ROOT TRAITS FOR EFFICIENT NITROGEN ACQUISITION AND
GENOME-WIDE ASSOCIATION STUDY OF ROOT ANATOMICAL TRAITS IN

MAIZE (Zea mays L.)

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By

Patompong Saengwilai

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The dissertation of Patompong Saengwilai was reviewed and approved* by the following:

Jonathan Lynch  
Professor of Plant Nutrition  
Dissertation Advisor  
Chair of Committee  

Kathleen Brown  
Professor of Postharvest Physiology  

Dawn Luthe  
Professor of Plant Stress Biology  

Surinder Chopra  
Associate Professor of Maize Genetics  

Teh-hui Kao  
Distinguished Professor of Biochemistry and Molecular Biology  
Head of The Intercollege Graduate Degree Program in Plant Biology  

*Signatures are on file in the Graduate School
Abstract

By 2050, the world population is predicted to reach 10 billion, almost double the current population. Feeding a growing population will become even more challenging. Since the advent of the Green Revolution in the 20th century, improved crop cultivars, chemical fertilizers, herbicides, and pesticides have been extensively used in order to increase agricultural productivity. As a result, agricultural food production was doubled within only four decades. Although several factors contributed to this doubling of world food production, a rapid increase in the use nitrogen (N) fertilizers seems to have played a major role. However, the continuous increase in the use of N fertilizers is causing detrimental impacts to the environment. For example, N fertilizers leached from agricultural areas to natural waters causes severe reductions in water quality by enhancing algal blooms, which disrupt normal functioning of ecosystems. Furthermore, nitrous oxide, which is a potent greenhouse gas, can be released to the atmosphere. Most importantly, the production of N fertilizer requires considerable energy from fossil fuels. As energy costs have risen in recent years, farmers face economic pressure from increasing N fertilizer costs. Therefore, relying on increasing N fertilization is not a sustainable solution for the future. We now need a “second green revolution” that will benefit resource-poor farmers by developing nitrogen efficient crop varieties that produce more food under low nitrogen conditions. A number of studies have shown that root traits play essential roles in N acquisition. Recently an ideotype of root traits for improved water and N acquisition has been proposed by Lynch (2013). Further research is needed to verify the physiological utility of these traits under low N conditions.

Understanding the genetic control of root traits is also essential for plant breeders to develop breeding strategies that maximize genetic gain at reasonable time and costs. Root anatomical traits influence transport of water and nutrients, root mechanical strength, and
interactions between roots and soil biota in rhizosphere. A number of experiments have shown significant benefits of root anatomical traits under biotic and abiotic stresses. Despite the high potential for improving crop performance and yield, few studies have been undertaken to characterize phenotypic variation and identify genetic control for root anatomical traits.

My research is focused on the physiological utilities of maize root traits specifically 1) root cortical aerenchyma (RCA), 2) number of crown roots (CN) and 3) root hair length for enhanced nitrogen acquisition. The results suggest that these traits may be promising breeding targets for enhancing nitrogen acquisition from low N soils. Another topic is to study phenotypic variation and genetic control of 20 root anatomical traits of the Wisconsin diversity panel. Single nucleotide polymorphism markers (SNPs), associated with the traits, were identified using Genome-Wide Association Study (GWAS). Molecular markers associated with these traits may be useful in plant breeding programs using marker-assisted selection. The results of this study will greatly improve our understanding of the phenotypic variation and genetic control of root anatomical traits in maize (*Zea mays* L.).
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Chapter 1

Introduction

Maize (*Zea mays* L.) is the most widely grown crop with a total production over 875 million tonnes in more than 176 million hectares worldwide (FAOSTAT, 2013). It serves as a staple food source in Latin America and Africa, and has become the most important materials for animal feed, industrial products, and biofuels. It is predicted that by 2050, we will need 70% more food than today to satisfy the demand of the future population. A large proportion of the increased demand will come from developing countries (FAO, 2013). Within a decade, the global demand of maize is expected to surpass the demand of wheat and rice and may account for more than half of the increased demand for cereals as a whole (UNDP, 2010). Since the availability of arable land and resources become more and more limiting, the necessary increase in maize production will require substantial changes in agronomic practices and methods for crop improvements.

Nitrogen Efficiency in Maize

Nitrogen efficiency (NE) can be defined in a variety of ways depending on the type of system. In cereal crops like maize, agronomic NE can be expressed as the ratio of grain yield to N supplied from both the soil and fertilizer applied (Moll et al., 1982). The global estimation of maize NE ranges from 25-50% (Raun & Johnson, 1999; Tilman et al., 2002) indicating the possibility for improvement. NE is complex and is influenced by interactions among several physiological and biochemical processes. In general, two major processes contribute to NE: N acquisition (NA, the ability of plants to acquire available N from the soil) and N utilization (NU,
the ability to use acquired N to produce grain yield). Therefore, a number of studies have been undertaken to understand physiological, biochemical, and genetic controls of NA and NU to identify the bottlenecks associated with NE.

Nitrogen Acquisition and Utilization

Sources of N in the soil include initially available N, net N mineralization from organic matter, biological nitrogen fixation, N inputs from irrigation water and atmosphere deposition (Ribaudo et al., 2011). Most of soil N is present in the form of complex organic molecules, which are converted to ammonium (NH₄⁺) through a process called mineralization. NH₄⁺ is then oxidized to nitrite (NO₂⁻) and to nitrate (NO₃⁻) through nitrification. These processes require the activities of soil microbes. Under oxygen limiting conditions, NO₃⁻ can be converted to nitrogen gases (N₂, N₂O, NO, NO₂) through a process known as denitrification. The availability of N to plants depends on the balance between the rates of mineralization, nitrification, and denitrification. N in the soil is extremely heterogenous and fluctuates greatly in space and time due to several factors such as soil microbial activity, temperature, precipitation, soil type, and soil pH (Ribaudo et al., 2011). Plants can uptake soil N in the form of NH₄⁺, NO₃⁻, and, to a lesser extent, amino acids. Under well-aerated agricultural soil, NO₃⁻ is the most abundant, while NH₄⁺ dominates under poorly aerated, submerged soil. N is delivered to plant roots through mass flow, which relies on transpiration and through diffusion, which depends on concentration gradient and diffusion coefficients. The diffusion coefficient of NO₃⁻ in the soil is approximately 1 x 10⁻¹⁰ m²·s⁻¹ while that of NH₄⁺ is 10 to 100 fold less (Owen and Jones, 2001). Due to its high diffusion coefficient, NO₃⁻ is not only readily available to plant roots, but it also leaches very quickly from the root zone. On the other hand, NH₄⁺ is relatively immobile in the soil and less easily lost through leaching.
Nitrogen acquisition occurs at the surface of plant roots. Root system architecture ensures adequate access to soil N while N transporters are responsible for the uptake process. When N is limiting, plants allocate more resources to the root system, resulting in an increase in the root to shoot ratio (Rufty et al., 1988). Elongation of the axial and the lateral roots is decreased under high N conditions. However, under low N conditions, a local supply of nitrate can significantly increase lateral root elongation (Chun et al., 2005; Wang et al., 2004). The uptake of NO$_3^-$ into roots is governed by two major transporter systems; a high-affinity transporter system (HATS) which operates at low NO$_3^-$ concentrations, and a low-affinity transporter system (LATS) which operates at high NO$_3^-$ concentrations (Miller and Cramer, 2004). NO$_3^-$ transporters have been identified and characterized in many species. Most of them have been found to belong to two distinct gene families: NRT1 and NRT2 (Forde and Clarkson, 1999; Forde, 2000). Like NO$_3^-$ transporters, many NH$_4^+$ transporter genes have been identified. Sequence comparisons have revealed two distinct groups: AMT1 and AMT2. Some AMTs are constitutively expressed (Suenaga et al., 2003), but for most the expression depends on NH$_4^+$ availability (Ludewig et al., 2002).

Once NO$_3^-$ is absorbed by roots, it is converted to NH$_4^+$ through reduction processes which take place in both roots and shoots. The reduction of NO$_3^-$ to NO$_2^-$ is catalyzed in the cytosol by the enzyme nitrate reductase (NR). After NO$_3^-$ reduction, NO$_2^-$ is translocated to the chloroplast where it is reduced to NH$_4^+$ by the enzyme nitrite reductase (NiR). NH$_4^+$ is then assimilated in the plastid or chloroplast primarily via GS/GOGAT cycles. Glutamine synthetase (GS) catalyzes the production of glutamine by amination of glutamate whereas glutamine:oxoglutarate aminotransferase (GOGAT) catalyzes the formation of glutamate by transferring an amide group from glutamine to 2-oxoglutarate. Alternative pathways of N assimilation are also proposed via the enzyme glutamate dehydrogenase (GDH) and asparagine synthetase (ASN) (Becker et al., 2000).
Nitrogen Acquisition and Utilization Efficiency

Several studies have evaluated the relative importance of NA efficiency (NAE) and NU efficiency (NUE) for grain yield production at low N input. High grain yield under low and medium N conditions was associated with high N uptake, whereas high yield under high N conditions was linked to ability to utilize N accumulated in the plants (Moll et al., 1982). A study including 35 maize hybrids has shown a significant positive correlation between NAE and NUE (Mi et al., 2005). Based on a phenomenological equation grain yield is ultimately limited by NA and the yield can be no greater than that allowed by NA regardless of improvements in crop mass accumulation or in biological pathways (Sinclair and Vadez, 2002). These evidences suggest that NAE is critical for improving NE under low N conditions. Interestingly, Moll et al. (1982) have shown that variation in NE of eight maize hybrids was due mainly to NUE under low N conditions. In addition, quantitative genetic studies in maize have shown that variation of NA before and after flowering was less than that of NU at low N input (Gallais and Hirel, 2004). Thus, NUE can also be important in determining NE under low N conditions. These contradictory results reported in the literature may be explained by variation in the nature of sample genotypes and testing environments.

Regardless of whether NAE or NUE contributes most to NE, improving NUE in maize would be difficult since NUE in maize is already high as shown by its high N harvest index (Sinclair and Vadez, 2002). In fact, no significant relationship between grain yield and N harvest index was found among maize hybrids (Wang et al., 2005). Furthermore, attempts to improve NE by genetic manipulation of nitrogen assimilation enzymes such as NR and GS/GOGAT have not been successful under field conditions. The results even showed that the yield response of crop plants to additional N levels was not limited by these N assimilation enzymes (Andrews et al., 2004). Most importantly, maize has a long breeding history, with most selection goals aiming for
a higher yield response to high N supplied, thus, NUE has already been subject to indirect selection. Unlike NUE, NAE has not yet been subject to indirect selection. Root biology research has indicated that root traits are direct targets for improving NAE (Chun et al., 2005). A number of studies have found genotypic variation in root physiology, anatomy, morphology, and architecture in maize (Tuberosa et al., 2003; Zhu et al., 2010; Zhu et al, 2005; Zhu & Lynch, 2004; Zhu et al., 2010). Moreover, quantitative trait loci (QTL) controlling several root traits have been identified (Mano et al., 2007; Tuberosa et al., 2003; Uga et al., 2008; Yan et al., 2004; Zhu, et al., 2005b). Therefore, ample opportunity exists for enhancing NAE through conservation and modern plant breeding by selection for good root traits in maize.

Maize Root System and Root Traits For Nitrogen Acquisition

Maize Root System

The root system of maize is composed of embryonic and post-embryonic root systems. The embryonic root system consists of two distinct root classes: a primary root and a variable number of seminal roots. The post-embryonic root system consists of roots that are formed at consecutive shoot nodes and lateral roots, which initiate from the pericycle of all root classes (Figure 1.1). The shoot-borne roots that are formed below ground are called crown roots whereas those that are formed above ground are called brace or prop roots (Hochholdinger, 2009). The anatomical organization of all root classes is the same (Figure 1.2). Maize roots have a central cylinder called a protostele with many xylem arms. The outermost part of the stele is surrounded by one layer of the ground tissue, endodermis, which connects to several parenchymatous cell files in the cortex region. The cortex is surrounded by a layer of epidermal cells, which consist of root hair- forming cells (trichoblasts) and non-root hair-forming cells
(atrichoblasts). In old roots, the epidermis is replaced by the lignified, suberized exodermis, which is a layer of tissue in the outermost part of the cortex. Like other monocotyledonous roots, maize roots do not exhibit secondary growth (Hochholdinger et al., 2004; Hochholdinger, 2009).

**Root Traits for Efficient Nitrogen Acquisition**

Plants express an array of root traits to help them cope with edaphic stress. In general, beneficial root traits are the traits that enhance soil exploration, resource exploitation, and exclusion/detoxification of toxic minerals (Lynch, 2007). Several lines of evidence have shown the growth/yield benefits of specific root traits under nutrient and water stress. Under phosphorus (P) stress, root traits that allow topsoil foraging have been shown to be beneficial since P is immobile in soil and more P is available in the topsoil (Lynch and Brown, 2001). Such root traits includes root architectural traits such as a shallow root system (root growth angle) (Zhu et al., 2005b) and enhanced lateral rooting (lateral root branching) (Zhu and Lynch, 2004), root anatomical traits such as high root cortical aerenchyma (RCA), a tissue containing air space in cortex (Esau, 1977), and long, densely spaced root hairs (Zhu et al., 2010). Under drought, traits that allow roots to explore the soil to greater depths would be beneficial since under water-limiting conditions water is more abundant in deep soil profiles. Such traits include a sparsely branched axile root system (Hund et al., 2009) and high RCA (Zhu et al., 2010).

For nitrogen, NH$_4^+$ and NO$_3^-$ are the most dominant available forms acquired by maize. These two forms, however, differ in their mobility in the soil. Thus, roots traits for efficient acquisition of NO$_3^-$ and NH$_4^+$ should be ones that consider the different spatial and temporal distribution of both nutrients in the soil. NO$_3^-$ is highly mobile in the soil. In agricultural systems, mineralization of organic matter and/or the application of nitrogen fertilizer at the beginning of the growing season cause a pulse of available NO$_3^-$ which may exceed the need of seedlings and
leach away from the root zone. Therefore, root traits that allow rapid root growth, enhance soil exploration at a great depth, as well as increase nitrate transport into the root surface should be beneficial for efficient NO$_3^-$ acquisition. Conversely, NH$_4^+$ is relatively immobile and moves toward the roots mainly by diffusion (Barber, 1995). The uptake of immobile NH$_4^+$ can exceed the rate of diffusion, thus, creating depletion zones around the roots. In an environment more dominated by the NH$_4^+$ fraction of available N, long and dense root hairs seem to benefit NH$_4^+$ acquisition like they do for P acquisition (Jungk, 2001). Root traits such as high RCA or low LCA may also benefit both NO$_3^-$ and NH$_4^+$ uptake by minimizing root metabolic costs and allowing greater soil exploration. Recently an ideotype for improved N acquisition by maize roots has been proposed called ‘Steep, Cheap and Deep’ (Lynch 2013). This ideotype consists of architectural, anatomical, and physiological traits that could increase rooting depth and thereby N capture in leaching environments. ‘Steep’ refers to architectural traits and ‘Cheap’ refers to traits that reduce the metabolic cost of soil exploration.

**Utilities of Maize Root Anatomical Traits**

Considerable phenotypic variation of root anatomical traits has been observed in maize and its relative, teosintes (Burton et al, 2013; Lynch, 2013; Weerathaworn et al, 1992). Root anatomical traits have the potential to improve tolerance to environmental stresses. For instance, root cortical aerenchyma (RCA) enhances tolerance to hypoxic conditions by facilitating root aeration and maintaining an aerobic condition around root zone (Jackson and Armstrong, 1999; Mano et al., 2006). In addition, RCA can be induced by drought and suboptimal availability of nitrogen, phosphorus, and sulfate (He et al., 1996; Drew et al., 2000; Bouranis et al., 2003; Evans, 2003; Fan et al., 2003; Zhu et al., 2010a). In these conditions, plants benefit from RCA by reduced root metabolic cost and by nutrients reabsorbed from lysing tissues (Fan et al., 2003; Zhu
et al., 2010a; Postma and Lynch, 2011). Currently, attempts to breed for aerenchyma are being done by interspecific introgression between teosintes and maize (Mano & Omori, 2013). Roots with small living cortical area (LCA) and small diameter have low root respiration and maintenance costs. These traits are related to drought tolerance in maize (Jaramillo et al., 2013). Cortical components such as cortical cell size, number of cortical cells, and number of cortical cell files could affect root thickness and the length and tortuosity of pathways by which water and nutrients travel through the root (Esau, 1967; Marschner, 1995). Stele traits are very important for the absorption and translocation of water. The size and number of xylem vessels influence their hydraulic conductivity. Water in xylem vessels is assumed to flow according to Poiseuille–Hagen law with a hydraulic conductivity is proportional to the fourth power of vessel’s radius. Narrow xylem vessels have increased resistance to water transport and loss and this trait has been used in plant breeding for water use efficiency (Richards & Passioura, 1989).

**Quantitative Genetic Studies of Root Anatomical Traits**

In the past decades, linkage analysis has been used to identify quantitative trait loci (QTLs) associated with phenotypic variation of root anatomical traits such as stele and xylem vessel diameter in rice (Uga et al., 2008), root cortical aerenchyma in Zea species (Mano & Omori, 2013; Mano et al., 2007; Mano & Omori, 2008) and root cellular development in tomato (Ron et al., 2013). However the progress in identifying genes underlying quantitative agronomic traits has been very slow because of limitations in allelic diversity and resolution in available genetic resources. The resolution of QTLs identified by linkage mapping is usually poor (10 to 20 cM intervals) because of the limited number of recombination events occurring during the construction and analysis of large families from two inbred lines (Flint-Garcia et al., 2005; McMullen et al., 2009). Additionally, linkage analysis has small number of alleles, and
population specific results, which hinder gene discovery and limit implementation of the results to other populations with different backgrounds (Flint-Garcia et al., 2005; McMullen et al., 2009).

By comparison to other agronomic traits, anatomical studies are laborious and difficult to quantify in a large number of samples. Most research is usually focused on one trait and phenotypic evaluations are simplified by ranking instead of quantitative measurements. Even though these methods have been successfully used to identify QTLs controlling anatomical traits such as aerenchyma (Mano et al., 2007; Mano and Omori, 2008; Mano and Omori, 2009), obtaining actual quantitative measurements of the traits would allow better phenotypic precision and may increase the likelihood of finding molecular markers that could be used in breeding.

To improve efficiency and accuracy of phenotyping anatomical traits, a program called RootScan has been recently developed for semi-automated image analysis of root cross-sections (Burton et al., 2012). RootScan allows the measurements of 16 primary anatomical traits and a multitude of their combinations, including: cell size, area, cortical components, xylem vessel area, root cortical aerenchyma, etc. within 1-3 min per image. This makes comprehensive quantitative genetic studies of root anatomical traits possible for the first time.

Recently another approach called “Association mapping” also known as “linkage disequilibrium (LD) mapping” has become increasingly popular among plant scientists. Association mapping exploits historical and evolutionary recombination events at the population level, which increases mapping resolution, reduces research time, and results in more allelic diversity than linkage mapping (Flint-Garcia et al., 2005). Association mapping has been successfully used for many crops including maize to dissect genetic control of quantitative traits such as flowering time (Thornsberry et al., 2001), leaf architecture (Tian et al., 2011), carotenoid accumulation in the endosperm (Harjes et al., 2008), and maysin synthesis in maize silk (Szalma et al., 2005). Together with high throughput phenotypic technologies, association mapping is an ideal approach for candidate gene discovery for root anatomical traits in maize.
Research Objectives

The objectives of this research were to examine the physiological utility of root traits anatomical, architectural, and morphological traits for efficient nitrogen acquisition from low N soils and to identify molecular markers associated with variation of root anatomical traits in maize (*Zea mays* L.). Specifically, the following hypotheses were tested in chapter 2, 3, and 4:

Chapter 2: Root cortical aerenchyma (RCA) enhances N acquisition by reducing root metabolic costs, permitting greater rooting depth and enhanced N acquisition under suboptimal nitrogen conditions.

Chapter 3: Maize genotypes with fewer crown roots (low CN) could explore soils at greater depth resulting in greater nitrogen acquisition, growth, and yield than genotypes with many crown roots (high CN) under low N conditions.

Chapter 4: Maize genotypes with long root hairs are better at acquiring ammonium from the soil, thereby having greater vegetative growth and yield than genotypes with short root hairs under low N conditions.

In Chapter 5, phenotypic variation and heritability of 20 root anatomical traits of field-grown maize were observed and single nucleotide polymorphism markers (SNPs) associated with variation of traits were identified by Genome-Wide Association Study (GWAS).
Figure 1.1: A maize root system showing the primary root, seminal roots, crown roots, lateral roots, and mesocotyl.
Figure 1.2: Cross-section of a second whorl crown root from a field-grown maize showing stele, meta xylem vessels, endodermis, cortex aerenchyma lacunae, and epidermis. The root was collected 8 cm away from the basal region at flowering stage (63 days after planting).
Citations


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Chapter 2

Root cortical aerenchyma enhances nitrogen acquisition from low nitrogen soils in maize (Zea mays L.)

Patompong Saengwilai¹, Eric A. Nord², Kathleen M. Brown¹,² & Jonathan P. Lynch¹,²

¹Intercollege Graduate Degree Program in Plant Biology, The Pennsylvania State University, University Park, PA 16802, USA.

²Department of Plant Science, The Pennsylvania State University, University Park, PA 16802, USA.

Abstract

Suboptimal soil nitrogen (N) availability is a primary constraint for crop production in developing nations, while in rich nations intensive N fertilization carries substantial environmental and economic costs. Understanding root traits that enhance nitrogen acquisition is therefore of considerable importance. Recent studies using functional-structural modeling predict that one such root trait, root cortical aerenchyma (RCA), which is tissue containing enlarged gas spaces found in the root cortex, could be an adaptive trait for N acquisition by maize. In this study we evaluated the utility of RCA for nitrogen acquisition by physiological comparison of maize genotypes with contrasting RCA phenotypes. Maize Recombinant Inbred Lines (RILs) of the Intermated B73 x Mo17 (IBM) population contrasting in RCA were grown under suboptimal and adequate N availability in greenhouse mesocosms and in the field in the USA and South Africa. Nitrogen stress increased RCA formation at 35 days after planting in mesocosms by 200% and at flowering in the fields by 90-100%. High RCA was associated with substantially reduced root
segment respiration in mesocosms and in the field. In the field, high RCA genotypes had 30% greater rooting depth ($D_{95}$) than low RCA genotypes at flowering. High RCA genotypes had greater shoot mass than low RCA genotypes under low N conditions by 66% in mesocosms, by 52% at the field in South Africa and by 31% in Pennsylvania. RCA formation correlated with 70% increase in grain yield under low N conditions in the field in Pennsylvania. Our results are consistent with the hypothesis that RCA improves plant growth under N limiting conditions by decreasing root metabolic costs, enhancing soil exploration in deep soil strata, and thereby increasing N acquisition at greater depths. Although potential fitness tradeoffs of RCA formation are poorly understood, increased RCA formation appears be a promising breeding target for enhancing nitrogen acquisition in low N soils.
Introduction

Nitrogen (N) deficiency is one of the most limiting factors in maize production worldwide (Ladha et al., 2005). In developing countries such as those in sub-Saharan Africa, less than 20 Kg N ha\(^{-1}\) is applied to cornfields of smallholder farmers due to inaccessibility to N fertilizers or expensive costs of the inputs (Azeez et al., 2006; Worku et al., 2007). In developed countries intensive N fertilization is used to maintain satisfactory yield (Tilman et al., 2002). In the USA, N fertilizers are the greatest economic and energy costs for maize production (Ribaudo et al., 2011). However less than half of nitrogen applied to crops is taken up, while the remaining N becomes a source of environmental pollution (Raun and Johnson, 1999; Smil, 1999; Tilman et al., 2002). For example, nitrogen and phosphorus effluents into marine systems from agriculture cause eutrophication and hypoxic zones (Diaz and Rosenberg, 2008; Robertson and Vitousek, 2009). Nitrate contamination in surface water and groundwater systems poses serious health risks such as methemoglobinemia and N-nitroso-induced cancers (UNEP, 2007). Emission of nitrous oxides (N\(_2\)O) from agricultural activities contributes to ozone damage and global warming (Kulkarni et al., 2008; Sutton et al. 2011). Furthermore, the production of nitrogen fertilizers requires considerable energy from fossil fuels, and since energy costs have risen in recent years, farmers face economic pressure from increasing nitrogen fertilizer costs. It is estimated that a 1% increase in crop nitrogen efficiency could save more than 1 billion US dollars annually worldwide (Kant et al., 2011). Therefore, even a small improvement in nitrogen efficiency would have significant positive impacts on the environment and the economy.

Soil nitrogen is heterogeneous and dynamic. The availability of soil N to plants depends on the balance between the rates of mineralization, nitrification, and denitrification. These processes are determined by several factors including soil composition, microbial activity, soil
temperature, and soil water status (Miller and Cramer, 2004). The predominant form of soil nitrogen available to plants in most agricultural systems is nitrate, which is highly soluble in water and thus mobile in the soil (Barber, 1995; Marschner, 1995). Mineralization of organic matter and/or the application of nitrogen fertilizer at the beginning of the growing season followed by precipitation and irrigation cause a pulse of nitrate which may exceed the need of seedlings and leach away from the root zone (Lynch, 2013). Therefore, increasing root production to explore deep soil strata could benefit nitrogen acquisition. However, the structural investments and metabolic expenditures of root systems are substantial and can exceed half of daily photosynthesis (Lambers et al., 2002). Full consideration of the costs and benefits of root systems is therefore crucial for identifying root traits to improve crop production especially in water and nutrient deficient environments (Lynch, 2007). Taking rhizoeconomics of root systems and spatiotemporal availability of nitrogen in the soil into account, (Lynch, 2013) proposed a root ideotype for enhanced N acquisition in maize called “steep, cheap, and deep”, in which ‘steep’ refers to architectural traits and ‘cheap’ refers to traits that reduce the metabolic cost of soil exploration. One element of this ideotype is abundant root cortical aerenchyma.

Root Cortical Aerenchyma (RCA) consists of enlarged air spaces in the root cortex (Esau, 1977). RCA is known to form in response to hypoxia and the role of RCA in improving oxygen transport to roots of many plant species under hypoxic conditions has been well researched (Vartapetian and Jackson, 1997; Jackson and Armstrong, 1999; Mano and Omori, 2007; Mano and Omori, 2013). Interestingly, RCA can also form in response to drought and edaphic stresses such as nitrogen, phosphorus (P), and sulfur deficiencies (Bouranis et al., 2003; Drew et al., 1989; Fan et al., 2003; Zhu et al., 2010), which suggests that the benefit of RCA extends beyond facilitating oxygen transport. Several lines of evidence suggest that RCA enhances root metabolic efficiency under stress. Fan et al (2003) found that RCA formation significantly reduced root segment respiration and phosphorus (P) requirements of root tissue, which allowed greater shoot
growth in soils with low phosphorus availability. Under drought, maize (*Zea mays* L.) genotypes with high RCA formation had greater root length, deeper rooting, better leaf water status, and 8 times greater yield than closely related genotypes with low RCA (Zhu et al., 2010a). Effects of RCA on root respiration were more pronounced for large-diameter roots compared to small-diameter roots (Jaramillo et al., 2013). Results from the functional-structural plant model *SimRoot* showed that RCA formation could be an adaptive response to deficiency of N, P, and K by decreasing the metabolic cost of soil exploration. By reducing root respiration, RCA decreases the carbon cost of soil exploration, and by decreasing the N and P content of root tissue, RCA permits internal reallocation of nutrients to growing root tissue, which is particularly beneficial under conditions of low N and P availability (Postma and Lynch, 2011b). Under suboptimal P availability, RCA increased growth of a simulated 40 day-old maize by 70% (Postma and Lynch, 2011a). In the case of nitrogen, RCA increased the growth of simulated maize plants up to 55% in low N conditions, and plants benefit from RCA more in high N leaching environments than low N leaching environments (Postma and Lynch, 2011b). In addition, the formation of RCA decreases critical soil nutrient levels, defined as the soil fertility below which growth is reduced, suggesting that cultivars with high RCA may require less fertilizer under non-stressed conditions. These *in silico* results suggest that RCA has potential utility for improving crop nutrient acquisition in both high- and low-input agroecosystems.

The overall objective of this research was to assess the utility of RCA for nitrogen acquisition in maize under nitrogen-limiting conditions. We employed maize recombinant inbred lines (RILs) with a common genetic background but contrasting in RCA formation under nitrogen stress to test if RCA formation in maize lines grown on low N soils is associated with reduced root respiration, greater rooting depth and enhanced N acquisition.
Materials and Methods

Greenhouse mesocosm study

Plant materials

Six maize RILs from the Intermated B73 and Mo17 (IBM) population were compared. Previous screening indicated that RILs 337, 133, 177 had low RCA, and RILs 196, 199, 345, had high RCA under low N conditions.

Experimental design

The experiment was a randomized complete block design. The factors were two nitrogen regimes (high and low nitrogen conditions), six RILs, and four replicates over 4 blocks. Planting was staggered one day between replicates with time of planting as a block effect.

Growth conditions

Plants were grown in a greenhouse located on the campus of The Pennsylvania State University in University Park, PA, USA (40°48′N, 77°51′W), with a photoperiod of 14/10 h at 28/24 °C. Seeds were soaked for 1 h in a fungicide solution consisting of benomyl (Benlate fungicide, E.I. DuPont and Company, Wilmington, DE, USA) and 1.3 M metalaxyl (Allegiance fungicide, Bayer CropScience, Monheim am Rhein, Germany) and then were surface-sterilized in 10% NaOCl for 1 min. The seeds were pre-germinated in rolled germination paper (Anchor Paper Company, St. Paul, MN, USA) soaked with 0.5 mM CaSO₄ and placed in darkness at 28°C in a germination chamber for two days. At planting, the plants were transferred to mesocosms
consisting of PVC cylinders 15.7 cm in diameter and 160 cm in height. The mesocosms were lined with transparent high-density polyethylene film to facilitate root sampling at harvest. The growth medium consisted of a mixture (volume based) of 50% medium size (0.5 – 0.3 mm) commercial grade sand (Quikrete Companies Inc., Harrisburg, PA, USA), 35% horticultural vermiculite, 5% Perlite (Whittemore Companies Inc., Lawrence, MA, USA) and 10% topsoil. The topsoil was collected from the Russell E. Larson Agricultural Research Center in Rock Springs, PA (Fine, mixed, semiactive, mesic Typic Hapludalf, pH ≈ 6.7, silt loam). Thirty-three liters of the mixture was used in each mesocosm to ensure the same bulk density of the media. One day before planting the mesocosms were saturated with 5 liters of a nutrient solution adjusted to pH 6. The nutrient solution for the high N treatment consisted of (in µM): NO₃ (7000), NH₄ (1000), P (1000), K (3000), Ca (2000), SO₄ (500), Mg (500), Cl (25), B (12.5), Mn (1), Zn (1), Cu (0.25), Mo (0.25) and FeDTPA (100). For the low N treatment, NO₃ and NH₄ were reduced to 70 and 10 µM, respectively. Two germinated seeds were sown per mesocosm and were thinned after 4 days to one plant per mesocosm. Plants were watered every other day with 100 ml of deionized water. Environmental data were collected hourly in the greenhouse using a HOBO U10-003 data logger (Onset Corporation, Pocasset, MA, USA). Soil solutions were collected at 20 cm depth intervals weekly using a micro-sampler 2.5 mm in diameter and 9 cm in length (Soilmoisture Equipment CORP., Santa Barbara, CA, USA). The solutions were stored at -80 °C until processing. The concentrations of nitrate in the solutions were determined using vanadium (III) chloride protocol according to (Doane and Horwáth, 2003).

**Root sampling, root segment respiration and root distribution in mesocosms**

Shoots and roots were harvested at 35 d after planting. At harvest, the polyethylene liners were removed from the mesocosms and laid on a root washing station. Root segments were
collected 20-24 cm from the base of the primary, seminal, and second whorl crown roots. The samples were stored in 75% EtOH at 4°C until processing and analysis. For root distribution studies, the liners were divided into 20 cm segments starting from the base of the shoot. Roots were cut and separated from each segment by carefully washing with tap water. The roots were preserved in 75% EtOH. Total root lengths were obtained by scanning and analyzing using WinRHIZO Pro (Régent Instruments, Québec City, Québec, Canada). For respiration measurements, three 6 cm root segments of second whorl crown roots were excised 20 cm away from the base of the root and lateral roots were removed with a Teflon-coated blade. Twenty minutes after excision, the samples were placed in a chamber connected to a Li-6200 IRGA (LI-COR, Lincoln, NE, USA). The temperature of the chamber was maintained at 27°C using a water bath. Carbon dioxide evolution from the root segments was recorded every 5 seconds for 180 seconds. After the respiration measurements, the root segments were stored in 75% EtOH for anatomical analysis.

RCA measurement

Root cross-sections were obtained by free hand-sectioning using Teflon-coated double-edged stainless steel blades (Electron Microscopy Sciences, Hatfield, PA, USA). The root sections were examined on a Diaphot inverted light microscope (Nikon, Chiyoda-ku, Japan) at 2.8x magnification. Three sections were selected as subsamples for image capture. The microscope was fitted with a black and white XC-77 CCD Video Camera Module (Hamamatsu, Iwata-City, Japan). ImageMaster 5.0 software (Photon Technology International, Birmingham, NJ, USA) was used to capture and save images. Analysis of images was performed in MatLab 7.6 2008a (The MathWorks Company, Natick, MA), using RootScan which is a program for semi-
automated image analysis of anatomical traits in root-cross sections (Burton et al., 2012). RCA was expressed as percentage of the root cortical area.

**Shoot dry weight and plant nitrogen status**

One day prior to harvest, leaf gas exchange of the second youngest fully expanded leaves was measured with a LI-6400 Infrared Gas Analyzer (LI-COR, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1200 µmol photons m⁻² s⁻¹ and constant CO₂ concentration of 400 ppm. At harvest, 6-mm diameter leaf discs were collected from the second youngest fully expanded leaves for chlorophyll measurement. Chlorophyll was extracted in 80% acetone. The concentrations of chlorophyll a and b in the extracts were determined at the wavelength of 663.2 and 646.8 nm with a spectrophotometer (Lichtenthaler and Buschmann, 2001). Shoots were dried at 60 °C for 72h prior to dry weight determination. The shoots were ground and 2-3 mg ground tissues were used for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer, Shelton, CT, USA).

**Field studies**

**Field conditions, experimental design, and plant materials**

Experiments were carried out during February to April in 2010 at Alma, Limpopo province, South Africa (SA) (24°33' 00.12 S, 28° 07'25.84 E, 1235 masl) and during June - August in 2011 at the Russell Larson Research and Education Center of the Pennsylvania State University in Rock Springs, PA, USA (PA) (40°42'37".52 N, 77°57'07".54 W, 366 masl). The soils at the experimental sites were a Clovelly loamy sand (Typic Ustipsamment) in Alma and
Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalf) in Rock Springs. Based on soil analysis at the beginning of growing season, N fertilizers were applied at the rate of 30 kg N/ha 5 times until flowering resulting in 150 kg N/ha in total for high N plots at Alma. Low N plots received 30 kg N/ha only at the beginning of growing season. At Rock Springs, fields were amended with 915 g/m² of sawdust to immobilize soil N. High N plots were fertilized with 150 Kg N/ha of urea while low N plots did not receive any N fertilizer. In both environments, soil nutrient levels of other macro and micronutrients were adjusted to meet the requirements for maize production as determined by soil tests. Pest control and irrigation were carried out as needed. Based on previous experiments conducted in the field (Saengwilai et al., unpublished), six IBM RILs consisting of low RCA RILs (1, 157, and 177) and high RCA RILs (31, 34, and 338) were planted at Alma and ten IBM RILs consisting of low RCA RILs (1, 85, 97, 157, and 165) and high RCA RILs (56, 82, 224, 284, and 353) were planted at Rock Springs. The experiments were arranged in a split-plot design with the two nitrogen treatments as the whole plot factor, and genotype as the split-plot. Five-row plots of each genotype (six meters long) were randomly assigned within each whole plot. Row width was 75 cm, and distance within a row was 23 cm, resulting in a planting density of 5.80 plants m⁻². The plants were harvested at 9 weeks after planting (flowering stage) at the SA and the PA field.

**Root sampling, root segment respiration and root distribution in the field**

At harvest, three 4 cm root segments of second whorl crown roots were excised from 8-12 cm away from the base of the root and lateral roots were removed with a Teflon-coated blade. The three root segments were placed in a tube chamber connected to a LI-6400 IRGA (LI-COR, Lincoln, NE, USA) The temperature of the chamber was maintained at 27°C using a water bath. Carbon dioxide evolution from the root segments was recorded every 5 seconds for 180 seconds.
After the respiration measurements, the root segments were stored in 75% EtOH for anatomical analysis.

For root distribution, soil cores were taken within a planting row midway between two plants by soil coring equipment (Giddings Machine Co., Windsor, CO, USA). The cores were divided into 10 cm segments and roots were extracted from each soil segment.

Root length was obtained as previously mentioned for mesocosm samples. Percentages of root length at each depth were calculated in each soil core. Depth above which 95% of root length is located ($D_{95}$) was calculated by linear interpolation between the cumulative root lengths (Trachsel et al., 2013).

**Shoot dry weight, chlorophyll measurements, and tissue nitrogen content**

One day prior to harvest, leaf gas exchange of the ear leaves was measured with a Licor-6400 Infrared Gas Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1800 µmol photons m$^{-2}$ s$^{-1}$ and constant CO$_2$ concentration of 360 ppm. At Rock Springs, 6-mm diameter leaf discs were collected from the ear leaves for chlorophyll measurement. Chlorophyll was extracted in 80% acetone. The concentrations of chlorophyll a and b in the extracts were determined at the wavelength of 663.2 and 646.8 nm with a spectrophotometer (Lichtenthaler and Buschmann, 2001). Shoots were dried at 60° C for 72h prior to dry weight determination. The leaves and stems were ground and 2-3 mg ground tissues were taken for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer, Shelton, CT, USA).
**Statistical analysis**

Statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012). Linear mixed effect models were fit using the function lme from the package nlme (Pinheiro et al., 2012) and a two-way ANOVA were used for comparisons between high and low RCA groups (or individual RILs), nitrogen levels and the interaction between these main effects. A protected least significant difference post hoc (a=0.05) test and Tukey’s Honest Significant Difference method (a=0.05) were used for multiple comparisons. Correlations and linear regressions were carried out between shoot and root traits with RCA and root respiration and between RCA and yield.

**Results**

**RCA formation and nitrogen stress**

Nitrogen (N) stress significantly increased RCA of plants grown in mesocosms (GH) by an average of 200% at 35 d after planting (DAP). The increase in RCA was significant in all root classes: seminal roots (218%, p<0.001), crown roots (74%, p=0.454), and primary roots (62%, p=0.015) (Figure 2.1). N stress did not affect root diameter, cortical cell file number, and diameter of xylem in all root classes (Table 2.1). The differences between RCA phenotypes were accentuated by low N treatment. Low RCA RILs (133, 177, and 337) averaged 5% RCA, while high RCA RILs (196, 199, and 345) averaged 18% RCA under low N conditions (Figure 2.2).

At the field site in South Africa (SA) N stress increased RCA of the plants by an average of 102% at flowering. Low RCA RILs (1, 157, and 177) averaged 9% RCA, while high RCA RILs (31, 34, and 338) averaged 19% RCA under N stress (Figure 2.2). At the field site in Pennsylvania (PA) N stress increased RCA of the plants by an average of 94% at flowering. Low
RCA RILs (1, 85, 97, 157, and 165) averaged 5% RCA, while high RCA RILs (56, 82, 224, 284, and 353) averaged 16% RCA under N stress. RCA of High RCA RILs was significantly greater than that of low RCA RILs under low N conditions in all environments (p<0.05, Figure 2.2).

**RCA and root respiration**

RCA reduced respiration of root segments both in the mesocosms and in the field (Figure 2.3). In the mesocosms increased RCA correlated with 4-fold reduction in root segment respiration (Figure 2.3). In the field, nitrogen stress reduced root respiration per unit of root length by 54% (Figure 2.4). Under N stress, high RCA RILs had 42% less specific root respiration per unit of root length than the low RCA RILs (Figure 2.4).

**RCA and root growth**

In mesocosms N stress reduced the average total root length of the plants by 42%. High RCA RILs had 35% greater total root length than the low RCA RILs under low N conditions (p<0.05) (Figure 2.5). In addition, N stress increased rooting depth (D₉₅) by 29%. D₉₅ of high RCA RILs was 15% greater than that of low RCA RILs under low N conditions (Figure 2.6). In the field site in South Africa (SA), D₉₅ of high RCA RILs was significantly greater than that of low RCA RILs (p<0.05) at flowering. Under low N conditions, D₉₅ was 35 cm for high RCA RILs and 27 cm for low RCA RILs (Figure 2.6).
Photosynthesis, nitrogen acquisition, and shoot mass

Under low N conditions in mesocosms the chlorophyll content of high RCA RILs was 22% greater than that of low RCA RILs (Figure 2.7A). Nitrogen stress reduced leaf photosynthetic rates on average by 8%. The high RCA RILs had 22% greater photosynthetic rates than the low RCA RILs under low N conditions (Figure 2.7B). Nitrogen stress reduced the shoot biomass of the plants by 58%. Under N stress, high RCA RILs had 66% more shoot mass and 68% greater tissue N content at 35 DAP compared with low RCA RILs (Figure 2.8). In the field in SA N stress reduced shoot mass of the plants by an average of 35% at flowering. The high RCA RILs had 52% greater shoot mass and 81% greater tissue N content than low RCA RILs at flowering under low N conditions (Figure 2.8). In the field in PA stress reduced shoot mass of the plants by an average of 36% at flowering. The high RCA RILs had 31% greater shoot mass and 28% greater tissue N content than low RCA RILs under low N conditions (Figure 2.8). RCA also correlated with 70% increase in grain yield under low N conditions (Figure 2.9).

Discussion

Our results are consistent with the hypothesis that RCA enhances N acquisition by reducing root metabolic costs, permitting greater rooting depth and enhanced N acquisition under suboptimal nitrogen conditions. We demonstrated that N stress induced RCA expression in both greenhouse (GH) and field (SA and PA) conditions. This effect was stronger in maize lines with high RCA formation under high N (Figure 2.2). High RCA RILs also had substantially reduced root segment respiration (Figure 2.4). Under suboptimal N availability, high RCA RILs had greater rooting depth than low RCA RILs by 15% in GH and 31% in SA field (Figure 2.6). Additionally high RCA RILs had greater shoot biomass than low RCA RILs under low N
conditions in all environments observed (Figure 2.8). At the field site in PA, RCA associated with 70% increased in grain yield under low N conditions (Figure 2.9).

We found that variation in RCA formation exists in maize RILs under unstressed conditions and the amount of RCA could be induced by suboptimal availability of N. These results are consistent with other studies (He et al., 1992). Interestingly not all RILs increased RCA in response to N stress, particularly low RCA RILs (Figure 2.2). The segregation of degree of RCA formation in response to N stress suggests that breeders could select for genotypes with consistently high or low or plastic RCA. The utility of phenotypic plasticity of RCA is currently unknown but genetic control and the utility of plastic traits such as root hair length have been documented in maize (Zhu et al., 2010).

Root respiration associated with growth, maintenance, and ion uptake are major components of root metabolic costs (Lambers et al., 1996; Lynch & Ho, 2005). Simulation studies have shown that respiration by root maintenance alone could cost up to 37% of cumulative photosynthesis. Without root maintenance respiration, simulated plants had up to 72% enhanced growth under nutrient limiting conditions (Postma and Lynch, 2011a; Postma and Lynch, 2011b). In this study, we found that high RCA RILs had lower root segment respiration than low RCA RILs under both stressed and non-stressed conditions (Figure 2.4). The formation of RCA in maize replaces metabolically active cortical tissue with air space and thus reduces root maintenance costs. We also found that high RCA genotypes had greater total root length under N stress in the mesocosms (Figure 2.5). These results were consistent with responses found under suboptimal availability of phosphorus and water (Fan et al., 2003; Zhu et al., 2010a). The results support our hypothesis that reduced root maintenance respiration costs allows high RCA RILs to support a larger root system and have greater soil exploration than low RCA RILs. An additional benefit of RCA is reallocation of nutrients from cortical tissue, which is predicted by simulation modeling to be an important function in N and P deficient plants (Postma and Lynch, 2011b).
Benefits of RCA from reallocation of N can be indirectly measured by comparing the amount of N in root tissues of high and low RCA RILs. This study is currently in process.

In an earlier study Fan et al. (2003) showed that 20% RCA reduced root respiration by 50% in seminal root segments in maize. In our study, we found that around 30% RCA is needed to reduce root respiration of crown root segments in half (Figure 2.3). Crown and seminal root anatomy is fundamentally similar but differs in size and number of cells; crown roots tend to have greater diameter, number of cortical cell layers, and cortical area (Burton et al., 2013). It has been shown that root respiration is substantially influenced by living portions of the root segments such as living cells in the cortex (Jaramillo et al., 2013) and possibly living cells in the stele. Since crown roots have larger living portions than seminal roots, we would expect that more RCA would be required in order to significantly affect root respiration in crown roots.

Distribution of roots in soil influences nutrient and water acquisition efficiency. For example, shallow rooting is beneficial for acquisition of topsoil-available nutrients such as phosphorus and potassium (Lynch and Brown, 2001), while deeper rooting allows plants to acquire highly mobile resources such as water and nitrate before it is lost from the root zone (Ho et al., 2005; Kristensen & Kristensen, 2000; Postma & Lynch, 2011; Zhu et al. 2010). Under low N conditions high RCA RILs had greater rooting depth ($D_{95}$) in the mesocosms and in the field (SA) than the low RCA RILs (Figure 2.6). Since the high RCA RILs had lower metabolic costs for root maintenance compared to the low RCA RILs, the high RCA RILs may be able to support more root growth resulting in greater rooting depth, which could enhance nitrogen acquisition in low N soils. Enhanced nitrogen acquisition in deep soil profile resulted in increase in tissue N content, chlorophyll content, and photosynthesis, which benefits overall plant growth and yield (Figure 2.7,2.8,2.9).

In the field, we found that the utility of RCA was greater in loamy sand at SA than in silt loam at PA. Although the relative reduction in shoot mass was similar between sites, plants in SA
were 2.5 times smaller than plants at PA under low N conditions (Figure 2.8), which indicated that they suffered from greater stress. At flowering, shoot biomass of high RCA RILs in SA was 52% greater than that of low RCA RILs whereas shoot biomass of high RCA RILs in PA was only 31% higher than that of low RCA genotypes. In high leaching environments such as the loamy sand in SA, the benefit of increased rooting depth could be more pronounced since nitrate leaching is more rapid in coarser soils. These results are consistent with simulation results (Postma and Lynch, 2011b).

It is important to note that selection for high RCA, may indirectly select for greater ethylene sensitivity, which may affect other adaptive root traits. In this study, we carefully observed and compared other root traits such as angle, number of crown roots and root branching under high and low N conditions. We found no significant difference for other root traits between high and low RCA RILs (Table 2.1). Taken together, we conclude that the results observed in this study are primarily due to contrasting RCA phenotypes.

RCA occurs through programmed cell death, yet little is known regarding molecular regulation of RCA formation. Microarray analysis showed that at least 575 genes might be involved in RCA formation under water logging conditions (Rajhi et al., 2011). To date, the only gene involved in RCA formation that is characterized and cloned is *XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET1)*. Expression of *XET1* gene has been shown to be induced under water logging conditions (Saab and Sachs, 1996; Subbaiah and Sachs, 2003). Traditional linkage analysis has been used to identify QTLs for maize× *Zea nicaraguensis* mapping populations, (Mano et al., 2007; Mano and Omori, 2008) and in recombinant inbred populations (Burton, 2010). However, no other genes associated with aerenchyma formation in non-stress environments were reported in these studies. Recently, association study, utilizing ancestral recombination events in natural populations to make marker-trait associations, has become popular among plant scientists (Flint-Garcia et al., 2005). Together with gene expression studies,
association mapping can facilitate discovery for RCA-forming genes and improve our understanding of genetic control of RCA.

Knowledge of interactions among agronomic traits is essential in developing ideotypes for nutrient efficient crops. Interactions among root traits could result in synergistic or antagonistic effects on resource acquisition. For example, increased adventitious rooting in common bean reduced growth of lateral roots arising from the tap and basal roots, which resulted in reduced P acquisition in low P soils (Walk et al. 2006). Whereas under low P conditions, common bean gains more benefit from having long root hair length combined with shallow root angle than having each trait alone (Miguel, 2011). As for RCA, simulation modeling predicted synergism between RCA and lateral root branching density in maize under low P conditions (Postma and Lynch, 2011b). Under low N conditions, RCA benefits metabolically costly root traits such as the number of crown roots because more crown root number allows greater volume of soil exploration at expense of root growth and maintenance (York et al., 2013). Since RCA reduced root metabolic costs for root growth in general, we propose that RCA may also be synergetic with root traits that enhance soil exploration in different soil domains such as root angle.

Substantial genetic variation for RCA exists in maize and its relatives in genus Zea (Burton et al., 2013). This suggests that there may be costs for RCA. It has been shown that RCA contributed to reduced root hydraulic conductivity in maize roots under low P conditions (Fan et al., 2007). RCA formation also inhibits radial transportation of nutrients such as phosphate and calcium (Hu et al., 2013) In addition, RCA may affect root pathogenicity and colonization of microbes. For example, in wheat, cultivars with high root cortical cell death are more susceptible to common root rot (Deacon et al., 1982). Conversely RCA formation may reduce colonization of beneficial fungi such as mycorrhizal fungi since they rely on living cortical cells. RCA may also affect mechanical strength of roots especially in plant species that lack a structural support in
the outer part of cortex (Striker et al., 2007). The cost/benefit of RCA and its interactions with other root traits are likely to be complex and may differ in different environments. This merits research.

There has been increasing evidence suggesting that increased RCA enhances water and nutrient acquisition in drought and edaphic stress (Fan et al., 2003; Zhu et al., 2010a; Postma and Lynch, 2011a; Postma and Lynch, 2011b). To our knowledge this paper is the first to empirically demonstrate the benefit of RCA for N acquisition in low N soils. Genetic variation of RCA was found in several important agronomic species such as wheat, barley, sorghum, rice, common bean, and maize (Colmer 2003; Fan et al. 2003; Haque, et al. 2012; Liljeroth 1995; Promkhambut et al. 2011; Zhu et al. 2010) making RCA amenable to plant breeding. We suggest that increased RCA formation may be a promising breeding target for enhancing nitrogen acquisition from low N soils.
Figure 2.1: Production of root cortical aerenchyma as percent of cortical area in three root classes of maize 35 days after planting (DAP) under high N and low N conditions in soil mesocosms. Data shown are means of 6 genotypes with 4 replicates ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 2.2: Production of root cortical aerenchyma between high RCA and low RCA maize RILs under high N and low N conditions at 35 DAP in soil mesocosms (GH) and at 63 DAP in the field at South Africa (SA) and Pennsylvania (PA). The data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05) compared within each location.
Figure 2.3: Correlation of root segment respiration and percentage of root cortical aerenchyma. Root segment respiration is negatively correlated with RCA in soil mesocosms ($r = -0.67$, $p < 0.001$) and in the field ($r = -0.8469$, $p < 0.001$).
Figure 2.4: Specific root respiration per length of high and low RCA genotypes at 35 day after planting (DAP) in both high and low N conditions in the mesocosms. Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).
Figure 2.5: Total root length of high and low RCA RILs at 35 DAP under high and low N conditions in mesocosms. Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).
Figure 2.6: Rooting depth ($D_{95}$) of maize lines at 35 DAP in mesocosms and 63 DAP in the field in South Africa under low N conditions. Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05) within the experiment.
Figure 2.7: Chlorophyll concentration (7A) and photosynthesis rate (7B) of high and low RCA RILs at 35 DAP in both high and low N conditions in mesocosms. Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).
Figure 2.8: Relative shoot biomass under high N and low N conditions at 35 DAP in soil mesocosms (GH) and at flowering (63 DAP) in the field at South Africa (SA) and Pennsylvania (PA). The data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05) compared within each location. Base line for shoot mass of GH=1.77g, SA=75.28g, PA=159.08g)
Figure 2.9: Correlation of yield and percentage of root cortical aerenchyma (% of cortex) under high (not significant) and low N (r=0.40, p=0.05) conditions in the field in Pennsylvania.
Table 2.1: Root anatomical traits of different root class at 35 days after planting in the mesocosms. Data shown are means of 4 replicates of six RILs grown under high and low N conditions. “ns” indicates that the values are not significant at p=0.05.

<table>
<thead>
<tr>
<th>Root class</th>
<th>Treatment</th>
<th>RCA (%)</th>
<th>Root diameter (mm)</th>
<th>Cortical cell file number</th>
<th>Meta xylem diameter (mm)</th>
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</thead>
<tbody>
<tr>
<td>Primary</td>
<td>High N</td>
<td>8.42</td>
<td>0.77</td>
<td>6.42</td>
<td>0.070</td>
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<td></td>
<td>Low N</td>
<td>13.67</td>
<td>0.72</td>
<td>6.32</td>
<td>0.070</td>
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<tr>
<td></td>
<td>p value</td>
<td>0.02</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Seminal</td>
<td>High N</td>
<td>3.49</td>
<td>0.63</td>
<td>6.40</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>Low N</td>
<td>11.13</td>
<td>0.63</td>
<td>6.12</td>
<td>0.067</td>
</tr>
<tr>
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<td>p value</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Crown</td>
<td>High N</td>
<td>6.91</td>
<td>0.77</td>
<td>7.20</td>
<td>0.078</td>
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<tr>
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<td>Low N</td>
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<td>7.00</td>
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</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.02</td>
<td>ns</td>
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Chapter 3

Low crown root number enhances nitrogen acquisition from low nitrogen soils in maize (Zea mays L.)

Patompong Saengwilai\textsuperscript{1}, Xiaoli Tian\textsuperscript{2,3}, and Jonathan Paul Lynch\textsuperscript{1,2}

\textsuperscript{1}Intercollege Program in Plant Biology, The Pennsylvania State University, University Park, PA 16802, USA

\textsuperscript{2}Department of Plant Science, The Pennsylvania State University, University Park, PA 16802, USA

\textsuperscript{3}State Key Laboratory of Plant Physiology and Biochemistry, and Department of Agronomy, China Agricultural University, Beijing 100193, China

For correspondence: E-mail jpl4@psu.edu

Abstract

In developing nations, low soil nitrogen (N) availability is a primary limitation to crop production and food security, while in rich nations, intensive N fertilization is a primary economic, energy, and environmental cost to crop production. It has been proposed that genetic variation for root architectural and anatomical traits enhancing exploitation of deep soil strata could be deployed to develop crops with greater N acquisition. Here we provide evidence that maize (Zea mays L.) with few crown roots (crown root number: CN) have greater N acquisition from low N soils. Nitrogen stress significantly decreased CN in greenhouse mesocosms and in field studies in the United States and South Africa. Maize lines with low CN had 45% greater rooting depth in low N soils than high CN genotypes. Deep injection of $^{15}$N-labeled nitrate showed that low CN genotypes acquired more N from deep soil strata than high CN genotypes, resulting in greater photosynthesis and total nitrogen content. Under low N, low CN genotypes
had greater biomass than high CN genotypes at flowering (85% in the field study in the US and 25% in South Africa). In the US, low CN genotypes had 50% greater yield under N stress compared to high CN genotypes. To our knowledge, this is the first report of the utility of CN for nutrient acquisition. Our results indicate that CN deserves consideration as a potential trait for genetic improvement of nitrogen acquisition from low N soils.
**Introduction**

Maize (*Zea mays* L.) is one of the world’s most important crops and is a staple food in Latin America and Africa. Maize production requires a large amount of fertilizer, especially nitrogen. In the USA, N fertilizers represent the greatest economic and energy costs for maize production (Ribaudo et al., 2011). However, on-farm studies across the North-central USA revealed that more than half of applied N is not taken up by maize plants and is vulnerable to losses from volatilization, denitrification, and leaching, which pollute air and water resources (Cassman, 2002). Conversely, in developing countries suboptimal nitrogen availability is a primary limitation to crop yields and therefore food security (Azeez et al., 2006). Increasing yield in these areas is an urgent concern since chemical fertilizers are not affordable (Worku et al., 2007). Cultivars with greater nitrogen acquisition from low N soils could help alleviate food insecurity in poor nations as well as reduce environmental degradation from excessive fertilizer use in developed countries.

The two major soil N forms available to plants are ammonium and nitrate. Nitrate is the main N form in most maize production environments (Miller and Cramer, 2004). Nitrate is highly mobile in soil and the spatiotemporal availability of soil N is rather complex. In the simplest case nitrogen fertilizers applied to the soil and/or nitrogen released from mineralization of soil organic matter are rapidly converted to nitrate by soil microbes. After irrigation and precipitation events, nitrate moves with water to deeper soil strata. Leaching of nitrate from the root zone has been shown to be a significant cause of low recovery of N fertilizer in commercial agricultural systems (Cassman et al., 2002; Raun & Johnson, 1999). Differences in root depth influence the ability of plants to acquire N. Studies using $^{15}$Nitrogen ($^{15}$N) labeled nitrate placed at different soil depths showed that only plants with deep rooting can acquire N sources from deep soil strata, which would otherwise have been lost through leaching (Kristensen & Thorup-Kristensen, 2000;
Kristensen & Thorup-Kristensen, 2004). Therefore selection for root traits enhancing rapid deep soil exploration could be used as a strategy to improve crop N efficiency.

The maize root system consists of embryonic and post-embryonic components. The embryonic root system consists of two distinct root classes: a primary root and a variable number of seminal roots formed at the scutellar node. The post-embryonic root system consists of roots that are formed at consecutive shoot nodes and lateral roots, which are initiated in the pericycle of all root classes. Shoot-borne or nodal roots that are formed below ground are called “crown roots” whereas those that are formed above ground are designated “brace roots” (Hochholdinger, 2009). While the primary root and seminal roots are essential for the establishment of seedlings after germination, nodal roots and particularly crown roots make up most of the maize root system and are primarily responsible for soil resource acquisition later in development (Hoppe et al., 1986).

Lynch (2013) proposed an ideotype for superior N and water acquisition in maize called “Steep, Cheap and Deep (SCD)”, which integrates root architectural, anatomical, and physiological traits to increase rooting depth and therefore capture of N in leaching environments. One such trait is crown root number (CN). CN is an integrated trait consisting of the number of belowground nodal whorls and the number of roots per whorl. The crown root system dominates resource acquisition during vegetative growth after the first few weeks and remains important during reproductive development (Hochholdinger et al., 2004). CN in maize ranges from 5 to 50 under fertile conditions (Trachsel et al., 2011). At the low end of this range, crown roots may be too spatially dispersed to sufficiently explore the soil. There is also a high risk for root loss due to herbivores and pathogens. If roots are lost in low N plants, there may be too few crown roots left to support the nutrient, water, and anchorage needs of the plants. At the high end, a large number of crown roots may compete with each other for water and nutrients as well as incur considerable metabolic costs for the plant (Figure 3.1). The SCD ideotype proposes that there is an optimal number of crown roots (CN) for N capture in maize (Lynch, 2013). Under low N conditions,
resources for root growth and maintenance are limiting, and nitrate is a mobile resource that can be captured by a dispersed root system. Optimal CN should tend toward the low end of the phenotypic variation to make resources available for development of longer, deeper roots rather than more crown roots. We hypothesized that in low N soils, maize genotypes with fewer crown roots (low CN) could explore soils at greater depth resulting in greater nitrogen acquisition, growth, and yield than genotypes with many crown roots (high CN).

The objective of this study was to test the hypotheses that (i) low CN genotypes have greater rooting depth than high CN genotypes in low N soils. (ii) Low CN genotypes are better at acquiring deep soil N than high CN genotypes. (iii) Low CN genotypes have greater biomass and yield than high CN genotypes in low N conditions. Recombinant Inbred Lines (RILs) contrasting in CN were planted in greenhouse mesocosms and in fields in the USA and South Africa under high and low nitrogen conditions. ¹⁵Nitrogen was employed to investigate nitrogen acquisition from deep soil strata.

Materials and Methods

Greenhouse mesocosm study

Plant materials

Based on the results of screening experiments in mesocosms in the USA and in the field in South Africa, recombinant Inbred Lines (RILs) IBM123 and IBM133 from the intermated B73 and Mo17 (IBM) population (Lee et al., 2002; Sharopova et al., 2002) and OHW3, OHW61, OHW74, and OHW170 from the cross between OH49 and W99 (OHW) contrasting in crown root number were selected for this study.
Experimental design

The greenhouse experiment was a randomized complete block design. The factors were two nitrogen regimes (high and low nitrogen conditions), six RILs, and four replicates. Planting was staggered one week between replicates with time of planting as a block effect.

Growth conditions

Plants were grown during October 13 to December 8, 2010 in a greenhouse located on the campus of The Pennsylvania State University in University Park, PA, USA (40°48′N, 77°51′W), with a photoperiod of 14/10 h at 28/24 °C (light/darkness). Seeds were soaked for 1 h in a fungicide solution containing benomyl (Benlate fungicide, E.I. DuPont and Company, Wilmington, DE, USA) and 1.3 M metalaxyl (Allegiance fungicide, Bayer CropScience, Monheim am Rhein, Germany) and then were surface-sterilized in 10% NaOCl for 1 min. The seeds were pre-germinated in rolled germination paper (Anchor Paper Company, St. Paul, MN, USA) soaked with 0.5 mM CaSO$_4$ and placed in darkness at 28°C in a germination chamber for two days. At planting, the plants were transferred to mesocosms consisting of PVC cylinders 15.7 cm in diameter and 160 cm in height. The mesocosms were lined with transparent high-density polyethylene film to facilitate root sampling at harvest. The growth medium consisted of a mixture (volume based) of 50% medium size (0.3 to 0.5 mm) commercial grade sand (Quikrete Companies Inc., Harrisburg, PA, USA), 35% horticultural vermiculite, 5% Perlite (Whittemore Companies Inc., Lawrence, MA, USA) and 10% topsoil. The topsoil was collected from the Russell E. Larson Agricultural Research Center in Rock Springs, PA (Fine, mixed, semiaactive, mesic Typic Hapludalf, pH ≈ 6.7, silt loam). Thirty-three liters of the mixture were used in each mesocosm to ensure the same bulk density of the medium. One day before planting, the
mesocosms were saturated with 5 liters of a nutrient solution adjusted to pH 6. The nutrient solution for the high N treatment consisted of (in µM): NO$_3$ (7000), NH$_4$ (1000), P (1000), K (3000), Ca (2000), SO$_4$ (500), Mg (500), Cl (25), B (12.5), Mn (1), Zn (1), Cu (0.25), Mo (0.25) and FeDTPA (100). For the low N treatment, NO$_3$ and NH$_4$ were reduced to 70 and 10 µM, respectively, and K$_2$SO$_4$ was used to replace K and SO$_4$. Each mesocosm received two seeds and after 4 days they were thinned to one plant per mesocosm. Plants were watered with 75 ml of deionized water every 2 days. Soil solutions were collected at 20 cm depth intervals weekly using a micro-sampler 2.5 mm in diameter and 9 cm in length (Soilmoisture Equipment CORP., Santa Barbara, CA, USA). The solutions were stored at -80 °C until processing. The concentrations of nitrate in the solutions were determined using the vanadium (III) chloride protocol according to Doane et al. (2003).

**Root harvest**

The plants were harvested at 28 days after planting. At harvest a polyethylene liner in each mesocosm was carefully removed and placed on a root washing station. The liners were divided into 20 cm segments starting from the base of the shoot. Media were carefully removed and the deepest layer reached by the roots was recorded for primary, seminal, and crown root classes. CN in each nodal whorl and root branching were counted. The roots were cut, separated from each segment, and preserved in 75% EtOH. Total root lengths were obtained by scanning and analyzing using the WinRhizo software (WinRhizo Pro, Régent Instruments, Québec City, Québec, Canada).
Shoot dry weight and plant nitrogen status

One day prior to harvest, leaf gas exchange of the first and the second youngest fully expanded leaves was measured with a Licor-6400 Infrared Gas Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1200 µmol photons m\(^{-2}\) s\(^{-1}\) and constant CO\(_2\) concentration of 400 ppm. Shoot carbon assimilation was measured with a Licor-6200 Infrared Gas Analyzer (Li-Cor Environmental Inc, Lincoln, NE, USA). In short, a 36.5 liter (28 x 28 x 46.5 cm) transparent acrylic chamber was placed around a plant. The base of the chamber was split to fit a stem of a plant. The air space around the stem and the base of the chamber was filled with modeling clay and sponges to separate the shoot from the growth media. The chamber connected to the Li-6200 with polyethylene tubing 0.03 liter in volume. Carbon dioxide exchange was measured for two minutes for each plant. Shoots were dried at 60 °C for 72h prior to dry weight determination. The shoots were ground and 2 to 3 mg of ground tissue was taken for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer).

Field studies

Field conditions, experimental design, and plant materials

Experiments were carried out during February to April in 2011 (SA2011) and 2012 (SA2012) at Alma, Limpopo province, South Africa (24°33’ 00.12 S, 28°07’25.84 E, 1235 masl) and during June - October in 2011 (US2011) at the Hancock Agricultural research station of the University of Wisconsin in Hancock, WI, USA (44°07’56”.74 N, 89°30’43”.96 W, 331 masl). The soils at the experimental sites were a Clovelly loamy sand (Typic Ustipsamment) in Alma and a Plainfield loamy sand (mixed, mesic Typic Udipsamment) in Hancock. In SA2011 and
SA2012 N fertilizers were applied at the rate of 30 kg N/ha for 5 times until flowering resulting in 150 kg N ha\(^{-1}\) in total for well-fertilized plots. The low N plots received 30 kg N ha\(^{-1}\) only at the beginning of the growing season. In US2011 the well-fertilized plots were amended with 103 kg N ha\(^{-1}\) at planting and at four weeks after planting resulting in a total of 206 kg N ha\(^{-1}\) while the low N plots were amended with 34 kg N ha\(^{-1}\) at the beginning of the cropping season only. In all environments, soil nutrient levels of other macro- and micronutrients were adjusted to meet the requirements for maize production as determined by soil tests. Pest control and irrigation were carried out as needed.

**Plant material**

The same six RILs used in the greenhouse experiment were used in SA2011. Different sets of genotypes were planted at US2011 and SA2012. These genotypes were selected based on previous screening in US field (Saengwilai et al., unpublished). Seven RILs consisting of IBM1, IBM9, IBM13, IBM77, IBM133, IBM165, and IBM187 were used in SA2012. RILs from the IBM populations; IBM10, IBM85, IBM218 and from the cross between NY821 and H99 (NyH) population; NYH76, NYH57, NYH212 were used in US2011. In each location the experiment was arranged in a split-plot design replicated four times with high and low N treatments. Four sections adjacent to each other in the field containing both high and low N treatments were assigned as blocks. Genotypes were randomly assigned to five-row plots. Each row was 4.5 m long. The distance between rows was 75 cm and within a row was 23 cm, resulting in a planting density of 6 plants m\(^{-2}\). The plants were harvested at flowering, 9 weeks after planting in SA2011 and SA2012 and 8 weeks after planting in US2011.
Root harvest

Evaluation of crown roots was carried out based on shovelomics (Trachsel et al., 2011). Three representative plants were selected for excavation in each plot. The selection was based on height, presence of bordering plants, and general appearance that represented individuals in the plot. At harvest roots were excavated using spades. A large portion of soil was removed from roots by carefully shaking. The remaining soil was removed by soaking the roots in diluted commercial detergent followed by vigorously rinsing at low pressure with water. Because three representative roots within a plot usually appear to be homogeneous, only one root was selected for phenotyping. Crown root number (CN) was measured by counting half of the root system. Assuming that the maize root system is symmetrical, CN was multiplied by two to obtain the total CN prior to data analysis. Data on other root traits such as root angle, diameter, and branching were also collected and included in the analyses when needed.

Rooting depth and $^{15}$N injection

Rooting depth was measured at flowering by soil coring (Giddings Machine Co., Windsor, CO, USA). Soil cores were taken within a planting row midway between two plants. The diameter of soil cores was 5.1 cm. The cores were divided into 10 cm segments and roots were extracted from each soil segment. Root lengths were obtained by scanning and analysis using WinRhizoPro (Régent Instruments, Québec, Québec City Canada). Percentages of root length at each depth were calculated in each soil core. Depth above which 95% ($D^{95}$) of root length is located was calculated by linear interpolation between the cumulative root lengths (Trachsel et al., 2013).
The ability of roots to acquire N in deep soil layers was studied by deep injection of $^{15}\text{NO}_3^-$ in SA2012. PVC pipes with a length of 75 cm and a diameter of 5 cm were used for $^{15}\text{NO}_3^-$ injection. Three representative plants were selected and the injections were done at a midway between adjacent plants within a planting row. Each plot received two injections. Prior to the injections, a soil auger was used to excavate a cylinder of soil to a depth of 50 cm. A PVC pipe was inserted into the hole and the $^{15}\text{NO}_3^-$ solution was poured into the hole. Each plot had 5 mL of K$^{15}\text{NO}_3^-$ solution (0.46 mg $^{15}\text{N}$ mL$^{-1}$, 98% $^{15}\text{N}$ enriched) injected into each of two holes. Following the injection each hole was filled with sand to prevent roots from growing down the hole. Seven days after $^{15}\text{NO}_3^-$ injection, the shoot biomass of the selected plant was harvested for $^{15}\text{N}$ and total N analysis.

**Shoot dry weight and tissue nitrogen content**

In SA2011 and SA2012 one day prior to harvest, leaf gas exchange of the ear leaves was measured with a Licor-6400 Infrared Gas Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1800 µmol photons m$^{-2}$ s$^{-1}$ and constant CO$_2$ concentration of 360 ppm. In all experiments, shoots were dried at 60 ºC prior to dry weight determination. The leaves and stems were ground and 2-3 mg of ground tissue were analyzed for tissue nitrogen content using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer).$^{15}\text{N}$ in plant tissue was analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility, University of California at Davis, USA (http://stableisotopefacility.ucdavis.edu/).
Statistical analysis

Statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012). Linear mixed effect models were fit using the function lme from the package nlme (Pinheiro et al., 2012) and two-way ANOVA were used for comparisons between high and low CN groups (or individual genotypes), nitrogen levels and the interaction between these main effects. ANCOVA was performed using the lm function to test effects of CN and N treatments on response variables. The protected least significant difference post hoc ($\alpha=0.05$) test and Tukey’s Honest Significant Difference method ($\alpha=0.05$) were used for multiple comparison tests.

Results

N stress effects on CN

In mesocosms, nitrogen stress reduced crown root number by 26% ($p<0.001$) at 28 days after planting (DAP). The CN ranged from 3 to 9 under low N conditions. The six genotypes responded differently to N stress. OHW3, OHW74, OHW 61, and IBM133 showed significant reduction in CN whereas OHW 170 and IBM 123 maintained their CN under low N conditions (Figure 3.2). Nitrogen stress reduced the average crown root whorl number from 2.75 to 2.13 ($p<0.05$; Figure 3.3A). Nitrogen stress did not affect the number of roots in the first whorl but significantly reduced the number of roots of the second, third, and forth whorl ($p<0.05$; Figure 3.3B).

At the field site in the USA (US2011), N stress reduced CN by 21% at flowering. The CN ranged from 24 to 44 under low N conditions. The reduction of CN was more pronounced in NYH57 and NYH76 than other genotypes (Figure 3.4A).
At the field site in South Africa in 2011 (SA2011), N stress reduced CN by 9% at flowering. The CN ranged from 21.5 to 35.5 under low N conditions. The six genotypes were grouped as high or low CN based on the mean difference in CN under low N conditions. The high CN genotypes consisted of IBM123, OHW3, and OHW170. The low CN genotypes consisted of IBM133, OHW61, and OHW74. Nitrogen stress significantly reduced CN in both high CN and low CN genotypes (p<0.05). On average high CN genotypes had 10 more crown roots than low CN genotypes under low N conditions (Figure 3.4B). A different set of genotypes was planted at the field site in South Africa in 2012 (SA2012). In 2012 the CN ranged from 30 to 46.5 under low N conditions. There was no significant effect of N stress on the average CN of these genotypes. However, the genotypes were significantly different in their CN under low N conditions (Figure 3.4D).

**CN effects on rooting depth and N acquisition**

In mesocosms the genotypes were grouped into high CN and low CN genotypes based on the average value of CN. The high CN genotypes consisted of OHW 170, OHW3, and IBM133. The low CN genotypes consisted of OHW61, OHW74, and IBM123. We found that primary roots, seminal roots, and crown roots of low CN genotypes had greater rooting depth (p<0.05) than those of high CN genotypes (Figure 3.5A).

In SA2011 N stress slightly increased maximum rooting depth (D95) from 30.5 to 37.2 cm but the effect was not significant. Low CN genotypes had significantly greater rooting depth than high CN genotypes (Figure 3.5B) under low N conditions. The low CN genotypes had a D95 value of 34.4 cm whereas for high CN genotypes the D95 value was 26.7 cm (p<0.05, Figure 3.5B). In SA2012 Low CN genotypes again had significantly greater rooting depth than high CN genotypes (Figure 3.6A). To investigate whether low CN genotypes were better at acquiring N from deep
soil strata, we injected $^{15}$N-labelled nitrate in the soil at a depth of 50 cm. One week after the $^{15}$N application we found that low CN genotypes had greater $^{15}$N content in shoot tissues than high CN genotypes under low N conditions (Figure 3.6B).

**CN effects on plant growth and yield**

In mesocosms N stress reduced shoot mass by an average of 45%. Shoot biomass and leaf photosynthetic rate were affected by CN (Table 3.1,3.2). ANCOVA and correlation analyses showed that under low N conditions, plants with low CN had greater leaf photosynthetic rates, canopy photosynthetic rates, tissue N content, and shoot mass, than plants with high CN (Table 3.1,3.2). There was no significant relationship between these variables and CN under high N conditions (data not shown).

In the field trials N stress reduced shoot mass by an average of 20% in SA2011 and by 24% in SA2012. ANCOVA and correlation analyses showed that under low N conditions, low CN genotypes had greater leaf photosynthetic rates, tissue N content, and shoot dry weight than plants with high CN at SA2011 (Table 3.1,3.3). There was no significant relationship between these variables and CN under high N conditions (data not shown).

In US2011 N stress reduced shoot mass by 34% at flowering (8 weeks after planting). Grain yield was reduced by 39% in low N soils. ANCOVA and correlation analyses showed that under low N conditions, low CN genotypes had greater tissue nitrogen content and shoot dry weight than high CN genotypes (Table 3.1,3.3). Low CN genotypes had greater relative grain yield than high CN genotypes under low N conditions (Figure 3.7).
Discussion

We demonstrate that low crown root number (CN) improves nitrogen acquisition by enhancing deep soil exploration in low N soils. Nitrogen stress significantly reduced CN in both greenhouse and field studies in 2011 (Figure 3.2,3.3,3.4). Maize lines with low CN had greater rooting depth than high CN genotypes (Figure 3.5,3.6) and acquired more $^{15}\text{N}$ labeled nitrate applied in deep soil in the field (Figure 3.6). Low CN genotypes had greater tissue nitrogen content and shoot biomass than high CN genotypes under low N conditions in all environments tested (Figure 3.6,Table 3.1). Finally, low CN genotypes had significantly greater relative grain yield than high CN genotypes in the field under low N conditions (Figure 3.7).

In the greenhouse we used mesocosms to create nitrogen-leaching environments comparable to conditions in well-drained agricultural soils. The mesocosms also permit a detailed investigation of root distribution by depth since entire root systems can be extracted. In field trials we applied a small amount of N fertilizer at the beginning of the growing season to the low N plots to support plant establishment and to create a pulse of N through the course of the growing season. CN was significantly reduced by N stress in soil mesocosms and in the field (Figure 3.2,3.3,3.4). The experiment in the mesocosms showed that reduced CN was attributable to decreased crown root whorl number and decreased number of roots per whorl (Figure 3.3A,3.3B). Nitrogen stress did not affect the number of roots of the first whorl, which is the earliest to emerge from the stem node, suggesting that plants may exhaust seed N reserves prior to or during the development of the second whorl crown roots.

We found that high CN genotypes had shallower primary, seminal, and crown roots than low CN genotypes under low N conditions (Figure 3.5). The results suggest that there may be compensation between the number of crown roots and growth of different root classes. These results are consistent with the reports in other crop species. In wheat and barley, the removal of
nodal roots stimulates the growth and activity of the seminal roots (Krassovsky, 1926). In common bean, increased carbon allocation to adventitious roots was related to decreased allocation to tap and basal roots, which affected total root length, soil exploration, and P acquisition under suboptimal P conditions (Walk et al., 2006), and removal of a specific root class led to an increase in the relative proportion of the remaining root classes (Rubio and Lynch, 2007). In maize the majority of axial roots in the root system are crown roots. The diameter of crown roots of the third whorl and subsequent nodes are much larger than that of the primary and seminal roots, and these roots are thus a greater sink for photosynthates. High CN genotypes must maintain growth and development of many crown roots, which would constrain the growth and elongation of crown roots and other root classes, resulting in shallower root systems compared to those of low CN genotypes (Figure 3.5,3.6). In addition, competition among roots within the root system for soil resources is greater in high CN genotypes, especially for a mobile resource like nitrate. The effect of reduced CN on soil exploration and N acquisition could result from reduced root competition for internal and external resources, as proposed by Lynch (2013).

We investigated the ability of low CN genotypes to take up N from deep soil layers in the field in SA2012 by injection of $^{15}$N-labelled nitrate in the soil at 50 cm depth within a planting row adjacent to the plants. We found that low CN genotypes had greater $^{15}$N uptake than high CN genotypes (Figure 3.6B). Soil nitrate analysis showed that nitrate was indeed more abundant in deep soil layers than in topsoil at the time of harvest (data not shown), thus, deep-rooting low CN genotypes are able to acquire N deep in the soil profile better than high CN genotypes. The ability to explore soils at greater depth and acquire N from N source in deep soils means that low CN plants have greater usage of N and thus have better N efficiency than high CN genotypes. Low CN plants could also reduce N leaching, thereby reducing environmental pollution.

Photosynthesis directly influences growth and yield of crop plants (Gastal and Lemaire, 2002). The rate of photosynthesis depends on content of N in the leaf tissue because
photosynthetic proteins, including Rubisco and light harvesting complex proteins, represent a large proportion of total leaf N (Evans, 1983). We found that low CN genotypes had greater tissue N content, which resulted in greater photosynthetic rates, and shoot biomass than high CN genotypes in greenhouse and field studies (Table 3.1, 3.2, 3.3). In US2011, the low CN genotypes also had 50% greater relative yield compared to high CN genotypes in low N soils (Figure 3.7). This is important especially for developing countries where yield of maize is less than 10% of its yield potential (Lynch, 2007).

Considering the range of reported CN in field-grown plants of 5-50 (Trachsel et al., 2010) and 10-32 (Bayuelo-Jiménez et al., 2011), our range of CN (20-45) falls between the medium to high range of phenotypic variation observed in maize. We propose that in extremely low CN phenotypes, roots may be too spatially dispersed to sufficiently acquire soil resources and such plants may be susceptible to lodging (Hetz et al., 1996). Additionally, plants with very low CN may be at risk of root loss due to herbivores and pathogens. This is particularly important for low-input agroecosystems where root survivorship is low. In this case the optimum number of CN would be large enough to allow rapid recovery from root damage but not too large to compete for internal and external resources. The optimum range of CN is likely to be dependent upon soil type and the severity of biotic and abiotic stresses. We anticipate that the optimum range of CN is also at the low end of the range of variation under drought and is likely to be greater in low density plantings, in fine-textured soils with slow leaching, and in soils with suboptimal availability of immobile nutrients such as phosphorus (P) and potassium (K), which are abundant in the topsoil. Greater CN may be beneficial to plants in low-input systems in which N continues to be available in the topsoil as a result of mineralization of organic matter (Poudel et al., 2001). However, many low-input systems are subject to drought in addition to suboptimal N availability. In this case, low CN enhancing deep soil exploration may be preferable to high CN since low CN
supports deep root system so the shallow portion of deep roots can acquire shallow N resources while the deep portion can explore deep soil for water resources.

Functional-structural modeling could be helpful in identifying optimum CN for specific environments as well as studying interactions between CN and other root traits. Recently, York et al. (2013) used the functional-structural plant model SimRoot, to observe interactions between CN and root cortical aerenchyma (RCA). They found that the synergistic effects of CN and RCA on plant growth were greater than the additive effects by 32% at medium N and by 132% at medium P. (York et al., 2013). In addition, an optimum number of crown roots can also interact with other traits enhancing deep soil exploration, such as steep root growth angle and few but long root branches, and may synergistically enhance resource acquisition under drought and suboptimal availability of mobile nutrients (Lynch, 2013).

The concept of optimum CN enhancing root growth and soil exploration under water and nutrient limiting conditions supports the rhizoeconomic paradigm, which considers the benefits and the costs of root traits as direct metabolic costs and as trade-offs and risks (Lynch and Ho, 2005; Nord and Lynch, 2009). We suggest that the optimum CN concept can be applied to other crop species in which nodal roots represent a major portion of the root system such as rice (Oryza sativa), wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) (Krassovsky, 1926; de Durlodot et al., 2007; Coudert et al., 2010).

Genotypic differences in crown root number have been reported in several crop species including maize and its relatives within Zea (Bayuelo-Jiménez et al., 2011; Burton et al., 2013; Lynch, 2013; Trachsel et al., 2010). Moreover, genes affecting CN expression have been isolated and characterized (Jenkins, 1930; Hetz et al., 1996) making CN a feasible target for plant breeding. To our knowledge, this is the first report of the utility of CN for enhancing nutrient acquisition in the literature. Our results support the hypothesis that CN is related to rooting depth
and soil N acquisition, and thus merits investigation as a potential element of more N-efficient cultivars.

Acknowledgements

We thank Dr. Kathleen M. Brown for her helpful review of the manuscript, and Bob Snyder, Bill Kojis, Curtis Frederick, and Johan Prinsloo for the management of the experiments in Hancock and Alma.
Figure 3.1: Visualization of maize root system of low and high crown root (CN) genotypes at 40 d after germination. Crown roots are colored in blue and seminal roots are in red. The number CN is 8 in the low CN genotypes and 46 in the high CN genotype (image courtesy of Larry M. York).
Figure 3.2: Crown root number of maize 28 days after planting under high N and low N conditions in soil mesocosms. Data shown are means of 4 replicates ± SE of the mean. Means with the same letters are not significantly different (p < 0.05).
Figure 3.3: Crown root whorl number (3A) and crown root number per whorl (3B) of maize 28 days after planting under high N and low N conditions in soil mesocosms. Data shown are means of genotypes with 4 replicates ± SE of the means. Means with the same letters are not significantly different (p < 0.05).
Figure 3.4: Crown root number of maize at flowering under high N and low N conditions at the fields in the USA in 2011 (4A), and in South Africa in 2011 (4B) and 2012 (4C). Data shown are means with 4 replicates ± SE of the means. Means with the same letters are not significantly different (p < 0.05)
Figure 3.5: Rooting depth of primary, seminal, and crown roots at 28 DAP under low N conditions in soil mesocosms compared between high and low CN within the same root class (5A) and D_{95} of maize at 9WAP under low and high N conditions at SA2011 field (5B). Data shown are means of 4 replicates ± SE of the mean. Different letters represent significant differences (p<0.05).
Figure 3.6: Correlations between 6A) crown root number and rooting depth ($R^2=0.53$, $p=0.04$), 6B) $^{15}$N in shoot ($R^2=0.35$, $p=0.02$), and 6C) shoot dry weight ($R^2=0.16$, $p=0.02$) at flowering under low N conditions in the field in South Africa (2012).
Figure 3.7: Correlation between crown root number and relative yield (lowN : highN) ($R^2=0.19$, $p=0.02$) under low N conditions in the field in the USA.
Table 3.1: Summary of correlation analysis (correlation coefficient and significant levels) between crown root number and parameter measured under low N conditions in six maize genotypes in soil mesocosms at 28 days after planting and in the field in South Africa and the USA in 2011.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mesocosms</th>
<th>South Africa</th>
<th>USA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canopy photosynthetic rate</td>
<td>0.26*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leaf photosynthetic rate</td>
<td>0.34*</td>
<td>0.31*</td>
<td>-</td>
</tr>
<tr>
<td>Tissue nitrogen content</td>
<td>0.23*</td>
<td>0.13*</td>
<td>0.13*</td>
</tr>
<tr>
<td>Shoot dry weight</td>
<td>0.23*</td>
<td>0.49**</td>
<td>0.22*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01

Table 3.2: Summary of ANCOVA model (F-value and degrees of freedom) of shoot traits at 28 day after planting as influenced by CN and N treatment in six maize RILs in greenhouse mesocosms.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Shoot weight</th>
<th>Photosynthesis Rate</th>
<th>Carbon Assimilation</th>
<th>Tissue N Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>22.31 (1,43)***</td>
<td>9.48 (1,43)**</td>
<td>6.51 (1,43)*</td>
<td>16.55 (1,44)***</td>
</tr>
<tr>
<td>N treatment</td>
<td>23.78 (1,43)***</td>
<td>31.62 (1,43)***</td>
<td>75.66 (1,43)***</td>
<td>29.15 (1,44)***</td>
</tr>
</tbody>
</table>
| CN×N treatment       | 4.89 (1,43)*     | 14.08 (1,43)***     | 0.20 (1,43)        | 2.79 (1,44)  
| R²                   | 0.66             | 0.53                | 0.63               | 0.49            |

*p<0.1, *p<0.05, **p<0.01, ***p<0.001,  
°p=0.1021. Degrees of freedom shown as (numerator, denominator)
Table 3.3: Summary of ANCOVA model (F-value and degrees of freedom) of shoot traits at flowering as influenced by CN and N treatment in six maize RILs in SA2011 and US2011 and in seven maize RILs in SA2012.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CN  treatment</td>
<td>3.19 (1,44) †</td>
<td>0.89 (1,52)</td>
<td>0.84 (1,44)</td>
<td>21.37 (1,44)***</td>
</tr>
<tr>
<td>N treatment</td>
<td>63.28 (1,44)***</td>
<td>33.53 (1,52)***</td>
<td>22.39 (1,44)***</td>
<td>14.34 (1,44)***</td>
</tr>
<tr>
<td>CN;N treatment</td>
<td>1.10 (1,44)</td>
<td>3.05 (1,52) †</td>
<td>3.62 (1,44) †</td>
<td>2.67 (1,44)</td>
</tr>
<tr>
<td>R²</td>
<td>0.59</td>
<td>0.39</td>
<td>0.34</td>
<td>0.49</td>
</tr>
</tbody>
</table>

†p<0.1, *p<0.05, **p<0.01, ***p<0.001, †p=0.1094. Degrees of freedom shown as (numerator, denominator)
Citations


Jenkins MT (1930) Heritable characters of maize. J Hered 79–81

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Chapter 4

Long root hairs enhance nitrogen acquisition from low nitrogen soils in maize (Zea mays L.)

Patompong Saengwilai\textsuperscript{1} and Jonathan P. Lynch\textsuperscript{1,2}

1. Intercollege Program in Plant Biology, The Pennsylvania State University, University Park, PA 16802, USA

2. Department of Plant Science, The Pennsylvania State University, University Park, PA 16802, USA

Abstract

Root hairs are known to facilitate the acquisition of immobile nutrients such as phosphorus and potassium in several crop species. However the agronomic value of root hairs for nitrogen (N) acquisition is poorly understood. In this study we utilized maize recombinant inbred lines (RILs) with contrasting root hair length to assess the utility of root hairs for N uptake in the greenhouse and in the field. A commercial nitrification inhibitor, nitrapyrin, was used to inhibit transformation of ammonium to nitrate. We found that neither N stress nor nitrapyrin treatment significantly affected root hair length in the greenhouse but N stress significantly reduced root hair length by 30\% in the field. In the greenhouse, long-haired RILs had 20\% greater biomass than short-haired RILs under low N conditions without nitrapyrin. In the field, long root hairs were associated with increased shoot mass and yield in low N without nitrapyrin treatment. Nitrapyrin treatment significantly increased shoot mass under high N conditions but slightly decreased under low N conditions. We also found a significant correlation between root hair length of plants in the greenhouse and in the field. We suggest that long root hairs enhance N
acquisition in low N soils by a combination of increasing root surface area for nitrate uptake and enhancing soil exploration beyond the depletion zone of ammonium and nitrate surrounding the root surface.
Introduction

Suboptimal nitrogen (N) availability is a major constraint for maize production, especially in smallholder farming where chemical fertilizers are unaffordable or unavailable for most farmers (Azeez, et al., 2006; Heffer & Prud’homme, 2009). Conversely, larger scale commercial farms utilize a considerable amount of N fertilizer to obtain economically satisfactory yield (Howarth et al., 2002; Ladha et al., 2005; Sutton et al., 2011). It is generally recognized that more than half of applied N is not taken up by crop plants but runs off or leaches away from farmlands causing air and water pollution (Baker et al., 2011; Cassman et al., 2002; Janzen et al., 2003; Sheldrick et al., 2002). Improving crop N acquisition is one of the keys to sustainably increase and maintain maize production while preserving environmental quality (Lynch, 2007; Tilman et al., 2002).

Acquisition of nutrients from soil depends on interactions between transport of nutrient from soil to root surface and nutrient uptake by the roots. Transport from soil to the root occurs through mass flow driven by transpiration and diffusion, which depends largely on concentration gradients and the mobility of nutrients in the soil (Barber, 1995). Uptake of immobile nutrients such as phosphorus (P) and potassium (K) at the root surface may exceed the rate of diffusion of nutrients in soil, resulting in depletion zones surrounding the roots. Root traits that expand the soil volume subject to the depletion of nutrients can, therefore, enhance acquisition of the nutrients. Root hairs, subcellular extensions of root epidermal cells, are beneficial in this regard. Long root hairs have been shown to facilitate P acquisition under low P conditions in both computer simulated plants (Bouldin, 1961; Ma et al., 2001) and in greenhouse and field-grown crops including maize (Zhu et al., 2010), common bean (Miguel, 2004), wheat and barley (Gahoonia & Nielsen, 1997; Gahoonia et al., 1997). Root hairs also release P-mobilizing exudates to enhance P availability in rhizosphere (Brown et al., 2012; Ryan & Delhaize, 2001).
To date, there has been much more research focusing on the utility of root hairs for P and K acquisition than for N acquisition. This is primarily due to the fact that availability of nitrate, a predominant N form in most agronomic soil, is not limited by diffusion, and thus N depletion around the root surface is considered to be negligible. However, root hairs may be beneficial for acquisition of nitrogen in the environments where ammonium, an immobile N form, represents a main N source such as anaerobic soils (Miller and Cramer, 2004). Recently long root hairs have been proposed as part of an ideotype for improved N acquisition by Lynch (2013). According to Lynch (2013), combination of long root hairs with other root traits including shallow growth angle, thin diameter, and root cortical aerenchyma will allow for efficient topsoil foraging for immobile nutrients including ammonium as well as for mobile nutrients such as water and nitrate that are not yet taken up by plants or lost to the environment while the primary root is penetrating into deep soil strata early in seedling development. In addition, long root hairs may also benefit plants during a period right after maize fields are fertilized with reduced forms of nitrogen fertilizers, such as urea or ammonia, which are commonly used in agriculture (Ribaudo et al., 2011). This is important since long root hairs may help seedlings to establish more quickly than short root hairs, which will benefit subsequent growth and development especially in N-limiting environments.

The objective of this study was to assess the utility of root hair length for ammonium acquisition. Maize Recombinant Inbred Lines (RILs) of the intermated B73 x Mo17 (IBM) population with contrasting root hair length were planted in the greenhouse and in the field under low and high nitrogen conditions. A commercial nitrogen inhibitor, nitrapyrin, was applied to half of each N treatment in the greenhouse and in the field to inhibit or minimize transformation of ammonium to nitrate creating ammonium-rich environments. We hypothesize that long-haired RILs are better at acquiring ammonium from the soil, thereby having greater vegetative growth and yield than short-haired RILs under low N especially with nitrapyrin treatments.
Materials and Methods:

Greenhouse study

Plant Materials

Six Recombinant Inbred Lines (RILs) from the inter-mated B73 x Mo17 (IBM) populations were used in this experiment. These RILs contrasted in root hair length. Short-haired RILs consisted of IBM43, IBM101, and IBM106. Long-haired RILs consisted of IBM111, IBM14, and IBM199.

Growth Conditions

The experiment was carried out in a greenhouse located on the campus of The Pennsylvania State University, University Park, Pennsylvania (40°48'N, 77°51'W) from March to April 2012. Seeds were germinated in darkness at 28 °C in rolled germination paper (Anchor Paper Company, St. Paul, MN, USA) moistened with 0.5 mM Ca\(_2\)SO\(_4\) for 3 days. Seedlings were planted in 10.5 L pots (Nursery Supplies Inc., Chambersburg, PA, USA) filled with a mixture of 50% sand, 35% vermiculite 5% perlite, and 10% topsoil (w/v). The topsoil was collected from the Russell E. Larson Agricultural Research Center in Rock Springs, PA (Fine, mixed, semiactive, mesic Typic Hapludalf, pH ≈ 6.7, silt loam). The experiment was arranged in a randomized complete block design with four replicates staggered one day between replicates. Planting dates and bench locations were considered as block effects. Plants were fertigated with a nutrient solution consisting of (in \(\mu\)M) P (1000), K (3000), Ca (2000), SO\(_4\) (500), Mg (500), CL (25), (B12.5), Mn(1), Zn(1), Cu(0.25), Mo(0.25) and DTPA-Fe (25). pH was adjusted to 6.0.
Ammonium sulfate \((\text{NH}_4)_2\text{SO}_4\) was used as the only nitrogen source. The nitrification inhibitor, nitrapyrin, \((2\text{-Chloro}-6\text{-}(\text{trichloromethyl}) \text{pyridine}; \text{INSTINCT, Dow AgroSciences, IN, USA})\), was employed to inhibit the transformation of ammonium to nitrate. INSTINCT contained 17.67\% active ingredient. There were two levels of N: high and low ammonium. Each level had two treatments: with and without nitrapyrin. High N treatments received 7000 µM while low N treatments received 700 µM of \((\text{NH}_4)_2\text{SO}_4\). The nitrapyrin treatments received 1 ppm of nitrapyrin at planting by mixing with the nutrient solution. Plants were grown under a photoperiod of 14/10 h at 28/24 °C (light/darkness) with total photosynthetically active radiation (PAR) of 1200 µmol photos m\(^{-2}\)s\(^{-1}\) during the light photoperiod.

**Data collection**

Plants were harvested at 5 weeks after planting. One day prior to harvest, leaf gas exchange of the second youngest fully expanded leaves was measured with a Licor-6400XT Infrared Gas Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) using a red-blue light at PAR intensity of 1200 µmol photons m\(^{-2}\)s\(^{-1}\) and constant CO\(_2\) concentration of 400 ppm. At harvest, 6-mm diameter leaf discs were collected from the second youngest fully expanded leaves for chlorophyll measurement. Chlorophyll was extracted in 80\% acetone and the concentrations of chlorophyll a and b in the extracts were determined at the wavelength of 663.2 and 646.8 nm with a spectrophotometer (Lichtenthaler and Buschmann, 2001). Shoots were dried at 60 °C for 72 h prior to dry weight determination. The shoots were ground and 2-3 mg of ground tissues were taken for tissue nitrogen analysis using an elemental analyzer (SeriesII CHNS/O Analyzer 2400, PerkinElmer). Whole root systems were carefully washed and preserved in 75\% EtOH at 4 °C prior to the evaluation of total root length and root hair length. Total root length was obtained by scanning and measuring with image analysis software (WinRhizo Pro, Régent Instruments, Québec, Québec City Canada). Roots were dried at 60 °C for 72 h prior to dry weight
determination. Soil media were collected and extracted in 2M KCl. The concentrations of ammonium and nitrate in soil media were determined by spectrophotometry (Doane and Horwáth, 2003).

**Root hair evaluation**

Three representative root segments were selected from primary and seminal roots of an individual plant. The roots were quickly dipped in 0.025% toluidine blue and carefully rinsed in deionized water prior to root hair examination. Root hairs were stained pinkish-blue whereas root surface remained unstained allowing for a clear observation of root hairs. For determination of root hair length the stained roots were observed at 35x magnification under a dissecting microscope (Nikon, SMZ-U, Japan.) equipped with a Hamamatsu Photonics XC77 charged-couple device (CCD) camera. A section of root with consistent fully elongated hairs was selected for image capture. Root hair length was quantified using a line tool in ImageJ version 1.47 (Abramoff et al., 2004). Five representative hairs per image were selected for length measurement. Root hair length was calculated by comparing number of pixels of a root hair to number of pixels per mm obtained from an image of a micrometer scale taken at the same magnification (1111.52 pixels mm⁻¹).

**Field study**

Eight RILs including the six RILs with contrasting root hair length previously characterized in the greenhouse study and two additional RILs; IBM27 and IBM7, were planted at the Russell E. Larson Experimental Farm of The Pennsylvania State University at Rock Springs, PA (40°42′37″.52 N, 77°57′07″.54 W, 366 masl) from June to October 2012. The soil at the experimental sites was a Hagerstown silt loam (fine, mixed, semiaactive, mesic Typic
Hapludalf). The experiment was a randomized complete block design with a split-split-plot arrangement of treatments and three replicates, with each replicate being an independent 1-acre field. RILs were randomly assigned to five-row plots. Each row consisted of 20 plants. The distance between rows was 75 cm and within a row was 23 cm, resulting in a planting density of 6 plants m$^{-2}$. Based on soil analysis at the beginning of growing season, entire fields were amended with 820 g m$^{-2}$ of sawdust to immobilize soil N. Each field was divided in half to make areas for high and low nitrogen. High N split-plots were fertilized with 155 Kg N ha$^{-1}$ of urea while low N split-plots did not receive any N fertilizer. Nitrapyrin was applied at the rate of 0.5 ml m$^{-2}$ to the half the area of each of high N and low N split-plots to create high and low N plus nitrapyrin treatments split-split-plots. Soil nutrient levels of other macro and micronutrients were adjusted to meet the requirements for maize production as determined by soil tests. Pest control and irrigation were carried out as needed. The plants were harvested at 9 weeks after planting to collect root hair length, leaf and stem biomass, and leaf area. Tissue nitrogen content was determined using an elemental analyzer (Series II CHNS/O Analyzer 2400, PerkinElmer). Soil was collected at 3, 6 and 9 week after planting using an auger. The soil column was divided into 4 segments; 0-15, 15-30, 30-45, and 45-60 cm depth. The soil was extracted in 2M KCl and stored at -80 °C. The concentrations of ammonium and nitrate in soil media were determined by spectrophotometry according to Doane & Horwáth, 2003. At maturity grain yield was collected from one full row excluding bordering plants in each plot.

**Statistical analysis**

Statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012). Linear mixed effect models were fit using the function lme from the package nlme (Pinheiro et al., 2012) and a two-way ANOVA were used for comparisons between long and short-haired groups (or individual RILs), nitrogen and nitrapyrin levels and the interaction
between these main effects. A protected least significant difference post hoc \( (\alpha=0.05) \) test and Tukey’s Honest Significant Difference method \( (\alpha=0.05) \) were used for multiple comparisons. ANCOVA was used to assess an influence of root hair length on shoot traits in the field. Correlations and linear regressions were carried out between shoot traits, yield, and root hair length in the field.
Results

Greenhouse study

Nitrogen and root hair length

At 35 d after planting (DAP), high nitrogen (N) treatments had 52% more ammonium and 42% more nitrate in soil media than low N treatments at 35 d after planting. Nitrpyrin did not affect ammonium concentration in high and low N soils but reduced nitrate availability by 50% in high N treatments and by 35% in low N treatments (Figure 4.1). Nitrpyrin increased the ratio of ammonium to nitrate from 0.29 to 0.55 under high N conditions and from 0.25 to 0.43 under low N conditions. Nitrpyrin significantly decreased total N availability under high N conditions by 39% and by 12% under low N conditions (Figure 4.1). Root hair length was significantly different among RILs (F=16.7637,p<0.0001). However, neither N stress nor nitrpyrin treatment significantly affected root hair length. Long-haired RILs had significantly greater root hair length than short-haired RILs in all treatments. On average root hair length of long-haired RILs was 23% greater than short-haired RILs (Figure 4.2).

Plant growth

Low N availability reduced shoot biomass by 34% at 35 DAP. Long-haired RILs had 20% greater shoot biomass than short-haired RILs in low N without nitrpyrin treatments (Figure 4.3). N stress reduced root biomass by 19%. Nitrpyrin treatment did not significantly affect root biomass. Long-haired RILs had slightly greater root biomass than short-haired RILs but the effect was not significant (Figure 4.4). Low N availability reduced total root length by 29%. There was no significant difference in total root length between long- and short-haired RILs compared
within each treatment (Figure 4.5). Low N availability increased root to shoot ratio by an average of 13% (F=17.2883, p<0.001). There was no significant difference in root to shoot ratio between long- and short-haired RILs compared within each treatment (Figure 4.6). We did not find any ammonium toxicity symptoms in high N plants in this experiment.

Field study

Soil ammonium and nitrate availability and root hair length

The availability of soil ammonium and nitrate in different soil depths at 3, 6, and 9 weeks after planting (WAP) was shown in Figure 4.7, 4.8, and 4.9. In general, ammonium was most abundant in the top 15 cm soil in all treatments at 3, 6 and 9 WAP, except in the low N with nitrapyrin treatment at 3 WAP where ammonium was most abundant at 45-60 cm soil depth (Figure 4.7). In high N plots, nitrate was most abundant in the topsoil while in low N plots nitrate was most abundant in subsoil at 15-30 cm depth except in low N with nitrapyrin treatment at 6 WAP where nitrate was more abundant in topsoil (Figure 4.8). Nitrate availability in topsoil significantly increased from 3 to 6 WAP in all treatments (p<0.05) but decreased from 6 to 9 WAP except in low N without nitrapyrin treatment (Figure 4.8).

At harvest ammonium availability was significantly greater in high N with nitrapyrin plots (Figure 4.9A). There was no significant difference in ammonium availability in subsoil at 15-30 and 30-45 cm depth. High N with and without nitrapyrin plots had greater ammonium availability in soil at 45-60 compared to low N with and without nitrapyrin plots (Figure 4.9A). Nitrate availability at harvest was greatest in the topsoil of high N with nitrapyrin plots and lowest in the topsoil of low N with nitrapyrin plots (Figure 4.9B). Nitrapyrin increased total N availability in high N soils by 39% but decreased total N availability in low N soil by 52%.
RILs differed in a degree of change in root hair length in response to N and nitrapyrin treatments. IBM RILs 199, and 15 maintained their ranking as long-haired in all treatments, IBM RILs 14, and 43 had short hairs, whereas root hair lengths of IBM RILs 101, 111, 27, 106 and 7 were not consistent among treatments (Figure 4.10). On average, low N availability decreased root hair length by 30%. Nitrapyrin treatments did not significantly affect root hair length (Figure 4.11A).

**Plant growth and yield**

Low N availability decreased shoot biomass at flowering by 51% and decreased yield by 30%. Nitrapyrin significantly increased shoot biomass at 9WAP by 10% but did not affect yield under high N conditions (Figure 4.11B and 4.11C). Plants in low N without nitrapyrin treatments had 36% greater yield than plants in low N with nitrapyrin plots (Figure 4.11C). Analysis of covariance (ANCOVA) demonstrated that the effect of root hair length on leaf weight, stem weight, leaf area, shoot weight, and yield was significant (Table 4.1). Stem weight, shoot weight, and yield were significantly affected by interaction between root hair length and N treatments (Table 4.1). Correlation analysis showed that root hair length was significantly associated with shoot biomass ($R^2=0.26$, $p<0.05$) and yield ($R^2=0.17$, $p<0.05$) in low N without nitrapyrin (Figure 4.12).
Discussion

Our results indicate that the effect of N stress on root hair length is not significant in the greenhouse but root hair length was significantly decreased by low N in the field. The effects of N stress on root hair length varied among RILs (Figure 4.10). Nitrapyrin treatments did not affect root hair length (Figure 4.2 and 4.11A). Long-haired RILs had greater growth in the greenhouse and in the field than short hairs RILs under low N conditions without nitrapyrin (Figure 4.3, 4.12 and Table 4.1). Long-haired RILs also had greater yield in comparison with short-haired RILs in the field under low N without nitrapyrin treatment (Figure 4.12B and Table 4.1). Nitrapyrin treatment significantly increased shoot mass under high N conditions but slightly decreased under low N conditions (Figure 4.11B).

In this present study, we assessed the value of root hair length for N acquisition by evaluating maize recombinant inbred lines (RILs) of the IBM population with contrasting root hair length. RILs are closely related genotypes with highly similar genetic background thereby minimizing the risk of effects from genetic interaction, epistasis, and pleiotropy which may confound the interpretation of the results (Zhu et al., 2005; Zhu et al., 2006). We found that these RILs had comparable root and shoot characteristics as evidenced by similar shoot dry weight, root dry weight, total root length, and root to shoot ratios under non-stress conditions in the greenhouse and similar shoot mass and yield in the fertile field. RILs of the same population has been used to dissect genetic control of root hair traits under differential P availability in maize as well as to investigate the utility of root hair length for P acquisition (Zhu et al., 2005; Zhu et al., 2010).

Many plant species including *Arabidopsis*, common bean, rape, spinach, tomato and maize increase root hair length in response to suboptimal phosphorus availability. (Foehse and Jungk, 1983; Bates and Lynch, 1996; Bates and Lynch, 2000; Miguel, 2004; Zhu et al., 2010) Increased root hair length has been shown to be an important strategy to enhance acquisition of
immobile nutrients particularly phosphorus (Bates & Lynch, 1996, 2000; Gahoonia et al., 1999; Gahoonia & Nielsen, 1998). For nitrogen, Foehse and Jungk (1983) found that rape, spinach, and tomato plants increased root hair length in response to N stress. In our study, we found that suboptimal N availability significantly reduced root hair length of maize plants in the field but not in the greenhouse. The reduction in root hair length in response to N stress is consistent with the study of Gaudin et al. (2011) using an aeroponic system. Root hair length of some RILs was not affected by N stress suggesting that there is variation in root hair length plasticity in response to N. The plasticity of root hair length in response to differential levels of nutrients has been shown in many plant species (Foehse and Jungk, 1983; Ewens and Leigh, 1985; Bates and Lynch, 1996). Intraspecific genetic variation for root hair length plasticity in response to P stress has been demonstrated in maize RILs of the IBM population (Zhu et al., 2010). It has been shown that maize RILs with root hair length plasticity, being short under high P and long under low P, had greater biomass allocation to roots, reduced root respiration, and greater shoot biomass under low P conditions than constitutively long haired RILs. The value of root hair length plasticity in response to N stress has not yet been reported and merits more research.

In the greenhouse, ammonium sulfate \((\text{NH}_4)_2\text{SO}_4\) was the sole nitrogen source. At harvest ammonium represented 25\% of total available N under low N and 30\% under high N conditions without nitrpyrin. We utilized a commercial nitrification inhibitor, nitrpyrin, to decrease the transformation of ammonium to nitrate (Abbasi et al., 2003; Adriaanse & Human, 1990; Ali et al., 2008). We found that media with nitrpyrin had less nitrate than media without nitrpyrin but did not differ in ammonium availability. This is unexpected but not surprising because the nitrification process in greenhouse media could be very slow and thus an addition of nitrpyrin did not significantly alter availability of ammonium. It is important to note that the greenhouse media contained 10\% field soil which could be contaminated with a small amount of nitrogen and soil microbes. Soil could also act as a buffer for nitrogen which maintained nitrogen availability in the media. In addition reduction in nitrate availability could be due to immobilization by other
soil microbes which may be present at a greater amount in nitrapyrin treatments. In the field ammonium was most abundant in the topsoil whereas nitrate availability was dynamic, with greater concentration in the topsoil under high N and greater concentration in the subsoil under low N conditions. Nitrapyrin preserved N in the topsoil in high N plots but depleted nitrate and total N availability in the topsoil in low N plots. These findings are consistent with those of Adrinnae and Human (1990). Our results confirm the benefit of nitrapyrin in high input soils but reveal a potential disadvantage in low N soils, as evidenced by reduced shoot mass and yield in low N soils compared to plants without nitrapyrin treatment (Figure 4.11B and 4.11C).

In maize, it has been shown that long root hairs do not incur significant metabolic costs in fertile conditions (Zhu et al., 2010). Interestingly, long root hairs could become less important or even detrimental to the plants when nutrient availability is low but not limited by diffusion. For example, Bates and Lynch (2000) demonstrated that the cost-benefit ratios, using respiration as a cost and P gain as a benefit, of Arabidopsis wildtypes were less than that of Arabidopsis mutants, rhd2, with no root hairs under low P condition in sand media. However, the cost-benefit ratios of the wildtypes were greater than that of the mutants in low P nutrient solutions where P is freely mobile. These results suggest that there may be a cost for root hair extension under suboptimal availability of nutrients, but those costs are outweighed by the benefits of enhancing P uptake in diffusion-limited environments. In our study we found that maize increased root to shoot ratio in response to N stress which is consistent with reports from maize and other plant species (Bonifas et al., 2005; Mcconnaughay & Coleman, 1999). However there was no significant difference in the proportion of biomass allocation to roots between long- and short-haired RILs under low N conditions. Although root dry weight of long-haired RILs was slightly greater than short-haired RILs under low N conditions, the effect was not significant. We also found that root hair length did not significantly effect total root length and total root weight in all treatments (Figures 4.4,4.5,4.6), therefore increased shoot mass under low N conditions found in the greenhouse was the result of having long root hairs and not increased total root length. Additionally the results
may indirectly suggest that root hair extension did not incur significant root construction costs under high or low N conditions.

Root hairs have long been believed to be less important for nitrogen acquisition because the availability of nitrate, a predominant form in most soil, is not diffusion-limited. Our results show that long root hairs enhance nitrogen acquisition from low N soils as evidenced by greater shoot biomass and yield in long-haired RILs when compared with short-haired RILs. In the environment where nitrate is a dominant form of soil N, overall N uptake may not be limited by diffusion. When the ammonium fraction is high, diffusion may play an important role in overall N uptake. In our study the concentrations of ammonium and nitrate in high N plots at harvest were in the range of typical agricultural soils reported in the literature (1 to 5 mM nitrate and 0.02-0.2 mM ammonium; Barber, 1995; Owen & Jones, 2001). In the field ammonium represented 5% of total soil available N in high N with and without nitrapyrin treatments. Interestingly, ammonium became more important in low N soils since it represented 20% of total soil available N in low N soil without nitrapyrin and 50% in low N soil with nitrapyrin treatments. However the increase in the percentage of ammonium in low N soil with nitrapyrin was attributable to a considerable decrease in nitrate concentration, which resulted in reduction in total N availability as well as average shoot biomass in comparison to the low N without nitrapyrin treatment. We found that shoot biomass and yield was correlated with root hair length in the low N treatment without nitrapyrin but not in the treatment with nitrapyrin. The results suggest that long-haired RILs benefit most in the environments where nitrate is also present in significant amounts. In addition, the treatment of nitrapyrin in low N soils may reduce total N available to plants resulting in a slight reduction in shoot mass and yield, as found in this study.

We speculate that root hairs could enhance nitrate uptake from low N soils by increased surface area, which is particularly beneficial when the concentration of nutrients at the root surface is low because the flux to the surface is then small, and a large absorptive area is required for to adequately supply the plant (Jungk, 2001). Additionally, electrophysiological evidence
indicates that high-affinity nitrate transporters in root hairs are greatly up-regulated under nitrate
deficiency (Meharg and Blatt, 1995), suggesting that long root hairs may increase
competitiveness for nitrate uptake under low N conditions. Moreover, increased expression of
nitrate transporters could enhance the rate of nitrate uptake at the root surface, which may exceed
the rate of nitrate supply from low N soil via mass flow of water, creating depletion zones of
nitrate. The benefit of long root hairs for nitrate uptake could then be realized in this senario.
Futher experiments using nitrate as the sole N source need to be carried out in order to assess the
benefits for nitrate uptake that plants gain by having long root hairs.

Quantitative genetic analysis indicated that genotypic variation in root hair length and
density in maize and common bean is controlled by several important QTLs (Yan et al., 2004;
Zhu et al., 2005) which suggests that root hair traits could be selected in crop breeding programs
through marker-assisted selection (MAS) as well as through direct phenotyping. We found that
there was a correlation between root hair lengths measured in soil media and in the field (Figure
4.13), which is consistent with previous report by Zhu et al. (2010). Therefore screening of a
large number of RILs can be done effectively and economically in a greenhouse or even in cigar
rolls (Zhu, 2003). Root hair traits could be visually evaluated and thus accessible to breeders in
developing countries (Vieira et al., 2007). We suggest that long root hairs could be used to
improve N acquisition in maize.
Figure 4.1: Concentrations of ammonium (a) and nitrate (b) in greenhouse media at 35 days after planting. Data shown are means of 8 replicates ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 4.2: Root hair length of long and short-haired genotypes in the greenhouse at 35 days after planting. Data shown are means of 4 replicates of 3 long-haired RILs and 3 short-haired RILs ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 4.3: Shoot dry weight of long and short-haired genotypes in the greenhouse at 35 days after planting. Data shown are means of 4 replicates of 3 long-haired RILs and 3 short-haired RILs ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 4.4: Root dry weight of long and short-haired genotypes in the greenhouse at 35 days after planting. Data shown are means of 4 replicates of 3 long-haired RILs and 3 short-haired RILs ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 4.5: Total root length of long and short-haired genotypes in the greenhouse at 35 days after planting. Data shown are means of 4 replicates of 3 long-haired RILs and 3 short-haired RILs ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 4.6: Root to shoot ratio of long and short-haired genotypes in the greenhouse at 35 days after planting. Data shown are means of 4 replicates of 3 long-haired RILs and 3 short-haired RILs ± SE of the means. Different letters represent significant differences (p<0.05).
Figure 4.7: Soil N-NH$_4^+$ content at different depth in the field. The soils were collected from high N (7A), high N+nitrapyrin (7B), low N (7C) and low N+nitrapyrin (7D) plots at 3, 6, and 9 weeks after planting.
Soil N-NO₃ content at different depth in the field. The soils were collected from high N (8A), high N+nitrapyrin (8B), low N (8C) and low N+nitrapyrin (8D) plots at 3, 6, and 9 weeks after planting.
Figure 4.9: Soil N-NH$_4$\(^+\) (9A) and N-NO$_3$\(^-\) (9B) at different depth in the field. The soils were collected at 63 days after planting from high N, high N+nitrapyrin, low N and low N+nitrapyrin plots. Data shown are means ± SE of the means. Different letters represent significant differences (p<0.05) compared within the same soil depth.
Figure 4.10: Root hair length of nine maize RILs grown under high N, high N+nitrapyrin, low N and low N+nitrapyrin conditions in the field. The plants were harvested at 63 days after planting. Data shown are means of 3 replicates ± SE of the means. Different letters represent significant differences (p<0.05) compared within the same genotype.

Figure 4.11: Root hair length (11A), shoot dry weight (11B) and yield (11C) of nine maize RILs grown under high N, high N+nitrapyrin, low N and low N+nitrapyrin conditions in the field at 63 days after planting. Data shown are means of 3 replicates ± SE of the means. Different letters represent significant differences (p<0.05)
Figure 4.12: Correlation between root hair length and shoot dry weight (12A; $R^2=0.26$, $p=0.004$) and between root hair length and yield per plant (12B; $R^2=0.17$, $p=0.027$) under low N without nitrapyrin conditions.
Figure 4.13: Correlation of root hair length in the greenhouse and in the field under high ($R^2=0.62$, $p<0.01$) and ($R^2=0.36$, $p=0.06$) low nitrogen conditions.
Table 4.1: Summary of ANCOVA model (F-value and degrees of freedom) of shoot traits at 63 d after planting and yield as influenced by root hair length (RHL) and N treatment in nine maize RILs in the field.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Leaf weight (g)</th>
<th>Stem weight (g)</th>
<th>Leaf area (m²)</th>
<th>Shoot weight (g)</th>
<th>Yield (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHL</td>
<td>136.67 (1.98)**</td>
<td>93.15 (1.98)**</td>
<td>102.83 (1.94)**</td>
<td>119.70 (1.98)**</td>
<td>9.71 (1.88)**</td>
</tr>
<tr>
<td>treatment</td>
<td>18.39 (3.98)**</td>
<td>29.68 (3.98)**</td>
<td>8.277 (3.94)**</td>
<td>31.36 (3.98)**</td>
<td>1.39 (3.88)</td>
</tr>
<tr>
<td>rep</td>
<td>8.85 (2.98)**</td>
<td>4.98 (2.98)**</td>
<td>14.79 (2.94)**</td>
<td>6.6829 (2.98)**</td>
<td>3.38 (2.88)*</td>
</tr>
<tr>
<td>RHL:treatment</td>
<td>1.36 (3.98)</td>
<td>4.13 (3.98)**</td>
<td>0.89 (3.94)</td>
<td>3.77 (3.98)*</td>
<td>2.35 (3.88)†</td>
</tr>
<tr>
<td>R²</td>
<td>0.66</td>
<td>0.65</td>
<td>0.59</td>
<td>0.68</td>
<td>0.16</td>
</tr>
</tbody>
</table>

†p<0.1, *p<0.05, **p<0.01, ***p<0.001. Degrees of freedom shown as (numerator, denominator)
Citations


Gahoonia TS, Nielsen NE (1998) Direct evidence on participation of root hairs in phosphorus (32P) uptake from soil. 147–152

Gahoonia TS, Nielsen NE, Lyshede OB (1999) Phosphorus (P) acquisition of cereal cultivars in the field at three levels of P fertilization. Plant Soil 211: 269–281


Chapter 5

Phenotypic variation and Genome-wide association study of root anatomical traits in maize (*Zea mays* L.)

*Patompong Saengwilai¹, Hannah Schneider², Eric A. Nord², James M. Johnson³, Jillian Foerster³, Shawn M. Kaeppler³, Kathleen M. Brown¹,², and Jonathan P. Lynch¹,²

1. Intercollege Program in Plant Biology, The Pennsylvania State University, University Park, PA 16802, USA

2. Department of Plant Science, The Pennsylvania State University, University Park, PA 16802, USA

3. Department of Agronomy, University of Wisconsin, Madison, WI 53706, USA

Abstract

Root anatomical traits influence the acquisition and transport of water and nutrients, the metabolic cost of root growth and maintenance, and the mechanical strength of the root system. A number of experiments have shown significant benefits of root anatomical traits under biotic and abiotic stresses. Despite the high potential for improving crop performance and yield, few studies have been undertaken to characterize genetic variation and identify quantitative trait loci (QTL) for root anatomical traits. Here we combined a laser ablation technique and *RootScan*, a semi automated image analysis program for root sections, to provide phenotypic data for a Genome-Wide Association Study (GWAS) in the maize lines of the Wisconsin Diversity Panel (WiDiv). Phenotypic analyses were performed for 20 root anatomical traits including cortical, aerenchyma, stele and xylem traits. The plants were grown in the field during 2011, 2012, and 2013. Traits were evaluated at anthesis. Significant phenotypic variation was found for each of
the traits. Variation of the anatomical traits ranged from 2-fold for diameter traits to 64-fold for aerenchyma area. The majority of anatomical traits were strongly correlated with root size. The distribution pattern of most traits was close to normal or slightly left-skewed. A principal component analysis (PCA) showed that three principle components (PC) contributed to 68.8 % of variation of root anatomical traits. PC1, the largest, is primarily size of root cross-section, cortex, and stele. PC2 is aerenchyma traits and cell size. PC3 is cortical cell component and the ratio of total cortical area to total stele area. Broad-sense heritability estimates were highest in the ratio of total cortical area to stele area (0.67) and lowest in size of cells the middle cortex (0.22). A total of 1140 significant Single Nucleotide Polymorphisms (SNPs) were identified for the 20 traits. Among these SNPs, 59 SNPs passed the chromosome-wide thresholds. Search for functional annotations and expression profiles of the SNPs and previously identified genes showed that several SNPs and their related genes were highly expressed in primary roots of maize seedlings. We propose and discuss some candidate genes for root anatomical traits. This study is the first to identify markers and candidate genes underlying variations in root anatomical traits in field-grown maize. The results will greatly improve our understanding of the phenotypic variation and genetic control of root anatomical traits. In addition, molecular markers associated with these traits may be useful in plant breeding programs using marker-assisted selection.
Introduction

Maize (Zea mays ssp. Mays) is the world’s most widely grown crop and is used as a staple food source especially in Latin America and Africa. It has been estimated that within a decade, the global demand for maize would account for more than half of the increased total demand for cereals (UNDP, 2010). This poses a challenge to maize breeders and farmers to increase maize production in order to meet future demand. Improving plant roots could lead to increased crop productivity since roots are fundamental for soil resource acquisition (Lynch, 2007). However metabolic costs of roots are substantial (Lambers et al., 1996; Lynch & Ho, 2005). Therefore knowledge of phenotypic variation and genetic control of root traits is crucial for plant breeders in order to develop breeding strategies that improve soil exploration with efficient root system.

Root anatomical traits are defined by the arrangement, size, and number of cells and tissues within root systems (Burton et al., 2013; Esau, 1977; Hochholdinger, 2009). In maize, the radial organization of roots can be divided into two major regions: the stele and cortex (Figure 5.1). The stele is the central part of the root containing vascular and parenchyma tissues. The arrangement of vascular cylinder in maize follows the polyarch organization typical of monocotyledonous plants (Esau, 1977). The outer most part of stele is enclosed by the pericycle, which is the site of lateral root initiation. The ground tissue consists of a single layer of endodermal cells and several layers of cortical tissues in cortex region. The endodermis contains the casparian strip, which limits radial movements of water and nutrients (Hose et al., 2001). The outermost part of cortex is bordered by a single layer of epidermal cells, which consist of root hair forming (trichoblast) and non-forming (atrichoblast) cells (Row and Reeder, 1957). The epidermis is replaced by lignified and suberized exodermis later in the development (Hose et al., 2001). In some varieties or under flooding, drought, and suboptimal nutrient conditions, tissues in cortical regions undergo programmed cell death forming air spaces called root cortical
aerenchyma (RCA) in the root cortex (Bouranis et al., 2003; Drew et a., 2003; Fan et al., 2003; He et al., 1996; Zhu et al., 2010).

Maize exhibits large phenotypic variation of root anatomical traits (Burton et al, 2013; Lynch, 2013; Weerathaworn et al, 1992). Several studies suggest that root anatomical traits have the potential to improve tolerance to edaphic stresses. For instance, RCA confers tolerance to hypoxic conditions by facilitating root aeration (Jackson and Armstrong, 1999; Mano et al., 2006). In addition, plants benefit from RCA by reallocating nutrients from lysed tissues and reducing root metabolic costs permitting enhanced soil exploration (Fan et al., 2003; Zhu et al., 2010; Postma and Lynch, 2011; Saengwilai, 2013). Roots with a small portion of cortex occupied by living cells defined as living cortical area (LCA) and small diameter have low root respiration and maintenance costs (Jaramillo et al., 2013). Cortical components such as cortical cell size, number of cortical cells, and number of cortical cell files could affect root thickness and the length and tortuosity of pathways by which water and nutrients travel through the root (Esau, 1967; Marschner, 1995). The size and number of xylem vessels influence their hydraulic conductivity. Water in xylem vessels is assumed to flow according to Poiseuille–Hagen law with a hydraulic conductivity is proportional to the fourth power of vessel’s radius. Therefore even a small change in the size of xylem vessel could dramatically affect the conductivity of water. Narrow xylem vessels have increased resistance to water transport and loss and this trait has been used in plant breeding for water use efficiency (Richards & Passioura, 1989).

In the past decade, linkage analysis has been used to identify a few quantitative trait loci (QTLs) associated with phenotypic variation of root anatomical traits such as stele and xylem vessel diameter in rice (Uga et al., 2008), root cortical aerenchyma in Zea species (Mano & Omori, 2013; Mano et al., 2007; Mano & Omori, 2008) and root cellular development in tomato (Ron et al., 2013). However, by comparison to other agronomic traits, little progress has been achieved in the characterization and identification of the genetic control of root anatomical traits. The main reasons are the difficulty in effectively and accurately phenotyping roots in a large
number of plants and the complex interactions between roots and surrounding rhizosphere. Additionally, linkage analysis has low resolution, small number of alleles, and population specific results, which hinder gene discovery and limit implementation of the results to other populations with different backgrounds (Flint-Garcia et al., 2005; McMullen et al., 2009). Association mapping utilizing historical and evolutionary recombination events to tackle marker-trait associations provides several advantages over traditional linkage analysis. Association mapping can be done using existing populations or diversity panels and thus save time for crossing to create mapping populations. Association mapping population consists of several diverse individuals thus increasing allelic diversity. Most importantly, association mapping has much higher resolution due to rapid linkage decay which can range from a few hundred base pairs (bp) in inbred lines of maize to 1-2 kbp in elite lines compared to 10-40 cM which could cover hundred of genes in linkage analysis (Flint-Garcia et al., 2003; Remington et al., 2001; Yu & Buckler, 2006). Therefore association mapping is an ideal approach for candidate gene discovery.

The objective of our study was to examine phenotypic variation of maize root anatomical traits and to identify markers associated with variation of root anatomical traits using Genome-Wide Association Study (GWAS). The study was carried out in 503 maize lines of the Wisconsin Diversity Panel (WiDiv). The lines were selected based on phenology, production of viable seed, agronomic suitability, uniformity, and pedigree information (Hansey et al., 2011). We combined a laser ablation technique, which facilitates root cross sectioning and imaging (see materials and methods) with a semi-automated image analysis program, RootScan (Burton et a., 2012) to use as a high-throughput phenotyping platform for root anatomical traits. The GWAS was performed using a set of 438,222 SNP markers derived from RNA-seq. We have discovered several significant SNPs associated with root anatomical traits, and several candidate genes were also identified.
Materials and Methods

Plant materials and growth conditions

Experiments were conducted during January to April of 2011, and 2012 and during November to February of 2013 at the Ukulima Root Biology Center (URBC) in Alma, Limpopo province, ZA (24°33′ 00.12 S, 28° 07′25.84 E, 1235 masl) using a randomized complete block design with two replications in each year. Each line was planted in a single row plot consisting of 20 plants per plot. Row width was 75 cm, and distance within a row was 23 cm. In all trials, soil nutrient levels of other macro and micronutrients were adjusted to meet the requirements for maize production as determined by soil tests at the beginning of the growing seasons. The trials were irrigated using a center pivot system. Pest control was carried out as needed.

Root sampling

Plants were harvested at 7-8 weeks after planting. Prior to root harvest, plant height and stem diameter at soil surface were measured. Root excavation was carried out based on shovelomics (Trachsel et al., 2011). Three representative plants were selected for excavation in each plot. The selection was based on height, presence of bordering plants, and general appearance that represented individuals in the plot. Root crowns were collected by carefully excavating a 15 cm radius around the stem to a depth of at least 15 cm. A large portion of soil was removed from roots by carefully shaking. The remaining soil was removed by soaking the roots in diluted commercial detergent followed by vigorously rinsing at low pressure with water. Because three representative roots within a plot usually appear to be homogenous, only one root was randomly selected from the uniform three roots for root sampling. Four cm root samples
were collected at 8 cm from the base of the second whorl crown roots. The samples were stored in 75% EtOH at 4°C until processing and analysis.

**Laser ablation and image capturing**

Root cross-sectional images were obtained using laser ablation technique. A root segment was placed on to a servo-driven stage (Aerotech Inc., Pittsburgh PA). The stage was set to automatically move into the camera and laser focal plane at the travel rate of 30 nm sec\(^{-1}\). The laser source was delivered by a high power Q-Switched Ultraviolet Laser (AVIA 355-7000, Coherent photonic group, CA) with a pulse repetition rate between 25 and 40kHz. The laser was coupled with a 10 mm aperture galvanometer scanner (SCANLAB, HurryScan, Saint Charles, IL) to quickly scan the beam along a line. The pulse duration of the laser was less than 30 ns and supplied pulse energy of approximately 150 microjoules. The images of root cross sectional surface were captured using a Canon EOS Rebel T3i 18 Mp camera with 65mm MP-E 1-5X variable magnification macro lens (Canon USA Inc., Melville, NY) to image each subsequent laser-illuminated slice allowing for a maximum theoretical resolving power of 800nm pixel\(^{-1}\).

**Quantification of root anatomical traits**

Root anatomical traits were quantified using a semi-automated image analysis program RootScan (Burton et al., 2012). RootScan separates different type of root tissues by using pixel thresholds. Primary measurements include area and count variables. The following measurements were made via pixel-counting: root cross sectional area (rxsa), aerenchyma area (aa), total stele area (tsa), total xylem vessel area (metva), median individual metaxylem area (medmetva). Pixel values were converted to mm or mm\(^2\), based on micrometer calibration (1173 pixels/linear mm). Cell size was estimated by median of cell objects within five equal radial bands of cortex region.
Mean of median cell size of the five bands (meancs) and mean of median cell size of three middle bands (meancsmidcs) represented cell size of root cross section in this study. Count data included number of cortical cells (ccc), cortical cell files (ccfn), and number of meta xylem vessels (nometv). Diameter traits including median individual metaxylem diameter (medmetvd), root cross sectional diameter (rxsd), and stele diameter (sd), were measured independently from area traits. Some of these primary measurements were used to calculate secondary measurements in RootScan: total cortical area (tca: rxs – tsa), percent aerenchyma (ratorxs: aa x 100/rxs, rcatotca: aa x 100/tca), cortical cell area (cca: non aa objects-noise), living cortical area (lca: tca-aa), ratios of stele to cross section area and stele to cortex area (tcatele), and water conductance of metaxylem (wmetv: the sum over all the fourth power of meta xylem vessel radii). All together 20 root anatomical traits were evaluated in this study.

**Statistical analysis**

Statistical analyses were performed using R version 2.15.1 (R Development Core Team, 2012). Linear mixed effect models were fit using the function lmer from the package lme4 (Maechler and Matrix, 2013). Allometric analysis was performed by plotting a linear regression of the logarithm of each trait against the logarithm of plant size derived from the multiplication of plant height and stem diameter, and recording the coefficient of determination ($R^2$) and the slope of the regression line (allometric scaling exponent, $\alpha$) (Niklas, 1994). Variation within each trait and among years was assessed using two-way analysis of variance. Repeatability in the NAM were calculated for each trait as:

$$R^2 = \frac{\sigma^2(G)}{\sigma^2(E) + \sigma^2(G)}$$

where $\sigma^2(G)$ is the genotypic variance and $\sigma^2(E)$ is the error variance (Fehr, 1987).

Broad-sense heritability ($H^2$) on an entry mean basis was calculated for each trait as:
where $\sigma^2(G)$ is the genotypic variance, $\sigma^2(GY)$ is the genotype by year variance, $\sigma^2(E)$ is the error variance, $r$ is the number of replicates per year, and $y$ is the number of year (Fehr, 1987)

A Pearson correlation and a principal component analysis were performed on the 3-year mean values of data for all the traits. Prior to the Genome-wide association analysis, square root transformation was performed on aa. Traits: cca, meansall, meansmidcs, medmtva, medmetvd, metva, nometv, and rxsa, were power-transformed using the lambda identified by Box-cox transformations.

### Genome-Wide Association Analysis

Genome-Wide Association Study (GWAS) was performed using a set of 438,222 SNPs derived from the RNAseq. GWAS was performed with the Genomic Association and Prediction Integrated Tool (GAPIT) package in R (Lipka et al., 2012). A Mixed Linear Model (MLM) was performed with no compression (Yu et al., 2006). The model could be described using the following equation:

$$y = X\beta + Wm + Qv + Zu + e$$

where $y$ is a vector of phenotypic observations; $\beta$ is a vector of unknown fixed effects other than the SNP under testing, $m$ is a vector of fixed marker effect (e.g. SNP), $v$ is a vector of subpopulation effects, $u$ is a vector of unknown random effects, $e$ is a vector of residual effects. $Q$ is an incidence matrix of principal component scores (eigenvectors) of marker-allele frequencies (Patterson et al., 2006). $X$, $W$ and $Z$ are incidence matrices of ones and zeros relating $y$ to $\beta$, $m$ and $u$, respectively. The covariance of $u$ is equal to $KVA$, where $K$ is the kinship matrix that was
estimated with a random set of SNPs according to the VanRaden method (VanRaden, 2008) and VA is the additive variance estimated with restricted maximum likelihood (REML). The kinship matrix estimation and the principal component (PC) analysis were performed with the GAPIT package. The optimum number of PCs/Covariates to include for each phenotype was determined by forward model selection using the Bayesian information criterion (BIC).

Differential stringency levels of the significance threshold were used in this study. To account for multiple testing, we used the SimpleM method, which applies a Bonferroni correction to the actual number of the effective number of independent tests which was calculated by considering the linkage disequilibrium between each pair of markers and applying principal component analysis to obtain the eigenvalues (Gao et al., 2008). The effective number of independent test corresponds to the number of eigenvalues necessary to explain 99.0% of the variance. In this study, the independent number of test was 172,573. The genome-wide threshold using the modified Bonferroni is 0.05/172,573 i.e. 2.9 x 10^{-7} for high stringency (ae = 0.05) and 0.1/172,573 i.e. 5.8 x 10^{-7} (ae = 0.1) for lower stringency threshold level. The effective number of tests per chromosome was used to determine the chromosome-wide threshold, which varied among chromosomes (Table 5.8). The minimum threshold of 1 x 10^{-4} was used as a baseline for SNP selections for each trait.

Functional annotations were explored using MapMan (Klie and Nikoloski, 2012) and maize genetics and genomics database (www.maizegdb.org). Expression pattern of gene models associated with the significant SNPs and their adjacent genes were examined using a comprehensive atlas of global transcription profiles across developmental stages and plant organs database (Sekhon et al., 2011).
Results

Phenotypic variation of root anatomical traits

Genotypes are significantly different in all root anatomical traits observed in this study. The minimum, median, mean, maximum, and range of each the traits is summarized in Table 5.2. In general, variation of the anatomical traits ranged from 2-fold for diameter traits including root cross sectional diameter (rxsd), stele diameter (sd), and median meta xylem diameter (medmetvd) to 64-fold for aerenchyma area (aa). The mean of cell size in the middle cortex (meancsmidcs) was slightly greater than the mean of cell size in the whole cortex (meancsall). Repeatability, broad-sense heritability ($H^2$), and ANOVA summaries for each trait are presented in Table 5.3. Among anatomical traits observed, cortical traits had relatively low repeatability and $H^2$ whereas the ratio of tca to tsa (tcatostele), aerenchyma and xylem traits had high repeatability and $H^2$ (Table 5.3).

Correlation among root anatomical traits was examined using Pearson correlation analysis (Table 5.4). In general, most traits were positively correlated with size of the root cross section. Area and diameter traits were highly correlated, for example root cross sectional area and diameter (0.78), total stele area and stele diameter (0.99), and median meta xylem area and diameter (0.97). Aerenchyma area was moderately correlated with root cross sectional area (0.54) and total cortical area (0.55) while percentage of aerenchyma was weakly correlated with cortical and stele traits with moderate negative correlations with cell size (-0.36 to -0.40).

The normality of distribution of each trait was assessed using Shapiro-Wilk normality test and the pattern of distribution was indicated by skewness and kurtosis values (Figure 5.2 and Table 5.5). The distribution pattern of most traits was close to normal or slightly left-skewed except for reatotca and rxsd, which had normal distribution. Among all the traits, the skewness value was greatest in wmetv followed by cca, tcatostele, and ccc. The distribution of all the traits
was leptokurtic (having a more acute peak around the mean and longer tail) with wmetv, tcatostele, medmetva, cca, and lca having the highest positive kurtosis values.

Relationships between root anatomical traits and plant size were observed and defined as either isometric or anisometric relationship (Table 5.6). Isometric relationships are those in which growth of a tissue is proportionate to increases in plant size. Anisometric relationships are those in which growth is not proportionate to increases in plant size. Based on comparison to plant size, relationships are considered to be isometric when scaling exponents ($\alpha$) are 0.5 for traits with linear dimension such as diameter, counts, percentages, and ratios, and 1 for area traits. Scaling exponents that deviate from these values indicate anisometric relationships. For all area traits, scaling exponent values were lower than the expected isometric value (1). For linear dimension traits, scaling exponent values of most traits were lower than the expected isometric value (0.5) except for those of cortical cell size.

Principal component analyses (PCA) were performed on the mean values of root anatomical traits across 3 years of data (Table 5.7). PCA showed that three principle components (PC) contributed to 68.8% of variation of root anatomical traits (Table 5.7). PC1, the largest, is primarily size of root cross-section, cortex, and stele. PC 2 is aerenchyma traits and cell size. PC3 is cortical cell component and the ratio of total cortical area to total stele area. Biplot of PC1 and PC2 showed that vectors of root anatomical traits were clustered into at least 4 groups according to size and direction of vectors (Figure 5.3). Traits that contributed mostly to PC1 clustered together in one group while traits in PC2 clustered on the opposite direction. PC3 traits could be separated into two groups: one with cortical cell area and cortical cell count and the other with the ratio of total cortical area to total stele area (Figure 5.3).
**Genome-wide association study of root anatomical traits**

Genome-wide association study (GWAS) of root anatomical traits was performed on mean and Best Linear Unbiased Prediction values (BLUP) of 3-year anatomical data using a set of 438,222 SNPs derived from RNA-seq. We found several significant SNPs associated with root anatomical traits. In summary, a total of 1140 SNPs were identified based on the minimum threshold value of $10^{-4}$. Among these SNPs, 59 SNPs passed the chromosome-wide thresholds (Table 5.9). Search for functional annotations of the SNPs indicated that majority of the detected SNPs correspond to hypothetical and uncharacterized proteins (Figure 5.3). When the SNPs were characterized for their functions based on Mapman database, we found that DNA and RNA regulation are the most prominent functions followed by metabolism and calcium signaling (Figure 5.4).

A total of 24 significant SNPs were identified on chromosomes 2, 3, 4, 6, 8 and 9 for cortical traits including cortical cell area, cortical cell count, cortical cell file number, living cortical area, cell size, area and diameter of root cross section, total cortical area, and the ratio of total cortical area to total stele area (Table 5.10). Three SNPs, rna2_4582259, rna2_4582471, and rna9_105528211, were identical between area and diameter of root cross section. The SNP, rna6_143357365, was identified for both cell size in the middle cortex and across the cross section. Some SNPs lie within the same gene model such as those for cortical cell file number on chromosome 6 and for area and diameter of root cross section on chromosome 2 and 9. Interestingly, the SNP, rna2_4582471 on chromosome 2 is located 33 kb upstream of *GRAS35* (GRMZM2G386362) a gene in GRAS transcription factor family which is *SCARECROW* homolog in rice (Figure 5.5a). The SNP, rna6_158225770, associated with variation in root cross-sectional diameter, is highly expressed in primary root of 6 day-old greenhouse-grown maize seedlings (Sekhon et al., 2011). This SNP corresponds to a probable *XYLOGLUCAN GLYCOSYLTRANSFERASE-7* (GRMZM2G074792) controlling cellulose synthesis (Figure 5.5a).
One SNP associated with cell size on chromosome 6, rna6_143357365, lies 39 kb downstream of GRMZM2G014300, which is homologous to \textit{SUPERSENSITIVE TO ABA AND DROUGHT 1 (SADI)} in \textit{Arabidopsis} (Figure 5.5b). The gene is also highly expressed in maize roots (Sekhon et al., 2011).

Three significant SNPs for aerenchyma traits, including area and percent aerenchyma, were found on chromosome 2 and 6. The SNP on chromosome 6 associated with variation in aerenchyma area, rna6_156217957, is located 167 kb downstream of GRMZM2G175870 encoding basic region/leucine zipper motif (bZIP) transcription factors (Figure 5.6a). The other two SNPs located on chromosome 2 are highly expressed in maize roots and lie within the same gene model, GRMZM2G083504, encoding Basic Helix-Loop-Helix (bHLH) transcription factors. In addition the marker rna1_188071906 identified with the threshold of $10^{-4}$ lies within a gene model AC234203.1_FGT011 encoding \textit{ETHYLENE-INSENSITIVE 3 LIKE (EIL3)}, which may be involved in ethylene signaling during aerenchyma formation (Figure 5.6b).

A total of 32 significant SNPs for stele and xylem traits were found on almost all chromosomes except on chromosome 1. There were no identical SNPs among the traits but some SNPs of the same trait lie within the same gene model such as rna7_121185578 and rna7_121186168 for meta xylem vessel number and rna10_88758826 and rna10_88760141 for the water conductance parameter. We found genes involving in cell wall synthesis such as \textit{XYLOGLUCAN GLYCOSYLTRANSFERASE-7} (GRMZM2G074792) for median meta xylem area (Figure 5.7a). In addition, some of the significant SNPs for the water conductance parameter are located close to water stress related genes such as \textit{RNA RECOGNITION WATER STRESS PROTEIN 1} (GRMZM2G019919) and \textit{SADI} (Figure 5.7b).
Discussion

In this present study, we examined phenotypic variation for 20 root anatomical traits and identified markers associated with the variation of the traits in 503 diverse inbred maize lines from the Wisconsin diversity panel (WiDiv). This panel represents a large variety of maize (Zea mays subsp. Mays) including non-stiff stalk synthetic, stiff stalk synthetic, tropical, subtropical, sweet corn, and popcorn (Hansey et al., 2011). GWAS revealed 1140 significant SNPs associated with root anatomical traits. The expression pattern and gene models around these significant SNPs were also examined.

Phenotypic variation in root anatomical traits

The results of this present study show that considerable variation exists for root anatomical traits in the WiDiv. Variation in cortical traits are particularly interesting because the cortex often comprises a major portion of the maize root (Figure 5.1) (Esau, 1977). Number and size of cortical cells contribute to variation in root thickness and help determine root metabolic costs. In maize, cortical cell size varies along the files and is the same within the radial ring creating a ring pattern that can be visually phenotyped. We found that the maximum value of root diameter was twice as great as the minimum value (Table 5.2). Variation in root diameter has been correlated with elongation rate and sink strength of roots (Cahn et al., 1989; Thaler & Pages, 1996). Large diameter roots have been associated with enhanced root penetration into compacted subsoil in monocots and dicots (Materechera et al., 1992) and have been proposed to be used in plant breeding for drought tolerance in rice (Zheng et al., 2000). However under suboptimal availability of water and nutrients, thin or small diameter roots would be beneficial by reducing the metabolic cost of constructing and maintaining these roots (Eissenstat, 1992). In addition, substantial variation found in living cortical area, cell size, number of cortical cells, and cortical
cell file number could have important consequences for root metabolic costs. For example, decreased living cortical area is associated with reduced root respiration (Jaramillo et al., 2013). Larger cells have a smaller ratio of cytoplasmic to vacuolar volume and thus reduced respiratory and nutrient requirements for root maintenance on a volume basis. Based on observed phenotypic variation, and potential functional significance, agronomic utility of these cortical traits merit further investigation.

Among cortical traits observed, the largest range of variation was found in aerenchyma area (Table 5.2). Aerenchyma is an important trait for plant breeding since it enhances tolerance to water logging by facilitating shoot-root oxygen transportation and maintaining aerobic conditions in the rhizosphere (Jackson and Armstrong, 1999). Plants also benefit from aerenchyma by reduced root metabolic cost and by nutrients reabsorbed from lysing tissues under drought, and suboptimal availability of nutrients (Fan et al., 2003; Zhu et al., 2010; Postma and Lynch, 2011). It has been documented that aerenchyma formation in maize is induced by flooding and edaphic stresses (Drew et al., 1989; Jackson and Armstrong, 1999; Drew et al., 2000; Bouranis et al., 2003). Attempts to breed for aerenchyma are being done by interspecific introgression between teosintes and maize (Mano & Omori, 2013). Interestingly variation in aerenchyma formation found in this study occurred in field grown plants under well-drain and fertile conditions. This result is consistent with those found in greenhouse-grown plants (Burton et al., 2013a; Burton et al., 2013b). Some genotypes in this study exhibited a high value of aerenchyma area as a percent of cortical area (49.9%) compared to those reported in literature (37.8% Burton et al., 2013; 30% Burton et al., 2012). These results suggest that considerable variation for aerenchyma exists in non-stressed maize and introgression for aerenchyma traits can be carried out within maize.

Stele traits are very important for the absorption and translocation of water. Number and size of xylem elements in the root are directly associated with water transport from root to shoot. In this study, the potential effect of differences in xylem diameter on hydraulic conductance per
vessel as reflected in the theoretical water conductance calculated with the Poiseuille–Hagen law was 16-fold (Tombesi et al., 2010). While large xylem vessels increase root water uptake, small xylem diameter increases root hydraulic resistance allowing plants to use water more efficiently as well as resistant to cavitation, and thus could be utilized in breeding for drought tolerance (Richards and Passioura, 1989; Richards et al., 2010).

Several root anatomical traits were highly correlated (Table 5.4) and clustered together in PCA (Figure 5.3). Generally, size-related traits such as area and diameter of the same tissue were highly correlated. Percentage of aerenchyma was highly correlated with aerenchyma area but weakly correlated with cortical and stele traits with moderate negative correlations with cell size (-0.36 to -0.40). Aerenchyma formation would be expected to affect number of cells and median value of cell size across root cortex since aerenchyma in maize begins to form in the middle cortical region where large cells are located. Therefore, breeders should take aerenchyma traits into account when selecting for reduced root metabolic costs using cell size and number of cortical cells. Trait correlations also indicate that our measurements could be redundant and thus efficiency of phenotyping could be improved by eliminating such redundant traits. However removing redundant traits should be done with caution because different measurements of the same tissue give distinct types of information. For example, while aerenchyma area and percent of aerenchyma are highly correlated and both indicate quantity of air space in the root, percent of aerenchyma takes cross sectional or cortical area into account. Thick roots may result in low percentage of aerenchyma but large aerenchyma area. In addition, correlations among traits could simplify phenotyping for selection in breeding. Weak correlations as seen between percentage of aerenchyma and most anatomical traits suggest that it could be possible to alter percent aerenchyma with minimal concomitant effects on the other traits.

Allometry indicates relationships between the growth of tissues or organs and increases in size if an organism (Niklas, 1994). In this study, we found that most traits had insignificant relationships with plant size except for cortical cell area, cortical cell count, cortical cell size, and
area and diameter of meta xylem vessels as shown (Table 5.6). This is very important since introgression of new traits to different maize cultivars could be complicated if the traits were influenced by plant size. For those with significant scaling exponent, the majority of the traits had the scaling exponent values lower than the expected isometric values except for cortical cell size. Based on these, as plant size increases, the proportion of these traits would be lesser than that predicted with isometric growth with an exception for cortical cell size in which the proportion would be similar to the predicted value.

Success in selection for complex traits can be achieved by determining strategies for selection that allows maximum genetic gain with use of minimum time and resources. Plant breeders use heritability estimates as guidelines to develop effective breeding strategies (Smalley et al., 2004). In our study, we examined repeatability and broad-sense heritability estimates ($H^2$) across 3 years for root anatomical traits. Root thickness and area of cortex had the lowest repeatability with moderate to low $H^2$. These traits are integrated traits consisting of cortical components such as cell size, number of cells, and number of cell files. Small changes in these components may dramatically affect size and area of root resulting in large variations between the two replicates within a year. Among the cortical components, cortical cell area had the lowest $H^2$ across 3 years. This is not surprising since genotypic variation of the traits across 3 years were only marginally significant and effects of environment and interactions between genotype and environment were significant (Table 5.3). Alternatively, low $H^2$ could be caused by inaccuracy of measurement methods and thus reflect a need to improve the method. High repeatability and $H^2$ as seen in aerenchyma and xylem traits indicates that variation in these traits is influenced mostly by genetic components and can be transmitted to succeeding generations. These traits are therefore good candidates for plant breeding.
Genetic control of root anatomical traits

Quantitative genetic study of root anatomical traits is usually limited to small sample size and few repetitions due to difficulties in evaluating a large number of genotypes. Most research is usually focused on one trait and phenotypic evaluations are simplified by ranking instead of quantitative measurements (Mano et al., 2007). Even though these methods have been successfully used to identify QTLs controlling anatomical traits such as aerenchyma, obtaining actual quantitative measurements of the traits should allow better phenotypic precision and may increase the likelihood of finding molecular markers that could be used in breeding. In addition, the ability to simultaneously phenotype multiple root anatomical traits from cross sections would allow the study of interactions between root traits.

A search for functional annotations and expression profiles of significant SNPs showed that several SNPs and their related genes were highly expressed in primary roots of maize seedlings (Sekhon et al., 2011), and that functions of some of the SNPs are potentially important for development of root anatomical traits. For example, one of significant SNPs associated with root cross sectional area is located upstream of GRAS-TRANSCRIPTION FACTOR-35 gene (GRMZM2G386362) in GRAS family. GRAS proteins are unique to plants and play important role in diverse processes such as gibberellin signal transduction, axillary meristem initiation, shoot meristem maintenance, phytochrome A signal transduction, and root radial patterning (Pysh et al., 1999). GRAS35 is homologous to SCARECROW (OsSCR; LOC_Os10g40390.1) in rice and ARABIDOPSIS HARRY MERISTEM 3 (AtHAM3; AT4G00150.1). The SCARECROW (SCR) gene is required for asymmetric cell divisions responsible for ground tissue formation of the root and shoot in Arabidopsis, maize and rice (Di Laurenzio et al., 1996; Kamiya et al., 2003; Lim et al., 2005). In Arabidopsis scr-1 mutants, cortex and endodermis are substituted with a single cell layer with mixed identity (Di Laurenzio et al., 1996). SCR is also expressed in the quiescent center, which may help maintain the stem cell identity of the surrounding initial cells (Sabatini et
Another homolog of GRAS35 is AtHAM. AtHAM was found to be involved in indeterminacy maintenance in shoot and root (Engstrom et al., 2011). In root, AtHAM is expressed in the meristem region including quiescent center, cortex/endodermal initials, and endodermis, cortex, and stele cell files as well as in differentiating and mature tissues especially in trichoblast, root hair forming epidermal cells. Mutations in AtHAM orthologs (Atham1,2,3) cause abnormalities in root development including reduced rates of cell division in root meristems, root bifurcation, and loss of indeterminacy. The Atham mutants had smaller root meristem diameter and fewer number of cells in radial tissue layers compared to wildtype (Engstrom et al., 2011). Although scr and ham mutants in maize and rice have not been reported, results of expression study and molecular complementation of SCR (Kamiya et al., 2003; Lim et al., 2000) and phylogenetic study of HAM (Engstrom et al., 2011) suggest that their functions may be similar in maize and rice.

For aerenchyma traits, QTLs for constitutive aerenchyma formation have been identified on chromosome 1, 5, and 8 in maize× Zea nicaraguensis mapping populations, (Mano et al., 2007; Mano & Omori, 2008). Additionally QTLs for aerenchyma traits were identified on chromosome 1,2,4,7, 8 and 9 in maize recombinant inbred populations by Burton et al. (2010). The authors reported one marker associate with a gene for peroxidase production located chromosome 2, which may be involved with program cell death in maize (Burton et al., 2010). No other genes of known association with aerenchyma formation in non-stress environments were reported in these studies. Here we have identified several significant SNPs for aerenchyma traits. Some of these are related with program cell death and ethylene signaling, which are known aspects of aerenchyma formation such as genes encoding Basic Helix-Loop-Helix (bHLH) and Basic region/leucine zipper motif (bZIP) transcription factors and EIL proteins. bHLH transcription factors belong to a family of transcriptional regulators found in eukaryotes. In animals, several functions of the bHLH family have been identified including control of cell proliferation and development of specific cell linages. However little is known about their roles in
plants. In maize a group of bHLH proteins interact with other groups of transcription factors such as R2R3-MYB proteins controlling anthocyanin production. In plants, bZIP transcription factors regulate processes including defense against pathogen, light and stress signalling, seed maturation and flower development. To date functions of bHLH and bZIP proteins in aerenchyma formation have not been reported. Interestingly, transcription modules composed of bHLH and bZIP transcription factors has been shown to regulate cell death and photooxidative response in Arabidopsis (Chen et al., 2013). In addition, bHLH proteins have been shown to be involved in programmed cell death of fat bodies in Drosophila (Liu et al., 2009) and of rice tapetum during late pollen development (Li et al., 2006). This evidence suggests that these transcription factors could be involved in aerenchyma formation. Another annotated gene found in this study is ETHYLENE INSENSITIVE 3-LIKE (EIL3). EIL3 belongs to the EIN3 family in Arabidopsis, which activates other transcription factors such as ethylene response factors (ERFs) and ethylene responsive DNA binding factors (EDFs) thereby regulating the expression of genes involved in the response to ethylene (Potuschak et al., 2003). The role of ethylene in aerenchyma formation in plant roots is well documented, therefore EIL3 could be a good candidate for further research (Evans, 2003; Gunawardena et al., 2001; He et al., 1996).

While studies in Arabidopsis have suggested genetic models and genes involved in xylem development of dicots, very few studies have been done in monocots. Recent studies using QTL analysis have identified QTLs for stele and xylem traits in rice and maize (Burton et al., 2010; Uga et al., 2008). The study in maize using recombinant inbred populations revealed four QTLs for stele area on chromosome 2,8,5 and 9 and five QTLs for xylem vessel area on chromosome 1,3, and 5. However no previously identified or candidate genes have been reported (Burton, 2010). In this present study, significant SNPs were found on almost all chromosomes except on chromosome 1. One of the SNPs for median meta xylem area, corresponding to XYLOGLUCAN GLYCOSYLTRANSFERASE-7 (GRMZM2G074792) was found on chromosome 6. This gene is also highly expressed in maize root seedlings. XYLOGLUCAN GLYCOSYLTRANSFERASE-7 is
involved in cell wall polysaccharide synthesis and modification (Reiter et al., 1997). Mutations in *Arabidopsis* cause alterations in cell wall monosaccharide compositions and a dwarfed phenotype (Reiter et al., 1997). Interestingly, the SNP for root cross-sectional diameter is also located close to this SNP. This could be expected since a significant positive correlation was found between the two traits. This result means that plant breeders could use root diameter or thickness as a selection index for xylem size. Selection for root thickness can be done without expensive lab equipment and thus accessible to farmers around the world. This method has been suggested in rice breeding for drought tolerance (Yambao et al., 1992).

Prior to this study, much research on genetic control of root traits has been carried out in artificial growth systems such as hydroponic and sand culture (Uga et al., 2008; Gaudin et al., 2011; Uga et al., 2011; Mace et al., 2012; Burton et al., 2013a). Although such research could provide useful information, the results may or may not be able to apply to plant breeding in actual agronomic systems due to the interactions between environment and genetic components. Therefore, the results of this present study are critical since the experiments were carried out in the field across multiple years.

While utilities of some of the traits observed have been reported in the literature (Richards and Passioura, 1989; Yambao et al., 1992; Zhu et al., 2010), some traits such as cortical cell file number (ccfn) and cell size (meancsall and meancsmidcs) are novel and could be important for plant breeding. Additionally, this study is the first to identify markers and candidate genes underlying variation in root anatomical traits in field-grown maize. Future research is needed to examine the potential of significant SNPs markers to be used in plant breeding using marker-assisted selection and to validate the effects of candidate genes and to understand the genetic control of root anatomical traits in maize.
Table 5.1: Root anatomical traits examined in this study.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Summation of all aerenchyma areas</td>
<td>mm²</td>
</tr>
<tr>
<td>cca</td>
<td>Summation of cortical cell area (tca-aa-cell wall area)</td>
<td>mm²</td>
</tr>
<tr>
<td>ccc</td>
<td>Total number of cells in cortical region</td>
<td>count</td>
</tr>
<tr>
<td>ccfn</td>
<td>Total number of radial cortical cell files</td>
<td>count</td>
</tr>
<tr>
<td>lca</td>
<td>Living cortical area (tca-aa)</td>
<td>mm²</td>
</tr>
<tr>
<td>meansall</td>
<td>Mean of median cell size in all cortical regions</td>
<td>mm</td>
</tr>
<tr>
<td>meansmides</td>
<td>Mean of median cell size in middle cortical regions</td>
<td>mm</td>
</tr>
<tr>
<td>rcatorxs</td>
<td>Root cortical aerenchyma as a percentage of root cross-sectional area</td>
<td>%</td>
</tr>
<tr>
<td>rcatotca</td>
<td>Root cortical aerenchyma as a percentage of total cortical area</td>
<td>%</td>
</tr>
<tr>
<td>rxsa</td>
<td>Root cross-sectional area</td>
<td>mm²</td>
</tr>
<tr>
<td>rxsd</td>
<td>Mean cross-section diameter that considers root cross section asymmetry</td>
<td>mm</td>
</tr>
<tr>
<td>tca</td>
<td>Total area of cortical region</td>
<td>mm²</td>
</tr>
<tr>
<td>tcatostele</td>
<td>Ratio between tca and tsa</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>sd</td>
<td>Mean stele diameter that consider root cross-section asymmetry</td>
<td>mm</td>
</tr>
<tr>
<td>tsa</td>
<td>Total area of stele region</td>
<td>mm²</td>
</tr>
<tr>
<td>medmetva</td>
<td>Median area of all meta xylem vessel</td>
<td>mm²</td>
</tr>
<tr>
<td>medmetvd</td>
<td>Median diameter of all meta xylem vessel</td>
<td>mm</td>
</tr>
<tr>
<td>metva</td>
<td>Summation of all meta xylem vessel areas</td>
<td>mm²</td>
</tr>
<tr>
<td>nometv</td>
<td>Total number of meta xylem vessels in stele</td>
<td>count</td>
</tr>
<tr>
<td>wmetv</td>
<td>Water flux parameter (Meta xylem vessels)</td>
<td>m⁴</td>
</tr>
</tbody>
</table>
Figure 5.1: Cross-section of a second whorl crown root from a field-grown maize plant at flowering stage obtained from laser ablation. Arrows and lines indicates aerenchyma lacunae, meta xylem vessels, stele, cortex, edodermis, and epidermis.
Table 5.2: Phenotypic variation of root anatomical traits for the Wisconsin diversity panel. Minimum, median, mean, and maximum values were based on data across 3 years.

### Cortical and aerenchyma traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Minimum</th>
<th>Median</th>
<th>Mean</th>
<th>Maximum</th>
<th>Range</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>0</td>
<td>0.17</td>
<td>0.18</td>
<td>0.64</td>
<td>64x</td>
<td>mm²</td>
</tr>
<tr>
<td>cca</td>
<td>0.06</td>
<td>0.35</td>
<td>0.38</td>
<td>1.61</td>
<td>29x</td>
<td>mm²</td>
</tr>
<tr>
<td>ccc</td>
<td>222</td>
<td>637.92</td>
<td>687.49</td>
<td>1669.67</td>
<td>8x</td>
<td>count</td>
</tr>
<tr>
<td>ccfn</td>
<td>5</td>
<td>8.89</td>
<td>8.89</td>
<td>12.67</td>
<td>3x</td>
<td>count</td>
</tr>
<tr>
<td>lea</td>
<td>0.32</td>
<td>0.7</td>
<td>0.73</td>
<td>1.71</td>
<td>5x</td>
<td>mm²</td>
</tr>
<tr>
<td>meanesall</td>
<td>11</td>
<td>225</td>
<td>221</td>
<td>558</td>
<td>51x</td>
<td>µm</td>
</tr>
<tr>
<td>meanesmides</td>
<td>11</td>
<td>224</td>
<td>228</td>
<td>657</td>
<td>63x</td>
<td>µm</td>
</tr>
<tr>
<td>rcatorxs</td>
<td>0</td>
<td>15.02</td>
<td>15.40</td>
<td>41.01</td>
<td>41x</td>
<td>%</td>
</tr>
<tr>
<td>rcatotca</td>
<td>0</td>
<td>19.24</td>
<td>19.36</td>
<td>49.87</td>
<td>50x</td>
<td>%</td>
</tr>
<tr>
<td>rxs</td>
<td>0.5</td>
<td>1.15</td>
<td>1.16</td>
<td>2.37</td>
<td>5x</td>
<td>mm²</td>
</tr>
<tr>
<td>rxsd</td>
<td>0.8</td>
<td>1.2</td>
<td>1.20</td>
<td>1.74</td>
<td>2x</td>
<td>mm</td>
</tr>
<tr>
<td>tca</td>
<td>0.41</td>
<td>0.91</td>
<td>0.93</td>
<td>1.92</td>
<td>5x</td>
<td>mm²</td>
</tr>
<tr>
<td>tcatotstele</td>
<td>1.9</td>
<td>3.71</td>
<td>3.83</td>
<td>9.14</td>
<td>5x</td>
<td>-</td>
</tr>
</tbody>
</table>

### Stele and xylem traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Minimum</th>
<th>Median</th>
<th>Mean</th>
<th>Maximum</th>
<th>Range</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>sd</td>
<td>0.35</td>
<td>0.55</td>
<td>0.56</td>
<td>0.81</td>
<td>2x</td>
<td>mm</td>
</tr>
<tr>
<td>tsa</td>
<td>0.09</td>
<td>0.24</td>
<td>0.25</td>
<td>0.51</td>
<td>5x</td>
<td>mm²</td>
</tr>
<tr>
<td>medmetva</td>
<td>0.002778</td>
<td>0.006005</td>
<td>0.006148</td>
<td>0.013696</td>
<td>5x</td>
<td>mm²</td>
</tr>
<tr>
<td>medmetvd</td>
<td>0.057253</td>
<td>0.088078</td>
<td>0.088674</td>
<td>0.134755</td>
<td>2x</td>
<td>mm</td>
</tr>
<tr>
<td>metva</td>
<td>0.021181</td>
<td>0.055855</td>
<td>0.057356</td>
<td>0.137727</td>
<td>7x</td>
<td>mm²</td>
</tr>
<tr>
<td>nometv</td>
<td>5.33</td>
<td>9.5</td>
<td>9.77</td>
<td>15</td>
<td>3x</td>
<td>count</td>
</tr>
<tr>
<td>wmetv</td>
<td>6.95E-18</td>
<td>4.13E-17</td>
<td>4.7545E-17</td>
<td>2.18E-16</td>
<td>31x</td>
<td>m⁴</td>
</tr>
</tbody>
</table>
Table 5.3: Repeatability and broad-sense heritability ($H^2$) of root anatomical traits for the Wisconsin diversity panel. Asterisks and dot represent significant levels of genotype (G), interaction between genotype and year (GxE), year (E), and interaction between replicates and year (RxE) from analysis of variance analysis: p<0.1 (.), p<0.05 (*), p<0.001(**), and not significant (ns).

<table>
<thead>
<tr>
<th>Year</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>3 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trait</td>
<td>Repeatability G</td>
<td></td>
<td>Repeatability</td>
<td></td>
</tr>
<tr>
<td>aa</td>
<td>0.38 *</td>
<td>0.45 **</td>
<td>0.43 **</td>
<td>0.50 **</td>
</tr>
<tr>
<td>cca</td>
<td>0.33 *</td>
<td>0.00 ns</td>
<td>0.44 **</td>
<td>0.32 .</td>
</tr>
<tr>
<td>ccc</td>
<td>0.51 *</td>
<td>0.53 .</td>
<td>0.47 **</td>
<td>0.38 *</td>
</tr>
<tr>
<td>cfn</td>
<td>0.49 .</td>
<td>0.18 .</td>
<td>0.24 .</td>
<td>0.43 **</td>
</tr>
<tr>
<td>lca</td>
<td>0.33 *</td>
<td>0.03 ns</td>
<td>0.31 **</td>
<td>0.38 **</td>
</tr>
<tr>
<td>meanesall</td>
<td>0.27 ns</td>
<td>0.29 .</td>
<td>0.41 **</td>
<td>0.30 *</td>
</tr>
<tr>
<td>meanesmides</td>
<td>0.26 ns</td>
<td>0.34 **</td>
<td>0.34 *</td>
<td>0.22 *</td>
</tr>
<tr>
<td>rcatom</td>
<td>0.22 ns</td>
<td>0.07 ns</td>
<td>0.13 ns</td>
<td>0.38 **</td>
</tr>
<tr>
<td>rxs</td>
<td>0.32 *</td>
<td>0.07 ns</td>
<td>0.14 ns</td>
<td>0.40 **</td>
</tr>
<tr>
<td>rxs</td>
<td>0.32 *</td>
<td>0.07 ns</td>
<td>0.15 ns</td>
<td>0.37 **</td>
</tr>
<tr>
<td>tca</td>
<td>0.35 **</td>
<td>0.34 **</td>
<td>0.58 **</td>
<td>0.67 **</td>
</tr>
<tr>
<td>sd</td>
<td>0.52 **</td>
<td>0.26 *</td>
<td>0.33 **</td>
<td>0.58 ns</td>
</tr>
<tr>
<td>tsa</td>
<td>0.55 **</td>
<td>0.24 *</td>
<td>0.32 *</td>
<td>0.56 **</td>
</tr>
<tr>
<td>medmetva</td>
<td>0.32 *</td>
<td>0.40 **</td>
<td>0.51 **</td>
<td>0.61 ns</td>
</tr>
<tr>
<td>medmetvd</td>
<td>0.40 *</td>
<td>0.42 **</td>
<td>0.51 **</td>
<td>0.55 .</td>
</tr>
<tr>
<td>metva</td>
<td>0.38 *</td>
<td>0.27 *</td>
<td>0.31 *</td>
<td>0.55 ns</td>
</tr>
<tr>
<td>nometv</td>
<td>0.24 ns</td>
<td>0.22 *</td>
<td>0.29 *</td>
<td>0.53 ns</td>
</tr>
<tr>
<td>wmetv</td>
<td>0.20 ns</td>
<td>0.31 *</td>
<td>0.31 *</td>
<td>0.40 **</td>
</tr>
</tbody>
</table>
Table 5.4: Pairwise correlation coefficients among root anatomical traits in the Wisconsin diversity panel. Correlation coefficients greater than 0.50 or lower than -0.50 are highlighted, and statistically significant relationships are indicated by an asterisk (p<0.05).

<table>
<thead>
<tr>
<th>Trait 1</th>
<th>Trait 2</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>cca</td>
<td>0.30</td>
<td>*</td>
</tr>
<tr>
<td>ccc</td>
<td>0.19</td>
<td>*</td>
</tr>
<tr>
<td>ccfn</td>
<td>0.17</td>
<td>*</td>
</tr>
<tr>
<td>lca</td>
<td>0.22</td>
<td>*</td>
</tr>
<tr>
<td>meancsall</td>
<td>-0.36</td>
<td>*</td>
</tr>
<tr>
<td>meancsmidcs</td>
<td>-0.40</td>
<td>*</td>
</tr>
<tr>
<td>medmetva</td>
<td>0.36</td>
<td>*</td>
</tr>
<tr>
<td>medmetvd</td>
<td>0.35</td>
<td>*</td>
</tr>
<tr>
<td>metva</td>
<td>0.44</td>
<td>*</td>
</tr>
<tr>
<td>nometv</td>
<td>0.19</td>
<td>*</td>
</tr>
<tr>
<td>rca</td>
<td>0.86</td>
<td>*</td>
</tr>
<tr>
<td>rcaottc</td>
<td>0.86</td>
<td>*</td>
</tr>
<tr>
<td>rxsa</td>
<td>0.54</td>
<td>*</td>
</tr>
<tr>
<td>rxsd</td>
<td>0.55</td>
<td>*</td>
</tr>
<tr>
<td>sd</td>
<td>0.43</td>
<td>*</td>
</tr>
<tr>
<td>tca</td>
<td>0.55</td>
<td>*</td>
</tr>
<tr>
<td>tcatostele</td>
<td>-0.09</td>
<td>*</td>
</tr>
<tr>
<td>tsa</td>
<td>0.42</td>
<td>*</td>
</tr>
<tr>
<td>wmetv</td>
<td>0.35</td>
<td>*</td>
</tr>
</tbody>
</table>
Table 5.5: Skewness, kurtosis, and p values for distributions of anatomical traits in the Wisconsin diversity panel. Explanations of abbreviations are listed in Table 5.1.

### Cortical and aerenchyma traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Skewness</th>
<th>Kurtosis</th>
<th>Shapiro-Wilk W test</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>0.8267149</td>
<td>1.3169114</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>cca</td>
<td>1.2179458</td>
<td>1.8622145</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ccc</td>
<td>1.0421273</td>
<td>0.9810162</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ccfn</td>
<td>0.0978493</td>
<td>0.7364366</td>
<td>0.0339</td>
</tr>
<tr>
<td>lca</td>
<td>0.889267</td>
<td>1.7491083</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>meancsall</td>
<td>0.0427501</td>
<td>0.4723199</td>
<td>&lt;0.0007</td>
</tr>
<tr>
<td>meancsmidcs</td>
<td>0.3536078</td>
<td>1.0933354</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>reatorxs</td>
<td>0.2241049</td>
<td>0.2917511</td>
<td>0.0308</td>
</tr>
<tr>
<td>reatotea</td>
<td>0.1826812</td>
<td>0.1885634</td>
<td>0.0808</td>
</tr>
<tr>
<td>rxsa</td>
<td>0.5683312</td>
<td>1.0566945</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>rxsd</td>
<td>0.1597844</td>
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<td>0.1315</td>
</tr>
<tr>
<td>tca</td>
<td>0.585038</td>
<td>1.0265671</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>tcato5stele</td>
<td>1.2020135</td>
<td>4.2674106</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

### Stele and xylem traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Skewness</th>
<th>Kurtosis</th>
<th>Shapiro-Wilk W test</th>
</tr>
</thead>
<tbody>
<tr>
<td>sd</td>
<td>0.492183</td>
<td>0.6832166</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>tsa</td>
<td>0.8856657</td>
<td>1.377295</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>medmetva</td>
<td>0.8069888</td>
<td>1.927201</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>medmetvd</td>
<td>0.5318525</td>
<td>1.1414004</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>metva</td>
<td>0.8904773</td>
<td>1.6428205</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>nometv</td>
<td>0.4977126</td>
<td>0.3280969</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>wmetv</td>
<td>2.2815706</td>
<td>7.9803821</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 5.6: Summary of allometric analysis of root anatomical traits showing $R^2$ value and slope of the regression line ("allometric scaling exponent", $\alpha$) for regression of logarithm of each trait against the logarithm of plant size derived from the multiplication of plant height and stem diameter. Based on comparison to plant size, relationships are considered to be isometric when scaling exponents ($\alpha$) are 0.5 for traits with linear dimension and 1 for area traits. “ns” indicates not significant at p=0.05.

**Cortical and aerenchyma traits**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Type</th>
<th>$R^2$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>area</td>
<td>0.000</td>
<td>-0.032</td>
</tr>
<tr>
<td>cca</td>
<td>area</td>
<td>0.017</td>
<td>-0.305</td>
</tr>
<tr>
<td>ccc</td>
<td>linear</td>
<td>0.022</td>
<td>-0.215</td>
</tr>
<tr>
<td>ccfn</td>
<td>linear</td>
<td>0.007</td>
<td>0.033</td>
</tr>
<tr>
<td>lca</td>
<td>area</td>
<td>0.002</td>
<td>0.035</td>
</tr>
<tr>
<td>meansall</td>
<td>linear</td>
<td>0.051</td>
<td>0.489</td>
</tr>
<tr>
<td>meanesmides</td>
<td>linear</td>
<td>0.046</td>
<td>0.512</td>
</tr>
<tr>
<td>rcatorxs</td>
<td>linear</td>
<td>0.001</td>
<td>-0.055</td>
</tr>
<tr>
<td>rcatotca</td>
<td>linear</td>
<td>0.001</td>
<td>-0.045</td>
</tr>
<tr>
<td>rxs</td>
<td>area</td>
<td>0.002</td>
<td>0.031</td>
</tr>
<tr>
<td>rxsd</td>
<td>linear</td>
<td>0.001</td>
<td>0.014</td>
</tr>
<tr>
<td>tca</td>
<td>area</td>
<td>0.001</td>
<td>0.027</td>
</tr>
<tr>
<td>tcatostele</td>
<td>linear</td>
<td>0.005</td>
<td>-0.047</td>
</tr>
</tbody>
</table>

**Stele and xylem traits**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Type</th>
<th>$R^2$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sd</td>
<td>linear</td>
<td>0.005</td>
<td>0.029</td>
</tr>
<tr>
<td>tsa</td>
<td>area</td>
<td>0.005</td>
<td>0.057</td>
</tr>
<tr>
<td>medmetva</td>
<td>area</td>
<td>0.018</td>
<td>0.109</td>
</tr>
<tr>
<td>medmetvd</td>
<td>linear</td>
<td>0.011</td>
<td>0.043</td>
</tr>
<tr>
<td>metva</td>
<td>area</td>
<td>0.014</td>
<td>0.109</td>
</tr>
<tr>
<td>nometv</td>
<td>linear</td>
<td>0.000</td>
<td>-0.002</td>
</tr>
<tr>
<td>wmetv</td>
<td>area</td>
<td>0.006</td>
<td>0.133</td>
</tr>
</tbody>
</table>
Figure 5.2: Phenotypic variation of 20 root anatomical traits in the Wisconsin diversity panel across 3 years.
Figure 5.3: Principle component analysis biplot of 20 root anatomical traits in the Wisconsin diversity panel. The x and y axes are components 1 and 2 respectively. The Biplot shows that vectors of root anatomical traits are clustered into at least 4 groups (red boxes) according to size and direction of vectors.
Table 5.7: Loading matrix from principle component analysis of 20 root anatomical traits in the Wisconsin diversity panel.

<table>
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<tr>
<th>Trait</th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
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<td><strong>Cortical and aerenchyma traits</strong></td>
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<td></td>
<td></td>
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<tr>
<td>aa</td>
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<td>-0.047</td>
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<td>0.389</td>
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<td>0.244</td>
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<td>-0.282</td>
<td>0.262</td>
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<td>-0.397</td>
<td>-0.178</td>
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<tr>
<td>meansmidcs</td>
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<td>-0.393</td>
<td>-0.144</td>
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<td>rcatorxs</td>
<td>-0.133</td>
<td>0.430</td>
<td>-0.135</td>
</tr>
<tr>
<td>rcatotca</td>
<td>-0.140</td>
<td>0.425</td>
<td>-0.174</td>
</tr>
<tr>
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<td>-0.134</td>
<td>0.110</td>
</tr>
<tr>
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<td>-0.128</td>
<td>0.107</td>
</tr>
<tr>
<td>tca</td>
<td>-0.301</td>
<td>-0.118</td>
<td>0.186</td>
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<tr>
<td>tcatostele</td>
<td>0.038</td>
<td>0.027</td>
<td>0.429</td>
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<tr>
<td><strong>Stele and xylem traits</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>sd</td>
<td>-0.303</td>
<td>-0.134</td>
<td>-0.171</td>
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<tr>
<td>tsa</td>
<td>-0.302</td>
<td>-0.138</td>
<td>-0.166</td>
</tr>
<tr>
<td>medmetva</td>
<td>-0.240</td>
<td>0.010</td>
<td>-0.169</td>
</tr>
<tr>
<td>medmetvd</td>
<td>-0.234</td>
<td>0.020</td>
<td>-0.173</td>
</tr>
<tr>
<td>metva</td>
<td>-0.287</td>
<td>-0.083</td>
<td>-0.234</td>
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<tr>
<td>nometv</td>
<td>-0.136</td>
<td>-0.124</td>
<td>-0.166</td>
</tr>
<tr>
<td>wmetv</td>
<td>-0.239</td>
<td>0.000</td>
<td>-0.181</td>
</tr>
</tbody>
</table>

| Standard deviation   | 2.854       | 1.743       | 1.605       |
| Proportion of variance | 0.407     | 0.152       | 0.129       |
| Cumulative proportion | 0.407       | 0.559       | 0.688       |
Table 5.8: Summary of number of imputed SNPs and effective number of test per chromosome. The effective number of test was determined through the SimpleM method and it corresponds to the number of eigenvalues necessary to explain 99.0% of the variance. The sum of the effective number of tests of each chromosome was used to calculate the genome-wide threshold and the effective number per chromosome was used to determine the chromosome-wise threshold.

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<th>Chr.</th>
<th>No. SNPs (imputed)</th>
<th>Effective No. tests</th>
<th>Simple M Threshold</th>
<th>α</th>
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<td>0.05</td>
<td>0.1</td>
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<td>5.43</td>
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<td>5.62</td>
<td>5.32</td>
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<td>48,557</td>
<td>18,778</td>
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<td>5.54</td>
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<td>53,361</td>
<td>20,292</td>
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<td>5.31</td>
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<td>35,890</td>
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<td>5.45</td>
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<td>14,723</td>
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<td>30,087</td>
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<td>5.08</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>438,222</strong></td>
<td><strong>172,573</strong></td>
<td><strong>Genome-wide</strong></td>
<td><strong>6.54</strong></td>
<td><strong>6.24</strong></td>
</tr>
</tbody>
</table>

The effective number of test was determined through the SimpleM method and it corresponds to the number of eigenvalues necessary to explain 99.0% of the variance. The sum of the effective number of tests of each chromosome was used to calculate the genome-wide threshold and the effective number per chromosome was used to determine the chromosome-wise threshold.
Table 5.9: Summary of the number of SNPs identified using the minimum threshold level of p values = $1 \times 10^{-4}$ and chromosome-wide thresholds (refer to Table 5.8).

<table>
<thead>
<tr>
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<th>p= $1 \times 10^{-4}$</th>
<th>Chromosome-wide</th>
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<td></td>
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<td>4</td>
<td></td>
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<td>tca</td>
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<tr>
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<td>4</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>561</strong></td>
<td><strong>24</strong></td>
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<tr>
<td><strong>Aerenchyma traits</strong></td>
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<td>rcatotca</td>
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<tr>
<td><strong>Total</strong></td>
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<tr>
<td>sd</td>
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<td>0</td>
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</tr>
<tr>
<td>tsa</td>
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Figure 5.4: Percentage of ontogenic categories of gene models according to Mapman database for root anatomical traits.
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<th>maf</th>
<th>P.value</th>
<th>Position</th>
<th>Chromosome</th>
<th>SNP</th>
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</thead>
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<td>19</td>
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<td>protein_degradation</td>
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<td>0.226</td>
<td>2.46E05</td>
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<td>16</td>
</tr>
<tr>
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<td>subtilases</td>
<td>0.04</td>
<td>0.226</td>
<td>2.46E05</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
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<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
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<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
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<td>0.009</td>
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<td>14</td>
<td>33</td>
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<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
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<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
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<tr>
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<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
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</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
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<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
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<tr>
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<td>0.009</td>
<td>2.57E02</td>
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<tr>
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<td>14</td>
<td>33</td>
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<tr>
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<td>33</td>
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<tr>
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<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
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<td>0.009</td>
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<td>14</td>
<td>33</td>
</tr>
<tr>
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<td>0.009</td>
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<td>14</td>
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<tr>
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<td>0.009</td>
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<td>14</td>
<td>33</td>
</tr>
<tr>
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<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
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<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
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<td>33</td>
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<td>Kinase VII</td>
<td>nucleotide metabolism</td>
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<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
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<td>nucleotide metabolism</td>
<td>0.00</td>
<td>0.009</td>
<td>2.57E02</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
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<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Kinase VII</td>
<td>nucleotide metabolism</td>
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<td>0.009</td>
<td>2.57E02</td>
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*Note: The P.value column represents the significance level of the association between the trait and the SNP. A lower P.value indicates a stronger association.*
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<thead>
<tr>
<th>Trait</th>
<th>SNP</th>
<th>Chromosome</th>
<th>Position</th>
<th>P.value</th>
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<th>Mapman term</th>
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*SNP* locations correspond to the Chr:1:19205000-19210000 position.
Figure 5.5: Manhattan plots of Genome Wide Association Analysis (GWAS) for (a) root diameter and (b) cell size. Horizontal dashed line is a minimum threshold ($p=1\times10^{-4}$). Selected significant SNPs and adjacent SNPs were highlighted in green and shown with their associated gene models.
Figure 5.6: Manhattan plots of Genome Wide Association Analysis (GWAS) for (a) aerenchyma area and (b) percent of aerenchyma. Horizontal dashed line is a minimum threshold ($p=1 \times 10^{-4}$). Selected significant SNPs and adjacent SNPs were highlighted in green and shown with their associated gene models.
Figure 5.7: Manhattan plots of Genome Wide Association Analysis (GWAS) for a) median meta xylem area and b) water conductance. Horizontal dashed line is a minimum threshold ($p=1 \times 10^{-4}$). Selected significant SNPs and adjacent SNPs were highlighted in green and shown with their associated gene models.


Fehr WR (1987) Principle of cultivars development. 1–465


in asymmetric cell divisions in rice plants. Plant J 36: 45–54


Maechler M, Matrix L (2013) Linear mixed-effects models using S4 classes.

Mano Y, Omori F (2013) Flooding tolerance in interspecific introgression lines containing
chromosome segments from teosinte (Zea nicaraguensis) in maize (Zea mays subsp. mays). Ann Bot 112: 1125–1139


Richards, Passioura (1989) A breeding program to reduce the diameter of the major xylem vessel in
the seminal roots of wheat and its effect on grain yield in rain-fed environments. Aust J Agric Res 40: 943–950


Chapter 6

Summary and Conclusions

Maize production requires a large amount of nitrogen (N) fertilizers, which is not affordable by most smallholder farmers in developing countries thereby posing threat to human food security. Although the benefits of adding N fertilizers to agricultural systems are straightforward, they are accompanied by substantial costs to human health and the environments. In my research, we have shown that root traits specifically high root cortical aerenchyma (RCA), small number of crown root (CN) and long root hairs enhanced N acquisition and increased maize yield in low N soils. In addition, we have identified significant SNPs markers using Genome-wide association study (GWAS) and discussed their potential candidate genes for 20 maize root anatomical traits. Knowledge of genetic control and physiological utilities of root traits is crucial to plant breeders for developing breeding strategies utilizing root traits for crop improvement.

In my research we found that maize recombinant inbred lines (RILs) with high RCA had greater growth and yield in low N soils than plants with low RCA. High RCA RILs had lower root respiration and greater rooting depth than low RCA RILs. This is evidence that RCA not only benefits plants under hypoxic conditions but also under drought and nutrient stresses including low nitrogen. Little is known regarding tradeoffs for having high RCA, but evidence suggests that there may be some disadvantages such as reduction in radial transport of nutrients and water. However, the fact that high RCA RILs performed better than low RCA RILs in the greenhouse and in the field under low N conditions indicates that benefits by having RCA outweigh costs of RCA under N stress. Lacking understanding of possible tradeoffs, we cautiously suggest that increased RCA formation may be a promising breeding target for enhancing nitrogen acquisition in low N soils.
This research is also the first to report the utility of CN (crown root number) in the context of enhancing nutrient acquisition. We found that low CN RILs had greater rooting depth and can acquire greater nitrogen from deep soil strata than high CN RILs, resulting in better growth and yield in the field. On the other hand, high CN RILs may be better at topsoil foraging, which may benefit acquisition of immobile nutrients such as phosphorus and potassium. We suggest that CN merits investigation as a potential element of more nutrient efficient maize cultivars.

This research proposes a novel idea about the role of root hair length for nitrogen acquisition efficiency. We found that long-haired RILs had better growth and yield maintenance under low N conditions. These results support the use of root hair length as a new breeding target for improved N acquisition in crops as well as challenge the paradigm of nitrogen uptake by plant roots, which has been traditionally believed to be not limited by diffusion. Additionally, screening of a large number of genotypes can be done effectively and economically in a greenhouse or even in cigar rolls. Root hair traits could be visually evaluated and thus accessible to breeders in developing countries. We suggest that long root hairs could be used to improve N acquisition in low N soils in maize.

Finally, we examined phenotypic variation for 20 root anatomical traits and identified markers associated with the variation of the traits in 503 diverse inbred maize lines from the Wisconsin diversity panel. GWAS revealed 1140 significant SNPs associated with root anatomical traits. Some significant SNPs are in proximity to genes of which function is known to be involved in root developments such as SCARECROW and ETHYLENE INSENSITIVE 3-LIKE (EIL3). Future research is needed to examine the potential of significant SNP markers to be used in plant breeding using marker-assisted selection and to validate the effects of candidate genes and to understand the genetic control of root anatomical traits in maize.
**Appendix A**

**Nutrient solutions used in greenhouse experiments**

**Appendix A Table 1: Nutrient solution used in mesocosms (chapter 2 and 3)**

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<th>Nutrient solution (High nitrogen treatment)</th>
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<td><strong>Macronutrient</strong></td>
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<tr>
<td>No. Stock conc. (M)</td>
<td>Chemicals</td>
<td>(ml)</td>
</tr>
<tr>
<td>1 1</td>
<td>KNO₃</td>
<td>3</td>
</tr>
<tr>
<td>2 1</td>
<td>Ca(NO₃)₂·4H₂O</td>
<td>2</td>
</tr>
<tr>
<td>3 0.5</td>
<td>MgSO₄·7H₂O</td>
<td>1</td>
</tr>
<tr>
<td>4 1</td>
<td>KH₂PO₄</td>
<td>1</td>
</tr>
</tbody>
</table>

| **Micronutrient**                          |    |       |
| No. Stock conc. (M) | Chemicals |       |
| 1 0.050 | KCl | 1 | 49.9 |
| 2 0.025 | H₃BO₃ | 1 | 25.0 |
| 3 0.002 | MnSO₄·H₂O | 1 | 2.0 |
| 4 0.002 | ZnSO₄·7H₂O | 1 | 2.0 |
| 5 0.001 | CuSO₄·H₂O | 1 | 0.5 |
| 6 0.001 | (NH₄)₆MO₇O₂₄·4H₂O | 1 | 0.5 |
| 7 0.1 | Fe-DTPA | 1 | 100.0 |

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<td>No. Stock conc. (M)</td>
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<td>(ml)</td>
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| **Micronutrient**                         |    |       |
| No. Stock conc. (M) | Chemicals |       |
| 1 0.050 | KCl | 1 | 49.9 |
| 2 0.025 | H₃BO₃ | 1 | 25.0 |
| 3 0.002 | MnSO₄·H₂O | 1 | 2.0 |
| 4 0.002 | ZnSO₄·7H₂O | 1 | 2.0 |
| 5 0.001 | CuSO₄·H₂O | 1 | 0.5 |
| 6 0.001 | (NH₄)₆MO₇O₂₄·4H₂O | 1 | 0.5 |
| 7 0.1 | Fe-DTPA | 1 | 100.0 |
Appendix A Table 2: Nutrient solution used in greenhouse experiment (chapter 4)

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<td>Stock conc. (M)</td>
<td>Chemicals</td>
<td>1L (ml)</td>
<td>Conc. (uM)</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>KH2PO4</td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>CaCL2</td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>2</td>
<td>MgSO4.7H2O</td>
<td>2</td>
<td>1000</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>(NH4)2SO4</td>
<td>0.25</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>2</td>
<td>K2SO4</td>
<td>2</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Micronutrient</strong></td>
<td>No.</td>
<td>Stock conc. (M)</td>
<td>Chemicals</td>
<td>1L (ml)</td>
<td>Conc. (uM)</td>
</tr>
<tr>
<td>1</td>
<td>0.050</td>
<td>1</td>
<td>KCL</td>
<td>1</td>
<td>49.9</td>
</tr>
<tr>
<td>2</td>
<td>0.025</td>
<td>1</td>
<td>H3BO3</td>
<td>1</td>
<td>25.0</td>
</tr>
<tr>
<td>3</td>
<td>0.002</td>
<td>1</td>
<td>MnSO2.H2O</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.002</td>
<td>1</td>
<td>ZnSO4.7H2O</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>0.001</td>
<td>1</td>
<td>CuSO4.H2O</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>0.001</td>
<td>1</td>
<td>(NH4)6MO7O24.4H2O</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>1</td>
<td>Fe-DTPA</td>
<td>1</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Appendix B

R code for statistics and GWAS analysis

Appendix B1: R code for statistics and quality control of data

##### Initial data quality ########
### descriptive statistics
library(Hmisc)
library(lattice)
describe(data)

### histograms of the data ###
pdf("histograms_of_rep_means.pdf",height=10,width=12)
for(i in 1:nrow(data)){
hist(data[,i], main=names(data)[i],xlab="measurement")
}
dev.off()

###################################################
### Analysis of Varaince ## Mixed Effects Model ###
###################################################
medianLacA
hist(data$medianLacA)
boxplot(data$medianLacA)
myboxplot<-boxplot(data$medianLacA)
outliers <- boxplot(data$medianLacA, plot=TRUE)
row.names(data$medianLacA %in% outliers,]
row.names(temp)
library(lme4)

do.call(lmer(),list(medianLacA~(1|genotype)+(1|Year)+(1|genotype*Year)+(1|Rep*Year),
data=data))

# LRT
fit.medianLacA_GxE<-update(fit.medianLacA, .~ - (1|genotype*Year))
anova(fit.medianLacA, fit.medianLacA_GxE) # LRT

fit.medianLacA_year<-update(fit.medianLacA, .~ - (1|Year))
anova(fit.medianLacA, fit.medianLacA_year) # LRT

# Entry #
(fit.medianLacA_GxE<-lmer(medianLacA~ (1|genotype) * (1|Year) * (1|genotype:Year) +
(1|Rep:Year), data=data))
fit.medianLacA_genotype<-update(fit.medianLacA_GxE, .~ - (1|genotype))
anova(fit.medianLacA, fit.medianLacA_genotype) # LRT

# G x E #
(fit.medianLacA_GxE<-update(fit.medianLacA, .~ - (1|genotype:Year))
anova(fit.medianLacA, fit.medianLacA_GxE) # LRT

# Year #
(fit.medianLacA_year<-update(fit.medianLacA, .~ - (1|Year))
anova(fit.medianLacA, fit.medianLacA_year) # LRT

# RANDOM EFFECTS

# Entry #
(fit.medianLacA_GxE<-lmer(medianLacA~ (1|genotype) * (1|Year) * (1|genotype:Year) +
(1|Rep:Year), data=data))
fit.medianLacA_genotype<-update(fit.medianLacA_GxE, .~ - (1|genotype))
anova(fit.medianLacA, fit.medianLacA_genotype) # LRT

# G x E #
(fit.medianLacA_GxE<-update(fit.medianLacA, .~ - (1|genotype:Year))
anova(fit.medianLacA, fit.medianLacA_GxE) # LRT

# Year #
(fit.medianLacA_year<-update(fit.medianLacA, .~ - (1|Year))
anova(fit.medianLacA, fit.medianLacA_year) # LRT

# RANDOM EFFECTS
anova(fit.medianLacA, fit.medianLacA_year)
# rep(year) #
fit.medianLacA_rep_year <- update(fit.medianLacA, .~ - (1|Rep:Year))
anova(fit.medianLacA, fit.medianLacA_rep_year)

############## Correlation across years ###############

y2010 <- data[which(data$Year == "2010"),]
y2011 <- data[which(data$Year == "2011"),]

# library(Hmisc)
# rcorr(cbind(y2008$medianLacA, y2009$medianLacA, y2010$medianLacA, y2011$medianLacA),
type="spearman")

# cor.test(y2008$medianLacA, y2009$medianLacA, method="spearman")
# cor.test(y2008$medianLacA, y2010$medianLacA, method="spearman")
# cor.test(y2009$medianLacA, y2010$medianLacA, method="spearman")
# cor.test(y2009$medianLacA, y2011$medianLacA, method="spearman")
# cor.test(y2010$medianLacA, y2011$medianLacA, method="spearman")

cor.test(y2010$medianLacA, y2011$medianLacA, method="spearman")

########## Broad sense heritibility ###########

medianLacA <- lmer(data$medianLacA ~ (1|genotype) + (1|Year) + (1|genotype:Year) +
                     (1|Rep:Year), data=data, na.action = na.omit))

genoxyear = 1.0987e-04
res = 9.8737e-05

(H_2 = 100 * geno / (geno + (genoxyear/2) + res/4)) # Entry-mean basis broad-sense heritability
## var(g) / var(g) + (var(gxe)/no_env) + (var(e)/no_reps x no_env)

### Residuals Outliers
library(influence.ME)
model.a_medianLacA <- lmer(medianLacA ~ (1|genotype) + (1|Year) + (1|genotype:Year) +
                          (1|Rep:Year), data=data)
alt.est.a_medianLacA <- influence(model.a_medianLacA, obs=TRUE)
cooks <- cooks.distance(alt.est.a_medianLacA)
plot(alt.est.a_medianLacA, which="cook", sort=FALSE, main="cook's distance plot of MedianLacA")

which(residuals(medianLacA)>0.10)
which(residuals(medianLacA)<(-0.10))

boxplot(residuals(medianLacA))
myboxplot <- boxplot(residuals(medianLacA))
myboxplot$sout
hist(residuals(medianLacA))
#### Mixed Model Assumptions ####

```r
df("widiv_medianLacA_residual_graphs.pdf", height=8, width=10)
par(mfrow=c(2,2))
plot(fitted(medianLacA), residuals(medianLacA), xlab="Predicted values", ylab="Residuals", main="Residual Plot of widiv medianLacA")
abline(h=0, col="red")
hist(resid(medianLacA), main="histogram of medianLacA residuals")
qqnorm(residuals(medianLacA), main="Residuals Q-Q Plot"); qqline(resid(medianLacA))
qqnorm(ranef(medianLacA)$genotype$("(Intercept)"), main="Genotypes Q-Q Plot"); qqline(ranef(medianLacA)$genotype$("(Intercept)"))
qqnorm(ranef(medianLacA)$"genotype:Year"$("(Intercept)"), main="Genotype by Year Q-Q Plot"); qqline(ranef(medianLacA)$"genotype:Year"$("(Intercept)"))
plot(alt.est.a_medianLacA, which="cook", sort=FALSE, main="cook’s distance plot of medianLacA")
dev.off()
```

####### BLUP OUTPUTS ##################

### This data frame contains the BLUPs of PH:

```r
medianLacA = ranef(medianLacA)$'genotype'
medianLacA$genotype = row.names(medianLacA)
names(medianLacA)[1] = "medianLacA"
medianLacA = medianLacA["genotype", "medianLacA"]
mean = mean(data$medianLacA, na.rm=TRUE)
options(digits=22)
medianLacA$medianLacA <- (medianLacA$medianLacA + mean)
write.csv(medianLacA, "widiv_medianLacA_Finalized_BLUPs.csv", row.names=FALSE)
```
Appendix B2: R code for GAPIT

# R code to use GAPIT on WIDIV inbred DATA

# Import library (each time to start R)
source("http://www.bioconductor.org/biocLite.R")  # These next two lines are commented
biocLite("multtest")
library(multtest)
library("gplots")
library("LDheatmap")
library("genetics")

# Import GAPIT
source("http://www.maizegenetics.net/images/stories/bioinformatics/GAPIT/gapit_functions.txt")

# Create myGAPIT directory under C drive and download tutorial data to it. Set it as
# working directory
setwd("/Users/jmjohnson26/Desktop/Khwan_GWAS")

# Tutorial 1: Basic Scenario
#----------------------------------------------------------------------------------------
myY <- read.table("GAPIT_WIDIV_PHENO_khwan_2010_2011.txt", head = TRUE)  # inputs the
phenotypic data ( requires a header)
myG <- read.table("GAPIT.RNAseq.hmp_486K_imputed.txt", head = FALSE)  # inputs the
genotypic data ( requires no header)
myKI <- read.table("GAPIT.Kin.VanRaden.Final.txt", head = FALSE)  # inputs a
# user created kinship (K matrix) (if you want to use one)
myCV <- read.table("GAPIT.Qmatrix.8.PC.txt", head= TRUE)  # inputs a
# user created covariate matrix (Q matrix)

# Step 2: Run GAPIT
myGAPIT <- GAPIT(
Y=myY,  # specifies your phenotype
G=myG,  # specifies your genotypic data
KI=myKI,  # specifies your kinship (if you input one, you need to
uncomment)
CV=myCV,  # specifies your covariate matrix (if you input one, you
need to uncomment)
kinship.algorithm = "VanRaden",  # specifies what algorithm to use for estimating
# kinship (if you don't specify a user created one)
kinship.cluster="average",  # specifies a clustering algorithm for compression
# kinship.group="Mean",  # specifies how to group them for compression
Model.selection = TRUE,  # specifies if you want GAPIT to choose the number of
principal components
SNP.test=TRUE,  # can use to supress the genomic selection part of the
SNP.impute = "Major",  # specifies how you want GAPIT to impute missing data
# the next two rows specify the number of groups you want
to use for compression (if you set them both equal to the number of individuals it turns
off compression)
group.from = 503,
group.to = 503,
P.CA.total=30,  # tells GAPIT the total number of PCA's you wish to use
SNP.Fraction=0.025,  # specifies to use a subset when calculating kinship and
PCA (speeds things up a bit)
)
Appendix B3: R code for Manhattan and QQ plots

```r
# source("http://people.virginia.edu/~sdt5z/0STABLE/qqman.r")

#### here is the source code to change how you'd like!

manhattan <- function(dataframe, colors=c("gray10", "gray90"), ymax="max", limitchromosomes=1:10, suggestiveline=-log10(5.623411e-07), genomewideline=-log10(5.623411e-07), annotate=NULL, ...) {

d=dataframe
if (!is.null(CHR) & "BP" %in% names(d) & "P" %in% names(d))) stop("Make sure your data frame contains columns CHR, BP, and P")
if (any(!is.na(limitchromosomes))) d=d[!CHR %in% limitchromosomes, ]

# remove na's, sort, and keep only 0<P<=1

d$logp = -log10(d$P)
d$pos=NULL
ticks=NULL
lastbase=0

numchroms=length(unique(d$CHR))

if (numchroms==1) {
  ticks=floor(length(d$pos)/2+1)
} else {
  for (i in unique(d$CHR)) {
    if (i==1) {
      d[d$CHR==i,]$pos=d[d$CHR==i,]$BP
    } else {
      lastbase=lastbase+tail(subset(d,CHR==i-1)$BP, 1)
      d[d$CHR==i,]$pos=d[d$CHR==i,]$BP+lastbase
    }
    ticks=c(ticks, d[d$CHR==i,]$pos[floor(length(d[d$CHR==i,]$pos)/2)+1])
  }
}

if (numchroms==1) {
  with(d, plot(pos, logp, ylim=c(0,ymax), ylab=expression(-log[10](italic(p)))),
      xlab=paste("Chromosome", unique(d$CHR), "position"), ...)
} else {
  with(d, plot(pos, logp, ylim=c(0,ymax), ylab=expression(-log[10](italic(p)))),
      xlab="Chromosome", xaxt="n", type="n", ...
  axis(1, at=ticks, lab=unique(d$CHR), ...)
  icol=1
  for (i in unique(d$CHR)) {
    with(d[d$CHR==i,],points(pos, logp, col=colors[icol], ...))
    icol=icol+1
  }
}

if (!is.null(annotate)) {
  d.annotate=d[which(d$SNP %in% annotate), ]
  with(d.annotate, points(pos, logp, col="green3", ...))
}
```

if (suggestiveline) abline(h=-log10(5.623411e-07), col="blue")
if (genomewideline) abline(h=-log10(5.623411e-07), col="red")

### Make a pretty QQ plot of p-values
qq = function(pvector, ...) {
  if (!is.numeric(pvector)) stop("D'oh! P value vector is not numeric.")
  pvector <- pvector[!is.na(pvector) & pvector<1 & pvector>0]
  o = -log10(sort(pvector, decreasing=F))
  e = -log10( ppoints(length(pvector)) )
  plot(e, o, pch=19, cex=1, xlab=expression(Expected~~-log[10](italic(p))),
       ylab=expression(Observed~~-log[10](italic(p))), xlim=c(0,max(e)), ylim=c(0,max(o)), ...)
  abline(0,1,col="red")
}

#infile
results <- read.table("C:/Users/jmjohnson26/Desktop/GWAS_Summaries/Inbred/manhattan plots/GAPIT.ear_leaf_no_manhattan.txt", header=T, stringsAsFactors=F)

### plotting
pdf("Widiv_ear_leaf_no_manhattan_plot.pdf", height=8, width=12)
manhattan(results, colors=c("black","#666666","#CC6600"), pch=20, genomewideline=F,
          suggestiveline=TRUE, main="Manhattan Plot of Widiv Ear Leaf Number")
dev.off()

### plotting with highlighted SNPs
#snps_to_highlight <- scan("http://www.StephenTurner.us/snps.txt", character())
#manhattan(results, annotate=snps_to_highlight, pch=20, main="Manhattan Plot")

### qq plots
qq(results$P)
VITA

Patompong Saengwilai

EDUCATION
• Ph.D., Intercollege Graduate Degree Program in Plant biology, 2013, Pennsylvania State University, University Park, PA
• B.Sc., Biology (Honors), Mahidol University, Bangkok, Thailand

RESEARCH EXPERIENCE
• Root traits for efficient nitrogen acquisition in Maize
• QTL and Association mapping in maize
• Aluminum-phosphate buffer for evaluation of Arabidopsis thaliana responses to low phosphorus
• Evaluation of root architectural and anatomical traits in rice and sorghum
• Phenotypic analysis of Arabidopsis mutants in candidate sperm-specific genes
• The effect of cadmium on Glutathione S-transferase activity in Targetes erecta
• Bioremediation of cadmium and arsenics

TALKS HELD AT CONFERENCES
• The ASA, CSSA, and SSSA Annual meeting 2012, oral presentation
• Plant Biology Seminar 2012, oral presentation
• ECHO Agricultural Conference 2010, oral presentation
• BioAsia2007, oral presentation
• The National Botany Conference 2007, oral presentation

POSTER PRESENTATIONS
• 55th Annual Maize Genetics Conference 2013, IL, USA
• International Society of Root Research (ISRR) 2012, Dundee, Scotland
• Undergraduate Exhibition 2012, PA, USA
• Plant Canada 2011, Nova Scotia, Canada
• Biannual Plant Biology Symposium 2011, PA, USA
• Science Exhibition 2011, PA, USA

HONORS AND AWARDS
• College of Agricultural Sciences Competitive Grants Award, 2012
• Royal Thai Government scholarship toward the completion of Ph.D., 2008-2013
• The Distinction program: Overseas Research Scholarship. Bangkok, Thailand 2006

TEACHING EXPERIENCE
• Plant nutrition, Pennsylvania State University State, USA, 2012
• Biology, Mahidol University, Thailand, 2007
• Biology, Bodindecha School (International program), Bangkok, Thailand, 2007

MEMBERSHIPS
• Crop Science Society of America
• American Society Of Plant Biologists
• Botany, Agronomy, Economic Botany, Kasetsart University, Thailand
• Academic Network for Thailand Development (ANTd), Thailand