral roots growing from the top 15 cm of the primary axial root (grey bars) and those growing from the remaining length (white bars) were assessed separately. Data are means ± standard error (n = 12-16; n = 7 for B73 -0.40 MPa treatment). Asterisks mark statistical differences between well-watered ($\Psi_w = -0.10$ MPa) and water-stressed ($\Psi_w = -0.25$ MPa) treatments for each genotype at p < 0.05 conducted by a student’s t-test.
primary root system. In the top 15 cm, FR697 showed a 28.9% increase in total lateral root length, with no modification of the number of first-order lateral roots. Lower on the primary axis there was also a trend of increased total lateral root length, as well as a 45.1% increase in the number of first-order lateral roots, primarily due an increase in the amount of newly emerging lateral roots several cm behind the primary axial root tip.

**First-order lateral root plasticity**

Due to the great variability of average lateral root length on a given axial root, a more specific sub-set of lateral roots was needed to study plasticity responses to mild water deficit conditions. The 10 longest first-order lateral roots on the top 15 cm of the primary axis were individually assessed for length, average diameter and root volume to determine a mean for each parameter per individual (Fig. 2-7 and Fig. 2-8). No change was observed in the average length, diameter, or volume of B73 first-order lateral roots over the range of water deficits tested (Fig. 2-8B, D, F). However, FR697 showed a large degree of plasticity in all parameters analyzed. Individuals grown at medium $\Psi_w$ values of -0.25 and -0.30 MPa had first order laterals that were up to 34% longer than the well-watered controls (Fig. 2-8A). It was observed that this enhancement was nearly constant over the entire population of sampled roots, showing that this response is not subject to influence by a few very long individuals (Fig. 2-9). Similar to the response of FR697 total lateral root length, the increased length of the first-order lateral roots
Figure 2-7. The 10 longest first-order lateral roots are a representative subset of total lateral root length

WinRhizo scans of representative FR697 (A, B) and B73 (C, D) plants grown at a medium $\Psi_w$ of -0.10 MPa (A, C) and -0.25 MPa (B, D). The dotted line in panel B depicts the 15-cm cut-off point that separated the two regions of analysis. Lateral roots highlighted in red show an example of the selection of the longest 10 first-order laterals in the upper profile. The scale bar = 5 cm.
Figure 2-8. Effects of mild water deficit on average length, diameter, and volume of first-order lateral roots

Average length, diameter, and volume of FR697 (A, C, E) and B73 (B, D, F) first-order lateral roots 9 DAT across the range of medium $\Psi_w$. ANOVA analyses are for each root parameter and genotype individually. Data are means ± standard error (n = 12-16; n = 7 for B73 -0.40 MPa treatment). Treatments denoted by different letters are statistically different at $p < 0.05$. No significant differences were seen for B73 lateral roots for any parameter across the range of water deficits tested.
The enhancement of FR697 first-order lateral root length induced by mild water deficit is a uniform response

The longest 10 first-order lateral roots per plant ranked shortest to longest for FR697 (A) and B73 (B). Black bars represent well-watered roots ($\Psi_w$ of -0.10 MPa) and red bars are roots grown at a $\Psi_w$ of -0.25 MPa. Collectively, the data were used to generate the means shown in Figure 2-8.
attenuated back to control levels at a $\Psi_w$ of -0.35 MPa and was maintained at -0.040 MPa, showing the dominance of this sub-set of lateral roots on total root length.

The average diameter of first-order lateral roots was significantly higher for FR697 plants grown in more stressed conditions, with roots growing at a medium $\Psi_w$ of -0.40 MPa showing an average diameter nearly 145% that of control roots (Fig. 2-8C). As a result, the volume of FR697 first-order lateral roots was the parameter most significantly affected by increasing water deficit, with roots of the -0.40 MPa treatment showing an average volume 230% larger than the well-watered controls (Fig. 2-8E).

**Dry-matter accumulation**

Shoot biomass declined with increasing water deficit for both genotypes. FR697 plants were slightly more sensitive to shoot growth inhibition, with a 44% decrease in leaf dry weight when grown at a medium $\Psi_w$ of -0.40 MPa compared to controls (Fig. 2-10A). Comparatively, B73 shoot dry weight was reduced 39% at this level of water deficit. In both genotypes the dry weight of the primary and seminal root systems showed no significant response to decreasing medium $\Psi_w$ (Fig. 2-10B, C). However, the dry weight of the nodal systems of both genotypes were reduced at a medium $\Psi_w$ of -0.40 MPa (Fig. 2-10D). Unfortunately, the subsets of the 10 longest first-order lateral roots were not assessed separately for biomass accumulation. However, the primary axial roots of both genotypes did not show any radial thinning over the range of mild water deficits tested (data not
Figure 2-10. Effects of mild water deficit on biomass accumulation

Biomass (dry weight) accumulation of FR697 (■) and B73 (○) shoots (A), primary root system (B), seminal root system (C), and the first group of nodal roots (Node 1) as influenced by decreasing medium $\Psi_w$. ANOVA analyses were conducted for each root category and genotype individually, with asterisks indicating treatments significantly different from their respective well-watered controls at $p < 0.05$. Data are means ± standard error ($n = 12-16$; $n = 7$ for B73 -0.40 MPa treatment).
shown), suggesting that there was not a direct tradeoff of axial vs lateral root radial expansion. Interestingly, the similarities in dry-weight accumulation between FR697 and B73 led to nearly identical root-to-shoot ratios across the $\Psi_w$ range tested, despite the differences in lateral root length and volume responses to mild water deficit conditions (Fig. 2-11).

**DISCUSSION**

**Suitability of various growth media for sustained mild water deficit experiments**

To accurately study maize lateral root growth in fine detail, careful attention was placed on environmental conditions and the growth media. Several commonly used growth media were assessed for their suitability in sustained $\Psi_w$ experiments. Large differences in water holding capacity of the media were observed. Sand could retain the least amount of water, with a holding capacity of 0.2 g H$_2$O g$^{-1}$ dried medium (Fig. 2-1A). Other media tested could hold considerably more water. Greens Grade, an artificial porous ceramic growth medium, was able to hold 5x the amount of water compared to sand, with an approximate $\theta_g$ of 1.0 g·g$^{-1}$ at capacity (Fig. 2-1B). Vermiculite, a growth medium extensively used in root research, held triple that amount with a $\theta_g$ of around 3.0 g·g$^{-1}$ at capacity (Fig. 2-1C). A high-porosity peat-based potting mix (Pro-Mix HP) could hold the largest amount of water out of all the media tested, retaining more than double the amount
Figure 2-11. Effects of mild water deficit on root-to-shoot biomass ratio

Effects of decreasing $\Psi_w$ on the root-to-shoot biomass (dry weight) ratio of FR697 and B73 seedlings. ANOVA analysis is for all treatments of both genotypes, with treatments of different letters being significantly different at $p < 0.05$. Data are means ± standard error ($n = 12-16$; $n = 7$ for B73 -0.40 MPa treatment).
of water compared to vermiculite and over 3500% the amount compared with sand, with a holding capacity equal to a $\theta_g$ of 7.0 g·g$^{-1}$ after drainage (Fig. 2-1D).

Furthermore, the dynamics of media $\Psi_w$ in relation to drying was also very diverse (Fig. 1A-D, Fig. 2-2). Sand was shown to be unsuitable for any prolonged mild deficit treatment due to the extremely limited amount of water it could retain (Fig. 2-1A) as well as the extremely narrow window of % of holding capacity (Fig. 2-2) and water content (Fig. 2-1A) useful in the desired range of water deficits. Greens Grade also holds a very small amount of water at full saturation, and has narrow windows of holding capacity and $\theta_g$ between the media $\Psi_w$ values of -0.25 and -0.40 MPa. Interestingly, Greens Grade reaches low levels of $\Psi_w$ when still at relatively high levels of % holding capacity. This is due to the extensive amount of capillary space within the ceramic particles (39% capillary pores), making it a suitable amendment for increased water retention but less practical for use on its own. Vermiculite could hold considerably more water and had a wider range of viable $\theta_g$ and % holding capacity relative to mild stress levels. When assessed on the basis of % holding capacity, vermiculite seemed to have the most optimal dynamics for mild water deficit experiments, with a large range of % capacity corresponding to a media $\Psi_w$ above -0.15 MPa, 28-100%, as well as a slow transition angle between $\Psi_w$ asymptotes (Fig. 2-2). However, the maximum amount of water held was only half that of Pro-Mix HP, which greatly limits its comparative ability to maintain media $\Psi_w$ in response to water loss via growth-sustaining uptake and evapotranspiration. Thus, the Pro-Mix HP medium was deemed to be the most suitable for sustained stable $\Psi_w$ studies on light-grown
transpiring plants, due to its high holding capacity and large window of $\theta_g$ available for experimentation over a range of mild stress conditions.

In conjunction with careful selection of growth media, experimental conditions were also highly controlled to increase the $\Psi_w$ stability of the system by reducing evapotranspiration. A very high RH regime was maintained, 90% during the light cycle and near saturation humidity during the dark. Growth under this regime reduced transpiration and allowed for a system in which photosynthetically active plants could be grown in an almost stable $\Psi_w$ environment. Increasing stress levels resulted in smaller leaf areas, which led to less water loss through transpiration, producing similar declines in medium $\Psi_w$ as shown by the relationships between $\Psi_w$ and $\theta_g$ (Fig. 2-1D). The $\Psi_w$ of each treatment decreased by only approximately 0.10 MPa at a depth of 15 cm throughout the duration of the experiments, creating a series of narrow overlapping windows of mild water deficit. Importantly, this relatively small decrease in $\Psi_w$ shows the stability of the experimental design to allow for the assessment of growth over a high-resolution series of specific, and reproducible, levels of water deficit.

In previous studies, the lack of high-resolution water stress conditions as well as the exclusion of very mild deficit levels could have potentially confused the understanding of results and hampered the synthesis of knowledge across species and growth media. Many studies have concluded that water deficit conditions repress lateral root growth (Pardales Jr and Yamauchi, 2003; Deak and Malamy, 2005; Ogawa et al., 2005; Xiong et al., 2006) or have no effect at mild levels before becoming inhibitory under more severe stress (van der Weele et al., 2000).
However, the range of stresses commonly tested often begins at levels already beyond the range of mild stress examined in the present study, potentially missing a window of enhancement. In soybean, it has been shown that the number of lateral roots in the primary root system can increase three-fold when grown at a very mild deficit level, a $\Psi_w$ of -0.16 MPa (Read and Bartlett, 1972). Furthermore, a dynamic growth pattern of lateral root enhancement has been reported for multiple genotypes of rice, peaking and attenuating to below control levels by a soil $\Psi_w$ of -0.20 MPa (Kano et al., 2011).

In this study, we show that in the Pro-Mix HP growth medium a $\Psi_w$ of -0.25 MPa results in the maximum enhancement of FR697 first-order lateral root length. The heightened levels of total lateral root length, as well as the average length of first-order lateral roots, declined back to well-watered levels at a $\Psi_w$ of -0.35 MPa. At this still relatively mild level of water deficit, both FR697 and B73 show no difference in lateral root parameters compared to their respective well-watered controls. By using a high-resolution series of $\Psi_w$ values across a range of mild water deficits, we were able to identify genotype-specific responses that may have been overlooked using many common methodologies.

**Genotypic differences in maize lateral root plasticity induced by mild water deficit**

Interestingly, the axial roots of both genotypes responded to the range of water deficits tested in a comparable way, while the lateral root plasticity responses were significantly different. It is important to note that there were no significant
reductions in the length of the primary axial roots at the levels of water deficit that elicit increased lateral rooting. It has been shown that axial root inhibition, either through decreased meristem activity or by damage or loss of the root tip, can promote lateral root growth and development, potentially via decreased flow of an axial root tip-sourced inhibitor of lateral root growth (Thimann, 1936; Böttger, 1974; Hinchee and Rost, 1986; Dubrovsky, 1997a, b; Ditengou et al., 2008). The lateral roots from the seminal axes behaved similarly for both genotypes but the primary system lateral roots exhibited significantly different responses to mild water stress. This shows that there is separate regulation of root plasticity between lateral roots of different axial root systems of the same individual. The average length of lateral roots from the primary axis was much longer than the reported 2.2 cm for first-order lateral roots growing from the maize seminal and nodal axes (Cahn et al., 1989). Similar to the results of Ito et al. (2006), we saw that first-order lateral roots of the FR697 maize primary axis were longer when grown under mild water deficit conditions (Fig. 2-8A). A lateral root phenotype of increased length isolated to the primary axial root could be an environmental adaptation aiding in seedling establishment in non-optimal conditions.

It has been reported that the elongation rate of maize first-order lateral roots increases in a linear relationship with root diameter (Cahn et al., 1989). However, in the current study, the $\Psi_w$ treatment that resulted in the longest FR697 lateral roots, -0.25 MPa, produced roots of the same width as well-watered controls. In fact, lateral roots grown at a media $\Psi_w$ of -0.25 MPa were longer and thinner than roots grown at a $\Psi_w$ at -0.30 MPa or lower at the end of the experiment (Fig. 2-
Furthermore, our results show an opposite response in root diameter as that reported for the primary root of maize, which showed progressive thinning over a wide range of increasing water deficits (Sharp et al., 1988). It is unknown if this was due to an intrinsic difference in response between the primary axial root and its corresponding laterals, or if the lateral roots examined would show similar thinning if grown under more severe levels of stress. In our system, the primary axial root showed no difference in diameter over the mild stress range tested for either genotype (data not shown), while a stress level of -0.20 MPa resulted in a significant decrease in diameter of primary roots in the Sharp et al. (1988) vermiculite-based experimental system. This could potentially be explained due to differences in the hydraulic contact between the roots and the media used.

Vermiculite is composed of very large particles compared to a maize seedling primary root and thus there would be a limitation in the amount of root-to-media contact due to relatively large interparticle space. In contrast, the Pro-Mix HP utilized in the present study is composed of a mix of particles of numerous sizes, including many very fine peat moss particles that greatly increase the hydraulic conductivity between the root and the soil. Furthermore, while similar levels of $\Psi_w$ were assessed between the experiments, as shown here, vermiculite and peat-based potting mixes have greatly different values of $\theta_g$ at common levels of $\Psi_w$. This difference between media could be an important aspect of the environment, potentially affecting the mobility of nutrients and the availability of oxygen. The differences in primary axial and lateral width responses seen between the experiments could also be due to the age difference of the plants, 48 h
compared to 11 days, respectively. Lastly, the thinning response seen in Sharp et al. (1988) may be characteristic of the WF9 x Mo17 hybrid genotype used, indicating further genotypic diversity between maize lines in primary root system responses to water deficit.

The corresponding dry weight data did not correspond well with whole root system length responses or with changes in the average length, diameter or volumetric responses of the first-order lateral roots (Fig. 2-8, Fig. 2-10). Most interesting is that, in FR697, there was no significant increase in dry weight between the -0.10 and -0.25 MPa treatments, despite there being 27% higher total lateral root length and no perceived difference in the average width of first-order lateral roots. These discrepancies suggest that other internal modifications related to water deficit were contributing more to weight, such as cell wall thickening, changes in cortical cell file number, cortical cell size, or formation of specialized internal structures such as aerenchyma (Zhu et al., 2010; Chimungu et al., 2014b, a; Lynch et al., 2014). However, the average width of lateral roots lower on the primary axial root was not assessed and could, like total lateral root length and first-order lateral number, differ in response from the lateral roots on the basal portion of the axis. The overall similarity of the root-to-shoot ratio exhibited by both genotypes, despite the drastic differences inherent in their root system sizes as well as lateral root responses to water deficit, may be indicative of an overarching regulatory process controlling the partitioning of plant biomass under water deficit conditions (Fig. 2-11).
An interaction between lateral root plasticity and determinate growth?

A possible explanation for the differences seen in first-order lateral root growth could be explained by their developmental program. Maize lateral roots are characterized by a growth pattern of short-lived elongation followed by cessation of growth (Fusseder, 1987). Examination of maize first-order lateral roots revealed that root apical meristems were lost as the root progressed through development, resulting in determinate root growth (Varney and McCully, 1991). Water stress has been shown to affect the duration of root growth of various plants. In *Pachycereus pringlei* the determinate developmental program of the primary axial root is accelerated in response to increasing water deficit, with an accompanying 1.5x increase in lateral root number per mm of root length (Dubrovsky and Gómez-Lomelí, 2003). This is an adaptive strategy, prioritizing lateral root growth to enhance root surface area and resource uptake in response to water deficit. A similar but less extreme response can be seen in *Opuntia ficus-indica* grown in both gradual and rapid dry-down experiments (Dubrovsky et al., 1998). It is possible that the mild water deficits in this study could have some interaction with the determinate root growth program, potentially delaying its onset, resulting in some of the observations seen here. This could potentially explain the discrepancies between total system length and dry weight data. If the FR697 lateral roots grown under well-watered conditions have reached their determinate length they would be expected to show signs of cell maturation, such as thickening of cell walls or incorporation of secondary metabolites such as suberin or lignin. It
is possible that such modifications could balance the weight added by continued cell production and elongation that occurs in roots grown at a $\Psi_w$ of -0.25 MPa.

These results show that a more detailed analysis of the perceived enhancement of lateral root growth in FR697 needs to be conducted across the course of root development. Work presented in the following chapter utilized a kinematic approach to expand upon the anatomical growth patterns along the lateral root growth zone. A spatio-temporal analysis of lateral root developmental plasticity induced by stable, reproducible levels of water deficit will allow an in-depth examination of the cellular mechanisms controlling lateral root responses to mild water stress.
REFERENCES


Chapter 3

Maize lateral root developmental plasticity induced by mild water stress II: Genotype specific spatio-temporal effects on determinate development
ABSTRACT

The capability of roots to mine the soil for available water is essential for survival and productivity, especially under water-limited conditions. The formation and elongation of lateral roots is an important process determining the architecture of the root system, and is highly plastic in response to soil drying. In plants, organ growth is initiated, maintained, and regulated through meristematic tissues. However, in some contexts, meristems are genetically encoded to stop the production of new cells, resulting in determinate growth. To explore the effects of water deficit on lateral root determinacy, we implemented a system allowing photosynthetically active growth in a near-stable water potential environment. Two inbred maize lines (B73 and FR697) with divergent lateral root responses to water deficit were assessed. First-order laterals of the water-deficit tolerant maize cultivar FR697 display an ability to delay the determinacy program when grown under a mild water deficit of -0.30 MPa. Maximum root elongation rates were maintained for nearly 2.5 days longer, and were still at 44% of maximum when well-watered laterals approached their determinate length. Maintenance of lateral root elongation resulted from sustained rates of cell flux and meristem activity. In addition, kinematic (spatio-temporal growth) analysis revealed that reductions in tissue expansion rates with aging were delayed by more than 2 days in the longitudinal, radial and tangential planes. This study reveals large genotypic variability in the interaction of water deficit with the developmental determinacy of maize lateral roots. Research focused on revealing the mechanisms of determinate growth, and examination of its suppression, will provide insights into
regulatory and maintenance programs of meristems with both determinate and indeterminate growth, as well as provide possibilities to modify the determinacy program if desired.
INTRODUCTION

In seed plants, growth is organized, regulated and maintained through meristematic tissues (Evert, 2006). The ratio between de novo meristem cell generation and subsequent division and differentiation of cells as they are displaced from the root tip allows for meristem maintenance and regulation. However, in some tissues meristems are genetically encoded to stop the production of new meristematic cells at a particular developmental age, resulting in determinate growth. Determinate root growth is the process in which root apical meristem (RAM) cells only divide for a certain duration before the RAM becomes exhausted and meristematic cells themselves undergo differentiation (Dubrovsky, 1997a; Shishkova et al., 2008). Furthermore, along with RAM exhaustion, it has been observed that final cell size also decreases in roots approaching determinacy (Dubrovsky and Gómez-Lomelí, 2003). Determinate root growth has been seen in many taxa, such as: Proteaceae (Purnell, 1960), Pteridophytes (Webster and MacLeod, 1996), Cactaceae (Dubrovsky, 1997a, b; Dubrovsky and Gomez-Lomeli, 2003), Fabaceae (Gladish and Rost, 1993), *Lolium perenne* L. (Zobel, 2013), *Zea mays* L. (Fusseder, 1987; McCully, 1987; Varney and McCully, 1991), *Arabidopsis thaliana* (Zhu et al., 1998), and many others.

Examination of first-order maize lateral roots revealed that the RAM was lost as development progressed, resulting in determinate growth (Fusseder, 1987; Varney and McCully, 1991). A maize lateral root that has reached the end of its determinate developmental program displays large anatomical deviations from an actively growing root, with fully elongated cells, root hairs and higher order lateral
branching immediately adjacent to the root tip (Varney and McCully, 1991). Furthermore, vasculature develops closer to the tip, with more late metaxylem open for conduction, and roots maintain their resource uptake functionality. These traits decrease the hydraulic isolation of the root tip which could be beneficial under water deficit conditions (McCully and Canny, 1985; Wang et al., 1994).

There have been a few reports in the literature of water deficit influencing the duration of root growth. In the cactus species *Pachycereus pringlei*, cessation of growth of the primary axial root is greatly accelerated, with an accompanying 1.5x increase in lateral root number, when grown on plates containing polyethylene glycol (Dubrovsky and Gómez-Lomelí, 2003). Furthermore, a different variety of cactus, *Opuntia ficus-indica*, showed a similar response when grown in both gradual and rapid dry-down vermiculite experiments (Dubrovsky et al., 1998). Furthermore, in these species, the growth first-order lateral roots are extremely short lived under water deficit. After only 2 d, lateral root growth stops and emergence of second-order branches can be seen shortly thereafter. The growth pattern exhibited by these cactus species prioritizes shallow lateral root growth at the expense of growth at depth, an adaptive strategy to maximize capture of rain water in an extremely water-limited environment. While the induction of lateral root growth has been seen in response to accelerated determinacy of the parent axial root, modifications of the determinate development of lateral roots themselves has not been assessed in respect to water deficit conditions.

In this report, we show that a large degree of variability exists between two maize genotypes in first-order lateral root responses to mild water deficit.
Kinematic analyses were used to obtain patterns of growth that consider the spatio-temporal variability of cell production and expansion within the lateral root growth zone. Examination of the longitudinal, radial and tangential planes revealed that the differences seen in longitudinal and volumetric expansion were related to the effects of mild water deficit on the determinate growth program.

**MATERIALS AND METHODS**

**Plant growth conditions**

Maize (*Zea mays* L. cvs B73 and FR697) seeds were surface sterilized with 5% bleach for 30 min and then rinsed thoroughly with tap water. Following sterilization, seeds were treated with fungicide (Spectracide Immunox® Multi-Purpose Fungicide, Spectrum Brands Holdings, Inc., Middleton, Wisconsin, U.S.A.) for an additional 30 min and then imbibed in 1 mM CaSO$_4$ for 23 h. After imbibition, seeds were transferred to moist germination paper wetted with 1 mM CaSO$_4$ and were allowed to germinate at 29°C in the dark at near saturation humidity. Seedlings with primary roots of 5-10 mm in length were selected and transplanted into 46 cm tall x 15 cm diameter Plexiglas tubes filled with Pro-Mix HP (Premier Tech, Québec, Canada) growth medium. To assure that adequate nutrition was available regardless of the added volume of water, the growth media used in experiments was first fully hydrated with nutrient solution to create uniform availability. The nutrient solution used was comprised of 0.50 mM KH$_2$PO$_4$, 0.50 mM MgSO$_4$·7 H$_2$O, 2.50 mM Ca(NO$_3$)$_2$·4 H$_2$O, 2.50 mM KCl, 0.31 mM EDDHA iron chelate (SPRINT® 138), 2.30 μM H$_3$BO$_3$, 0.90 μM MnSO$_4$· H$_2$O, 0.60 μM
ZnSO$_4$ $\cdot$ 7 H$_2$O, 0.10 μM Na$_2$MoO$_4$ $\cdot$ 2 H$_2$O, 0.11 μM NiCl$_2$ $\cdot$ 6 H$_2$O and 0.15 μM CuSO$_4$ $\cdot$ 5 H$_2$O (personal communication with Dr. Dale Blevins, University of Missouri). The final solution pH was adjusted to 5.6 with 10 mM NaOH. Following hydration, the medium was dried in a forced air oven held at 55°C for at least 48 h, desiccating to equilibrium with the oven-warmed air. The dried medium was then weighed and mixed with a pre-determined amount of deionized, distilled (DDI) water to generate a well-watered treatment with a water potential ($\Psi_w$) of -0.10 MPa and a mild water stress treatment of -0.28 MPa. Growth medium $\Psi_w$ was measured by means of isopiestic thermocouple psychrometry (Boyer, 1965). Eight seedlings were transplanted into each tube in a circular pattern, at an equal distance from the center and edge of the tube, at a depth of 5 cm.

After transplanting, tubes were weighed and then moved into a controlled environment growth chamber (Conviron PGW36) kept at a constant 29°C. The light intensity was 700 μmol m$^{-2}$ s$^{-1}$ PAR at canopy height, with a 14h/10h day/night cycle and a RH of 90% near saturation, respectively. Tube bases were placed in plastic buckets and were surrounded with moist cheesecloth to minimize evaporation from the tube bottoms. Each day tubes were weighed, controls were watered back to original weight and the cheesecloth around all tubes was remoistened. Water deficit treatments did not receive any watering for the duration of experiments.

Plants were harvested at 1, 2, 3, 5, 6, 7, 9 and 11 days after transplant (DAT) and the $\Psi_w$ of the media was assessed at a tube depth of 15 cm to confirm maintenance of media $\Psi_w$. Roots were then carefully washed free of all growth
medium and kept at 4°C until further analysis. Leaf area was obtained using a LI-COR LI 3000A portable leaf area meter. Following leaf area measurements, the leaves and stem of each individual were gathered together, dried in an oven at 55 °C until fully desiccated, and analyzed for shoot dry-matter accumulation.

**Root trait analysis from scans**

Clean root systems were analyzed for a variety of root traits by WinRHIZO equipment and software (Regent Instruments Inc.) using an image capture resolution of 800 dpi. Total root length of the primary and seminal systems was assessed and the length of the axial roots was subtracted, resulting in cumulative lateral root length for each system. The whole organ responses of the longest 10 first-order lateral roots growing from the top 15 cm of the primary axial root were assessed by individually analyzing 10 first-order branches, excluding all higher order laterals, to obtain an average length, diameter and volume per treatment. Following analysis, the primary and seminal root systems from each individual were separated, dried in an oven at 55 °C until fully desiccated, and analyzed for root dry-matter accumulation.

Two independent time-courses of first-order lateral root growth throughout development were used to model the relationship by non-linear regression. First-order lateral root elongation and acceleration rates was calculated as the first- and second-order derivatives of the sigmoidal function modeled by regression analysis of the length increase over time. Additionally, the change in width of first-order
lateral root growth zones were assessed by measuring the diameter every 300 μm from the root cap junction, using WinRHIZO scans for both time-courses, and were modeled by logarithmic regression.

**Optical clearing of first-order lateral roots**

A tissue clearing technique modified from Warner et al. (2014) was utilized to allow visualization of cortical cell lengths by optical sectioning. One first-order lateral root tip 0.5 cm in length was sampled at harvest from each FR697 and B73 plant grown under well-watered or mild water deficit conditions at 3, 5, 7, 9 and 11 DAT. Samples were stored in 70% ethanol at 4°C for further analysis. Root tip segments were then treated with 10% (w/v) KOH for 1 h at room temperature, followed by submergence in a clearing solution composed of 6 M urea, 30% (v/v) glycerol, and 0.1% (v/v) Triton X-100 for a minimum of 30 min.

**Analysis of cell length profiles**

Following clearing, unsectioned root tips were mounted on to slides using the clearing solution detailed above. Samples were observed using differential interference contrast (DIC) microscopy (Zeiss Axiovert) with a 40x water objective. A series of overlapping images of cortical files were collected along the lateral root growth zone beginning at the root cap junction, using a mounted camera (Leica DFC290 color camera) fitted to the microscope and connected to a PC running MetaMorph® software v.7.8.12. (Molecular Devices, LLC., Sunnyvale, CA,
U.S.A.). Photographs were taken every 150 μm until final cell lengths were reached (4000 μm from the root cap junction), and mosaic images were utilized to construct cell profiles. Cell lengths of cortical cells were measured every 150 μm from the root cap junction. The subsequent cell length profiles obtained were averaged across all laterals for each treatment and time point (n = 13-16).

**Kinematic equations from anatomical records**

Cellular displacement velocity, \( v_z \) (μm·h\(^{-1}\)) is a measurement of how fast a cell at a given position \( (z) \) is being displaced away from the root cap junction. Rates of cellular displacement were calculated using the average elongation rate for all first-order lateral roots per treatment and the final cell lengths obtained for each individual root,

\[
v_z = \frac{(c_z - c_f)}{ER}
\]

where \( c_z \) is the local cell length, \( c_f \) is the final cell length and ER is the root elongation rate. Anatomical records do not allow for accurate calculations of displacement velocity within the meristem (Silk et al., 1989). Therefore, velocity calculations began when cells were at least 2.5x the length of the shortest cells observed, an estimation of the end of the meristematic region (Erickson, 1961).

Cellular flux, \( f \) (cells·h\(^{-1}\)), as calculated here represents the rate at which fully elongated cells exit the growth zone per unit time and is proportional to rates of cellular production (Beemster and Baskin, 1998). Rates of cell flux were
calculated using the final cellular displacement velocity (i.e. the root elongation rate) and the final cell lengths obtained for each individual root and averaged per treatment,

$$f = \frac{v_f}{c_f}$$

(2)

**Longitudinal, radial plus tangential, and volumetric strain rates**

Longitudinal strain rates, $r_z$ (μm·h$^{-1}$·μm$^{-1}$), represent the change in displacement velocity with respect to distance along the root, $z$ (μm),

$$r_z = \frac{\partial v}{\partial z}$$

(3)

and were calculated as the derivatives of the sigmoidal functions fitted to the displacement velocity curves by non-linear regression (Beemster and Baskin, 1998).

The radial ($r$) plus tangential ($\theta$) strain rate, $r_{r+\theta}$ (μm$^2$·h$^{-1}$·μm$^{-2}$), is the rate of change in cross sectional area expansion with respect to distance from the root cap junction,

$$r_{r+\theta} = \left(\frac{2}{R_z}\right)(v_z)(\partial R/\partial z)$$

(4)

where $R$ is the root radius (μm) (Sharp et al., 1988; Meicenheimer, 2006). The change in radius ($\partial R$) along the root tip was calculated as the derivative of logarithmic functions fitted to the data by non-linear regression, utilizing root radius measurements from two independent time-courses.
The addition of the longitudinal and radial plus tangential strain rates gives the volumetric strain rate, $r_{total}$ (μm$^3$·h$^{-1}$·μm$^{-3}$), or the spatial distribution of volumetric expansion along the growth zone,

$$r_{total} = (\frac{\partial v}{\partial z}) + \left(\frac{(2/R_z)(v_z)(\partial R/\partial z)}{}\right) = r_z + r_{r+\theta}$$  \hspace{1cm} (5)

### Statistical Analysis

Student’s $t$-tests and ANOVA analyses were carried out at the 0.05 significance level in IBM SPSS Statistics 2017 (IBM Corporation, Armonk, New York, U.S.A.). Graph production, regression analysis, 95% confidence interval evaluation and derivative calculations were performed using Origin 2017 (OriginLab, Northampton, MA, U.S.A). Experimental sample sizes for plant growth assays were 10–16 individual plants, from two individual tubes of up to eight seedlings. Data presented for first-order lateral roots are the means generated by the longest 10 of each individual, averaged across all 10-16 plants per treatment.

### RESULTS

**A near-stable mild water deficit environment for the assessment of lateral root developmental plasticity**

To provide precise and reproducible levels of water deficit, fully desiccated media was adjusted to a $\Psi_w$ of -0.30 MPa by the addition of pre-calibrated amounts of water to specific levels of gravimetric water content ($\theta_g$). Furthermore, plants
were grown in controlled environment chambers held at 90% RH during the light cycle and near saturation humidity in the dark. These conditions greatly reduced evapotranspiration and allowed for up to 11 days of photosynthetically active growth in a near stable $\Psi_w$ environment (Table 1). The media $\Psi_w$ began to drop more quickly after 9 days of growth, showing a decrease of -0.05 MPa from 9 to 11 days, 2.5-fold the decrease seen from days 7 to 9. This more dramatic drop was due to the increased effect of changes in $\theta_g$ relative to $\Psi_w$ as the media dries (Chapter 2, Fig. 2-1). Even after supporting the growth of eight seedlings per tube for 11 days, the media $\Psi_w$ only decreased an average of -0.11 MPa, displaying the stability of the experimental system in controlling a sustained level of water deficit.

**Whole root system responses to mild water deficit**

The level of mild water deficit (MWD) used in this study, a growth medium $\Psi_w$ of -0.30 MPa, did not have a major effect on the elongation of the primary or seminal axial roots in either genotype (Fig. 3-1). Additionally, the average lengths of axial roots were similar between genotypes for the duration of the experiment. In contrast, there were many differences in total lateral length between the genotypes, both intrinsically as well as in response to MWD conditions (Fig. 3-2). The total lateral root length from B73 seminal axes was less than that of FR697 in the later stages of development under both treatments (Fig. 3-2). Neither of the genotypes showed any difference in seminal system lateral length under MWD conditions at 11 DAT, but from 6 to 9 DAT there was a trend of reduced total length. However, the lateral roots originating from the primary axial root showed a
Table 1. Maintenance of stable media $\Psi_w$ throughout the duration of lateral root development

Change in the $\Psi_w$ of medium growing FR697 or B73 seedlings. Transplant and harvest $\Psi_w$ values are the averages of two tubes per genotype, growing eight seedlings each. Harvest $\Psi_w$ was assessed at a tube depth of 15 cm, at 11 DAT. No significant difference in the amount of drying was seen between media growing FR697 and B73 seedlings. Data are means ± the range of the data.

<table>
<thead>
<tr>
<th>DAT</th>
<th>Transplant</th>
<th>FR697 Harvest</th>
<th>$\Delta \Psi_w$</th>
<th>B73 Harvest</th>
<th>$\Delta \Psi_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-0.28 ± 0.01</td>
<td>-0.30 ± 0.005</td>
<td>0.02</td>
<td>-0.30 ± 0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>-0.28 ± 0.01</td>
<td>-0.30 ± 0.01</td>
<td>0.02</td>
<td>-0.33 ± 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>-0.28 ± 0.01</td>
<td>-0.30 ± 0.01</td>
<td>0.02</td>
<td>-0.30 ± 0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>-0.28 ± 0.01</td>
<td>-0.31 ± 0.03</td>
<td>0.03</td>
<td>-0.31 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>7</td>
<td>-0.28 ± 0.01</td>
<td>-0.32 ± 0.03</td>
<td>0.04</td>
<td>-0.31 ± 0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>9</td>
<td>-0.28 ± 0.01</td>
<td>-0.33 ± 0.04</td>
<td>0.05</td>
<td>-0.34 ± 0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>11</td>
<td>-0.28 ± 0.01</td>
<td>-0.39 ± 0.07</td>
<td>0.11</td>
<td>-0.37 ± 0.07</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Figure 3-1. Timecourse of the effects of mild water deficit on axial roots

The average length of FR697 (A) and B73 (B) primary (squares) and seminal (circles) axial roots grown under well-watered conditions (WW, black) or a mild water deficit (MWD, red) at a $\Psi_w$ of -0.30 MPa. Data from two independent time-courses are plotted. Sigmoidal growth functions are fitted to the data by regression analysis with $R^2 > 0.96$ for all genotype x treatment combinations. Colored shading depicts the 95% confidence interval of data predicted by the regression analyses. Data are means ± standard error (n = 13-16).
Figure 3-2. Timecourse of the effects of mild water deficit on total lateral root length.

Total lateral root length of the FR697 (A) and B73 (B) primary (squares) and seminal (circles) root systems grown under well-watered conditions (WW, black) or a mild water deficit (MWD, red) at a $\Psi_w$ of -0.30 MPa. Data from two independent time-courses are plotted. Sigmoidal growth functions are fitted to the data by regression analysis with $R^2 > 0.94$ for all genotype x treatment combinations. Colored shading depicts the 95% confidence interval data predicted by the regression analyses. Data are means ± standard error ($n = 13-16$).
significantly different relationship, with B73 having a higher total length than FR697 for most of development. Like the lateral roots of the seminal axes, the total lateral root length of the B73 primary system was unperturbed by MWD (Fig. 3-2). In contrast, the total lateral length of the FR697 primary system was greatly enhanced under MWD conditions at 11 DAT, resulting in a 62.8% increase from 431 cm to 701 cm. Differences in lateral root length were seen from 7 DAT onward, with increasing divergence between lateral roots grown under well-watered (WW) and MWD conditions as time progressed.

**First-order lateral root length, elongation and acceleration influenced by mild water deficit**

The growth dynamics of the 10 longest first-order lateral roots from the primary axis were assessed throughout the course of determinate development (Fig. 3-3). Both genotypes had a similar growth response under WW conditions, with FR697 having shorter first-order lateral roots at nearly all timepoints (Fig. 3-3A, B). At 2 DAT, first-order lateral roots began to emerge from most individuals of both genotypes under all treatments. The lateral roots of both genotypes increased their acceleration to maximum rates at 3 DAT, and achieved their greatest elongation rates for WW controls at 4.5 DAT, with FR697 laterals growing 493 μm h⁻¹ and B73 laterals growing 749 μm h⁻¹ (Fig. 3-3 C-F). From this point in development, B73 first-order lateral roots grown under WW and MWD conditions acted the same, decelerating rapidly from peak elongation rates. At approximately 6.5 DAT, B73 lateral roots reached the maximum rates of deceleration and at 11
Figure 3-3. Timecourse of the effects of mild water deficit on first-order lateral root length.

Growth dynamics of the average length (A, B), elongation rate (C, D) and acceleration rate (e, f) of first-order lateral roots of FR697 (A, C, E) and B73 (B, D, F) grown under well-watered conditions (WW, black) or a mild water deficit (MWD, red) at a $\Psi_w$ of -0.30 MPa throughout development. Length data from two independent time-courses across development are plotted. Sigmoidal growth functions are fitted to the data by regression analysis with $R^2 > 0.98$ for all genotype x treatment combinations. Colored shading depicts the 95% confidence interval data predicted by the regression analyses. Lateral root elongation rate and acceleration rate profiles are the first and second derivatives of the regression analyses, respectively. The dotted line in panels (E) and (F) represents the point when lateral roots transition from acceleration to deceleration. Data are means ± standard error (n = 13-16).
DAT had almost ceased elongation. In contrast, at 5 DAT, FR697 lateral roots began to diverge in their growth pattern based on $\Psi_w$ treatment. Lateral roots grown under MWD continued to maintain positive rates of acceleration and increased their elongation rates for an additional 1.5 days, to a maximum of 546 $\mu$m h$^{-1}$ at 6 DAT. Lateral roots maintained rates of elongation equal to at least 100% of the maximum WW growth until 7 DAT, 2.5 days longer than controls, and were still at 45% of maximum rates at 11 DAT. It is interesting to note that while B73 lateral roots showed much higher maximum rates of elongation and acceleration compared to FR697, the lateral roots of both genotypes had the same duration of growth under well-watered conditions.

**Effects of mild water deficit on first-order lateral root diameter and volume changes associated with determinate growth**

The increase in diameter of first-order lateral roots was similar for both genotypes early in development, with a rapid increase from 2-3 DAT, just after primordia emerge (Fig. 3-4A, B). Similar to other traits, B73 lateral roots grew to a larger average diameter than those of FR697 over the same duration of growth, with a maximum of 0.46 mm and 0.37 mm for B73 and FR697, respectively. Interestingly, FR697 lateral root tips began to thin as they progressed through development, with a notable decrease in diameter by 11 DAT (Fig. 3-4A). When grown under MWD conditions the thinning phenotype of FR697 lateral roots was repressed, while the width of the lateral roots of B73 was unaffected (Fig. 3-4A, B).
Figure 3-4. Timecourse of first-order lateral root average diameter and volume influenced by determinacy and mild water deficit.

Growth dynamics of the average diameter (A, B) and volume (C, D) of first-order lateral roots of FR697 (A, C) and B73 (B, D) grown under well-watered (WW, black) and a mild water deficit (MWD, red) at a $\Psi_w$ of -0.30 MPa throughout development. Data from two independent timecourses are plotted. Sigmoidal growth functions are fitted to the data by regression analyses with $R^2 > 0.87$ for all genotype x treatment combinations. Data are means ± standard error ($n = 13-16$).
The average rate of volumetric expansion for lateral roots of both genotypes was slow at 2-3 DAT. Shortly thereafter, from 4-5 DAT, the period of greatest increase in root volume began for all treatments. There were no differences in the average volume of B73 lateral roots throughout development under MWD conditions. B73 lateral roots were still exhibiting significant expansion at the end of the experiment with volumes of 1.68 mm$^3$ and 1.73 mm$^3$ for lateral roots grown under WW or MWD conditions, respectively (Fig. 3-4D). However, large deviations in the volumetric increase of FR697 lateral roots were induced by MWD (Fig. 3-4C). FR697 lateral roots in WW conditions nearly ceased expanding at 8 DAT, with an average volume of 0.62 mm$^3$ at 11 DAT. In contrast, lateral roots grown under MWD continued to increase in volume at almost maximum rates until 9 DAT. At the end of the experiment expansion rates began to slow, with a final measured volume of 1.17 mm$^3$, approximately 90% larger than FR697 WW roots.

**Anatomical modifications of determinate growth influenced by mild water deficit**

Major modifications of cell length profiles occurred in first-order lateral roots of both genotypes under WW conditions as they approached the end of the determinate growth program (Fig 3-5A, C). Beginning at 9 DAT for FR697, and 5 DAT for B73, final cell size of first-order lateral roots began to decrease, and continued until there was a measured 23.4% drop in FR697 and a 19.5% drop in B73 at 11 DAT. Additionally, the average cell size near the root cap junction increased for both genotypes, with FR697 displaying this response again from 9
Figure 3-5. Effects of mild water deficit on the anatomical modifications associated with determinate development.

Changes in the cell length profiles along the growth zone of first-order lateral roots of FR697 (A, B) and B73 (C, D) grown under well-watered conditions (A, C) and a mild water deficit at a $\Psi_w$ of -0.30 MPa (B, D). Cell length profiles were assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT. One of the longest 10 first-order lateral roots was sampled from each individual ($n = 10-16$) and cortical cell lengths were assessed every 150 μm ($n = 3-10$). Data are means ± standard error.
DAT onward and B73 beginning at 7 DAT. The B73 lateral roots grown under MWD had a very similar cell length profile as lateral roots grown under WW conditions, with only minor deviations in the onset of final cell size reduction (Fig. 3-5D). Strikingly, when FR697 first-order lateral roots were grown under MWD conditions, all of the anatomical changes that occur during the normal determinate developmental program were repressed (Fig 3-5B).

**Cell flux patterns associated with determinate growth are affected by mild water deficit**

Changes in the elongation rates of first-order lateral roots throughout development were highly correlated with the dynamic responses of cell flux in both genotypes as they progressed through determinate growth (Fig. 3-6). Cell flux is a measurement of how many cells are leaving the growth zone per unit time, and under steady growth is an indicator of the rate of cell production (Silk et al., 1989; Beemster and Baskin, 1998). Similar to the responses observed in first-order lateral root length, there were significant genotypic differences in the response of cell flux across development when grown in a MWD environment. Under WW conditions, FR697 lateral roots had a very similar pattern of cell flux as that of B73 lateral roots across development, albeit with 20-30% lower levels at all timepoints. However, as seen in other parameters, B73 first-order laterals were non-responsive to the level of MWD tested, while FR697 laterals showed a dramatic interaction between determinate development and water deficit, as discussed below.
Figure 3-6. Genotypic variation in the effect of mild water deficit on cell flux throughout development.

Changes in the cell flux of first-order lateral roots of FR697 and B73 grown under well-watered (WW) conditions or a mild water deficit (MWD) at a $\Psi_w$ of -0.30 MPa. Cell flux was assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT. Asterisks denote significant differences between WW and MWD treatments within a genotype. One of the longest 10 first-order lateral roots was sampled from each individual ($n = 10-16$). Comparisons were conducted by a student’s $t$-test, $p < 0.001$. Data are means ± standard error.
Early after emergence, at 3 DAT, when first-order laterals were accelerating, relatively high rates of cell flux were observed, with 2.8 and 2.6 cells h\(^{-1}\) for FR697 lateral roots grown under WW and MWD conditions, respectively. Higher rates were seen in B73 lateral roots at this time, with 3.5 and 3.2 cells h\(^{-1}\) observed in WW and MWD conditions, respectively. After two additional days of growth, lateral roots of all treatments were near their maximum rates of elongation and displayed maximal rates of cell flux accordingly. FR697 lateral roots showed an increase to 4.4 and 4.5 cells h\(^{-1}\) for WW and MWD, respectively. Likewise, in B73 cell flux was increased to 5.6 cells h\(^{-1}\) under WW conditions and 5.7 cells h\(^{-1}\) when grown under MWD.

At 7 DAT cell flux declined approximately to the rate seen at 3 DAT for B73 lateral roots, 3.7 cell h\(^{-1}\), with a slightly higher flux of 4.5 cells h\(^{-1}\) seen under MWD. However, FR697 lateral roots maintained maximum rates of cell flux at 7 DAT under MWD, 4.3 cells h\(^{-1}\). This is in contrast to FR697 lateral roots grown under WW conditions, which showed a similar response as the B73 laterals, attenuating back to approximately the levels seen at 3 DAT, 2.5 cells h\(^{-1}\). As lateral roots progressed further through determinacy, cell flux continued to decrease, dropping to 1.8 and 2.1 cells h\(^{-1}\) at 9 DAT for B73 laterals grown under WW and MWD conditions, respectively. Similarly, the cell flux of FR697 WW laterals was reduced to 1.3 cells h\(^{-1}\) at 9 DAT. However, when grown under MWD conditions, FR697 lateral roots continued to maintain high rates of cell flux at 9 DAT, 3.0 cells h\(^{-1}\). This trend continued at 11 DAT, with rates of 0.7 and 1.9 cells h\(^{-1}\) for first-order lateral roots of FR697 grown under WW and MWD, respectively. Further reductions in
cell flux were observed in B73 first-order lateral roots at 11 DAT. However, unlike FR697, no differences were observed between treatments in B73 lateral roots, with rates of 0.9 and 1.1 cells h⁻¹ for WW and MWD, respectively.

**Spatio-temporal distribution of longitudinal expansion influenced by determinacy and mild water deficit**

If growth is steady for the duration it takes a cell to move through the growth zone, assuming stable rates of cell production and displacement, then anatomical records of cell length profiles can be used to estimate the distribution of longitudinal expansion rates along the growth zone (Silk et al., 1989). Displacement velocities were calculated and were modeled using a sigmoidal function fitted by non-linear regression (Fig. 3-7). The derivative of the regression function is the longitudinal strain rate \( \mu m \cdot h^{-1} \cdot \mu m^{-1} = h^{-1} \), representing the distribution of local relative elongation rate along the growth zone as a function of distance from the root cap junction. A dynamic response of the longitudinal strain profile is seen for both genotypes as lateral roots progress through determinacy (Fig. 3-8). Again, FR697 laterals displayed a large interaction between their determinate growth program and MWD conditions, while B73 lateral root longitudinal strain profiles were largely unaffected.

For simplification of comparisons, the WW growth dynamics for both genotypes will be presented first. The growth zone of B73 lateral roots was longer than that of FR697 early in development, 4000 μm and 3000 μm, respectively (Fig. 3-8A, C). For both genotypes under control conditions, peak longitudinal strain
Figure 3-7. Effects of mild water deficit on the reductions in displacement velocity associated with determinate growth.

Changes in displacement velocity along the growth zone of first-order lateral roots of FR697 (A, B) and B73 (C, D) grown under well-watered conditions (A, C) or a mild water deficit at a \( \Psi_w \) of -0.30 MPa (B, D). Displacement velocities were assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT. One of the longest 10 first-order lateral roots was sampled from each individual \( (n = 10-16) \). Sigmoidal growth functions are fitted to the data by regression analysis with \( R^2 > 0.98 \) for all genotype x treatment combinations. Colored shading depicts the 95% confidence interval data predicted by the regression analyses. Data are means ± standard error.
Figure 3-8. Genotypic differences in the effect of mild water deficit on reductions of longitudinal strain rate associated with determinate growth

Changes in the longitudinal strain rate profiles along the growth zone of first-order lateral roots of FR697 (A, B) and B73 (C, D) grown under well-watered conditions (A, C) or a mild water deficit at a $\Psi_w$ of -0.30 MPa (B, D). Strain rates were assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT, and were calculated as the first derivative of the sigmoidal growth function fitted to the displacement velocity profiles.
rates were at similar levels 3 DAT, 0.33 h\(^{-1}\) and 0.32 h\(^{-1}\), for FR697 and B73, respectively. FR697 lateral roots displayed maximum rates closer to the root cap junction than B73 at this time, 757 μm compared to 1157 μm. At 5 DAT, when roots were near maximum elongation rates (Fig. 3-3C, D), the highest local longitudinal strain rates throughout development were observed. An increase was seen in both genotypes, with B73 laterals showing a shift of peak rates closer to the root cap junction (Fig. 3-8C). The magnitude of increase was similar for both genotypes, resulting in peak rates of 0.47 and 0.44 h\(^{-1}\) for FR697 and B73, respectively. As lateral roots continued to age, larger differences were seen between genotypes. FR697 lateral roots at 7 DAT showed a substantial reduction in maximum longitudinal strain rates as the elongation of the roots began to decrease (Fig. 3-8A). In contrast, B73 lateral roots only showed a small decrease in peak rates; however, the maximum continued to shift closer to the root cap junction, moving from 985 μm to 710 μm, a response not seen in FR697 laterals (Fig. 3-8A, C). Furthermore, the size of the growth zone of B73 lateral roots was reduced at 7 DAT to roughly the same size as FR697 lateral roots, approximately 3000 μm. No further reductions in the length of the growth zone were seen in B73 lateral roots after this time. At 9 DAT both genotypes showed large reductions in maximum longitudinal strain rates. Additionally, in FR697 the location of the peak strain rate moved closer to the apex, resulting in a distance from the root cap junction much closer to that of B73, 530 μm compared to 740 μm. As lateral roots approached determinacy, very low longitudinal strain rates of 0.09 h\(^{-1}\) and 0.07 h\(^{-1}\) were observed for FR697 and B73, respectively (Fig 3-8A, C). Additionally, the peak
strain rate was shifted further toward the root cap junction in FR697 lateral roots, to 300 μm, and the length of the growth zone was reduced to nearly 50% of its maximum size, approximately 1500 μm in length.

When challenged with MWD conditions, B73 showed no major deviations in the dynamic changes seen in first-order lateral root longitudinal strain rates throughout determinate development (Fig. 3-8C, D). In contrast, the effects of MWD on FR697 lateral roots were similar to the changes seen in other parameters. Major deviations in the modification of longitudinal strain rates began at 7 DAT when lateral roots showed a much smaller reduction in peak rates compared to WW controls, with rates of 0.27 h⁻¹ and 0.42 h⁻¹ for WW and MWD, respectively (Fig. 3-8A, B). This trend continued at 9 DAT when lateral roots grown under MWD conditions maintained peak local rates of 55% of maximum levels, 0.28 h⁻¹, compared to the 23% of maximum rates seen in WW conditions, 0.10 h⁻¹ (Fig. 3-8A, B). At 11 DAT, when FR697 lateral roots were still growing at a considerable rate under MWD conditions (Fig. 3-3C), there was an accompanying maintenance of longitudinal strain rates at 34% of maximum levels, 0.17 h⁻¹ (Fig. 3-8B). Additionally, at 9 and 11 DAT, FR697 lateral roots did not show any shortening of the growth zone as seen in WW conditions. Taken together, these results show that MWD acts to delay the reductions in longitudinal strain rates of FR697 first-order lateral roots associated with determinate growth.
Spatio-temporal distribution of cross-sectional area expansion influenced by determinacy and mild water deficit

As seen for longitudinal expansion, there were significant differences between FR697 and B73 in the response of cross-sectional area expansion (\(\mu\text{m}^2 \cdot h^{-1} \cdot \mu\text{m}^{-2} = h^{-1}\)) of first-order lateral roots when grown under MWD conditions (Fig. 3-9 and 3-10). Lateral roots of FR697 plants grown under WW conditions showed a progressive thinning of the radius of the growth zone with aging, dropping from a maximum of 136 \(\mu\text{m}\) to 107 \(\mu\text{m}\) at 11 DAT (Fig. 3-9A), while under MWD conditions the thinning associated with determinate growth was completely repressed (Fig. 3-9B). In contrast, the increase in diameter along the B73 lateral root growth zone was non-responsive to both the effects of aging as well as MWD conditions (Fig. 3-9C, D).

Increases in root circumference are facilitated by both radial as well as tangential expansion, and thus, the spatial growth profile of the increase in cross sectional area along the growth zone comprises the radial plus tangential strain rates. This measurement is derived from both the rates of diameter increase along the growth zone, as well as the local rates of displacement velocity, and therefore combines the many differences seen in FR697 but not observed in B73 first-order lateral roots under MWD conditions.

Initial rates of expansion at 3 DAT were very different between genotypes under WW conditions (Fig. 3-10A, C). FR697 lateral roots showed greater peak rates than B73, 0.097 \(h^{-1}\) compared to 0.049 \(h^{-1}\), due to the lateral tips being more
Figure 3-9. Effects of mild water deficit on the diameter of the lateral root growth zone throughout determinate development.

Changes in diameter along the growth zone of first-order lateral roots of FR697 (A, B) and B73 (C, D) grown under well-watered conditions (A, C) or a mild water deficit at a $\Psi_w$ of -0.30 MPa (B, D). Diameter measurements were assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT. One of the longest 10 first-order lateral roots was analyzed from each individual ($n = 10\text{-}16$). Data from two independent time-courses are plotted. Logarithmic functions are fitted to the data by regression analysis with $R^2 > 0.87$ for all genotype x treatment combinations. Colored shading depicts the 95% confidence interval data predicted by the regression analyses. Data are means ± standard error.
Figure 3-10. Effects of mild water deficit on the radial plus tangential strain rate of the lateral root growth zone throughout determinate development.

Changes in the radial plus tangential strain rate profiles along the growth zone of first-order lateral roots of FR697 (A, B) and B73 (C, D) grown under well-watered conditions (A, C) or a mild water deficit at a $\Psi_w$ of -0.30 MPa (B, D). Strain rates were assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT.
tapered early in development (Fig. 3-9A, B and 3-10A, B). Furthermore, peak rates were observed closer to the root cap junction in FR697 than B73, at 970 μm and 1540 μm, respectively. Only minor changes in strain rates were seen in response to MWD at this early stage in development, with an approximate 12% reduction in the peak rates of FR697 being the most affected.

Maximum rates of radial plus tangential expansion were observed at 5 DAT for all genotype and stress combinations. The highest rates seen were in FR697 lateral roots grown under WW conditions, at 0.143 h⁻¹, nearly double the rate of 0.078 h⁻¹ seen in B73. When grown under MWD conditions, B73 lateral roots showed an increase in maximum rates at 5 DAT to 0.097 h⁻¹, while FR697 showed the opposite, displaying a decrease to 0.124 h⁻¹ (Fig. 3-10B, D).

At 7 DAT, FR697 WW lateral roots showed a substantial decrease in peak local radial plus tangential expansion rates to 0.062 h⁻¹, while B73 lateral roots were only slightly reduced to 0.084 h⁻¹. However, the location of peak local rates in B73 lateral roots shifted apically, from 1300 μm to 930 μm from the root cap junction (Fig. 3-10C). At this developmental stage, major differences began to arise between genotypes in their determinate development when exposed to MWD. While B73 lateral roots showed no modifications in the strain rate profile at 7 DAT under MWD, FR697 lateral roots maintained high rates of radial plus tangential expansion, with peak rates of 0.106 h⁻¹, 85% of the maximum MWD strain rate.

As the lateral roots continued to progress through development, many of the differences between genotypes lessened under WW conditions. FR697 laterals at 9 DAT showed further reductions in maximum rates to 0.033 h⁻¹, as well
as a peak shift apically to 540 μm. Under MWD conditions, FR697 lateral roots again showed a repression of the reductions in peak rates, sustaining a rate of 0.084 h⁻¹, 67.7% of the maximum compared to the 23.1% seen in lateral roots under WW conditions. Moreover, the shift of peak rates apically and the reductions of the growth zone were also repressed in FR697 under MWD conditions.

At the end of the experiment, at 11 DAT, no further reduction was seen in the peak rates of FR697 WW lateral roots, but there was further shortening of the growth zone, with peak rates at 330 μm. The relative maintenance of local rates continued for FR697 lateral roots under MWD at 11 DAT, with rates of 0.053 h⁻¹, 42 % of maximum, as well as continued maintenance of the length of the expansion region. The radial plus tangential strain rate of B73 lateral roots under WW conditions was reduced to the same level as FR697, a rate of 0.032 h⁻¹, at 9 DAT and was further reduced to 0.018 h⁻¹ at 11 DAT, with no major effect of MWD observed.

**Spatio-temporal distribution of volumetric expansion influenced by determinacy and mild water deficit**

Due to the extreme differences in dimensions regarding the length-to-width ratio of maize laterals roots, the rate of longitudinal strain dominates the effects on volumetric expansion \((\mu m^3 \cdot h^{-1} \cdot \mu m^{-3} = h^{-1})\). The volumetric expansion profiles for B73 acted almost identically to the behavior of longitudinal strain for WW and MWD treatments, with slightly increased values at every measurement (Fig. 3-11). The thinning effects seen in FR697 laterals under WW conditions
Figure 3-11. Genotypic differences in the effect of mild water deficit on reductions of volumetric strain rate.

Changes in the volumetric strain rate profiles along the growth zone of first-order lateral roots of FR697 (A, B) and B73 (C, D) grown under well-watered conditions (A, C) or a mild water deficit at a $\Psi_w$ of -0.30 MPa (B, D). Strain rates were assessed at 3 (black), 5 (red), 7 (blue), 9 (green) and 11 (pink) DAT.
compounded with the reductions seen in longitudinal strain, resulting in a volumetric strain rate profile that was more heavily impacted late in development under WW conditions. When grown under MWD, FR697 lateral roots maintained 36% of maximum rates of volumetric strain, compared to 20% for WW lateral roots at the end of the experiment. Furthermore, MWD repressed all reductions in the length of the expansion zone induced by determinate growth (Fig. 3-11A, B).

DISCUSSION

Genotype-specific whole root system responses to mild water stress

In both genotypes, the length of the axial roots was unaffected under the mild water stress conditions that resulted in enhanced lateral root growth, an important consideration when assessing lateral root responses to stimuli. An active RAM has been suggested as a source of an inhibitor of lateral root growth (Thimann, 1936; Böttger, 1974; Hinchee and Rost, 1986; Dubrovsky, 1997a, b; Ditengou et al., 2008), and it has been shown that axial root inhibition can promote lateral root growth and development. This response is well exemplified by a Sonoran Desert cactus, whose primary axial root ceases growth at only 5 mm in length to dramatically induce lateral root formation, an adaption to facilitate rapid establishment in extremely water-limited conditions (Dubrovsky, 1997a). Interestingly, in the current study, primary and seminal axial roots hinted at the potential to have extended periods of growth under MWD conditions (Fig. 3-1),
with higher slopes of root length increase between 9 and 11 DAT, but longer duration experiments would be needed to confirm this response.

Total lateral root length growing from both the primary and seminal axes of B73 was unresponsive to MWD (Fig. 3-2B). However, FR697 plants showed a significant increase in total lateral root length growing from the primary axis (Fig. 3-2A). While no differences in total length were observed for FR697 seminal lateral roots under MWD over the duration of the experiment, the function modeled by the regression analysis suggests that there could be a significant increase if grown for a longer period. This time sensitive response is also seen in the increase in primary axis lateral roots, with no differences apparent until 7 DAT. Experiments detailed in Chapter 2 revealed that the response to MWD seen in FR697 lateral roots only occurs over a narrow window of mild water stresses between -0.25 and -0.35 MPa. At more severe stress levels, no genotypic differences are detectable between FR697 and B73 total lateral root length when compared to their respective WW controls. An even more extreme example is seen in rice, when the entire response range of enhanced lateral rooting, and subsequent attenuation to below control levels, occurs at $\Psi_w$ above -0.20 MPa (Kano et al., 2011). Additionally, that study also utilized two rice varieties which vary in their response over this narrow window in a very similar manner to FR697 and B73, showing that genotypic variability in lateral root responses to mild water stress exists across species. The great degree of specificity in time and $\Psi_w$ range that results in increased lateral length demonstrates the necessity to study the effects of stress on growth across high-
resolution stress gradients, as well as throughout the course of development, to understand the mechanisms underlying alterations of net growth parameters.

Lateral root length along the primary root axis was very heterogenous (Chapter 2, Fig. 2-6). Ito et al. (2006) showed that first-order lateral roots of both maize and wheat had a wide range of duration of growth, leading to a mixture of lateral lengths interspersing along the primary axial root. To facilitate the study of first-order lateral root responses, a specific subset of laterals was chosen from each individual. The average length of the 10 longest laterals from the top 15 cm of the primary axis was found to be a good proxy of total lateral length (Chapter 2), showing similar growth dynamics, levels of enhancement induced by water deficit, and point in time when growth deviated from WW controls (Fig. 3-2 and Fig. 3-3).

**Genotypic differences in well-watered first-order lateral root growth and development**

Great variation between genotypes was seen in the whole-organ growth of first-order lateral roots as they progressed through their determinate growth program under WW conditions (Fig. 3-3 and Fig. 3-4). Interestingly, spatial growth profiles of lateral roots from both genotypes show similar responses to the cessation of growth as they progress through determinacy.

Average length and elongation rates were higher for B73 first-order lateral roots at most timepoints throughout determinacy. Furthermore, B73 lateral roots accelerated to maximum rates, and decelerated accordingly, at nearly 150% of the
rates shown by FR697 lateral roots (Fig. 3-3). Despite these differences, the total duration of lateral root growth was remarkably similar between genotypes. It is interesting to speculate that regardless of genotype or the speed of root growth, an overarching regulation of meristem longevity may be present in maize. The longest 10 first-order lateral roots from the primary axis began elongation by 2 DAT, and were nearly at their final length at 11 DAT (Fig. 3-3A, B). This shows a duration of at least 9 days as the period of determinate growth for WW primary axis first-order lateral roots of both genotypes tested, much longer than the reported 2.5 day duration of maize first-order lateral roots on seminal and nodal axial roots (Cahn et al., 1989).

Expansion in width of first-order lateral roots during development was also higher in B73 (Fig. 3-4B), leading to lateral roots with nearly 270% more volume than FR697 roots at the end of the experiment under well-watered conditions (Fig. 3-4D). As FR697 lateral roots approached determinacy, the average diameter decreased slightly from 9 DAT onward due to a tapering effect of new growth, a response not seen in B73 (Fig. 3-4 and Fig. 3-9). A similar response has been observed in maize primary roots that displayed a marked thinning response along the growth zone as the root aged (Fraser et al., 1990). The thinning of lateral roots with aging could provide potential benefits such as increased rates of radial water diffusion across the cortex, and if the stele is likewise reduced in diameter, an enhanced rate of axial water transport (Ahmed et al., 2016). In conjunction with the vasculature developing closer to the tip in roots that have reached determinacy,
this could be a method to conserve carbon resources while maintaining adequate water uptake (Varney and McCully, 1991; Wang et al., 1994).

When roots are growing at a steady rate, the quantity of cells within the RAM is more or less constant (Ivanov, 1981). However, when roots approach determinacy the number of meristematic cells begins to drop rapidly (Dubrovsky, 1997b). The changes in cell length profiles of WW FR697 and B73 lateral roots were similar throughout development, despite differences in growth rates (Fig. 3-3 and Fig. 3-5). Final cell size was observed to be reduced with aging, and longer cells were found near the root tip, suggestive of decreased cellular displacement. Cellular flux was seen to decrease as lateral roots aged, with similar magnitudes of change between genotypes, but higher net values for B73 at all timepoints (Fig. 3-6). The higher rate of cell flux, together with slightly longer final cell lengths, are responsible for B73 first-order lateral roots growing faster than those of FR697.

The effects of determinate growth can best be assessed by examination of the spatio-temporal kinematics of the growth zone. The growth zone of the maize lateral root was approximately 0.4 mm in length, much shorter than the reported 12 mm of the maize primary root (Sharp et al., 1988). The dynamic changes in the longitudinal strain rate were similar between B73 and FR697 laterals under WW conditions, with only slight differences in the timing of the shortening of the growth zone and the reduction in maximal local growth rates (Fig. 3-8A, C). In contrast, due to the thinning effect of FR697 and not B73 lateral roots with aging, as well as how heavily tapered FR697 lateral root tips are early in development, drastic differences were seen in the radial plus tangential strain rates of the growth zone.
Maximal local rates were more than double for FR697 lateral roots early in development (3-5 DAT), with similar levels to B73 as lateral roots approached their determinate length. Interestingly, the length of the growth zone was longer for radial plus tangential compared to longitudinal growth, as lateral roots of all treatments were still increasing in width past 4000 μm from the root cap junction (Fig. 3-10).

The dynamic changes in volumetric strain rates of WW lateral roots were again similar between genotypes early in development, with B73 having a longer zone of volumetric expansion at all timepoints (Fig. 3-11A, C). Significant differences in volumetric strain rate between genotypes were observed at 9 and 11 DAT under WW conditions. The compounding effects of thinning, and the dramatic shortening of the longitudinal growth zone seen in FR697, resulted in extremely low rates of volumetric strain along the growth zone of first-order lateral roots.

Apart from the thinning phenotype displayed by FR697 (Fig. 3-9), both genotypes showed similar kinematics under WW conditions regarding changes related to determinate development (Fig. 3-8, Fig. 3-10 and Fig. 3-11). This once more draws an interesting contrast between the inherent differences in net growth between FR697 and B73, and the similarities of their determinate developmental processes.
Delayed determinate growth of FR697 first-order lateral roots induced by mild water deficit conditions

The multitude of dynamic growth changes that occur in FR697 first-order lateral roots throughout determinate development were highly affected by the level of MWD tested. When grown under such conditions the period of elongation was increased, maintaining maximum growth rates for approximately 2.5 d longer than WW controls (Fig. 3-3C). Moreover, at the end of the experiment, lateral roots grown under MWD conditions were still at 44% of maximum rates of elongation when WW control lateral roots were approaching the end of their determinate growth. Furthermore, the thinning phenotype displayed by FR697 lateral roots was also repressed under MWD conditions, resulting in roots with nearly 90% larger volumes compared to WW controls at 11 DAT (Fig. 3-4A, C and Fig. 3-9A, B). In contrast, Sharp et al. (1988) showed that the primary root of the maize seedling showed dramatic thinning at a similar level of water deficit. Discrepancies between results could potentially be accounted for by differences in the growth media used, the age and genotypes of the plants, or innate differences in radial growth responses to MWD between axial and lateral roots. Additionally, the level of soil compaction has been shown to heavily influence the relationship between root width, internal anatomy, and the water deficit level of the growth media in maize, rice (Oryza sativa L.), pea (Pisum sativum L.), and cotton (Gossypium hirsutum L.) (Iijima et al., 2007). This important aspect should not be overlooked when comparing radial growth responses to water deficit across studies, as different
growth media may have very different water content to mechanical strength relationships.

Large interactions were seen between the anatomical modifications associated with aging in FR697 first-order lateral roots and their responses to MWD. Lateral roots grown under MWD did not show any inhibition of final cell length, nor the increase in cell size near the root cap as seen in WW roots (Fig. 3-5A, B). Larger cells near the apex are suggestive of decreased levels of cell production, as cells elongate to full size while being displaced less by new cell expansion. The changes in duration of lateral growth under MWD were, in part, the result of maintenance of higher rates of cell flux throughout development (Fig. 3-6). Measurements of cell flux are an indicator of levels of net cell production but cannot distinguish between rates of new cell formation from initials, and the number and frequency of subsequent cell divisions within the meristem (Beemster and Baskin, 1998). In the maize primary root, axial root inhibition due to MWD was shown to be mostly due to decreased cell expansion rates; however, rates of cell production were affected at more severe levels of stress around -0.80 MPa (Sharp et al., 1988; Fraser et al., 1990; Sacks et al., 1997). Rates of both cell expansion as well as production are heavily reduced in primary roots of cacti in response to moderate and severe levels of water stress (Dubrovsky et al., 1998; Dubrovsky and Gómez-Lomelí, 2003). In contrast, cell flux was shown to increase in response to mild water deficit in arabidopsis primary roots early in development (van der Weele et al., 2000), although effects on duration of root growth were not assessed.
Strain rate profiles revealed that MWD resulted in a large degree of maintenance regarding the progressive inhibition of longitudinal, radial plus tangential and volumetric expansion rates associated with the later stages of WW determinate growth (Fig. 3-8A, C; Fig. 3-10A, C; Fig. 3-11A, C). Longitudinal strain rates were similar to lateral roots grown under WW conditions at 3-5 DAT, but significantly higher maximum local rates were maintained at 7–11 DAT (3-8A, C). Furthermore, MWD entirely repressed the reduction in growth zone length at all measured timepoints. The shift in the kinematic profiles again suggests an approximate 2.5 d delay of the onset of determinacy, as the longitudinal strain rates for lateral roots grown under MWD at 9 DAT and WW lateral roots at 7 DAT were approximately the same. Furthermore, lateral roots grown under MWD at 11 DAT showed maximum rates significantly higher than WW laterals at 9 DAT. This is once again in contrast to the responses of the primary axial root of maize grown at similar levels of water stress (Sharp et al., 1988), which showed a decline in both the maximum local longitudinal expansion rates as well as the length of the growth zone. Similarly, in *P. pringlei* and other cacti, the length of the growth zone is inhibited under progressive water stress (Dubrovsky and Gómez-Lomelí, 2003). Interestingly, like in the maize primary axial root (Sharp et al., 1988), there was complete maintenance of local longitudinal growth rates for all genotypes and levels of stress in the most apical portion of the growth zone across development (Fig. 3-8).

Under MWD conditions, local rates of cross-sectional area expansion, or the radial plus tangential strain rate, along the growth zone of FR697 first-order
lateral roots were even more resilient than longitudinal strain rates to the reductions that occur with determinate growth under WW conditions. At 7 DAT, rates were still at 85% of maximum, compared to 43% in WW control roots. Moreover, at 11 DAT lateral roots grown under MWD displayed peak rates of 42% of maximum, and showed no indication of the shortening of the length of the growth zone as seen in WW conditions. In the case of radial plus tangential dimensions, the strain rate profiles suggest a more substantial delay of determinate growth than the 2.5 days observed for average length as well as longitudinal strain rates (Fig. 3-10A, B). It is curious why maintaining high rates of circumferential expansion would be beneficial under water-limited conditions. It is possible that the inhibition of thinning is to maintain the surface area of the root, preventing reductions in the amount of root-soil contact and impairment of hydraulic conductivity. Another possibility is that MWD conditions are triggering responses in the plant associated with resistance to soil compaction. Water content and the mechanical strength of the soil are heavily interrelated, and it would be logical for the responses to one stress to be primed if the other is encountered.

The summation of the longitudinal and radial plus tangential strain rates gives the volumetric stain rate, or the relative rate of volumetric expansion along the growth zone (Fig. 3-11). As FR697 first-order lateral roots showed dynamic responses to MWD in both components of volumetric strain rate (Fig. 3-10A, B; Fig. 3-11A, B), effects were amplified, highlighting the differences in response between genotypes. Under MWD, FR697 lateral roots maintained the length of the zone of volumetric expansion for the entirety of the experiment, while maintaining
significantly higher rates of local volume increase from 7 DAT onward compared with WW control roots. Again, this is in sharp contrast to the results of the primary root in maize, which showed reduction in both longitudinal and radial plus tangential strain rates induced by water deficit, leading to a compounding inhibition of volumetric expansion along the growth zone (Sharp et al., 1988).

Possible mechanisms of interaction between mild water deficit and determinate growth control

Several studies have assessed the cellular and genetic basis for root determinacy. Transcription factors PLETHORA1 (PLT1) and PLT2, WUSCHEL-RELATED HOMEobox 5 (WOX5), SHORT ROOT (SHR) and SCARECROW (SCR) have been shown to be involved in preserving quiescent center (QC) identity and are necessary for indeterminate root growth (Benfey et al., 1993; Scheres et al., 1995; Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004; Lucas et al., 2011). Expression of the transcription factors SCR and SHR, which play essential roles in meristem patterning, have been shown to be responsive to water stress in arabidopsis as well as several crop species (Llave et al., 2002; Schafleitner et al., 2007; Ma et al., 2010; Zhou et al., 2010; Kantar et al., 2011; Wang et al., 2011). Additionally, WOX5, a critical transcription factor that maintains the QC and the surrounding stem cells in an undifferentiated state, has also been shown to be down regulated in response to water-limited conditions (Huerta-Ocampo et al., 2011).
Furthermore, the phytohormone auxin is a crucial element serving several roles in meristem development and functionality. PIN FORMED 4 (PIN4) is an auxin efflux protein that is expressed in the QC and surrounding cells and is required for correct RAM patterning along with PIN FORMED 3 (PIN3) (Friml et al., 2004). Transport of auxin into the RAM is facilitated via PIN FORMED 2 (PIN2), suggesting a vital role in regulation of RAM length. Many studies of transgenic plants with aberrant auxin transport or patterning report development of roots ending in premature RAM consumption (Stepanova et al., 2008). It has been shown that auxin levels increase with water stress in the maize primary root as growth is inhibited (Ribaut and Pilet, 1994), and a multitude of auxin-inducible genes and proteins are upregulated in a region-specific manner in the growth zone of maize and soybean primary roots (Poroyko et al., 2007; Spollen et al., 2008; Yamaguchi et al., 2009). The flow of auxin through the root is the signal for many diverse physiological responses to the environment and is likely a key factor coordinating the interaction of determinate growth and plasticity to water stress.

It has been suggested that an alternate path of determinate root growth regulation exists, controlled by FOLYLPOYGLUTAMATE SYNTHETASE 1 (FPGS1), an enzyme involved in vitamin B9 metabolism (Srivastava et al., 2011; Reyes-Hernández et al., 2014). Tetrahydrofolates (THFs) are a group of B9 vitamins that are involved in the metabolism of folic acid derivatives, which are essential for synthesis of critical cellular compounds such as amino acids, nucleic acids, tRNA and pantothenate (Hanson and Roje, 2001; Jabrin et al., 2003). It has been shown that folate derivatives are required at high levels in actively growing

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tissue such as the RAM. Analysis of overall folate content in *Pisum sativum* showed five-fold higher levels in the root tip compared to mature root tissue (Jabrin et al., 2003). Furthermore, both transcript levels and protein abundance of Dihydropteron Pyrophosphokinase-Dihydopteroate Synthase (HPPK-DHPS), the enzyme responsible for the first two reactions in the production of THFs, was enriched specially in pea root tips (Jabrin et al., 2003).

Mutations of *AtFPGS1* result in RAM exhaustion, aberrant QC divisions and determinate root growth. Additionally, arabidopsis plants grown on medium containing methotrexate, a powerful inhibitor of folate synthesis, resulted in determinate roots and similar QC defects (Srivastava et al., 2011). Expression analysis studies revealed that the activation of cellular division in the QC facilitated by FPGS1 is independent of auxin gradients and RAM regulatory elements such as PLT, SCH, SCR and WOX5 (Reyes-Hernández et al., 2014). Furthermore, studies of *mko2*, an *AtFPGS1* mutant, showed an expansion of the promoter activity domain of QC-specific transcription factors, suggesting a role of FPGS in QC quiescence. Together, the evidence suggests two roles for FPGS involved in determinate root growth; a requirement in maintaining the stem cell niche, as well as a role in folate metabolism leading to the production of critical compounds required for cell division.

Several reports have shown an interaction between the expression patterns of genes involved in folate metabolism and abiotic stress. FPGS has been shown to be differentially regulated in response to heat stress in cotton (Demirel et al., 2014). Furthermore, there was a 1.6-fold increase of THF levels in the leaves of
water stressed rice (Shu et al., 2011). In contrast, it has been shown that THF levels were unaffected by salt stress in arabidopsis cell culture (Kim et al., 2007). Two other enzymes involved in folate metabolism, FORMATE DEHYDROGENASE (FDH) and SERINE HYDROXYMETHYLTRANSFERASE (SHMT), showed altered expression in response to several stresses including hypoxia, wounding, chilling, and drought in potato (*Solanum tuberosum* L.) leaves (Hourton-Cabassa et al., 1998). It would be interesting to see if the expression of FPGS1, or other components of the THF metabolic pathway, are differentially regulated in association with the delayed determinacy of maize lateral roots grown under mild water deficit conditions.

**CONCLUSIONS**

Taken together, the results demonstrate that a mild water deficit at a $\Psi_w$ of -0.30 MPa acts on FR697, but not B73, first-order lateral roots to delay the normal determinate growth program, allowing for maintained rates of longitudinal, radial plus tangential and volumetric expansion, potentially for optimum seedling establishment and resource uptake. To our knowledge, this is the first report of water stress acting to delay the determinate growth program of a plant organ. Future studies focusing on the mechanisms underlying delayed determinate root growth could provide information pertaining to meristem organization and longevity, as well as shed light on the regulation of environmentally induced lateral root plasticity.


Chapter 4

Summary and Future Directions
Summary

In this dissertation, a detailed evaluation of several growth media was conducted for suitability of use in sustained water potential experiments. A medium selected for its high water-holding capacity, and tolerance to reductions in Ψ<sub>w</sub> via water loss, was utilized in developing conditions for photosynthetically active growth in a stable Ψ<sub>w</sub> environment. Two maize genotypes with contrasting lateral root responses to mild water deficit, FR697 and B73, were assessed for the level of plasticity of multiple root categories. It was seen that B73 lateral roots were largely unresponsive to the range of deficits tested. In contrast, FR697 showed an enhancement of total lateral root length of the primary root system when grown at water potentials of -0.25 and -0.30 MPa. Furthermore, FR697 showed longer and wider first-order lateral roots at these stress levels, resulting in substantially larger volume under MWD compared with well-watered conditions.

Evaluation of the effects of MWD on the determinate developmental program of FR697 primary axis first-order lateral roots showed that there was an enhancement of the duration of growth, delaying determinacy approximately 2.5 days. Manifestations of maintained growth were examined on an anatomical and kinematic basis along the lateral root growth zone. Results showed that MWD suppressed the cessation of elongation that occurs under normal determinate development. Maintained rates of elongation were due to a combination of sustained cell flux and final cell length. Furthermore, the thinning that occurs along the growth zone of FR697 lateral roots under normal determinate development, a trait not seen in B73, was also suppressed in response to MWD conditions.
Analysis of the spatial profile of local expansion rates along the growth zone revealed that FR697 lateral roots maintained high rates of growth in the longitudinal, radial, and tangential planes for a longer duration under MWD conditions. Much work is still to be done to understand the complex interactions between determinate root growth and lateral root plasticity induced by water deficits.

**Potential benefits of delayed determinate growth in water-limited conditions**

The delay of determinacy exhibited by FR697 first-order lateral roots under MWD conditions has many potential benefits related to improved tolerance to water stress. As soil dries, capillary forces greatly increase, leading to large reductions in hydraulic conductivity. Continued growth of the lateral roots under MWD conditions allows for exploration of new areas of the soil profile, bringing the root closer to new sources of water. As only slightly more intense levels of deficit result in abolishment of this phenotype, the response seems to be a useful adaptive strategy, that can be easily repressed if conditions become too extreme. Furthermore, first-order lateral growth may be maintained under MWD to allow for more opportunities for higher order branching. Increasing the number of root tips could also lead to greater capacity for resource uptake, soil exploration and increased environmental perception. However, in the experiments presented in
this dissertation, enhanced production of secondary laterals was not noticeable by eye at the end of experiments, and lateral root primordia were not assessed.

Additionally, maintaining active meristematic growth also maintains the production of mucilage by root cap border cells (Vermeer and McCully, 1982). Mucilage sheaths on root tips increase the hydraulic connectivity at the root-soil interface and facilitate water uptake via increasing the water content of the rhizosphere (McCully, 1999; Carminati et al., 2010; Ahmed et al., 2014). Furthermore, mucilage has been shown to ease root growth through soil, and its production is increased in response to mechanical impedance (Barber and Gunn, 1974; Iijima and Kono, 1992; Iijima et al., 2004). These traits could be particularly useful in environments that experience a large degree of soil hardening in water-limited conditions.

**Radial anatomical modifications induced by mild water deficit and the consequences on lateral root mechanical strength**

An in-depth assessment of radial anatomy would add a significant component to the understanding of how first-order lateral roots respond to water stress throughout determinate development. In adverse conditions, it is known that cortical cells can be modified in several ways to reduce the metabolic costs of respiration and promote continued growth. Formation of aerenchyma, increasing cortical cell size and reducing the number of cortical cell files are some ways to achieve this “cheaper” root growth (Iijima et al., 2007; Zhu et al., 2010; Chimungu
et al., 2014b, a; Lynch et al., 2014). It would be interesting to know what anatomical modifications are responsible for the thinning phenotype seen in FR697 lateral roots, and if the suppression of thinning under MWD simply prevents the changes, or affects radial anatomy in a distinct way. Furthermore, this type of analysis would also allow assessment of secondary cell wall thickening and other modifications, such as suberin or lignin deposition. An experiment to see if first-order laterals increase the production of such compounds as they progress through determinate development would be useful to determine many structural aspects related to water movement and mechanical strength.

It has been observed that plant roots thicken when challenged with hard or compacted soils to provide mechanical strength to the root axis (Materechera et al., 1991; Materechera et al., 1992). In natural and agronomic settings, it is common for soil water deficits to interact with mechanical stresses, either in the form of compaction or root penetration into hard soil (Bengough et al., 2011). Soil strength generally increases rapidly with decreasing water content, as increasing capillary forces drive more negative matric potentials (Whiteley et al., 1982; Whalley et al., 2005). Increases in soil strength of an order of magnitude are common between soil water potentials of -5 kPa to -1.5 MPa (Bengough et al., 2006). It has been shown that even soils considered to be relatively hydrated (up to -0.10 MPa) can provide mechanical impedance to root elongation via water films connecting adjacent soil particles (Whalley et al., 2005). The growth of several crop species has been shown to be heavily impacted by physical resistance, including wheat (Atwell, 1990; Merotto Jr and Mundstock, 1999), barley (Wilson et
al., 1977; Goss and Russell, 1980), peanut and cotton (Taylor and Ratliff, 1969) and maize (Mirreh and Ketcheson, 1973; Veen, 1982; Veen and Boone, 1990). Furthermore, soil compaction has been shown to affect the elongation of pea primary roots unevenly along the growth zone, similar to maize growth under water deficit conditions, with maintenance of local elongation rates in the most apical portion (Sharp et al., 1988; Croser et al., 2000; Bengough et al., 2006).

In modern agriculture, compaction and soil tension effects are aggravated through the use of heavy machinery utilized in farm settings (Bengough et al., 2011). Additionally, in times of fluctuating soil moisture, such as pre-planned or natural wetting-drying cycles, soil shrinking and swelling may result in mechanical stress being applied to root systems (Striker et al., 2007). Soil shrinkage has been shown to exert 120 – 200 kPa of pressure on root systems, resulting in root collapse and the impairment of resource uptake (Richards and Greacen, 1986; Bengough et al., 2006; Striker et al., 2007).

The durability of plant organs is a function of anatomical organization and the composition of structural elements (Niklas, 1992; Aranwela et al., 1999). Fortification of organs can be achieved by formation of tissue with high physical resistance underneath the epidermis, such as sclerenchyma, lignin or the presence of dense multiseriate rings of cells in the outermost cortex (Striker et al., 2007). Furthermore, internal anatomy, as well associated mechanical properties, can change with organ size (Niklas, 1992; Aranwela et al., 1999; Genet et al., 2005), and many stresses including flooding, water stress, and soil compaction have been shown to alter root dimensions (Veen, 1982; Sharp et al., 1988; Fraser
et al., 1990; Materechera et al., 1992; Merotto Jr and Mundstock, 1999; Visser et al., 2000; Kirby and Bengough, 2002; Iijima et al., 2007).

Many of the traits which may reduce metabolic costs under water deficit conditions may also diminish durability. Aerenchyma formation can create a large degree of variation in root internal structure (Justin and Armstrong, 1987), leading to decreases in root mechanical strength required to combat soil compression (Engelaar et al., 1993; Striker et al., 2006). Additionally, while larger cells per unit volume may reduce metabolic costs associated with respiration, tissues composed of smaller diameter cells have a higher density of cell walls, which enhances their stiffness and strength, providing greater resistance to deflection and buckling compared to tissue composed of larger cells (Anten et al., 2005; Weijschedé et al., 2008; Chimungu et al., 2014a).

In addition to assessment of radial anatomy, evaluation of the resultant effects on mechanical durability of lateral roots would be useful to understand potential tradeoffs of the enhanced duration of growth induced by mild water deficit. Root compression experiments have been used to examine the mechanical strength of roots and determine the force required to buckle or collapse the root (Striker et al., 2006). It would be interesting to examine whether lateral roots that have reached the end of their determinate growth are more durable due to complete cell maturation and cell wall thickening all the way to the apex. Furthermore, the thinning observed in well-watered lateral roots of FR697 may be the result of a reduction in average cell size with a maintenance of cell file number. If this is the case, the roots would be significantly denser as more of the cross-
sectional area is composed of cell wall tissue. It is hypothesized that this would add considerable durability to the root tissue and may be an additional beneficial trait of FR697 to adapt to the environment that B73 lacks. Examination of how root resistance to compression is affected by the anatomical modifications associated with delayed determinacy could prove useful for the targeted development of plants in areas commonly affected by a combination of water deficit and mechanical impedance.

**Spatio-temporal analysis of the genetic control of delayed lateral root determinacy induced by mild water deficit**

It would be highly informative to make use of the kinematic profiles to assess the spatial changes in the growth zone for targeted tissue sampling. Selection by this method would allow analogous tissue to be sampled across the course of determinate development as well as in response to water deficits, without error introduced by erroneous tissue sampling (Poroyko et al., 2007; Spollen et al., 2008). Furthermore, using this approach, tissue segments at different distances along the growth zone could be selected specifically for longitudinal or radial + tangential growth, providing insight into the regulation and potential tradeoffs of length versus radial expansion involved in lateral root environmental plasticity.

Genetic data gathered would shed light on the changes that occur throughout determinate growth, as well as provide insight into the genetic control of the delayed determinacy response induced by MWD. It has been proposed that
there are two separate processes controlling determinate root growth, one being the complex network of hormonal fluxes, genes and transcription factors regulating meristem maintenance (Jiang and Feldman, 2005; Shishkova et al., 2008), and a second path regulated by FOLYL POLYGLUTAMATE SYNTHETASE1 (FPGS1). It has been shown that FPGS1 is involved in a process regulating the “indeterminacy-to-determinacy switch” via C1 folate metabolism. Impaired functionality of FPGS1 results in activation of the cell cycle in the QC and meristem exhaustion (Reyes-Hernández et al., 2014). It is not clear which of these control programs are involved in the constitutive determinate growth process of maize lateral roots, or how MWD would act to delay the developmental mechanisms involved.

Expression analysis of genes encoding transport proteins could provide insight into the alterations of resource allocation to lateral roots approaching determinate length. It would be interesting to examine whether larger changes are seen in expression of auxin transport proteins, which would influence RAM maintenance mechanisms, or in transporters involved in carbon fluxes, which could limit the meristem to its own local carbon supply for essential folate metabolism. Furthermore, in the event that maize lateral root determinate development is not regulated via C1 metabolism, major alterations of the import of carbon must still be involved in the transition to non-growing tissue. Import of carbon is clearly still required for maize lateral roots that have reached determinate length, as the roots are still functional in water uptake and support the development of root hairs and higher order lateral roots (Varney and McCully, 1991).
Genotypic variation in hydrotropism and hydropatterning

It is an intriguing possibility that in addition to having a highly plastic root system to water deficit, FR697 lateral roots could also have a superior capacity to sense water gradients in the soil. Recent work has been focused on the field of hydrotropism (Eapen et al., 2005; Kobayashi et al., 2007; Cassab et al., 2013) and hydropatterning (Bao et al., 2014; Robbins and Dinneny, 2015; Robbins and Dinneny, 2017) to understand how a plant can sense available water, grow in the direction of availability and allocate resources efficiently to suit the environment. It would be useful to develop a way to introduce moisture patches into the media of the controlled $\Psi_w$ system to examine if hydrotropism affects the interaction between determinate root growth and plasticity to MWD. Most intriguing would be if a lateral root experiencing delayed determinacy under MWD conditions could quickly switch back to a determinate developmental program upon directing its growth toward a zone of sufficient moisture. Furthermore, while B73 showed no major interactions between the levels of MWD tested and lateral root development, robust hydrotropism or hydropatterning responses could exist, adding an additional layer of complexity in respect to the differences in lateral root growth exhibited by these genotypes.

An additional question is, to what extent can roots that have reached their determinate length sense their environment? The classical interpretation is that the root tip is the portion of the root responsible for environmental sensing. However, when a lateral root has reached its determinate length it is completely devoid of a root cap as well meristematic tissues, and the root tip may even abscise (Varney
and McCully, 1991; Dubrovsky, 1997). Interestingly, it has been recently shown that the cortex of the elongation zone is responsible for hydrotropic sensing and directional growth (Yamazaki et al., 2012; Dietrich et al., 2017). It is an intriguing notion that while a root that has reached its final length could no longer itself grow in response to hydrotropism, it could still potentially send signals to divert nearby roots towards the area of moisture.

**Real-time observations of growth and resource allocation during mild water deficit-induced delayed determinacy**

It would be very interesting to utilize techniques that allow for the visualization of changes in water and carbon fluxes into first-order laterals throughout development, and to assess how MWD affects these processes during delayed determinacy. This could be done using a wide variety of methods such as utilization of deuterated water or nuclear magnetic resonance imaging to follow the flow of water in real-time (Scheenen et al., 2000; Ahmed et al., 2016). Recently, imaging techniques have been developed to measure both the architecture of the root system and the flux of resources through the plant simultaneously in a non-invasive way. Positron emission tomography (PET) can be used to study the flow and allocation of resources in real-time by use of radioisotopes, and can be paired with magnetic resonance imaging (PET-MRI), X-ray computed topography (PET-CT) or gel-based optics to simultaneously visualize the development of root architecture (Jahnke et al., 2009; Garbout et al., 2012; Wang et al., 2015; Topp et
al., 2016; van Dusschoten et al., 2016). Unfortunately, such techniques do not have the resolution to monitor changes in resource distribution along the lateral root growth zone. Nevertheless, alterations in the flux of resources along the lateral root axes could be visualized throughout determinate growth, as well as the extent of the modified allocation during delayed determinacy under MWD conditions.

**Alternative growth conditions**

A concern of any study is if the results are in some way reliant on an unseen environmental condition or circumstance and are therefore nontransferable to other settings, natural or artificial. It would be interesting to assess how specific the delayed determinacy response of FR697 lateral roots under MWD conditions is to the highly controlled environmental conditions presented here. Preliminary experiments showed that similar responses to water deficit, or lack thereof, were seen in lateral roots of both genotypes when grown in vermiculite, turface, Greens Grade and sand compared to Pro-Mix HP. However, variations of soil hardness through compaction could alter the normal determinate growth program. It is known that roots can thicken when challenged with hard soils to provide durability and aid in continued elongation. How would this type of environment affect the thinning phenotype displayed by FR697 lateral roots under normal determinate development? If the observed thinning does not occur in compacted media, then how would MWD conditions, which suppress the thinning in our system, alter the radial dimensions of the lateral roots? Furthermore, it is possible that lateral roots
have specific anatomical or physiological adaptations to mechanical impedance, which may take priority over the lateral root growth responses to MWD examined in this study.

Additionally, unlike the growth media assessed in this study, most settings for plant growth are composed of an extremely heterogenous environment. The lateral root growth dynamics presented here could be dramatically altered if the growth medium was comprised of a mixture of nutrient patches, moisture gradients, or areas of differential mechanical impedance. It is likely that under such circumstances lateral root development would not occur in a uniform manner, but would respond to local stimuli throughout the medium, potentially forming an uneven root system that does not represent the idealized architype. Furthermore, it is important to look at multiple stresses together as very rarely in nature is the plant only affected by a single growth limitation. Experiments aimed at addressing if the results described in this study would still hold true with the addition of a secondary stress such as salt, heavy metals, toxins or pathogens could give information on how highly the plant prioritizes the water deficit-induced delay of determinacy, compared to responding to other adverse conditions.

Finally, experimental results obtained from laboratory seedling systems do not always correlate with those of field-grown root systems (Watt et al., 2013). It is important to assess if the lateral root responses to MWD reported here are also seen in agricultural environments at this stage of development. Field studies comparing the growth responses of these genotypes utilizing a rainout shelter, or
another form of irrigation management, with controlled-environment conditions, would greatly support the findings presented here.

**Extension of findings to older plants, different root systems and other species**

While the water deficit-induced lateral root responses demonstrated in this dissertation would benefit the plant regarding seedling establishment, if they were to translate to roots of a more mature maize plant it would greatly enhance the capacity of the roots to adaptive to their environment. It would be intriguing to see if the lateral roots growing more apically on the primary axial root show the same responses as the subset from the basal 15 cm. Additionally, while there was no increase in total lateral root length on the seminal axes in response to MWD for either genotype, a trend was observed which hinted that enhanced rooting may be observed in a longer experiment. Moreover, the age of the plants used in this study did not allow for the analysis of lateral root responses to MWD on the nodal axes. However, it has been reported that lateral roots of the maize nodal axes do have a determinate developmental program, with a duration of growth of approximately 2.5 days (Varney and McCully, 1991). It would be a useful addition to be able to compare lateral root growth between all root categories at various depths. However, growth conditions able to maintain near-stable levels of stress for longer than the 11 days examined here would be required.
An additional genotype, Mo17, was tested in preliminary experiments and did not display the enhancement of growth exhibited by FR697, exhibiting growth similar to that of B73 (Fig. A-3). However, a study of a much larger and more genetically diverse group of maize genotypes, such as the NAM (nested association mapping) lines (McMullen et al., 2009), would be beneficial to determine how much genotypic variability is found in maize regarding lateral root determinacy and plasticity to water stress. Furthermore, other species should also be assessed over a high-resolution series of mild water deficits to determine if this trait is specific to a certain type of plant, e.g. grasses, or conserved across broader groups. As discussed in previous chapters, many crop species have been observed to possess longer lateral roots when grown under some level of water deficit. However, most studies assessed growth at a single time point and not the effects on the organ throughout the entire course of development. Therefore, it is possible that many of the previous reports did indeed show the product of delayed determinacy. However, without a detailed examination of the lateral root responses of several diverse species throughout development, the prevalence of MWD-induced delayed determinacy remains to be determined.


APPENDIX

Figure A-1. Non-transpiring system comparison of lateral root responses to decreasing growth medium water potential.

Effects of nearly halting transpiration on the growth of the primary and seminal root systems over a range of decreasing growth medium $\Psi_w$. Non-transpiring plants were grown as described in Chapter 2, except as follows. Tubes were 76 cm tall and filled to 46 cm with pre-calibrated Pro-Mix BX growth medium. The remaining 30 cm of the tubes created microcosms for leaf growth and were sealed with plastic wrap. Slits were made in the plastic wrap to allow gas exchange and moistened cheese cloth was placed atop each microcosm to maintain a high internal RH at near saturation. Plants were grown for 9 DAT and root traits were analyzed using WinRHIZO equipment and software. Data are means ± standard error (n = 6-8 plants).
Figure A-2. Ineffectiveness of Greens Grade and sand to maintain a stable water potential.

Transplant and harvest water potentials of Greens Grade and sand after supporting the growth of maize seedlings for 9 Days. Harvest $\Psi_w$ measurements were taken at a tube depth of 15cm. Both media showed an approximate decrease in $\Psi_w$ of -0.30 MPa at the end of the experiment. Data are means of two independent tubes measured at transplant as well as at harvest for both media.
Figure A-3. Water deficit-induced lateral root plasticity of Mo17.

Primary and seminal root system responses of the inbread maize line Mo17 to decreasing $\Psi_w$ of Pro-Mix BX growth medium. Plants were grown for 9 DAT in the non-transpiring system described in Figure A1. Root traits were analyzed using WinRHIZO equipment and software. Data are means ± standard error ($n = 3$-4 plants).
Figure A-4. Enhancement of FR697 lateral root length in response to mild water deficit in other growth media.

Total lateral root length response of the primary root system to decreasing vermiculite and Greens Grade media water potential. Plants were grown for 9 DAT in the non-transpiring system described in Figure A1. Root traits were analyzed using WinRHIZO equipment and software. Data are means ± standard error (n = 4-8 plants).
VITA

Tyler G. Dowd was born in Boulder Colorado to Linda and David Dowd on November 18th, 1987. During his youth, he spent a great deal of time outdoors in the forests of the Rocky Mountains and begin to become very interested in the trees and plant life in the surrounding areas. In 2006, he enrolled in courses at Colorado State University in Fort Collins Colorado to begin the formal study of biology. During his undergraduate carrier, he served as an intern at the Colorado State Herbarium and graduated with a Bachelor of Science degree in Biological Sciences: Concentration Botany. Following graduation, Tyler got a position as a lab technician in the Adams/ Demmig-Adams ecophysiology lab at the University of Colorado at Boulder. In 2012, he joined the Division of Plant Science at the University of Missouri in a collaborative project between the laboratories of Dr. Robert E. Sharp and Dr. David M. Braun to research environmental plasticity of maize lateral roots to water-limited environments. His dissertation for the degree of Ph.D. in Plant, Insect and Microbial Sciences was titled “Delayed Maize Lateral Root Determinacy Induced by Mild Water Deficit”.