EFFECT OF NITROGEN FORM AND GENETIC VARIATIONS OF THE ASPARAGINE-CYCLING PATHWAY ON MAIZE EAR GROWTH

BY

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THESIS

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Abstract

The positive growth response of maize ears to nitrate supplementation declines during late vegetative development, whereas ammonia addition continues to promote ear growth through the reproductive phase. The underlying genetic and physiological mechanisms for this phenomenon are currently unknown. Previous studies with DuPont Pioneer found that in the maize line Gaspé Flint, ear growth no longer responded to additional nitrate 20 days after emergence, but a subsequent addition of ammonia significantly increased ear dry weight as well as the transport amino acids asparagine and glutamine. This nitrogen-induction experiment was duplicated, and root, shoot, and ear tissues were profiled via RNA-Seq to characterize responses to ammonia or nitrate at four or eight hours after nitrogen treatment. Results suggest an upregulation of amino acid assimilation and metabolism genes in ammonia-treated plants. Illinois High Protein (IHP) differs from other genotypes in exhibiting an increased rate of nitrogen uptake, vigorous ear growth under low N conditions, very high levels of asparagine, but a reduced response to ammonia. These observations suggest that the mechanism for ammonia-induced ear growth in some genotypes may already be active in IHP even without ammonia supplementation. Compared to many other maize genotypes, IHP has functional variants of the two asparagine-cycling genes, Asparagine synthetase (AS) and Asparaginase (ASNase). We examined the effect of these two genes on ear growth using a range of populations that contain variants of these genes. Greater AS expression was associated with improved ear growth, whereas very low ASNase activity was associated with decreased developing ear size.
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CHAPTER 1: COMPARING GENE EXPRESSION OF AMMONIUM AND NITRATE-TREATED MAIZE VIA RNA-SEQ

Introduction

Impact of Nitrogen on Maize Production

Nitrogen is arguably the single most important input in agricultural maize production today. Along with hybrid development, application of nitrogen has led to the dramatic increase of maize yield since the 1930s. Depending on soil type and environment, maize yield response to an adequate supply of nitrogen can be as much as 150% (O’Neill et al., 2004). Industrially produced nitrogen fertilizer became possible with the development of the Haber-Bosch process in the early 20th century. The process produces ammonia (NH₃) from atmospheric nitrogen (N₂) and hydrogen gas (H₂) obtained from natural gas. Ammonia originally used for munitions production became widely available for agricultural use following World War II. Today, about 46% of nitrogen used as a fertilizer in the United States is utilized for maize production (USDA, 2011). United States nitrogen application rate has increased substantially in the last 50 years. Average maize nitrogen rate has increased nearly 2.5-fold since 1964 (USDA, 2011). This increase in nitrogen rate has coincided with a 152% increase in maize yield in the same time period (USDA, 2013). Although increased nitrogen application has resulted greater maize yields, it has also resulted in greater economic and energy inputs in the current agricultural maize system.
Aside from economic effects, nitrogen application has significant environmental impacts. Several studies have shown that nitrogen use efficiency in maize and other cereal crops is as low as 30-50% (Cassman et al., 2002; Smil, 1999; Raun & Johnson, 1999; Balasubramanian et al., 2004). The remaining applied nitrogen is lost to the environment through nitrate leaching, erosion, denitrification, volatilization, and microbial immobilization. As much as 78 million tons of applied nitrogen is entering the environment every year due to crop production (FAO, 2013). This nitrogen has been implicated in a variety of environmental problems. The hypoxic “dead zone” of the Gulf of Mexico has been linked to deposition of nitrate from the Mississippi River basin (Rabalais et al., 2002). Diaz and Rosenberg (2008) report more than 400 waterway systems containing dead zones, with the number of systems affected continuing to rise dramatically. Inorganic nitrogen application also contributes to an increase in atmospheric greenhouse gasses. About 3.15 million tons of nitrous oxide (N₂O) is released into the atmosphere every year, which is significant due to N₂O being 296 times more potent as a greenhouse gas per unit mass than carbon dioxide (CO₂) (Snyder et al., 2009). Excessive nitrate in drinking water has also been implicated in infant methaemoglobinemia (blue baby syndrome) and digestive tract cancers, though these claims are still disputed (Powlson, 2008).

**Nitrogen Uptake, Assimilation, and Utilization**

Inorganic nitrogen produced from the Haber-Bosch process is in the form of NH₃, though it can be readily converted into other forms, such as urea, ammonium nitrate, and aqua ammonia. Once applied to aerobic soils, however, NH₃ and urea are readily converted to nitrate (NO₃⁻) by soil microbes. Ureases catalyze the reaction of urea and water into NH₃ and
CO₂. NH₃ then can react with water to produce an ammonium ion (NH₄⁺) and a hydroxide ion (OH⁻). This step is essential to keeping nitrogen in the soil due to NH₃ being a gas that will volatilize into the atmosphere if not converted into the ion form. Once in this form, nitrification by soil microbes can convert it to NO₃⁻. NH₃ is first converted into nitrite (NO₂⁻) via the *Nitrosomonas* genera of bacteria. Following this conversion, *Nitrobacter* bacteria further oxidizes NO₂⁻ into NO₃⁻. These microbial conversions result in soil heterogeneity for inorganic nitrogen form. The concentration of NO₃⁻ in many agricultural soils has been reported at 1-5 mM, whereas NH₄⁺ is generally at lower concentrations of 20-200 µM, though this can vary greatly based on a variety of factors (Owen & Jones, 2001). Although NO₃⁻ is generally more available to the plant due to higher concentrations and being transported through mass flow, when it is available, the plant prefers NH₄⁺ to NO₃⁻ (Macduff & Jackson, 1991).

Plants have both a high affinity and low affinity transport system for uptake of NH₄⁺ and NO₃⁻ into plant root cells (Crawford & Glass, 1998; von Wirén et al., 2000). High affinity NH₄⁺ and NO₃⁻ transporters (HATS) are active when their respective substrate is low. They act as scavengers by efficiently taking up nitrogen when the nutrient is scarce. When the HATS are saturated at higher concentrations of NH₄⁺ and NO₃⁻, low affinity transport systems (LATS) become the predominant form of inorganic nitrogen transport. This system allows the plant to respond to a wide range of NH₄⁺ and NO₃⁻ concentrations that may be present in the soil solution.

Assimilation of NO₃⁻ taken up by the roots is predominantly carried out in the shoot tissues, especially in soils with high concentrations of NO₃⁻ (Andrews, 1986). Whether in the root or shoot, NO₃⁻ is reduced in the cytoplasm to NO₂⁻ by nitrate reductase (NR). NO₂⁻ is then
further reduced in the plastid to NH$_4^+$ by nitrite reductase (NiR). NH$_4^+$ taken up by the root is largely assimilated in root tissue, possibly due to the toxic effects of NH$_4^+$ at high concentrations (Kiyomiya et al., 2001). It has been shown, however, that plants do have some ability to transport free NH$_4^+$ ions through the xylem to shoot tissues, though at a much lower level than NO$_3^-$ (Schjoerring et al., 2002).

NH$_4^+$ is assimilated into organic nitrogen by way of the GS/GOGAT cycle. Glutamine synthetase (GS) catalyzes the reaction of glutamate and NH$_4^+$ into glutamine. Glutamate synthase, also known as glutamine-2-oxoglutarate aminotransferase (GOGAT), catalyzes the transfer of an amide group on glutamine to 2-oxoglutarate, resulting in two molecules of glutamate. This glutamate may then be cycled back into the assimilation of NH$_4^+$ by GS. Plants have two forms of both GS and GOGAT. GS1 is localized to the cytoplasm, whereas GS2 is a chloroplast-localized enzyme. GS1 has been found to be the dominant GS enzyme in the root, and GS2 is the major GS enzyme in plant leaves (Miflin and Lea, 1980). These localizations suggest that GS2 is the primary enzyme for assimilation of NH$_4^+$ reduced from NO$_3^-$ in the chloroplast, and that GS1 is the major inorganic nitrogen assimilator in the root. GOGAT has an Fd-dependent and an NADH-dependent form. Both are plastid specific, though Fd-GOGAT is found in chloroplasts and is the major form of GOGAT in the leaves, while NADH-GOGAT is found primarily in tissues that are non-photosynthetic. This indicates that GS2 and Fd-GOGAT are the major system of NH$_4^+$ assimilation in leaves and other photosynthetic plant tissue, and GS1 and NADH-GOGAT function as the main NH$_4^+$ assimilation pathway in root tissues.

Glutamate dehydrogenase (GDH) catalyzes the reversible reaction of glutamate to 2-oxoglutarate and NH$_4^+$. GDH was originally believed to be the primary assimilation route of
NH$_4^+$.

Because GDH has a high $K_m$ for NH$_4^+$ and mutant studies have shown there is no evidence of GDH assimilation of NH$_4^+$, GDH is believed to be primarily a catabolic enzyme (Lam et al., 1996). Some evidence, though, has been given for GDH acting as an assimilator of NH$_4^+$ in times of stress, such as toxic levels of NH$_4^+$ (Skopelitis et al., 2006).

Following assimilation, amino acids are transported to sink tissues. Depending on the developmental stage, this may be developing leaves, roots, flowers, or seeds. The predominant forms of transport amino acids are glutamine, glutamate, asparagine, and aspartate. Asparagine biosynthesis in plants is catalyzed by Asparagine synthetase (AS), which transfers an amino group from glutamine onto aspartate to produce a molecule of asparagine and glutamate. Aspartate is synthesized by Aspartate aminotransferase through the transamination of glutamate and oxaloacetate, forming aspartate and 2-oxoglutarate. Once these four transport amino acids reach their sink tissue, they are utilized in the biosynthesis of many other amino acids, nucleotides, and metabolites. These free amino acids can also be incorporated into proteins to carry out a wide range of functions, including enzymatic, structural, transport, and storage. Around 80% of plant nitrogen in the leaf is stored in the chloroplasts (Kant et al., 2011). A large portion of this is stored in the carbon fixing enzyme Rubisco, along with other abundant enzymes such as PEP carboxylase and GS. As leaves senesce these proteins are hydrolyzed into free amino acids by proteases and become a major source of nitrogen for seed development.

**Effect of Nitrogen Form on Plants**

There have been a wide range of studies analyzing how inorganic nitrogen supplementation affects the physiology, metabolism, gene expression, and regulatory network
of plants. There have also been numerous studies on how the two plant-available forms of inorganic nitrogen, \(\text{NO}_3^-\) and \(\text{NH}_4^+\), differ in their effect on plants, however less is known on the differences of their effect on gene expression. Physiological studies on the effect of these two nitrogen forms suggest unique systems of uptake, assimilation, and utilization that likely extend to modifications of the transcriptome.

An early study in tomato showed that excessive \(\text{NH}_4^+\) has a negative effect on plant growth and development, especially vegetative biomass (Clark, 1936). Many studies since have come to similar conclusions (Guo et al., 2007). This is likely due to plants accumulating toxic levels of \(\text{NH}_4^+\) from numerous applications of high concentrations of solely \(\text{NH}_4^+\). Conversely, some studies have shown increased biomass of \(\text{NH}_4^+\)-treated plants in maize (Lewis et al., 1989), rice (Qian, 2004), and blueberry (Claussen & Lenz, 1999). While it is not clear whether \(\text{NO}_3^-\) or \(\text{NH}_4^+\) is more beneficial to plant growth, a large body of evidence exists supporting a mixed system of both \(\text{NH}_4^+\) and \(\text{NO}_3^-\) for maximum growth and yield in a wide variety of crops (Below, 2002). Because \(\text{NH}_4^+\) is largely assimilated in root tissue where it is taken up and most \(\text{NO}_3^-\) is exported to the leaves for assimilation, providing both forms of nitrogen may allow for a greater amount of nitrogen uptake and assimilation efficiency than either form by itself.

\(\text{NO}_3^-\) and \(\text{NH}_4^+\) also exhibit distinct effects on photosynthesis and the carbon-nitrogen balance. \(\text{NO}_3^-\) reduction in leaves consumes NADPH produced by the electron transport chain, which competes with \(\text{CO}_2\) fixation for NADPH consumption (Gerendás et al., 1997). Along with potentially reducing carbon fixation, \(\text{NO}_3^-\) reduction results in a 143% greater photoenergy cost for plants supplied with \(\text{NO}_3^-\) rather than \(\text{NH}_4^+\) (Raven, 1985). \(\text{NH}_4^+\) assimilated in the roots has
also been implicated in deregulating feedback inhibition of photosynthesis by acting as a sink for carbon skeletons produced in leaves (Hall et al., 1984; Claussen & Lenz, 1995).

Water uptake and utilization has also been shown to differ in plants treated with the two forms of inorganic nitrogen. Yin and Raven (1998) showed that water use efficiency increased in plants supplied with NH$_4^+$· However, some species show increased water uptake when supplied with NO$_3^-$ rather than NH$_4^+$ (Chance III et al., 1999). Reduction of osmotic regulation in the leaf may be one factor that causes reduced water uptake in NH$_4^+$-treated plants, due to reduced uptake of other cations such as K$^+$, Ca$^{2+}$, and Mg$^{2+}$ (Raab & Terry, 1995). Transpiration rate in NH$_4^+$-treated plants, though, is equal or greater than that of NO$_3^-$-treated plants (Høgh-Jensen & Schjoerring, 1997). Guo et al. (2002) demonstrated that the source of the differences in water uptake were in the roots, not the shoot, and that reduction of the activity of aquaporins in plant treated with NH$_4^+$ were likely involved. NH$_4^+$-treated maize has been shown to respond more favorably to drought by having higher turgor pressure and chlorophyll concentrations compared to water-stressed maize treated with NO$_3^-$ (Mihailović et al., 1992).

**Effect of Nitrogen Form on Ear Growth in Gaspé Flint**

Gaspé Flint is a fast-growing, dwarf maize variety with only a 60 day life cycle that is well suited for controlled growth chamber experiments (Figure 1; Hourcade et al., 1986). DuPont Pioneer performed several experiments testing how changes in nitrogen form and concentration affected ear growth in Gaspé Flint across a range of developmental stages. The study found that plants grown with 1mM KNO$_3$ and subsequently switched to 2mM KNO$_3$ at or before 18 days after emergence (DAE) will exhibit ear growth of a plant grown normally at 2mM.
KNO₃ (Figure 2). Following 18 DAE, though, the switch to 2mM KNO₃ ceases to have an effect, and ear dry weight is equivalent to that of a 1mM KNO₃-grown plant. Conversely, plants grown with 2mM KNO₃ and switched to 1mM KNO₃ at or before 18 DAE have ear growth as if always supplied with the lower concentration, while after 20 DAE the decrease in nitrogen fails to have an effect.

At 23 DAE, approximately three days before anthesis, ear dry weight in Gaspé does not respond to changes in KNO₃ concentration. However, a switch at this time point from either 1mM or 2mM KNO₃ to 1mM NH₄Cl results in a significant increase in ear dry weight. Switching from 1mM to 10mM KNO₃ after 20 DAE has little to no effect on ear growth, whereas switching to 1mM NH₄Cl roughly doubles the ear growth rate compared to 1mM KNO₃-grown plants (Figure 3). This shows that NH₄⁺ has the potential to positively affect maize ear growth during late vegetative and early reproductive growth stages, even after there is no response to NO₃⁻.

Switching to NH₄Cl results in decreased shoot dry weight as well as increased shoot total nitrogen, though neither are significant (Table 1). Ear dry weight and total nitrogen are significantly greater in plants switched to NH₄Cl than those continuing to receive KNO₃ at both 1mM and 2mM concentrations. Shoot amino acid concentrations are notably altered when plants are switched to an NH₄Cl solution (Figure 4). Total amino acids are approximately doubled. Specifically, asparagine concentrations increase by as much as 10x, and glutamine concentrations are increased nearly 5x. The dramatic increase of these two transport amino acids in the shoot suggests NH₄⁺ is being assimilated and then transported as amino acids much more rapidly than NO₃⁻. Switching the nitrogen source to NH₄Cl during vegetative stage of growth does not result in an increase of asparagine and glutamine concentrations. This
indicates that the developing reproductive structures are acting as sinks that preferentially drive the assimilation of NH$_4^+$.

The objective of the following research is to reproduce the above nitrogen switching experiment at 23 DAE when NH$_4^+$ shows a significant positive effect on ear growth and amino acid concentrations, while NO$_3^-$ does not. The goal is to profile the transcriptome of both NH$_4^+$ and NO$_3^-$-treated plants to better understand the underlying genetic mechanism behind this physiological phenomenon. Understanding the genetic basis of this occurrence can give better insight into the best practices for timing and form of nitrogen application, as well as targets for genetic modification in improving nitrogen use efficiency in maize.

**Figure 1.** Growth of Gaspé Flint. Gaspé Flint at 23 days after emergence (DAE) corresponds to the early VT maize growth stage (A). 27 DAE corresponds to roughly the R2 maize growth stage (B).
Figure 2. Effect of switching nitrogen concentrations on ear dry weight. Filled black squares represent the ear dry weight of maize plants grown with 1mM KNO$_3$ and subsequently switched to 2mM KNO$_3$ across a range of time points. Empty squares represent the ear dry weight of maize plants grown with 2mM KNO$_3$ and subsequently switched to 1mM KNO$_3$ across a range of time points. The filled triangle represents the ear dry weight of maize grown with 1mM KNO$_3$ and subsequently switched to 1mM NH$_4$Cl at 23 days after emergence. The empty triangle represents the ear dry weight of maize grown with 2mM KNO$_3$ and subsequently switched to 1mM NH$_4$Cl at 23 days after emergence. Data generated by DuPont Pioneer.
Figure 3. Ear growth rate of maize with three different nitrogen sources. All plants were grown with 1mM KNO$_3$ up to 22 days after emergence and then switched to 10mM KNO$_3$, 1 mM NH$_4$Cl, or remained with 1mM KNO$_3$. Data generated by DuPont Pioneer.

Table 1. Effect of nitrogen form and concentration on plant biomass and nitrogen. Plant dry weight, plant total nitrogen, ear dry weight, ear total nitrogen, and ear to shoot ratio was measured for six different nitrogen switching treatments at 27 days after emergence. Plants were initially grown with either 1mM KNO$_3$ or 2mM KNO$_3$ and subsequently switched to either 1mM KNO$_3$, 2mM KNO$_3$, or 1mM NH$_4$Cl. Letters in bold denote significant differences. Data generated by DuPont Pioneer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant Dry Weight (g)</th>
<th>Plant Total Nitrogen (µmole)</th>
<th>Ear Dry Weight (g)</th>
<th>Ear Total Nitrogen (µmole)</th>
<th>Ear:Shoot Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mM KNO$_3$ → 1mM KNO$_3$</td>
<td>4.11</td>
<td>2457.76</td>
<td>.193 b</td>
<td>201.91 b</td>
<td>0.046959</td>
</tr>
<tr>
<td>1mM KNO$_3$ → 2mM KNO$_3$</td>
<td>4.02</td>
<td>2248.99</td>
<td>.212 b</td>
<td>206.21 b</td>
<td>0.052736</td>
</tr>
<tr>
<td>1mM KNO$_3$ → 1mM NH$_4$Cl</td>
<td>3.83</td>
<td>2670.45</td>
<td>.262 a</td>
<td>313.93 a</td>
<td>0.068407</td>
</tr>
<tr>
<td>2mM KNO$_3$ → 2mM KNO$_3$</td>
<td>4.32</td>
<td>2758.16</td>
<td>.208 b</td>
<td>189.61 b</td>
<td>0.048148</td>
</tr>
<tr>
<td>2mM KNO$_3$ → 1mM KNO$_3$</td>
<td>4.21</td>
<td>2630.92</td>
<td>.193 b</td>
<td>169.87 b</td>
<td>0.050923</td>
</tr>
<tr>
<td>2mM KNO$_3$ → 1mM NH$_4$Cl</td>
<td>3.79</td>
<td>3217.92</td>
<td>.297 a</td>
<td>345.42 a</td>
<td>0.078364</td>
</tr>
</tbody>
</table>
Figure 4. Proportional change in shoot amino acids in response to changes in nitrogen treatments. Levels of total amino acids, asparagine (asn), and glutamine (gln) are shown as a relative amount among the six different treatments. Plants were initially grown with either 1mM KNO$_3$ or 2mM KNO$_3$ and subsequently switched to either 1mM KNO$_3$, 2mM KNO$_3$, or 1mM NH$_4$Cl. Data generated by DuPont Pioneer.

Materials and Methods

Plant Growth and Sampling

Twenty-eight pots of Gaspé Flint were grown in a single growth chamber. Plants received sixteen hours of light daily. Temperature was set to 30°C and 25°C during light and dark, respectively. Humidity was controlled at 60%. Two seeds of Gaspé were planted in each 6” pot containing Turface. All plants were watered once daily for one minute with Modified Hoagland’s solution containing 2mM MgSO$_4$, 0.5mM KH$_2$PO$_4$, 83 ppm Sprint 330, 3mM KCl, 1µM ZnSO$_4$, 1µM MnCl$_2$, 3µM H$_3$BO$_4$, 0.1µM CuSO$_4$, 0.1µM NaMoO$_4$, and 1mM KNO$_3$ as the nitrogen source. Each pot was thinned to one plant per pot one week following planting. Following thinning, watering was increased to twice daily with the same solution as listed above. A random design was used to determine which pots will receive each nitrogen form and at what time point they will be sampled. Twenty-three days after emergence, at roughly the
early VT growth stage, ten of the plants were watered with a Modified Hoaglands solution containing NH$_4$Cl as the nitrogen source rather than KNO$_3$. The remaining plants still received the solution containing KNO$_3$. Five plants were sampled just before the nutrient solution was added. Ten plants were sampled at both four hours and eight hours after nutrient application: five that received the NH$_4$Cl solution and five that received KNO$_3$. Roots, shoots, and ears of each plant were sampled and individually flash frozen in liquid nitrogen.

**RNA Extraction and Sequencing**

The seventy-five samples above were ground to powder using mortar and pestle, while remaining frozen with liquid nitrogen. RNA was extracted from the samples with Trizol and chloroform, pelleted with isopropanol, and rehydrated in 100 µl of RNase-free water. Samples were treated with DNase to remove any contaminating DNA. RNA purity and concentration were assessed using gel electrophoresis and a NanoDrop 1000. 5 µg of RNA from three of the five biological replicates of each treatment was then pooled. The three replicates chosen were based on RNA quality and concentration.

cDNA of the pooled RNA was produced with reverse transcriptase by the W.M. Keck Center at the University of Illinois. The cDNA of the fifteen treatments were then sequenced on an Illumina HiSeq 2000 by the Keck Center. Fifteen libraries were generated from 100-bp sequences (Table 2). Sequences were aligned to the cDNA sequences of the maize working gene set using Novoalign. Gene fragments per kilobase of transcript per million mapped reads (FPKM$s$) were calculated using Cufflinks. Libraries were then transferred to Microsoft Excel for analysis.
Table 2. RNA-Seq library overview. The sampling time, tissue, nitrogen treatment and sequence reads of each library are given. Because of sequencer error of four libraries in the first sequencing run, all libraries were sequenced a second time.

<table>
<thead>
<tr>
<th>Library</th>
<th>Time Point</th>
<th>Tissue</th>
<th>Nitrogen Treatment</th>
<th>Sequence Reads (Run 1)</th>
<th>Sequence Reads (Run 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 hours</td>
<td>Shoot</td>
<td>-</td>
<td>NA</td>
<td>12681500</td>
</tr>
<tr>
<td>2</td>
<td>0 hours</td>
<td>Root</td>
<td>-</td>
<td>12382769</td>
<td>13262231</td>
</tr>
<tr>
<td>3</td>
<td>0 hours</td>
<td>Ear</td>
<td>-</td>
<td>9223675</td>
<td>9981868</td>
</tr>
<tr>
<td>4</td>
<td>4 hours</td>
<td>Shoot</td>
<td>Nitrate</td>
<td>10552802</td>
<td>11309374</td>
</tr>
<tr>
<td>5</td>
<td>4 hours</td>
<td>Root</td>
<td>Nitrate</td>
<td>NA</td>
<td>11260470</td>
</tr>
<tr>
<td>6</td>
<td>4 hours</td>
<td>Ear</td>
<td>Nitrate</td>
<td>10621648</td>
<td>11573347</td>
</tr>
<tr>
<td>7</td>
<td>4 hours</td>
<td>Shoot</td>
<td>Ammonium</td>
<td>11884087</td>
<td>12794699</td>
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<tr>
<td>8</td>
<td>4 hours</td>
<td>Root</td>
<td>Ammonium</td>
<td>8229808</td>
<td>8876691</td>
</tr>
<tr>
<td>9</td>
<td>4 hours</td>
<td>Ear</td>
<td>Ammonium</td>
<td>NA</td>
<td>8151867</td>
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<td>10</td>
<td>8 hours</td>
<td>Shoot</td>
<td>Nitrate</td>
<td>11847871</td>
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<tr>
<td>11</td>
<td>8 hours</td>
<td>Root</td>
<td>Nitrate</td>
<td>7262848</td>
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<td>12</td>
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<td>Ear</td>
<td>Nitrate</td>
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<td>11323357</td>
</tr>
<tr>
<td>13</td>
<td>8 hours</td>
<td>Shoot</td>
<td>Ammonium</td>
<td>11093192</td>
<td>11887813</td>
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<tr>
<td>14</td>
<td>8 hours</td>
<td>Root</td>
<td>Ammonium</td>
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<td>11300835</td>
</tr>
<tr>
<td>15</td>
<td>8 hours</td>
<td>Ear</td>
<td>Ammonium</td>
<td>11043816</td>
<td>11832951</td>
</tr>
</tbody>
</table>

Expression Analysis

Due to sequencer error, several libraries during the first round of sequencing either failed or exhibited low quality data. Because of this, 11 out of the 15 treatments had two technical replicates, while the remaining 4 libraries had only one sequenced replicate. Libraries with two replicates were not found to be significantly different in number of sequences read. On a gene-by-gene basis, 88.13-91.07% of genes had less than a two-fold difference in expression when comparing the two library replicates, showing the replicates were roughly similar for the majority of expressed genes. With this information in mind, gene expression was averaged between libraries with two technical replicates.

To control for fold change values being overly inflated due to very low FPKM values, a minimum baseline of 20 FPKM was set for all genes when determining differential expression.
A 1.5x fold change cutoff was set for establishing differential expression. To be considered differentially expressed, a gene of a specific nitrogen treatment must be over or under-expressed compared to both the 0-hour time point of the corresponding tissue, as well as the same tissue of other nitrogen treatment.

Gene ontology analysis was performed using agriGO (Du et al., 2010). Parametric Analysis of Gene Set Enrichment (PAGE) was used to analyze log2 transformed fold change of genes. Alpha was set at 0.05 and Hochberg FDR was utilized as the multi-test adjustment method. Both Plant Metabolic Network (http://www.plantcyc.org) and MapMan software (Thimm et al., 2004) were utilized to investigate gene expression in specific metabolic pathways.

**qRT-PCR**

RNA expression of two glutamine synthetase genes was quantified using MJ Opticon II qRT-PCR system. Original RNA samples were reverse transcribed to cDNA using the Bio-Rad iScript™ cDNA Synthesis Kit. The following 20 µL reaction mixture was used: 10 µL SYBR® Select Master Mix (Applied Biosystems), 7 µL H2O, 2 µL 1:10 dilution of cDNA, 1 µL forward and reverse primers (Invitrogen). Reaction mixes were subjected to 43 cycles of cDNA amplification, followed by a standard melting curve. Primers amplifying glyceraldehyde 3-phosphate dehydrogenase were used as the relative expression control in the study. Cycle threshold (C_T) values were extracted to Microsoft Excel for analysis.

Primers of glutamine synthetase 6 (Gln6, grmzm2G050514) and glutamine synthetase 1 (Gln 1, grmzm2G098290) were used (Church, 2008). Primers for Gln6 result in a 146 bp product and have the following sequences:
Results and Discussion

Library Summary Statistics

On average over 3.9 million reads mapped to unique maize genes. These reads mapped to an average of 18,513 different genes in each treatment, resulting in an average FPKM of 212. The majority of genes were found to be expressing across all three tissues, though many genes were unique to specific tissues or only two of the three tissues (Figure 5A). Shoot and root tissues shared the greatest number of expressed genes. Likely due to being tissues that are not directly connected, the roots and ear share the fewest number of genes. The nitrogen treatment and time point treatment showed similar patterns of gene presence and absence (Figure 5B & C). The before nitrogen and 0-hour time point treatments showed the least similarity compared to the NO$_3^-$ and NH$_4^+$ treatments and the 4-hour and 8-hour time points, respectively. This suggests that nitrogen induction, regardless of the specific source, has a more similar pattern of gene expression than plants not treated with nitrogen.
**Differential Gene Expression**

The number of differentially expressed genes that respond uniquely to $\text{NH}_4^+$ and $\text{NO}_3^-$ supplementation is relatively similar. Roughly two hundred genes are over or under-expressed with $\text{NH}_4^+$ induction in each tissue at both the 4 and 8-hour time points (Figure 6A). Only nine genes are shown to respond to $\text{NH}_4^+$ in multiple tissues, and no genes respond similarly in both root and ear tissue. This pattern is comparable in genes responding to nitrate at both the 4 and 8-hour time points (Figure 6B). Two to three hundred genes respond to nitrate in each tissue. Again only a handful of genes respond to nitrate in multiple tissues. There is also still no overlap in the ear and root tissues, suggesting the root and ear are more dissimilar in gene expression and response compared to the other two tissue comparisons.

Because only two to three hundred genes responded to either nitrogen treatment at both time points, it is likely that the 4 and 8-hour time points are relatively unique in their developmental response to nitrogen induction. Looking at only one time point at a time, gene response to either nitrogen source increases significantly (Figure 7). Gene response in specific
tissues ranges from eleven hundred to over six thousand. There is also much more overlap in
gene response in multiple tissues as well as overlap in all three tissues. The significant
differences in nitrogen response at the two time points suggest that differential gene
expression should be analyzed separately for the two time points rather than analyzing only
“high confidence” genes that respond similarly in both time points.

Figure 6. Number of genes in each tissue differentially expressed at both the 4 and 8 hour time points
to either ammonium (A) or nitrate (B). To be considered differentially expressed the gene in a specific
nitrogen treatment must be 1.5x over or under-expressed compared to both the 0-hour time point of
the corresponding tissue, as well as the same tissue of other nitrogen treatment.
Figure 7. Number of genes in each tissue differentially expressed in ammonium-treated plants 4 hours after induction (A), ammonium-treated plants 8 hours after induction (B), nitrate-treated plants 4 hours after induction (C), and nitrate-treated plants 8 hours after induction (D). To be considered differentially expressed the gene in a specific nitrogen treatment must be 1.5x over or under-expressed compared to both the 0-hour time point of the corresponding tissue, as well as the same tissue of other nitrogen treatment.

Gene Ontology Analysis

Gene ontology (GO) was analyzed for the over-representation of upregulated or downregulated genes in certain annotation groups. This analysis was applied to the log2 transformed fold change of each nitrogen treatment at both time points compared to the corresponding 0-hour time point. In general, the root had a greater number of GO terms that were upregulated, whereas GO analysis of shoot and ear tissues showed more terms being downregulated. In the root a number of interesting GO terms were upregulated. Several terms
involving nitrogen, amine, and amino acid metabolism are upregulated in both \(\text{NH}_4^+\) and \(\text{NO}_3^-\) treated plants. Amino acid biosynthesis, however, is only significantly upregulated in \(\text{NH}_4^+\)-treated plants (Figure 8). More specifically, glutamine biosynthesis is only upregulated in \(\text{NH}_4^+\)-treated plants at the 4 hour time point. Additionally, the GO terms “response to oxidative stress” and “antioxidant activity” are upregulated in \(\text{NO}_3^-\)-treated plants. This suggests high levels of \(\text{NO}_3^-\) are a source of oxidative stress on plants. No nitrogen or amino acid terms are upregulated in the shoot, though nitrogen compound biosynthesis is upregulated in the ear.

**Figure 8.** A selection of upregulated gene ontology terms in the root. Each annotation term is divided into the following four treatments in that order: ammonium-treated plants 4 hours after induction, ammonium-treated plants 8 hours after induction, nitrate-treated plants 4 hours after induction, and nitrate-treated plants 8 hours after induction.
Upregulation of Amino Acid Metabolism in Ammonium-Treated Plants

Gene ontology analysis showed evidence for an upregulation of genes in the roots involved in amino acid metabolism in NH$_4^+$-treated plants. This coincides with a physiological increase of total amino acid concentrations as well as the individual amino acids asparagine and glutamine in NH$_4^+$-treated plants, thus further analysis of the upregulated amino acid metabolism genes throughout the plant may give better insight into the observed increase in amino acids and ear growth rate. Using MapMan to analyze differential expression in amino acid metabolism pathways, twenty-three genes involved directly in amino acid metabolism were found to be upregulated specifically in NH$_4^+$-treated plants (Table 3). None of the twenty-three genes are upregulated in more than one tissue, although there are several genes coding for the same enzymatic function in multiple tissues.

Six genes are upregulated in root tissues (Figure 9A). Three of these genes are likely directly involved in the primary uptake and assimilation of NH$_4^+$. The first, a gene coding for a high affinity transmembrane ammonium transporter protein, is likely dealing with the large influx of NH$_4^+$ into the root. Increased production of this protein in the root may allow the plant to both take up more NH$_4^+$ as well as more rapidly transport the ion throughout the root system for assimilation. The upregulation of two cytosolic glutamine synthetase genes suggests the root is the primary site for initial NH$_4^+$ assimilation into organic nitrogen. GS is the major path of plant nitrogen assimilation, thus upregulation of these two genes in the root may be a key mechanism by which NH$_4^+$-treated plants are able to more efficiently utilize nitrogen.

The remaining three genes play a role in glutamate metabolism. Glutamate dehydrogenase catalyzes the reversible deamination of glutamate, forming 2-oxoglutarate and
$\text{NH}_4^+$ – It is unclear in this scenario of excess $\text{NH}_4^+$ whether this upregulated enzyme catalyzes the forward or reverse reaction. Studies have consistently shown that the deamination of glutamate is the normal direction of this reaction in plants (Lam et al., 1996). If this is the case, GDH may be playing a role in the recycling of $\text{NH}_4^+$ in the roots. However, certain studies also suggest that GDH may assimilate $\text{NH}_4^+$ in cases of high stress such as high concentrations of $\text{NH}_4^+$ (Skopelitis et al., 2006). If this is the case, GDH may be acting as an alternate path of assimilation in ammonia-treated plants. Upregulated glutamate decarboxylase catalyzes the breakdown of glutamate to GABA and CO$_2$. GABA production has been shown to be induced by an $\text{NH}_4^+$ treatment (Roberts et al., 1992), and the increase of GABA production in the root may be linked to plant stress response (Kinnersley & Turano, 2000). Finally, a gene coding for the enzyme phenylalanine ammonia-lyase is upregulated in the root of $\text{NH}_4^+$-treated plants. Phenylalanine ammonia-lyase breaks the amino acid phenylalanine down into trans-cinnamate and $\text{NH}_4^+$. This further supports the possibility that $\text{NH}_4^+$ is being recycled in the roots even after the initial assimilation.

Shoot tissues treated with $\text{NH}_4^+$ showed the fewest number of upregulated genes involved specifically in amino acid metabolism (Figure 9B). Aspartate-semialdehyde dehydrogenase is upregulated. The enzyme produces a metabolite in aspartate metabolism that is a precursor to the amino acids lysine, methionine, isoleucine, and threonine. The remaining four upregulated genes all produce transporter proteins: one ammonium and three amino acid transporters. Although much of the $\text{NH}_4^+$ was assimilated in the root, it is likely a smaller amount is still exported from the root to the shoot, so ammonium transporters continue to play a role in the shoot. Amino acids, though, are likely the major form of
transported nitrogen in the shoot. This is evident in three amino acid transporters being upregulated. Though there is clear evidence for the upregulation of both NH$_4^+$ and amino acid transport, other areas of amino acid metabolism show little to no effect. These nitrogen metabolites are likely being transported to sink tissues such as the ear, tassel, and developing leaves, although less seems to be utilized in developed shoot tissues.

In the ear two more amino acid transporters are upregulated in NH$_4^+$-treated plants, indicating the ear is a sink for assimilated nitrogen (Figure 9C). This is additionally supported by the upregulation of several other amino acid metabolism genes. An upregulated urease enzyme suggests a recycling of nitrogen into a more usable form in the ear. Urease breaks down urea into CO$_2$ and NH$_3$, a more metabolically available form of nitrogen. Nitrogen recycling is also supported by the upregulation of a cytosolic GS gene in the ear, though unassimilated NH$_4^+$ transported from the roots could also be inducing increased GS activity.

Aside from these genes, eight others involved in amino acid metabolism are upregulated. They are involved in the biosynthesis of glycine, cysteine, methionine, lysine, threonine, isoleucine, and histidine, as well as the breakdown of leucine. The upregulation of these genes show that not only nitrogen transport and assimilation are active in the ear, but the utilization of these nitrogen compounds for growth and metabolism is occurring as well.
Table 3. Amino acid metabolism genes upregulated in ammonium-treated plants in at least one time point. Gene identifier (GRM), enzyme function, tissue, and FPKM of the five nitrogen and time point treatments for each gene are given.

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Figure 9. Pathways upregulated in ammonium-treated plants. Pathways of genes upregulated in ammonium-treated plants in the root (A), shoot (B), and ear (C) are highlighted in red. Double-sided arrows next to NH$_4^+$, NO$_3^-$, and AAs (amino acids) denote transporters for that metabolite. Highlighted amino acid names signify an upregulated gene involved in the biosynthesis or metabolism of that amino acid.
qRT-PCR

Gene expression quantification via qRT-PCR was conducted on the GS genes *Gln6* and *Gln1*. Expression of *Gln6* was studied to verify the expression trends of the same gene in the above RNA-Seq study and to validate the RNA-Seq study as a whole in assessing RNA abundance. *Gln1* is the only plastid-localized GS gene and is known to be highly expressed in the shoot. The gene was either very lowly expressed or completely absent in the above RNA-Seq libraries, thus qRT-PCR was used to assess the gene’s abundance.

qRT-PCR revealed similar trends of *Gln6* expression as in the RNA-Seq study (Figure 10A). The gene was highly expressed in the root, though expression was very low in shoot and ear tissues. Like RNA-Seq study, the gene was upregulated in the NH$_4^+$-treated roots compared
to both the NO₃⁻-treated roots at the same time point and the 0-hour control. This result confirms the overexpression of *Gln6* in the roots of NH₄⁺-treated plants and further validates genes found to be differentially expressed in the above RNA-Seq study.

*Gln1* was found to be moderately expressed in all tissues, unlike what was found in the RNA-Seq study (Figure 10B). Expression was highest in shoots and lowest in the ear. Expression of the gene appears to be downregulated in the ear with both NH₄⁺ and NO₃⁻ application. Expression is also downregulated in the roots, though more so in NH₄⁺-treated plants. The gene is somewhat upregulated in the shoots of NO₃⁻-treated plants. This suggests that more nitrogen, in the form of NO₃⁻, is reaching the shoot chloroplasts for assimilation in NO₃⁻-treated plants.

**Figure 10.** Gene expression via qRT-PCR of *glutamine synthetase 6* (A) and *Glutamine synthetase 1* (B).
Conclusions

The above results support NH$_4^+$ and NO$_3^-$ having distinct systems of uptake, assimilation, transport, and utilization. Most of the NO$_3^-$ taken up by the plant is sent to the leaves for assimilation or storage in the vacuole. Once assimilated, much of this is incorporated into proteins, especially for photosynthesis, in the leaves where it was assimilated. Much of this incorporated nitrogen is not available again for ear growth until leaf senescence when proteins are broken down to amino acids for remobilization to the ear and other developing tissues. Because of this, increased concentrations of NO$_3^-$ have little immediate effect on the developing ear growth rate. Much of the NH$_4^+$ entering the roots is assimilated in the roots into amino acids to avoid the toxic effects of high concentrations of NH$_4^+$. Some of this NH$_4^+$ may be transported to the shoot, but is largely transported in amino acid form, especially glutamine,
asparagine, glutamate, and aspartate. These amino acids can be transported directly to the ear for utilization in growth and development of the reproductive structure. These two proposed systems of uptake, assimilation, and transport support the observation of significantly increased ear growth in NH₄⁺ treated plants.

Increased expression of GS in the roots shows both that the root tissue is the primary site of NH₄⁺ assimilation and that increasing this nitrogen assimilating enzyme is one of the main routes to quickly alleviate the high concentrations of NH₄⁺ entering the roots. Increased GS expression in the ear, along with several other amino acid metabolism genes, shows that a significant portion of the nitrogen in NH₄⁺-treated plants is reaching the ear for utilization. These findings show that GS and the assimilation of nitrogen in NH₄⁺-treated plants may be one of the limiting factors for nitrogen utilization by the maize ear.

Consequently, increasing the expression of GS in maize may be a potential route to improving its nitrogen use efficiency and possibly its overall grain yield; however, as this study shows, timing and form of nitrogen application are important factors that must be considered in conjunction with GS expression. Several previous studies have already reported that overexpression of cytosolic GS is a viable route to increasing plant nitrogen use efficiency and grain yield. Knockout mutant studies of GS in maize and rice have shown that grain development is significantly negatively affected when certain cytosolic GS genes are not expressed (Martin et al., 2006; Tabuchi et al., 2005). Several cytosolic GS overexpression studies have also shown enhanced grain yield and plant biomass in maize, wheat, and tobacco (Martin et al., 2006; Habash et al., 2001; Fuentes et al., 2001; Oliveira et al., 2002). These
overexpression studies especially stress increased yield and plant biomass under low nitrogen conditions.

Although these studies have demonstrated overexpressed GS could potentially have a commercial impact on nitrogen use efficiency, there are currently no commercial transgenic crops with improved nitrogen use traits. Because of the high variability and complexity of nitrogen status, moisture, and weather in the field, successful transgenic studies in the greenhouse or growth chambers may not translate well to the field. Maize with improved yield and nitrogen use efficiency through the overexpression of GS would need to be coupled with the correct nitrogen management to realize its full potential in the field. As this study has shown, both the form and timing of nitrogen are crucial factors that would affect the outcome of a maize plant overexpressing GS in the field. The availability of NH$_4^+$ as a nitrogen source is critical because NO$_3^-$ is most often assimilated by the chloroplastic form of GS. This would both severely reduce the effectiveness of an overexpressed cytosolic GS and would tie up more nitrogen in leaf proteins rather than being immediately available for use by the ear. Additionally, NH$_4^+$ would be most effective during reproductive development. Without the ear as a sink tissue, NH$_4^+$ assimilated in the roots would largely follow the same fate as NO$_3^-$ by being shipped to the shoot for incorporation into leaf proteins.
CHAPTER 2: EFFECT OF ASPARAGINE-CYCLING PATHWAY ON MAIZE EAR GROWTH

Introduction

The Asparagine-Cycling Pathway

The asparagine-cycling pathway is the main pathway that controls the interconversion of the amino acids asparagine and aspartate (Figure 11). Additionally, the pathway serves as a secondary pathway for cycling of glutamine and glutamate. The pathway is composed of two enzymes: Asparagine synthetase (AS) and Asparaginase (ASNase). AS catalyzes the transamination of an amide group from glutamine onto aspartate, resulting in the amino acids asparagine and glutamate. Four genes for AS have been identified in maize (Todd et al., 2008). These four genes all have differing gene expression profiles across tissues and developmental time points, suggesting unique roles in asparagine production for the four genes in maize. AS has also been shown to be highly light-sensitive, being expressed much more abundantly in the dark (Lam et al., 1994). ASNase catalyzes the breakdown of asparagine into aspartate and NH$_4^+$.

There have been two genes of ASNase identified in maize. ASNase has been found to be more active in developing tissues, such as young leaves, roots, and developing seeds (Michalska et al., 2006). Because transported asparagine supplies a large portion of the nitrogen in these developing tissues, ASNase works to convert the amino acid into a more metabolically active form.
Asparagine, the product of AS, is an important transport and storage amino acid in plants (Sieciechowicz et al., 1988). It is more efficient than glutamine as a nitrogen transporter, as it has a higher nitrogen to carbon ratio. Asparagine is also a relatively inert and stable amino acid, making it an important nitrogen storage compound. The two resulting metabolites from ASNase are much more active in plant metabolism and growth. \( \text{NH}_4^+ \) can be recycled back into glutamine where it can be utilized in the biosynthesis of many different nitrogen-containing compounds. Aspartate can also be utilized for the metabolism of many compounds, including the direct biosynthesis of the amino acids lysine, methionine, isoleucine, and threonine.

**Figure 11.** The asparagine-cycling pathway. Asparagine synthetase drives the production of asparagine, which is utilized for nitrogen transport and storage. Asparaginase breaks asparagine down into forms more available for metabolism and growth.

**Illinois Protein Selection Strains in Relation to the Asparagine-Cycling Pathway**

Illinois High Protein (IHP) and Illinois Low Protein (ILP) are maize lines that have gone through over 110 cycles of recurrent selection for grain protein concentration since 1896 (Lucas et al., 2013). Through selection IHP has increased in grain protein from about 10% to over 32%, while selection in ILP has reduced grain protein to 4%. Though plants were only selected for grain protein, several other nitrogen-related traits have been concurrently altered in IHP and
ILP as well. These two populations clearly show grain protein is inversely correlated with both grain starch and kernel size, which has led to greater grain yield in ILP. IHP takes up more total nitrogen from the soil than ILP and has greater nitrogen remobilization and partitioning to the developing ear (Below et al., 2004). Though IHP is shorter and more prone to lodging, it also has a larger root system, which likely aides in its enhanced nitrogen uptake (Lohaus et al., 1998). Hybrids created by crossing inbred IHP and ILP lines to a common parent also showed unique nitrogen characteristics (Uribelarrea et al., 2004). Grain protein responds increasing nitrogen supplementation in IHP hybrids, whereas no response to nitrogen is seen in ILP hybrids.

Similar to NH$_4^+$-treated plants from chapter 1, IHP hyper-accumulates the amino acid asparagine. Leaf and phloem tissues show greater than a tenfold increase in asparagine concentration in IHP relative to ILP (Lohaus et al., 1998). Maize asparagine is highly responsive to supplemental nitrogen and has been shown to be a marker of plant nitrogen (Seebauer et al., 2004). This accumulation of asparagine in IHP is attributed to unique variants of the AS and ASNase genes (Church, 2008). Asparagine synthetase 3 (AS3) is normally expressed only in the dark since it is diurnally regulated. However, AS3 in IHP is highly expressed for both RNA and protein throughout light and dark periods. RNA expression gives the opposite result for ASNase in IHP. The gene is essentially a knockout in IHP and has little to no expression throughout the plant. The combination of alleles for these two genes together results in the very high accumulation of asparagine in IHP, whereas ILP has low asparagine concentrations due to low AS and high ASNase (Figure 12).
Figure 12. Complement of Asparagine synthetase (AS) and Asparaginase (ASNase) alleles in Illinois High Protein (IHP) and Illinois Low Protein (ILP). IHP has high expression of daytime AS and very low expression of ASNase, resulting in high accumulation of asparagine. ILP has low expression of AS and high expression of ASNase, resulting in a higher rate of asparagine breakdown and aspartate and NH$_4^+$ production.

Functional Variation of the Asparagine-Cycling Pathway in Maize

The upregulation of asparagine in NH$_4^+$-treated plants (Chapter 1) and in IHP suggests that these two scenarios may have a similar underlying genetic mechanism. If this is the case, AS would be expected to be upregulated and ASNase should be downregulated in NH$_4^+$-treated plants in the previous RNA-Seq study. Unfortunately, Gaspé Flint has the light regulated version of AS, and that experiment was conducted in full light. If AS is upregulated in NH$_4^+$-treated maize, the experiment would likely need to be performed in the dark for genotypes that have a light-sensitive AS allele. ASNase was somewhat downregulated, although it was downregulated in both the NH$_4^+$ and NO$_3^-$ nitrogen treatments.

Because of the demonstrated association of increased asparagine concentration with increased ear growth, it would be interesting to determine if the two genes controlling asparagine levels, AS and ASNase, play a role in ear growth, even though they were not up or downregulated in NH$_4^+$-treated plants in the previous experiment. The goal of the following experiment is to utilize functional variants of AS and ASNase in maize to determine if either of the two genes play a role in ear growth. To be able to test the effect of these two genes on ear growth, maize germplasm with alleles that clearly vary in expression are necessary.
As stated previously, IHP and ILP have distinct alleles for both genes. For AS many maize inbreds also have either the IHP or ILP allele. The maize inbred B73 shares the lower expressing ILP allele, whereas Mo17 is highly expressing like IHP (Figure 13). Sequenced by Farag Ibraheem, the highly expressing IHP allele of AS was found to have an insertion in its promoter sequence with an additional G-box promoter sequence that may contribute to its increased expression. qRT-PCR confirmed these expression trends for IHP, ILP, B73, and Mo17 (Figure 14). Three variants of ASNase have been identified. Like AS, ILP shares its highly expressing allele with B73. However, IHP and Mo17 differ in their ASNase alleles. IHP has little to no expression of ASNase, possibly due to both an insertion and a deletion in its promoter region. Mo17 also has an insertion in its promoter region, though it only has moderately reduced expression compared to IHP and B73. Using populations containing these variations of AS and ASNase, it is possible to test the role these genes play on ear growth.

**Figure 13.** Allele variants of Asparagine synthetase and Asparginase. Asparagine synthetase (A) has a lower expressing ILP/B73 allele and a higher expressing IHP/Mo17 allele. Asparaginase (B) has a highly expressing ILP/B73 allele, a very low expressing IHP allele, and a moderate expressing Mo17 allele. Green rectangles represent G-box promoter sequences. Yellow triangles represent insertion sequences. Brackets represent deleted sequence. Arrows represent the transcribed portion of the gene.
Figure 14. Gene expression via qRT-PCR of the inbreds B73, Mo17, ILP, and IHP for Asparagine synthetase (A) and Asparaginase (B). Blue bars with a minus (-) symbol denote being grown with no supplemental nitrogen. Red bars with a plus (+) symbol denote being grown with high nitrogen supplementation.

Materials and Methods

Plant Materials and Experimental Design

Four sets of material were utilized to test the effect of AS and ASNase on ear growth. A subset of 20 lines from the intermated B73 x Mo17 recombinant inbred line (IBMRI) population were used (Lee et al., 2002). Both inbreds and hybrids crossed to LH82 were examined. Hybrids were developed by Jessica Bubert at the University of Illinois in 2011. Plants were grown during the 2013 growing season in single-row, paired high and low nitrogen plots. Each hybrid plot was 5.3 m long with .76 m spacing between rows. Each inbred plot was 3.66 m long with .76 m spacing between rows. Hybrid plots were hand-planted at 30 plants per plot, for a density of about 74,000 plants ha⁻¹. Inbred plots were hand-planted at 20 plants per plot, for a density of about 70,000 plants ha⁻¹. Hybrids were blocked by three replicates, and inbreds were blocked by two replicates. Hybrids and inbreds were grouped by maturity to facilitate sampling.
and phenotyping and also to avoid shading stress between rows. No supplemental nitrogen was added to low nitrogen plots. High nitrogen plots received 224 lbs ha\(^{-1}\) of supplemental nitrogen. Nitrogen was applied at the V4 growth stage as granular ammonium sulfate in two bands adjacent to the plots. Paired nitrogen plots were chosen to minimize spatial variation of nitrogen in the field.

The second population was a selection of 20 inbred parents from the maize nested association mapping (NAM) population and ex-Plant Variety Protection (ex-PVP). The following inbreds were tested: O1DFA3, 91CSV-1, B73, B97, CML103, FBPL, Ky21, LH1, LH123, LIZL5, Mo17, MS71, Oh43, Oh7B, PH207, PHG35, PHG47, PHG84, PHJ40, and PHZ51. These inbreds represent a diverse set of maize germplasm. Again, both inbreds and hybrids crossed to LH82 were tested. Hybrids were made by Jessica Bubert at the University of Illinois in 2011 growing season, and the material was grown for analysis in 2013. The experimental design was the same as the IBMRI population listed above.

Illinois protein strain recombinant inbreds (IPSRI) were also tested. The IPSRI population was developed by intermating the IHP and ILP lines. The IPSRIs were developed by the Dudley and Moose labs at the University of Illinois (Dudley et al., 2007; Lucas et al., 2013). 150 IPSRI lines were grown in 2013. Single-row plots at the inbred dimensions listed above were grown without supplemental nitrogen application. 15 seeds were planted per plot, giving a density of about 52,500 plants ha\(^{-1}\). The IPSRIs were again grouped by maturity to facilitate sampling and avoid shading stress.

The final set of material was an [IRHP1 x (IHP1 x IRHP1)] BC1S1 population. The population was by crossing IHP1 to IRHP1, crossing this again to IRHP1, and finally making a self
of this backcross. Illinois Reverse High Protein (IRHP) is a line derived from IHP by selecting for low protein out of the high protein population. It is genetically similar to IHP, though it shares the ILP alleles of AS and ASNase. This population was developed by Christine Lucas at the University of Illinois in 2009 and 2010. 70 single-row BC1S1 plots were grown at the inbred dimensions listed above in 2012. 20 plants were planted per plot at a density of about 70,000 plants ha⁻¹. Plots were not grown with supplemental nitrogen application. Plots were ordered by random design.

**Genotyping of Asparagine Synthetase and Asparaginase**

Because it was a segregating population, genotyping for each plant was conducted in the [IRHP1 x (IHP1 x IRHP1)] BC1S1 population for the AS and ASNase genes. Approximately eight leaf discs from 1186 plants were hole-punched into 1.1 mL tubes. Leaf tissue was lyophilized and ground to powder using two steel balls in each tube and a GenoGrinder 2000 at 1500 rpm. DNA was extracted using a modified version of the PUREGENE protocol by Quiagen. Leaf powder was treated with cell lysis buffer and protein precipitate solution. DNA was precipitated with isopropanol and rinsed with 70% ethanol. DNA pellets were rehydrated with 100 µL of DNase-free water.

Primers designed to genotype AS and ASNase were created by Farag Ibraheem and Yuhe Liu of the University of Illinois, respectively. The AS primers anneal to the promoter region of the gene. IHP alleles result in 448 and 778 bp bands following PCR, while ILP alleles have a single 557 bp band. The primer sequences are as follows:

**Forward**- 5’-CTCAACTCATCGGCACAGACTTGCATC - 3’

**Reverse**- 5’- TCGAATTTATCCTTCTACAACCCCAATC - 3’
ASNase primers anneal to the 3’ UTR region of the gene. IHP alleles result in 309 bp bands following PCR, whereas ILP alleles have a 294 bp band. The primer sequences are as follows:

Forward: 3’-TTAAACACATGGCAATCGCAGGAT -5’
Reverse: 5’-TCATGGAGTACAAGGGCCTGCC-3’

The same PCR protocol was used for both genes. Initial denaturation was 94°C for 2 minutes. Following that was 40 cycles of denaturation at 94°C for 30 seconds, annealing of primers at 53°C for 1 minute, and elongation of nucleotides at 72°C for 1 minute. The program ended at 72°C for 7 minutes. Polymorphisms were determined using gel electrophoresis and a UV light box to illuminate ethidium bromide intercalated in the DNA products. 1% agarose gels were used to detect polymorphisms for AS. Higher concentration 3% gels were used to identify polymorphisms for ASNase because the PCR products for the two alleles only have a 15 bp difference in size.

Genotype information of AS and ASNase was already available for most samples in the IBMRI, IPSRI, and NAM/ex-PVP populations.

**Plant Sampling and Phenotypic Measurements**

Plants were sampled for ear traits at the R1 and R6 growth stages. The R1 growth stage in maize corresponds to pollination, and R6 is maturity. At the R1 stage earshoots were sampled just after emergence of the silks. Earshoots were bagged to prevent pollination of ears. For the IBMRI, IPSRI, and NAM/ex-PVP inbred populations, two earshoots were sampled from each row. 361 earshoots were sampled from the [IRHP1 x (IHP1 x IRHP1)] BC1S1 population based on the genotype information of individual plants. Earshoots were lyophilized,
and the weight and length of each earshoot were measured. Individual free amino acid concentrations were measured for the inbreds and hybrids of the IBMRI and NAM/ex-PVP populations. Lyophilized earshoots were ground to powder with a steel ball and a GenoGrinder 2000 at 1500 rpm. Free amino acids of approximately 60-70 mg of tissue were extracted with 500 mL of trichloroacetic acid. Samples were filtered into 96-well plates and run on a 1200 Agilent HPLC for amino acid quantification. HPLC analysis was adapted from a protocol developed by Agilent (Woodward et al., 2007). Amino acids were derivatized with o-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC). Metabolites were separated using a 4.6 x 50 mm, 1.8 micron particle size Zorbax Eclipse Plus C18 column (Agilent PN-959941-902). A diode array detector is used to detect and quantify amino acids. Chemstation (Agilent) was the software used to run the machine, visualize the chromatograms, and export the data.

At maturity 190 ears were sampled from the IRHP1 x [IHP1 x IRHP1] BC1S1 population. Grain weight, cob weight, kernel weight, and kernel number were measured. Grain nitrogen, starch, and oil were also measured with a Perten DA 7200 Near Infrared (NIR) Analyzer. Four ears were sampled from each row of the IBMRI and NAM/ex-PVP populations. Ears were dried, and grain weight, cob weight, kernel weight, and kernel number were measured. Grain nitrogen, starch and oil were measured with a Perten DA 7200 Near Infrared (NIR) Analyzer.
Results and Discussion

**Ear Size at Anthesis**

The IBMRI population was tested for the effect of nitrogen rate, AS, ASNase, and the interaction of nitrogen with the two genes (Table 4). Nitrogen did not have a significant effect on the earshoot weight or length of IBMRI inbreds, though IBMRI hybrids had significantly longer earshoots at high nitrogen supplementation. The IBMRI population was tested for the difference between B73 and Mo17 alleles for each gene. The higher expressing Mo17 allele for AS had a significantly greater earshoot length than the B73 allele in the IBMRI inbreds. The effect of ASNase was significant for earshoot weight. Earshoots with the moderately expressing Mo17 allele had a greater earshoot weight than those with the highly expressing B73 allele. In the IBMRI hybrids, the higher expressing Mo17 allele for AS had both a greater length and weight than the B73 allele (Figure 15). Again, earshoots with the moderately expressing Mo17 allele for ASNase had a greater earshoot weight than those with the highly expressing B73 allele.

The diversity NAM/ex-PVP population was also tested in both inbreds and hybrids. There was no significant response to nitrogen in the earshoots for both the inbreds and hybrids. The IHP and B73 alleles of both AS and ASNase were tested for their effect on earshoot traits. Earshoots with the highly expressing IHP allele of AS had both a significantly greater length and weight (Figure 16). Earshoots with the highly expressing B73 allele of ASNase had both a significantly greater length and weight compared to the non-expressing IHP allele. Neither gene had a significant effect on earshoot size in the NAM/ex-PVP hybrids.
The IPSRI population was tested for the effect of the IHP and ILP alleles of AS and ASNase on earshoot size. Earshoots with the highly expressing IHP allele of AS had a greater earshoot length than the lower expressing ILP allele. Alleles of ASNase were not significantly different for earshoot length or weight.

Because it was a segregating population, the IRHPx(IHP1xIRHP1)BC1S1 population was tested for the effect of IHP, ILP and heterozygous alleles in AS and ASNase on earshoot size. Alleles were not significantly different for AS for earshoot length or weight. The ILP allele of ASNase was significantly greater in earshoot weight and length than both the IHP and heterozygous allele. The IHP and heterozygous allele were not significantly different from each other.

When looking at the trends for the effect of AS on earshoot length and weight, the higher expressing allele, Mo17 or IHP, is greater than the lower expressing allele, B73 or ILP, in every case that it is significant. This suggests that greater production of asparagine promotes earshoot growth and development. The trend for the effect of ASNase on earshoot growth is not quite as clear. Earshoots with the lower expressing Mo17 allele of ASNase have a greater earshoot weight than their higher expressing B73 counterparts in the IBMRI populations. However, earshoots with the non-expressing IHP allele have a significantly lower earshoot weight and length in the NAM/ex-PVP inbred and IRHPx(IHP1xIRHP1)BC1S1 populations. This result suggests a complete knockout of ASNase may be detrimental to earshoot growth, though only a partial reduction in ASNase does not have a negative effect.
Table 4. Effect of nitrogen, Asparagine synthetase, and Asparaginase on earshoot weight and length. Nitrogen and allelic trends are shown when significant. N*AS and N*ASNase are interaction terms between nitrogen rate and the alleles of the two genes. NS denotes not significant. Het denotes heterozygote. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.

<table>
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<th>Background Trait</th>
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<td>NS</td>
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<td>Earshoot Weight</td>
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<td>NS</td>
<td>ILP &gt; het = IHP *</td>
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</table>

Figure 15. Effect of Asparagine synthetase (AS) and Asparaginase (ASNase) variants on earshoot weight (A) and length (B) in the IBMRI hybrids. Weight is measured in grams and length is measured in millimeters. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.
Figure 16. Effect of Asparagine synthetase (AS) and Asparaginase (ASNase) variants on earshoot weight (A) and length (B) in the NAM/ex-PVP inbreds. Weight is measured in grams and length is measured in millimeters. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.

R1 Earshoot Amino Acids

Twenty-one amino acids were profiled in each sample via HPLC: the twenty common proteinogenic amino acids and hydroxyproline. For brevity only the four amino acids involved directly in the asparagine-cycling pathway (asparagine, aspartic acid, glutamine, and glutamic acid) and the total amino acid concentration will be presented in this study (Table 5). Individual amino acids were analyzed as their ratio of the total amino acid pool to control for concentration variation among samples. Amino acid profiles were analyzed in the IBMRI and NAM/ex-PVP populations in both inbreds and hybrids. The same model was applied as for earshoot size at anthesis.

Amino acids did not respond to nitrogen in the inbreds of either the IBMRI or NAM/ex-PVP populations. Hybrids of the two populations, though, showed the same trends. Asparagine, glutamine, and total amino acids responded positively to high nitrogen supplementation. Aspartic acid did not significantly respond to nitrogen. Glutamic acid concentration has a negative response to high nitrogen supplementation. This trend is likely
due to high glutamine production from glutamic acid when excess inorganic nitrogen is present in the plant.

Allelic differences in AS did not show a significant effect on amino acids in most cases, though an effect was observed for asparagine in the IBMRI and NAM/ex-PVP inbreds. Earshoots with the lower expressing B73 allele of AS had a greater asparagine concentration than the higher expressing Mo17 allele. The opposite trend was observed in the NAM/ex-PVP inbreds. Earshoots with the higher expressing IHP allele had a greater asparagine concentration than the B73 allele. These contradicting results and the fact that they are only significant at the 0.1 P>F level suggest that they may not be exhibiting a true effect of AS on asparagine concentration.

ASNase exhibited an effect on amino acid concentrations in the IBMRI population. Asparagine had a significantly greater concentration in earshoots with the lower expressing Mo17 allele than earshoots with the B73 allele in IBMRI hybrids. Aspartic acid showed the same trend in the IBMRI inbreds. Decreased expression of ASNase intuitively increases the concentration of asparagine through limiting the breakdown of the amino acid. However, decreasing ASNase and the breakdown of asparagine into aspartic acid also has the effect of increasing aspartic acid in the IBMRI inbreds. Possibly, decreasing ASNase and increasing asparagine may also inhibit synthesis of asparagine. Aspartic acid would then have a route to accumulate through production by the enzyme aspartate aminotransferase. Glutamine had a significantly greater concentration in earshoots with the B73 allele than earshoots with the Mo17 allele in IBMRI hybrids. Greater expression of ASNase results in the release of larger amounts of NH$_4^+$ that can be reassimilated into glutamic acid, forming glutamine. Total amino
acids are also significantly greater in earshoots with the B73 allele in both the IBMRI inbreds and hybrids. Because glutamine is usually the most abundant amino acid found in the maize earshoots, greater concentrations of the amino acid in earshoots with the B73 allele likely resulted in greater total amino acids as well.

There were also a few cases of an interaction effect between nitrogen and the two genes. Earshoots with the B73 allele of AS in the NAM/ex-PVP hybrids respond positively to nitrogen for glutamine concentration. Earshoots with the IHP allele do not respond to nitrogen, though they have high concentrations of glutamine at both nitrogen levels. This suggests that allelic differences in AS may play a role in how glutamine responds to nitrogen. In the IBMRI hybrids an interaction effect of nitrogen and ASNase was observed in both asparagine and glutamic acid concentrations. Allelic differences of ASNase did not have an effect on asparagine concentrations at low nitrogen. At high nitrogen the Mo17 allele of ASNase resulted in a greater concentration of asparagine than the B73 allele. A similar trend was observed for glutamic acid concentration. However, the allelic difference was observed under low nitrogen conditions and high nitrogen resulted in no allelic effect. These patterns suggest ASNase may only effect amino acid concentrations at certain levels of nitrogen supplementation.


Table 5. Effect of nitrogen, Asparagine synthetase, and Asparaginase on amino acid concentrations. Nitrogen and allelic trends are shown when significant. N*AS and N*ASNase are interaction terms between nitrogen rate and the alleles of the two genes. NS denotes not significant. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.

<table>
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<th>Background Trait</th>
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<tr>
<td>Aspartic acid</td>
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<td>Glutamine</td>
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<tr>
<td>Glutamic acid</td>
<td>NS</td>
</tr>
<tr>
<td>Total Amino Acids</td>
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</tr>
</tbody>
</table>

| IBMRI Hybrids    | Nitrogen         | Asparagine Synthetase | Asparaginase | N*AS | N*ASNase |
| Asparagine       | High > Low **    | NS                    | Mo17 > B73 • | NS   | Mo17 Hi N > B73 Hi N > B73 Lo N = Mo17 Lo N ** |
| Aspartic acid    | NS               | NS                    | NS           | NS   | NS       |
| Glutamine        | High > Low **    | NS                    | B73 > Mo17 • | NS   | NS       |
| Glutamic acid    | Low > High **    | NS                    | NS           | NS   | Mo17 Lo N > B73 Lo N > B73 Hi N = Mo17 Hi N * |
| Total Amino Acids| High > Low **    | NS                    | B73 > Mo17 • | NS   | NS       |

| NAM/ex-PVP Inbreds | Nitrogen         | Asparagine Synthetase | Asparaginase | N*AS | N*ASNase |
| Asparagine        | NS               | IHP > B73 •           | NS           | NS   | NS       |
| Aspartic acid     | NS               | NS                    | NS           | NS   | NS       |
| Glutamine         | NS               | NS                    | NS           | NS   | NS       |
| Glutamic acid     | NS               | NS                    | NS           | NS   | NS       |
| Total Amino Acids | NS               | NS                    | NS           | NS   | NS       |

| NAM/ex-PVP Hybrids | Nitrogen         | Asparagine Synthetase | Asparaginase | N*AS | N*ASNase |
| Asparagine        | High > Low **    | NS                    | NS           | NS   | NS       |
| Aspartic acid     | NS               | NS                    | NS           | NS   | NS       |
| Glutamine         | High > Low **    | NS                    | NS           | NS   | IHP Lo N > B73 Lo N * |
| Glutamic acid     | Low > High •     | NS                    | NS           | NS   | NS       |
| Total Amino Acids | High > Low **    | NS                    | NS           | NS   | NS       |

Grain and Cob Weight

Mature ear traits were measured in the IBMRI, NAM/ex-PVP, and IRHPx(IHP1xIRHP1)BC1S1 populations. Grain weight and cob weight were measured in all sets of materials, and kernel weight and kernel number were also determined for the hybrids and backcross populations (Table 6). Nitrogen was shown to have a significantly positive effect for all four traits in the hybrids. Cob weight in the IBMRI inbreds was the only other trait to show a significantly positive response to nitrogen.
Significant effects on ear traits by variants of AS were observed in the IBMRI hybrids and the NAM/ex-PVP inbreds. Ears with the highly expressing Mo17 allele of AS had a greater grain weight in the IBMRI hybrids (Figure 17A), as well as a greater cob weight in both the IBMRI hybrids (Figure 17B) and the NAM/ex-PVP inbreds. This is the same trend observed in the developing earshoots, which again supports the hypothesis that increased AS expression is beneficial to ear growth and development. When the grain weight of the IBMRI hybrids is broken down into its component parts kernel weight and kernel number, kernel weight is found to be the component contributing to greater grain weight in IBMRI hybrids with the Mo17 allele (Figure 17C). This suggests the gene contributes more to grain fill than the number of developed kernels per ear.

ASNase also shows mature ear trait trends similar to those shown for the earshoots at anthesis. Ears in the IBMRI inbreds with the moderately expressing Mo17 allele have a greater cob weight than ears with the highly expressing B73 allele. Conversely, ears with the very low expressing IHP allele in the NAM/ex-PVP inbreds and the IRHPx(IHP1xIRHP1)BC1S1 population results in decreased ear trait measurements. Cob weight is reduced in ears with the IHP allele in the NAM/ex-PVP inbreds. The IHP variant also fall to the bottom for grain weight, cob weight, and kernel weight in the IRHPx(IHP1xIRHP1)BC1S1 population. Like the earshoots, a complete knockout of ASNase may be a detriment to ear growth.
Table 6. Effect of nitrogen, Asparagine synthetase, and Asparaginase on mature ear traits. Nitrogen and allelic trends are shown when significant. N*AS and N*ASNase are interaction terms between nitrogen rate and the alleles of the two genes. NS denotes not significant. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.

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Figure 17. Effect of Asparagine synthetase (AS) and Asparaginase (ASNase) variants on grain weight (A), cob weight (B), and kernel weight (C) in the IBMRI hybrids. Weight is measured in grams. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.

Grain NIR Analysis

Near infrared analysis was used to determine grain protein, starch, and oil concentration. The IBMRI, NAM/ex-PVP, and IRHPx(IHP1xIRHP1)BC1S1 populations were all tested for the effect of nitrogen and variants of AS and ASNase on these grain components (Table 7). Protein, starch, and oil were all highly responsive to nitrogen in the IBMRI and NAM/ex-PVP hybrids. Protein positively responded to supplemental nitrogen, whereas starch and oil exhibited a negative response to nitrogen. Protein also had a significantly positive response to nitrogen in the IBMRI and NAM/ex-PVP hybrids.
Variants of AS did not show an effect on grain composition in any background. Only oil in the NAM/ex-PVP inbreds showed an allelic difference for ASNase. However, there were several significant interactions between nitrogen and either AS or ASNase. A nitrogen and AS interaction was observed in the starch of IBMRI hybrids and oil of NAM/ex-PVP hybrids. In both cases allelic differences were only detected at low nitrogen. Interaction of nitrogen with ASNase shows the opposite trend. Allelic differences for starch in the IBMRI hybrids and protein and oil in the NAM/ex-PVP hybrids were only observed at high nitrogen. These results suggest variants of AS and ASNase may only affect grain composition at certain nitrogen levels. At low nitrogen, AS looks to play a role in grain nitrogen traits, whereas ASNase may only be important when plants have an abundance of nitrogen.

Table 7. Effect of nitrogen, Asparagine synthetase, and Asparaginase on grain composition. Nitrogen and allelic trends are shown when significant. N*AS and N*ASNase are interaction terms between nitrogen rate and the alleles of the two genes. NS denotes not significant. •, *, and ** denote a P>F less than 0.1, 0.05, and 0.01, respectively.
Conclusions

The above results make a strong case for increased AS expression being beneficial to maize ear growth and development. Developing earshoots with the highly expressing allele of AS resulted in significantly greater earshoot length than the lower expressing allele in four of the six materials tested. Additionally, the same trend is observed for mature grain and cob traits in the IBMRI hybrids and NAM/ex-PVP inbreds. Increasing AS expression in the maize plant results in greater production of asparagine, which is vital for nitrogen transport to sink tissues. AS has been shown to play an important role in the senescence and remobilization of nitrogen to developing tissues (Gaufichon et al., 2010). Even within sink organs, AS has been demonstrated as a source of nitrogen remobilization to developing kernels (Cañas et al., 2010).

Transgenic approaches to overexpressing AS have resulted in higher nitrogen in the seeds and greater tolerance to low nitrogen conditions in Arabidopsis (Lam et al., 2003). Also, in tobacco overexpressing AS resulted in increased asparagine concentrations and improved growth (Brears et al., 1993). These studies and the one presented above demonstrate the usefulness of developing commercial maize with increased AS production, whether it is through transgenic or conventional routes, to increase nitrogen utilization as well as possibly overall yield.

ASNase did not show quite as strong trends as AS in this study, though a negative effect of the IHP allele was observed in two sets of materials in both the developing earshoot and the mature ear. This suggests that very low or no expression of ASNase may be detrimental to ear growth and development. Although asparagine production appears to be beneficial to maize growth, lacking the means to break it down likely has several negative effects. Accumulation of
asparagine in maize also leads to protein accumulation in the seed, which is inversely correlated with yield. Also, a lack of ASNase would decrease the amount of metabolically available nitrogen since it would be tied up as asparagine. To get a clearer understanding of the role of ASNase in maize ear growth, a set of material with a cleaner background would likely be needed to observe the effect of ASNase variants. Near isogenic lines or transgenic over or under-expression of the gene would be two routes to further study the effect of ASNase while removing much of the background genetic variation.
REFERENCES


APPENDIX: SUPPLEMENTARY DATA FILES

Supplemental File 1: Earshoot dry length and dry weight data of the IBMRI inbreds, IBMRI hybrids, NAM/ex-PVP inbreds, NAM/ex-PVP hybrids, IPSRIs, and IRHPx(IHP1xIRHP1)BC1S1 populations. Bar charts comparing allelic effects of AS and ASNase in each background can be found within each tab of the file. File name: Supplemental File 1 (Earshoot Size).xlsx

Supplemental File 2: Earshoot amino acid data of the IBMRI inbreds, IBMRI hybrids, NAM/ex-PVP inbreds, and NAM/ex-PVP hybrids populations. Asparagine, aspartic acid, glutamic acid, and glutamine data is given as the ratio of the total free amino acid pool. Total amino acids is in the unit parts per million (PPM). Bar charts comparing allelic effects of AS and ASNase in each background can be found within each tab of the file. File name: Supplemental File 2 (Earshoot Amino Acids).xlsx

Supplemental File 3: Grain weight, cob weight, kernel weight, and kernel number data of the IBMRI inbreds, IBMRI hybrids, NAM/ex-PVP inbreds, NAM/ex-PVP hybrids, and IRHPx(IHP1xIRHP1)BC1S1 populations. Bar charts comparing allelic effects of AS and ASNase in each background can be found within each tab of the file. File name: Supplemental File 3 (Grain & Cob).xlsx

Supplemental File 4: Grain protein, starch, and oil data of the IBMRI inbreds, IBMRI hybrids, NAM/ex-PVP inbreds, NAM/ex-PVP hybrids, and IRHPx(IHP1xIRHP1)BC1S1 populations. Bar charts comparing allelic effects of AS and ASNase in each background can be found within each tab of the file. File name: Supplemental File 4 (Grain NIR).xlsx