Surveying Genetic and Phenotypic Variation for Response to Density Stress in Maize, Wheat and Barley

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Abstract

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Increasing plant population density can increase biomass and grain yield on a per area unit basis, however this relationship is curvilinear where eventually a high plant density will overwhelm the tolerance to density stress and yield per unit area will decrease. The main objective of this thesis is to gain an understanding of the genetic and morphological responses to density stress. In the first part of this thesis, we examined differential gene expression in seedlings of four barley genotypes and five maize genotypes grown at low and high densities. A microarray analysis approach was employed and identified 219 and 35 transcripts differentially expressed in barley and maize, respectively, with little gene expression patterns overlap among genotypes, indicating that these genotypes respond in very different ways and may have different mechanisms to deal with density stress. In the second part of this thesis, we examined multiple genotypes of maize, wheat, and barley planted at three different densities with the highest density being up to three times the normal density. A combined genotype analysis for each species showed that the highest plant density had the greatest biomass yield m^{-2} for wheat and maize while barley had no significant difference among the three densities for biomass yield m^{-2}. For grain yield m^{-2}, barley had the lowest yield at the highest plant density, wheat had no difference among the densities and maize had the greatest grain yield m^{-2} in the high plant density.

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Chapter One:

Literature Review

Introduction

Plant population density stress elicits a competitive response between neighboring plants for sunlight. Plants detect future crowding from their neighbors by sensing a decrease in the ratio of red to far-red (R:Fr) light using photoreceptors called phytochromes (Ballare, 1999; Smith, 1982). Once neighboring plants are detected, plants begin a shade avoidance response to outgrow their neighbors by reallocating their resources into stem elongation, longer leaves, and early flowering to capture the most sunlight for seed development (Ballare et al., 1991; Smith and Whitelam, 1997). This interplant competition to collect sunlight causes a decrease in biomass per plant and grain yield per plant as plant density increases.

Utilization of Plant Density Stress Tolerance

Even though biomass and grain yield are decreased on a per plant basis as density increases, biomass and grain yield can be increased on a per area unit basis (Duncan, 1958; Hashemi et al., 2005; Silva et al., 2007; Tollenaar, 1989). This relationship is curvilinear where eventually a high plant density will overwhelm the tolerance to density stress and yield per unit area will also decrease (Cox, 1996; Hashemi et al., 2005; Kirby, 1967; Sarlangue et al., 2007; Silva et al., 2007; Spink et al., 2000). Because of this quadratic relationship, there is an optimum plant density with the maximum yield per unit area. However, the optimum plant density varies depending on the genotype. Sarlangue et al. (2007) estimated the optimum plant density for grain yield m^{-2} for hybrids KWS Romario (early maturing hybrid) and DK688 (late maturing hybrid) was 12.75 and 11

plants m^{-2}, respectively. Hashemi et al. (2005) found that the optimum plant density for biomass yield m^{-2} ranged between 9 and 12 m^{-2} for three different hybrids.

Plant breeders understand this relationship and have been breeding for more density tolerant plants to increase the optimum plant density and further increase biomass and grain yield per unit area (Cox, 1996; Hashemi et al., 2005; Sarlangue et al., 2007; Tollenaar and Wu, 1999; Widdicome and Thelen, 2002). In maize, plant density has increased from 39,520 seeds ha^{-1} in the 1960's to 66,690 seeds ha^{-1} in 2005 by increasing planting rates and reducing row spacing resulting in an increase of 21% in grain yield per hectare (Cardwell, 1982; Elmore and Abendroth, 2006). Modern hybrids have been selected to intercept and utilize photosynthetically-active radiation more efficiently than older hybrids by having a more compact canopy structure (Subedi et al., 2006), a higher leaf area index after silking (Tollenaar and Aguilera, 1992), and higher leaf photosynthesis rates (Dwyer et al., 1991). Leaf and root angles have also been shown to play an important role in plant density stress tolerance (Duvick and Cassman, 1999; Fellner et al., 2003; Hammer et al., 2009). An increase in leaf angle results in a uniform distribution of sunlight throughout the plant canopy. From 1961 to 1991, there has been a 122% change in leaf angle (Duvick and Cassman, 1999). By using a systems modeling approach, it has been shown that an increase in root angle results in increased biomass and yield at higher planting densities (Hammer et al., 2009).

The effectiveness of selection on plant density tolerance has been characterized in many studies. One study by Sangoi et al. (2002) compared grain yield of three hybrids: Agroceres 12 (double cross hybrid, 1970's), Agroceres 303 (double cross hybrid, 1980's) and Cargill 929 (single-cross hybrid, 1990's) at four plant densities: 25,000,

50,000, 75,000 and 100,000 plants ha^{-1}. All three hybrids showed a curvilinear response to grain yield per plant. The older varieties yielded more grain ha^{-1} at the lowest density, but as the density increased, the newest variety (Cargill 929) had the greatest increases in grain yield ha^{-1}.

The majority of previous studies only employed plant populations relatively close to the normal range (i.e. 3 to 12 plants m^{-2} for maize, 200 to 640 for wheat and barley (Conry, 1998; Geleta et al., 2002; Spink et al., 2000; Turk et al., 2003; Whaley et al., 2000; Wiersma, 2002)). However, a study increasing plant density to 20 plants m^{-2} found the optimum biomass and grain yield to be at the highest plant density used in maize (Edwards et al., 2005). In addition, two spring wheat cultivars were found to have their highest grain yield at 675 seeds m^{-2} (Faris and DePauw, 1981). In these two studies, the full potential of these genotypes was not seen due to insufficient increases in plant population density.

Plant architecture and planting date also impact density stress tolerance. A study by Faris and DePauw (1981) found that the optimum density for grain yield for two semi dwarf wheat varieties was 675 seeds m^{-2} and 462 m^{-2} and for a tall variety was approximately 362 m^{-2}, indicating that semi dwarf varieties are more tolerant to density stress. Wiersma (2002) planted seven varieties at multiple planting rates and two planting dates two to three weeks apart. When combining all cultivars, the optimum planting density for the first and second planting dates was 371 plants m^{-2} and 386 plants per m^{-2}, respectively. These results indicate that the timing of planting plays a role in density stress tolerance.

Morphological Differences Due to Plant Density Stress

Many vegetative characteristics are altered by plant density stress. Stem diameter, leaf width and leaf length have been shown to decrease as plant density increases (Smith and Whitelam, 1997). Stem elongation is a common classic response to shade avoidance, but interestingly, it depends on the genotype. In corn, Silva et al. (2007) found no difference in height using cultivar Centalmax (open pollinated variety), whereas other studies have found an increase (Modarres et al., 1998) and decrease (Silva et al., 2003) in plant height. Turk et al. (2003) found a decrease in plant height in winter barley. There is also a reduction in tiller number related to plant density stress in wheat (Spink et al., 2000; Casal, 1988) and barley (Davis and Simmons, 1994; Kirby and Faris, 1972).

Total biomass per plant is reduced during density stress. Sarlangue et al. (2007) examined maize hybrids differing in maturity planted at 5, 8, and 15 plants m^{-2}. Total biomass per plant had a negative curvilinear response with a high correlation ($r^2 > 0.86$) with plant density. However, each genotype exhibited a different degree of severity to planting density. Biomass per plant decreased more rapidly for Hybrid DK688 (late maturing hybrid) as plant density increased whereas hybrid KWS Romario (early maturing variety) had a more gradual decrease.

Grain yield on a per plant basis is also reduced with plant density stress. Borras et al. (2002) evaluated maize hybrids grown at 3, 9, and 12 plants m^{-2}. The reduction in grain yield was greater from 3 to 9 plants m^{-2} than 9 to 12 m^{-2} showing a curvilinear response. There are three main ways to reduce grain yield per plant in maize, wheat and barley: lower seed number per ear or spike, a decrease in fertile ears or spikes, and lower

seed weight. In maize, it has been shown that plant density stress increases the anthesis-silking interval (ASI) causing a reduction in the amount of pollen successfully pollinating the flower (Hashemi-Dezfouli and Herbert, 1992; Tollenaar et al., 1997). This delay in pollination reduces the number of kernels per ear or can cause completely barren ears. Hashemi-Dezfouli and Herbert (1992) reported that the increase in barren ears was positively correlated with an increase in ASI. Although there have been no studies looking at ASI in wheat or barley, both species also exhibit a lower number of seeds per spike as density increases (Conry, 1998; Spink et al., 2000) and a reduction in number of tillers per plant ultimately giving a lower number of spikes per plant (Conry, 1998; Spink et al., 2000). Plants can also reduce grain per plant due to high density stress through low kernel weight. Spink et al. (2000) and Whaley et al. (2000) each conducted a study on winter wheat and identified significant decreases in thousand grain weight. Studies in maize have found a similar response for kernel weight (Hashemi-Dezfouli and Herbert, 1992; Westgate et al., 1997).

Density stress can also alter the chemical composition of the crop. It has been reported in winter wheat that due to the increase in stem growth allocation, plants grown at higher densities have a lower concentration of potassium and nitrogen in the tillers (Sato et al., 1993). This change in potassium and nitrogen concentrations has a detrimental effect as it increases the risk of frost damage. In maize, high plant densities reduces the quality of corn silage by decreasing the nutrition content (Graybill et al., 1991), increasing neutral detergent fiber (NDF) and decreasing crude protein (CP) concentrations (Cox and Cherney, 2001). Not only is the quality of biomass reduced,

grain protein content can also be reduced as plant density increases in maize (Borras et al., 2002) and wheat (Geleta et al., 2002).

Quantitative Trait Loci for Plant Density Stress Tolerance

Quantitative trait loci (QTL) mapping can identify areas of the genome that are related to plant density stress tolerance. While QTL mapping does not identify specific genes, mapping can be helpful in breeding by identifying relevant genomic regions for use in marker-assisted selection or genomic selection. In maize, it has been difficult to detect QTL related to plant density stress even in studies with sufficient power to detect effects (Gonzalo et al., 2007). However, a total of 14 significant locus by density interactions and 30 QTLs have been identified for traits such as grain yield per plant, kernel number, anthesis to silking interval (ASI), and plant height related to plant density stress (Gonzalo et al., 2006, 2009). A study by Gonzalo et al. (2006) used eight segmental introgression lines (SIL) derived from a cross of B73x Tx303 and also crossed the SILs to Mo17 to examine hybrid performance. The SILs and hybrids were planted at densities of 50,000 and 100,000 plants per hectare and locus by density interactions were examined. A total of 14 significant locus by density interactions were identified: three loci for grain yield per plant, kernel number, and ASI; two for days to anthesis; and one for growth rate, ear height, and plant height. None of the 14 significant locus by density interactions for a trait were significant in both hybrids and inbreds. Out of the eight SILs that were examined in this study, five were associated with two or more QTL for different traits.

In another QTL analysis study, Gonzalo et al. (2009) identified QTLs related to plant density stress using 186 recombinant inbred lines from the cross of B73 xMo17 (developed by Charles Stuber and Lynn Senior at North Carolina State University). The population was planted at densities of 50,000 and 100,000 plants per hectare. They identified 30 QTL with significant marker main effects: eight for plant height; five for ASI; six for percent barrenness (infertile ears) and fertile ears per plant; three for yield per plant; and two for days to anthesis. Many of the barrenness QTLs overlapped with QTLs of other traits: three mapped to the same position as ears per plant, four mapped with ASI, and one mapped with yield per plant.

Maternal effects, cytoplasmic effects and parent-of-origin effects related to plant density stress have been examined on reciprocal crosses (Gonzalo et al., 2007). A total of 92 reciprocal crosses from RILs from the IBM population were created by backcrossing the RILs to either B73 or Mo17 and crosses were made in both directions. The reciprocal crosses were grown at two densities (50,000 and 100,000 plants per hectare) and four traits were evaluated: plant height (V7 stage, V12 stage and final height), days to anthesis, days to silking, and kernel weight. In the early stages of plant growth, plant density increased maternal effects for all four traits. However, maternal effect did not account for all of the variation for these traits. Although multiple QTL have been identified an understanding of the underlying genes governing the response to density stress tolerance is needed.

Differentially Expressed Genes Related to Plant Density Stress

Plant density stress can impact gene expression. Variation in gene expression in two maize hybrids grown at low and high planting densities has been examined (Guo et al., 2004). They used two Pioneer® hybrids 3306 (developed in 1960's) and 3394 (developed in 1990's). These two hybrids were chosen because there was a shift in how hybrids were selected between the 1960's and 1990's. The newer hybrids, represented by 3394, were selected to be more density tolerant and were provided with additional levels of nitrogen applications. Only two (lipid transfer protein and pro-rich protein) of the 15 transcripts examined were differentially expressed between low and high densities and only in hybrid 3394. Although limited to only two genotypes and 15 genes, this study provides evidence for allelic variation for density stress response.

In another experiment, heterosis was studied by examining genome-wide changes in gene expression in several hybrids grown at different plant densities (Guo et al., 2006). The GeneCalling mRNA profiling method (described in Shimkets et al., 1999) was employed to examine the effects of density-stressed environments on hybrids 3394 and 3306. The hybrids were grown at three different plant densities: 4,000, 18,000, and 35,000 plants per acre. The proportion of genes with paternally-biased allele expression increased as the plant density increased. They were also able to demonstrate that the more density tolerant hybrid, 3394, had a greater percentage of genes with a mid-parent expression compared to less density tolerant hybrid 3306. While this method allowed the entire maize transcriptome to be analyzed, GeneCalling does not allow identification of the gene transcripts.

Gene expression profiling has been conducted in *Arabidopsis thaliana* (Devlin et al., 2003) for response to shade in young seven-day old seedlings. The genotypes used were wild type, *phyB* and *phyA phyB* double mutants. Plants were grown in white light and then transferred to either white light or low R:Fr light. Tissue was collected 1 hour and 24 hours after transplant to look at early and delayed responses to shade. In total, they identified 301 genes related to shading such as photosystem I, photosystem II, proline-rich protein, and six transcription factors encoding for indole-3-acetic acid (IAA).

DNA methylation can alter gene expression by gene silencing and may be affected be environmental stresses such as increasing plant densities. Tani et al. (2005) compared DNA methylation patterns of inbreds and hybrids planted at low and high densities. In the 20 loci studied, they found that inbreds had a significant increase in DNA methylation between low and high densities whereas hybrids had no differences between low and high densities.

One additional area of study to note is shade avoidance syndrome, which is a competitive response to outgrow neighboring plants for sunlight. As the R:Fr ratio decreases, which indicates shading by neighboring plants, plants begin to elongate stems, reduce tillering and flower earlier to compete for the most sunlight. Much work has gone into identifying how plants detect their neighbors and the biological pathways used in the shade avoidance syndrome (Ballare, 1999; Ballare et al., 1990; Ballare and Casal, 2000; Casal et al., 1996; Devlin et al., 1999; Kasperbauer, 1992; Kasperbauer and Karlen, 1994; Maddonni et al., 2001; Rajcan, 2004).

Monocots have three phytochromes: *PHYA, PHYB* and *PHYC* (Mathews and Sharrock, 1996, 1997). In Arabidopsis and maize, *PHYB* has been shown to be the

dominant photoreceptor acting in the shade avoidance response (Devlin et al., 2003; Filiault et al., 2008; Sheehan et al., 2007). In Arabidopsis, both *PHYA* and *PHYC* help regulate *PHYB* and inhibit the shade avoidance response (Franklin et al., 2003). *PHYA* also promotes flowering by sensing day length (Reed et al., 1994) and transgenic rice overexpressing *PHYA* had a reduction in plant height and an increase in panicles per plant (Garg et al., 2006). *PHYC* has also been identified as a cryptochrome blue light receptor (Franklin et al., 2003). *PHYA* is light-liable and has been shown to be differentially expressed when comparing light grown and dark grown 10 day old maize seedlings (Sheehan et al., 2004). In the same maize seedlings, *PHYC* had no change in expression whereas *PHYB* was differentially expressed in light versus dark grown maize seedlings (Sheehan et al., 2004).

In Summary

Gene Expression Profiling Related to Plant Density Stress

Genome-wide expression analysis related to plant density stress using microarrays has not been previously conducted. The use of microarrays allows a sensitive evaluation of the level of transcript accumulation. Contig sequences and annotations also permits comparison of gene lists either obtained from this study or from previous studies. In the first part of this study, transcript accumulation changes were examined in both maize and barley seedlings planted at low and high densities to identify genes responsive to plant density stress. We used five maize inbred lines and four barley varieties. The main objective was to identify genes differentially expressed between low and high densities in maize and barley seedlings. We were particularly interested in quantifying the transcriptional response to density stress at early stages as well as comparing the identity of altered transcripts in two monocot species.

Field Evaluations for Biomass and Grain Yield Due to Plant Density Stress

Many morphological field evaluations have been conducted on plant density stress response in maize, wheat and barley, but these studies were limited to a few genotypes in each study. In the second part of this study, phenotypic responses to plant density stress for fourteen barley, sixteen wheat and nine maize genotypes were examined in the field at plant populations up to three times the normal planting rate. Our main objective is to determine the optimum planting density for each genotype for grain and biomass yield per hectare.

Chapter Two:

Gene Expression Profiling for Response to Density Stress in Maize and Barley

Introduction

Plant population density stress elicits a competitive response between neighboring plants for sunlight. Plants detect crowding from their neighbors by sensing a decrease in the ratio of red to far-red (R:Fr) light using photoreceptors called phytochromes (Ballare, 1999; Smith, 1982). Once neighboring plants detect each other, a shade avoidance response is initiated to outgrow their neighbors by reallocating their resources into stem elongation, longer leaves, and early flowering to capture the most sunlight for seed development (Ballare et al., 1991; Smith and Whitelam, 1997). Other vegetative and reproductive traits such as stem diameter, leaf width, leaf length, number of tillers, biomass per plant and grain per plant are reduced (Casal, 1988; Hashemi-Dezfouli and Herbert, 1992; Smith and Whitelam, 1997).

Even though biomass and grain yield are decreased on a per plant basis as density increases, yield can be increased on a per area unit basis (Duncan, 1958; Hashemi et al., 2005; Silva et al., 2007; Tollenaar, 1989). This relationship is curvilinear where eventually a high plant density will overwhelm the tolerance to density stress and yield per unit area will also decrease (Cox, 1996; Hashemi et al., 2005; Kirby, 1967; Sarlangue et al., 2007; Silva et al., 2007; Spink et al., 2000). Because of this quadratic relationship, there is an optimum plant density with the maximum yield per unit area. However, the optimum plant density varies depending on the genotype. Plant breeders have understood this relationship and have been breeding for more density tolerant plants to push the optimum plant density and further increase grain yield per unit area (Cox, 1996; Hashemi et al., 2005; Sarlangue et al., 2007; Tollenaar and Wu, 1999; Widdicome and Thelen, 2002). In maize, plant density has increased from 39,520 plants/ha in the 1960's to

66,690 plants/ha in 2005 by both increasing planting rates and reducing row spacing, resulting in an increase of 21% in grain yield per hectare (Cardwell, 1982; Elmore and Abendroth, 2006). While density stress tolerance for yield has been a selected trait and numerous studies have looked at the morphological variation in response to plant density stress, an understanding of the genetics of density stress tolerance is limited and the linkage between density stress tolerance for yield and biomass has not been elucidated.

Quantitative trait loci (QTL) mapping has been used to identify regions of the genome that are related to plant density stress tolerance. In maize, it has been difficult to detect QTL related to plant density stress even in studies with sufficient power to detect effects (Gonzalo et al., 2007), however a total of 14 significant locus by density interactions and 30 QTLs have been identified for traits such as grain yield per plant, kernel number, anthesis to silking interval (ASI), and plant height related to plant density stress (Gonzalo et al., 2006, 2009). The effect of plant density stress on maternal effects has also been mapped using reciprocal crosses for four traits: plant height, days to anthesis, days to silking, and kernel weight (Gonzalo et al., 2007). Although multiple QTL have been identified an understanding of the underlying genes governing the response to density stress tolerance is needed.

Several groups have begun to assess transcriptional changes that are induced by density stress. One study assessed the allelic differences in transcript accumulation in two maize hybrids grown at high and low densities for fifteen transcripts (Guo et al. 2004). Only two (lipid transfer protein and pro-rich protein) of the 15 transcripts examined were differentially expressed between low and high densities and only differentially expressed in one hybrid. Although limited to only two hybrids and 15

genes, this study provides evidence for allelic variation for density stress response. In a genome-wide survey, Guo et al. (2006) used GeneCalling mRNA profiling (described in Shimkets et al., 1999) to determine if a density stressed environment alters gene expression patterns. The GeneCalling mRNA profiling method (described in Shimkets et al., 1999) was employed to examine the effects of density-stressed environments on two hybrids. As the plant density increased, the proportion of genes exhibiting the mid-parent expression value decreased and expression became biased towards the paternal allele. The two hybrids varied on the percentage of genes with a mid-parent expression values. While this method provides a genome-wide approach to study gene expression patterns it does not allow for identification and analysis of specific genes of known identity.

Gene expression profiling in *Arabidopsis thaliana* for response to shade in seven day old seedlings assayed expression in wild type, *phyB* and *phyA phyB* double mutant seedlings (Devlin et al., 2003). Plants were grown in white light and then transferred to either white light or low R:Fr light. Pattern fitting software, which can detect subtle gene expression changes, identified 301 genes related to shading such as photosystem I, photosystem II, proline-rich protein, and six transcription factors encoding for indole-3-acetic acid (IAA).

While *Arabidopsis thaliana* has been used to characterize phytochromes in dicot species (Devlin et al., 2003; Filiault et al., 2008; Franklin et al., 2003), we are just beginning to understand phytochromes in monocots. Monocots have three phytochromes: *PHYA, PHYB* and *PHYC* (Mathews and Sharrock, 1996, 1997). In Arabidopsis and maize, *PHYB* has been shown to be the dominant photoreceptor acting in

the shade avoidance response (Devlin et al., 2003; Filiault et al., 2008; Sheehan et al., 2007). In Arabidopsis, both *PHYA* and *PHYC* help regulate *PHYB* and inhibit the shade avoidance response (Franklin et al., 2003). *PHYA* also promotes flowering by sensing day length (Reed et al., 1994) and transgenic rice overexpressing *PHYA* exhibited a reduction in plant height and an increase in panicles per plant (Garg et al., 2006). *PHYC* has also been identified as a cryptochrome blue light receptor (Franklin et al., 2003). *PHYA* is light-liable and has been shown to be differentially expressed when comparing light grown and dark grown 10-day old maize seedlings (Sheehan et al., 2004). In the same maize seedlings, *PHYC* had no change in expression whereas *PHYB* was differentially expressed in light versus dark grown maize seedlings (Sheehan et al., 2004).

In this study, we examined the phenotypic response and transcript accumulation changes in maize and barley seedlings planted at low and high densities. We used five maize inbred lines and four barley varieties. The objectives of this study were 1) to evaluate the phenotypic response to density stress in barley and maize seedlings; 2) quantify the transcriptional response to density stress at early growth stages by identifying genes differentially expressed between low and high densities in seedlings of four barley genotypes and five maize genotypes; and 3) compare the identity of altered transcripts in two monocot species.

Materials and Methods

Plant material, experimental design and sampling.

Five maize genotypes (B73, Mo17, P39, Oh43 and Hp301) and four barley genotypes (Steptoe, Morex, Harrington and Baronesse) were used for plant density experiments. A description of all of the genotypes is located in Table 1. To assess morphological variation and to collect tissue samples for gene expression analyses for plants under plant density stress, individual experiments consisting of a randomized design with three biological replicates were conducted for both maize and barley. Initial experiments were conducted to identify the appropriate time points and plant densities for seedlings to exhibit morphological differences due to plant density stress. For the initial experiment, genotypes Mo17 and B73 for maize, and Harrington, Morex and Steptoe for barley were planted at densities ranging from 2 to 24 plants/pot (Tables 2 and 3). Plant height and stem diameter data were collected from 10 to 23 days after planting for maize and barley. Data on the number of tillers per plant was also recorded for barley. For maize, there were significant differences for both plant height and stem width at 14 days after planting between plant densities of 2 and 18 plants per pot (Table 2). For barley, there were phenotypic differences between 3 and 21 plants per pot at 15 days after planting (Table 3). Based on this preliminary data, we collected maize tissue at 12 days after planting using plant densities of 2 and 18 plants per pot. Barley tissue was collected 15 days after germination using plant densities of 3 and 21 plants per pot.

The maize experiment was conducted in a greenhouse at the Plant Growth Facility on the St. Paul campus of the University of Minnesota. For each biological replicate, there were three pots at the low density (2 plants/pot) and two pots at the high density (18

plants/pot) for each genotype. Five genotypes were used: B73, Mo17, P39, Oh43 and Hp301 (Table 1). Plants were grown in 50:50 mixture of compost:Metromix200 (Sun Gro Horticulture CM Ltd., Canada) in 24cm diameter pots spaced 10cm apart. Days were extended to 16 hours with 1000-Watt (530 to 710 $\mu Em^{-2}s^{-1}$) vapor lights and temperatures ranging from 22-32°C. Plants were watered thoroughly when needed. Phenotypic measurements were taken twelve days after planting on two plants per pot. Stalk diameter was taken 2cm above ground and plant height was from the soil to the tip of the highest leaf.

The barley experiment was conducted in growth chambers in Borlaug Hall on the St. Paul campus of the University of Minnesota. Each biological replicate consisted of four pots at the low density (3 plants/pot) and high density (21 plants/pot). Four genotypes were used: Steptoe, Morex, Harrington and Baronesse. To ensure uniform germination, seeds were cold treated in complete darkness for three days at 4°C, then placed for two days at room temperature. Germinated seeds were transplanted into 10x10cm pots with Metromix 200 and fertilized with Osmocote Slow Release fertilizer (14-14-14; Scott's Co., USA). Pots were placed 8cm apart and watered when needed. The plants were grown in the growth chamber at 20°C during the day and 18°C at night; days were extended to 16 hours with light intensity ranging from 280 to 300 $\mu Em^{-2}s^{-1}$. Phenotypic measurements were taken on two plants per pot fourteen days after transplant. Stem diameter was taken approximately 1cm from the soil, plant height was from the soil to the tip of the highest leaf, and the number of tillers per plant was counted.

Tissue samples were collected for the high and low density for the five maize inbred lines at twelve days after planting and for the high and low density at fifteen days

after transplanting for the four barley genotypes (note one day later than phenotypic measurements were taken). For each of the three biological replications, all above-ground biomass was collected from two plants per pot and pooled as one biological sample for each density/genotype and directly frozen in liquid nitrogen and stored at -80°C. A total of 30 tissue samples for maize and 24 tissue samples for barley were collected. Collection times were between 8:00 and 9:00AM.

Affymetrix GeneChip Arrays.

The GeneChip Maize Genome Array was developed with NCBI GenBank and *Zea mays* UniGene Build 42 EST sequences that were derived from multiple inbred lines (www.affymetrix.com). The oligonucleotide probe length is 25mer with 15 probe pairs per gene. The Maize Genome Array consists of 17,555 probe sets and represents 13,339 genes.

The Barley1 Affymetrix GeneChip was released in 2003 (Close et al., 2004). There are 22 probe pairs per gene, 11 perfect match and 11 mismatch. Probesets on the Barley1 Chip were derived from 350,000 ESTs from 84 cDNA libraries. The Barley1 Chip contains 22,792 probe sets representing 21,439 genes.

RNA Extraction and GeneChip hybridization.

For both maize and barley, tissue was ground in liquid nitrogen and RNA was extracted using the Trizol (Invitrogen, Carlsbad, CA, U.S.A) protocol. For barley, samples were further purified by digesting genomic DNA with a DNase treatment and using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). For maize, the samples were

further purified using a lithium chloride protocol. Briefly, an equal volume of 4M LiCl was added to the RNA sample and placed on ice for four hours. Samples were centrifuged for 10 minutes (12,000RPM) and the supernatant was removed and the pellet was resuspended in TE ph 8.0. For each RNA sample, 5µg of RNA was sent to the Biomedical Genomics Center at the University of Minnesota for RNA labeling, GeneChip hybridization and GeneChip scanning.

GeneChip data analysis.

The microarray data was normalized using GeneSpring GX 9.0 Software (Agilent Technologies, Santa Clara, CA) with the GC-RMA algorithm (Wu et al., 2004). Correlation coefficients among the three biological replicates ranged between 0.988 and 0.997 for barley, and 0.989 and 0.997 for maize. Clustering analysis for both maize and barley was performed by implementing a two-way ANOVA for genotype x density using a cutoff p-value <0.05 and no FDR correction to obtain all expressed genes. Hierarchical clustering was then conducted on the expressed genes using an Euclidean distance metric.

To identify differentially expressed gene transcripts, a ≥2 fold change and a p-value < 0.05 for a two sided t-test was implemented between low and high planting densities for each genotype. Very few genes were identified with a ≥2 fold change, therefore no false-discovery rate (FDR) was calculated. All CEL files were uploaded to the Plant Expression database (PLEXdb; www.plexdb.org). The accession numbers for the experiments are ZM27 and BB90 for maize and barley, respectively.

Validation of differentially expressed genes using Real-time RTPCR.

The barley microarray data was further validated and investigated by conducting another experiment related to plant density stress. The same procedures used for the tissue collection for the microarray experiments were followed with modifications. The genotype Steptoe was grown in a growth chamber at 3 (low) and 21 (high) plants per pot. Tissue was collected at 11, 15 (same as microarray experiment), and 19 days after germination. Tissues collected were the crown region and all other above-ground biomass (stem and leaves).

RNA was extracted from tissue as described above. Single strand cDNA was synthesized from 5ng of RNA using Superscript III One-Step RT-PCR kit (Invitrogen Corp., Carlsbad, CA). Primers were developed using Primer 3 (Rozen and Skaletsky, 2000) and analyzed with Beacon Designer (Premier Biosoft International, Palo Alto, CA) to test for secondary structures. Three primer pairs were developed for transcripts that showed differential accumulation in the low to high densities: contig14901_at (auxin-responsive protein), contig1689_at (lipid transfer protein (LTP)), and contig6484_at (NAC domain protein). Primers were also developed for contig baak4o13_s_at (Actin) to use as a positive control. Primers were designed with an amplicon size of approximately 200bp and Tm between 65 and 70°C. Primer sequences are shown in Table 4.

Real-time RTPCR was performed in an Applied Biosystems 7900HT Real-time RTPCR system (Applied Biosystems, Foster, CA) using SYBRgreen PCR Master Mix Kit (Applied Biosystems, Foster, CA) and 5ng of cDNA in a 20ml reaction volume. The two-step Real-time RTPCR program used was 95.0°C for 1 min; followed by 40 cycles of 95.0°C for 30 s; 68.0°C for 1 min; and then 72.0°C for 6 min.

Primer efficiencies for each primer were evaluated by performing a two-fold dilution series on a pooled cDNA sample in five dilutions: undiluted, 1/2, 1/4, 1/8, and 1/16. The cycle threshold (Ct) for each dilution were plotted to get a calibration line. Primer efficiencies were calculated using an equation $E = 2^{[-1/slope]}$ (Pfaffl, 2001). Acceptable primer efficiencies were in the range of 1.70-2.05. To ensure amplification of only one product, melting curves were visualized and PCR products were sequenced for each primer.

For each PCR run, three technical replicates for the gene of interest primers and two technical replicates for the control primers were performed for each sample. Replicates were averaged and fold changes (FC) were calculated using the equation

$$FC = \mathrm{Log}_2[(E_{\mathrm{gene}})^{\Delta \mathrm{Ctgene}}/(E_{\mathrm{con}})^{\Delta \mathrm{Ctcon}}]$$

Where ΔCt = (Ct cDNA from low density– Ct cDNA from high density); “gene” is the gene of interest and “con” is the control (equation from Pfaffl, 2001). This equation allowed for correction of starting template based on the Ct of the control gene.

Results and Discussion

Morphological differences due to plant density stress.

Initial experiments were conducted with maize and barley seedlings grown in standard greenhouse growth conditions at different planting densities to obtain an understanding of the parameters that play a role in the phenotypic effect of density stress upon seedlings. For both maize and barley, high planting densities affected plant height and stem width after 2-3 weeks of growth. Tables 2 and 3 show the phenotypic data in these initial experiments and demonstrate that plants show much greater morphological differences due to plant density stress as the plants develop.

For our subsequent experiments, we selected planting densities that resulted in phenotypic effects and a growth stage when these phenotypic effects were just beginning to be observed. Maize seedlings were grown at 2 (low) or 18 (high) plants per pot for 12 days and barley seedlings were grown at 3 (low) or 21 (high) plants per pot for 15 days after germination. These time points reflect a period just prior to phenotypic changes in maize and coincident with the time of phenotypic changes in barley.

Based on our initial density-stress trials in maize and barley (Tables 2 and 3), we expected stem width to show significant differences between 10 and 14 days after planting and plant height would exhibit no significant differences until 14-18 days after planting. In maize, we found that plant height exhibited significant ($p<0.05$) differences for 12 day-old B73, Oh43 and P39 seedlings. However, B73 decreased in height and P39 and Oh43 increased in height when comparing the high to the low densities (Table 5). For stem width, only B73 and P39 exhibited a statistical decrease in width in the high density.

In barley, we observed significant ($p<0.05$) reductions in stem width and number of tillers in the high density for all genotypes (Table 5). The differences in plant height were significantly different ($p<0.05$), but Baronesse, Morex and Steptoe increased plant height in high density, whereas height decreased for Harrington in high density (Table 5).

These morphological differences are consistent with morphological changes due to shade avoidance syndrome (Sawers et al., 2005). Previous work for RNA expression analysis on plant density stress in maize collected tissue at the V4 leaf stage (~3 three weeks after planting) with low and high density (Guo et al., 2004). The maize seedlings grown at high density displayed early senescence in the lower leaves compared to the low density, signifying that the plants were stressed at the high density.

In our morphological studies we observed significant differences in seedlings grown at low versus high densities (Table 5). One of our objectives was to study the transcriptional changes induced by density stress. Therefore, we chose to sample young seedlings prior to stages at which the morphological differences become pronounced. It is worth noting that the barley phenotypic responses in plants sampled for the expression profiling were more substantial than the maize phenotypic responses (Table 5). It is likely that some of the variation in directionality of density-stress response is likely due to the early sampling time point and if we had measured older seedlings we would have expected to observe consistent phenotypic changes across most genotypes.

Identification of transcripts that are differentially expressed in low and high density seedlings.

To identify genes differentially expressed between low and high planting densities, we performed transcript profiling in maize and barley using the Affymetrix - GeneChip® Maize Genome Array and the Affymetrix - GeneChip® Barley Genome Array. Seedlings of five maize genotypes (B73, Mo17, Oh43, P39 and Hp301) were sampled twelve days after planting and seedlings of four barley genotypes (Steptoe, Morex, Harrington and Baronesse) were sampled at fifteen days after germination. Microarray data were obtained for three biological replicates of pooled tissue for each genotype by treatment combination and were normalized with GC Robust Multi-array Average (GCRMA) using GeneSpring software.

Several approaches were used to demonstrate the quality of the expression profiling data. First, we examined the correlation coefficients among biological replicates. The correlation coefficients among biological replicates were greater than 0.988 for both species. We also noted that the expression profiles of plants of the same genotype grown at high and low density were highly correlated (greater than 0.988 in both species) which suggested that there are not major transcriptional changes induced by the density stress. Second, we performed a clustering analysis of all maize or all barley microarrays (Fig. 1) to assess the similarity of the expression profiles. The clustering analysis showed high levels of similarity for the expression profiles for each genotype but did not provide evidence for clustering by density treatment. Third, to ensure that that there was low variability among the biological replicates, we also looked at variation for each treatment/genotype by comparing transcript accumulation among genotypes. A

previous study identified 1,633 transcripts differentially expressed between 11- day old B73 and Mo17 seedlings (Stupar and Springer, 2006). If the variability among biological replicates was high, then comparing genotypes would result in a low number of transcripts differentially expressed being identified between any two genotypes. Transcripts were considered differentially expressed when a ≥2 fold change and an unpaired t-test with a p-value of <0.05. We were able to identify over 1,157 transcripts between any two genotypes in maize and 326 genes for barley (Table 6), suggesting relatively low variability among biological replicates and the ability to identify differentially expressed genes in each of the genotypes.

We proceeded to search for genes that exhibit variable transcript levels in maize or barley seedlings grown at low versus high density for each genotype. The use of standard approaches (unpaired t-test with an FDR of 5%, then a ≥2 fold change) did not identify any differentially expressed genes at low versus high density in barley or maize. This was not entirely surprising based upon the clustering and correlation results noted above. Therefore, we proceeded to implement a less-stringent approach in an attempt to identify density-stress responsive genes. In this approach, we first identified the subset of genes that exhibit ≥2 fold variation between low and high density and subsequently performed an unpaired t-test with a p-value of <0.05. This approach removed ~99% of the genes that did not show at least ≥2 fold change. The implementation of this method identified 219 unique transcripts in barley and 35 unique transcripts in maize (Tables 7 and 8, Fig. 2).

Characterization of differentially expressed genes.

For barley, 219 unique gene transcripts were identified as differentially expressed between the low and high densities (Table 7, Fig. 2). Steptoe had 144, Baronesse had 31, Harington had 74 and Morex had 33 genes differentially expressed. The four sets had seven genes in common (Table 7, Fig. 2). However, many genes overlapped with one other set. Baronesse had 18 (58%) genes in common with one other genotype, Harrington had 37 genes (50%), Morex had 15 (45%), and Steptoe had 40 (28%). When the four genotypes were pooled for a combined analysis, a total of 51 genes were differentially expressed between the low and high densities. Out of the 51 genes, 49 genes (96%) were in common with the list created from the individual genotype analysis.

For the 219 barley gene transcripts, we compared regulation of the transcript from all four sets from low density to high density (Table 7). Even if the gene transcript may not have been significant in the all set analysis, we still used the fold change to identify a consensus regulation for the gene transcript. A total of 32 (15%) transcripts showed a decrease in accumulation, whereas 53 (24%) showed an increase in accumulation. A total of 134 (61%) gene transcripts did not have all four genotypes with a fold change in the same direction. Interestingly, if Morex is removed from the analysis, another 96 transcripts have a consensus in decrease accumulation in the remaining three genotypes.

In maize, 35 gene transcripts were differentially expressed between the low and high densities (Table 8). B73 had 20, Hp301 had 5, Mo17 had 4, Oh43 had 3, and P39 had 3 genes differentially expressed. The five genotypes had no genes in common. When the four genotypes were pooled for a combined analysis, a total of 51 genes were differentially expressed between the low and high densities. Out of the 51 genes, 49

genes (96%) were in common with the list created from the individual genotype analysis. Using all five sets from low density to high density for comparing regulation, only three (9%) gene transcripts had an increase and three had a decrease in accumulation in all five genotypes.

For barley, annotation of differentially expressed transcripts identified up regulation (from low to high density) for genes encoding photosystem II 10kDa protein (1 contig), NAC domain protein (1 contig), proteins related to auxin (2 contigs), zinc finger proteins (2 contigs), phytochrome A (1 contig) and phytochrome B (1 contig). There was an abundance of histones (62 contigs) down regulated and found mainly differentially expressed in Steptoe (Table 7). For maize, annotation of the transcripts showed up-regulation of genes encoding an auxin family protein (1 contig), and a zinc finger protein (1 contig) (Table 8).

Many of the genes identified as differentially expressed are related with changes in light intensity, which decreases when plants are grown at high densities compared to low densities. All transcripts coding for photosystem II 10kDa protein were up regulated in the high density compared to low density. Variation in light intensity can rapidly change photosystem stoichiometry. Depending on the species, low-light growth (such as shading) can result in an increase or decrease in the photosystem II/photosystem I ratio (Kawamura et al., 1979; Sukenik, et al., 1987). An interesting result in the microarray analysis of both barley and maize is that only two genes encoding phytochromes were differentially expressed between the densities. The barley and maize microarray have six and four probesets for phytochromes, respectively. Based on previous studies, we would expect *PHYA* and *PHYB* to be differentially expressed (Sheehan et al., 2004), however,

we might not have been able to detect all of the probesets for *PHYA* and *PHYB* because we did not use extreme treatments of either complete darkness or low R:Fr light versus white light. Even though there should be a higher R:Fr ratio in the low density, there still is far red light for the plants to detect.

Hormonal pathways are associated with plant density stress. Auxins have been shown to be phytochrome-mediated and alter cell elongation, leaf angle (Sawers et al., 2005) and inhibits lateral shoot growth (Tian and Reed, 2001). We found one transcript related to auxin repressed protein in barley and an auxin family protein in maize. The growth stage we collected tissue was still in the vegetative stage. Phytohormones, especially auxins begin to accumulate at higher levels once the floral transition occurs (Sangoi et al., 2002). A later collection time may have identified more transcripts related to phytohormone pathways.

An abundance of histones (62 transcripts) were down regulated in high density and found mainly differentially expressed in Steptoe. One interesting study by Tessadori et al. (2009) investigated how light intensity controls chromatin compaction. Their results indicate that *PHYTOCHROME-B* and *HISTONE DEACETYLASE-6* are regulators for light-mediated chromatin compaction of the Nuclear Organizing Regions (NORs), indicating that plants grown with low-light have less compacted chromatin than plants grown with high-light.

Comparison of Gene Lists.

The barley and maize gene list were compared using Wu-Blast (Advanced Biocomputing, LLC, St. Louis, MO). To compare the lists, contig sequences from each

list were subjected to BLAST alignments against each other using tblastn. Two contigs matched between maize to barley: zm.1315.1.A1_at with contig3156_s_at (oxalate oxidase-like protein) and zm.17580.1.S1_at with contig5688_at (zinc finger protein). All E-Score values were less than 2.3E-28.

We also compared our gene list to genes that have been previously identified related to either plant density stress or shade tolerance. A lipid transfer protein has been previously identified to be differentially expressed in maize in low versus high density (Guo et al., 2004). We were able to identify three transcripts encoding for lipid transfer protein in barley. However, there was not a consensus on the direction of regulation. In Arabidopsis, gene expression profiling for shade avoidance identified 301 genes differentially expressed between white light and low R:Fr light grown seedlings. Based on annotation, we compared our lists and found transcripts in common coding for: beta-amylase, cytochrome P450, lipid transfer protein, peroxidase, protein kinase, syntaxin and zinc finger protein.

Validation of differentially expressed transcripts using Real-time RTPCR.

We were able to show that the microarray data was of high quality and that there were morphological differences among the two densities indicating that plants in the high density were indeed stressed. However, we were not able to identify many genes especially with substantial fold changes. Two possible reasons for very few genes differentially expressed are that tissue was collected too early or the tissue collected did not have the genes differentially expressed. The barley phenotypic responses in plants sampled for the expression profiling were more substantial than the maize phenotypic

responses (Table 5). Furthermore, the barley analysis identified genes differentially expressed with greater fold change differences compared to the maize analysis. This result shows that it is likely that if we had collected tissue from older seedlings we would observe greater fold change differences and possibly more genes being differentially expressed. We also pooled all above ground tissue for each sample. Different tissues could have different expression patterns related to density stress. By pooling all tissues we could have diluted changes in gene expression in specific tissues.

To explore these two issues, we further investigated transcript accumulation variation by conducting an independent experiment to collect new tissue samples for barley for multiple time points and two tissues. The genotype Steptoe was grown at low and high densities and tissue was collected at 11, 15 (same as microarray experiment), and 19 days after germination. The two tissues collected were the crown region (including the meristem) and all other above ground biomass, mainly stem and leaf tissue. Quantitative RT-PCR was performed to assess the expression levels for three transcripts that showed differential accumulation in the low to high densities: contig14901_at (auxin-responsive protein); contig1689_at (Lipid transfer protein (LTP)); and contig6484_at (NAC domain protein).

Quantitative RT-PCR analysis indicated that the microarray experiment and the quantitative RT-PCR experiment are consistent for the three gene transcripts tested (Fig. 3). For contig6484_at (NAC domain protein), microarray analysis indicated an up–regulation in high density treatments at 15 days after germination. The quantitative RT-PCR assay for above ground biomass tissue showed up-regulation of this transcript in high density treatments for all time points tested. However, there was no detectible

expression of this transcript at any time point or density treatment in crown tissue. Contig1689_at (lipid transfer protein), is down regulated in stem and leaf tissue at 15 days after germination in both microarray and real-time analysis. However, the other time points (11 and 19 days after germination) do not show consistent alterations in expression in stem and leaf tissue. The crown tissue had no difference in expression for any time point. The third gene, contig14901_at (auxin-responsive protein), exhibits up-regulation in the microarray data. Quantitative RT-PCR did not detect any difference in expression between the low and high densities at 11 and 15 days in either tissue, but gene expression was up-regulated in both tissues at 19 days. This delay in gene expression differences could be due to using different tissue than what was used for the microarray data or development may have been delayed in the new growth chamber experiment causing a delay in change of gene expression. These results show that the microarray data was reproducible and gene expression varies at different time points and in different tissues.

Conclusions

This study demonstrates that identifying all genes related to plant density stress may be a challenge. Even though we had good quality microarray data, we needed to employ to less stringent analysis to identify genes differentially expressed. Two possible reasons for very few genes differentially expressed in this study are that tissue was collected too early or we did not collect the tissue with differentially expressed genes. Future gene profiling of many time points (in the vegetative and reproductive stages) would give an even greater understanding of genes involved in density stress.

This study also revealed that there is little overlap for gene expression patterns among the genotypes, indicating that these genotypes respond in very different ways and may have different mechanisms to deal with the stress. This could be clearly seen with the barley genotype Steptoe and the abundance of histone genes differentially expressed compared to the other genotypes that did not have any differentially expressed. Another example for this is that Morex did not show any differences in gene expression between low and high density, but did show significant phenotypic differences when tissue was collected. Both Morex and Steptoe are both six row barley, however, Morex is relatively low tillereing and exhibits less yield than Steptoe, Harrington and Baronesse. It may be plausible that the higher yield in Harrington, Baronesse and Steptoe could be due to tolerance to plant density stress.

Table 1. Description of maize and barley genotypes used in the plant density experiments.

Genotype	Description	Publication
Maize		
B73	Dent, Iowa Stiff Stalk Synthetic (ISSS)	Russell, W.A. 1972. Registration of B70 and B73 Parental Lines of Maize (Reg. Nos. PL16 and PL17). Crop Sci. 12:721.
Mo17	Dent, Missouri Lancaster Sure Crop	Zuber, M.S. 1973. Registration of 20 Maize Parental Lines (Reg. Numbers PL 18 to 37). Crop Sci. 13:779a-780a.
Oh43	Dent, Lancaster Sure Crop Illinois	Bergquist, R.R. 1982. Registration of Maize Inbred LP Oh43 Rp_1^{Td} Germplasm (Reg. No. GP 93). Crop Sci. 22:451-452.
P39	Sweetcorn, Origin Unknown	Burr, H.S. 1943. Electrical correlates of pure and hybrid strains of sweet corn. Proc. Nat. Acad. Sci. USA. 29:163-166.
hp301	Popcorn, Indiana	USDA, ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)*. [Online Database] National Germplasm Resources Laboratory, Beltsville, Maryland. Available: http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1084096 (23 December 2009)
Barley		
Baronesse	2 Row Feed	Developed in Nordsaat, Germany and marketed in U.S. by WestBred, LLC (Bozeman, MT).
Harrington	2 Row Malt	Williams, K.J., J. Cheong, K. Willsmore, M.Cakir, and H.A. Wallwork. 2003. A comparison of the genetics of seedling and adult plant resistance to the spot form of net blotch (*Pyrenophora teres* f. *maculata*) Aust. J. Agric. Res. 54:1387-1394.
Morex	6 Row Malt	Rasmusson, D.C., and R.W. Wilcoxson. 1979. Registration of 'Morex' barley. Crop Sci. 19:293.
Steptoe	6 Row Feed	C.E. Muir and R.A. Nilan. 1973. Registration of 'Steptoe' barley. Crop Sci. 13:770.

Table 2. Maize seedling phenotypic responses to plant density stress.

Genotype	Days[†]	Height					Stem Width				
		Low[‡]	Medium		High		Low	Medium		High	
B73	10	28.3[§]	28.7		30.8	**	0.54	0.52		0.47	
	14	49.9	49.1	*	45.8	***	0.55	0.52	*	0.39	*
	18	68.0	60.9	***	52.6	***	0.54	0.49	**	0.36	*
	22	78.1	68.5	***	58.3	***	0.75	0.62	***	0.39	***
Mo17	10	23.1	24.4		26.1	*	0.47	0.49		0.45	
	14	45.5	47.3		46.9		0.44	0.49		0.41	
	18	66.9	63.4	*	58.1	**	0.42	0.46		0.39	
	22	82.4	73.7	*	66.7	**	0.64	0.61	***	0.46	**

[†] Days after planting.
[‡] Plant population. Plants were grown at 2 (low), 8 (medium) and 24 (high) plants per pot.
[§] Data correspond to average of three biological replications and is annotated as an overall mean.
Significance values using unpaired t-test of treatment (medium or high) versus low plant density: * = 0.05, ** = 0.01, *** < 0.0001.

Table 3. Barley seedling phenotypic responses to plant density stress.

		Plant Height			Stem Width			Number of Tillers		
Variety	**Days**[†]	**Low**[‡]	**High**		**Low**	**High**		**Low**	**High**	
Harrington	11	17.6[§]	19.4		0.16	0.16		0.0	0.0	
	15	28.6	28.8		0.21	0.19		0.0	0.0	
	19	39.4	37.6		0.29	0.17	**	1.8	0.0	*
	23	42.5	36.4	*	0.32	0.17	**	1.8	0.0	*
Morex	11	15.0	14.4		0.16	0.13		0.0	0.0	
	15	26.5	24.3		0.22	0.17		0.0	0.0	
	19	36.3	35.7		0.29	0.16	***	0.6	0.0	
	23	42.6	39.8		0.35	0.22	**	1.3	0.0	*
Steptoe	11	18.8	16.9	*	0.17	0.13	*	0.0	0.0	
	15	33.9	28.2	*	0.24	0.17	**	0.0	0.0	
	19	42.2	35.5	**	0.34	0.18	***	1.5	0.0	**
	23	43.7	38.3	*	0.35	0.20	**	1.8	0.0	**

[†] Days after germination.
[‡] Plant population. Plants were grown at 3 (low) and 21 (high) plants per pot.
[§] Data correspond to average of three biological replications and is annotated as an overall mean. Significance values using unpaired t-test of low versus high plant density: * = 0.05, ** = 0.01, *** < 0.0001.

Table 4. Primers designed for Real-Time RTPCR in barley.

Contig	Annotation	Product Size		Sequence	Tm (°C)
Contig14901_at	Auxin-responsive protein	245	F:	CCTACCGCCGCTGCTAGTGC	67.5
			R:	CTGCCGGTGTGCTTCTTGGA	67.0
Contig1689_at	Lipid transfer protein (LTP)	163	F:	TGCCCTGCGAGGAGTTCCAT	67.5
			R:	CGGAGCCGACAACACCAAGG	68.4
Contig6484_at	NAC domain protein	196	F:	AGGAGGAACGACGCCACCAC	67.0
			R:	AGGGGCGGCATAGTCGGAAG	67.8
baak4o13_s_at	Actin	197	F:	AAGAGATGGCGCAGATTTATACAGCA	66.1
			R:	GCAAACCAATAACGTACGGGGACA	67.7

Table 5. Seedling phenotypic responses to density stress.

Genotype	Height (cm)			Stem Width (cm)			Number of Tillers		
	Low[†]	High		Low	High		Low	High	
Barley									
Baronesse	31.9[‡]	33.8	**	0.24	0.21	***	1.3	0.2	***
Harrington	38.6	35.6	***	0.26	0.20	***	1.8	0.3	***
Morex	33.6	35.8	***	0.27	0.20	***	1.0	0.2	***
Steptoe	37.4	40.3	***	0.30	0.21	***	1.3	0.2	***
Maize									
B73	20.4	18.7	*	0.23	0.20	*		NA	
HP301	14.1	15.1		0.11	0.12			NA	
Mo17	12.2	13.2		0.21	0.20			NA	
OH43	11.5	13.7	*	0.24	0.23			NA	
P39	14.2	18.0	*	0.16	0.19	*		NA	

[†] Plant population: The barley experiment was planted at 3 (low) and 21 (high) plants per pot. The maize experiment was planted at 2 (low) and 18 (high) plants per pot.
[‡] Data correspond to average of three biological replications and is annotated as an overall mean. Phenotypic measurements were taken 14 days after germination for barley and 12 days after planting for maize.
Significance values using unpaired t-test of low versus high plant density: * = 0.05, ** = 0.01, *** < 0.0001.

Table 6. Number of transcripts exhibiting differential accumulation.

Genotype vs. Genotype		High Density		Low Density		High and Low Density	
		FC[†]	t-test[‡]	FC	t-test	FC	t-test
Barley							
Baronesse	Harrington	446	375	403	326	394	382
Baronesse	Morex	715	623	609	522	605	597
Baronesse	Steptoe	628	499	567	477	515	503
Harrington	Morex	904	795	1890	1870	697	671
Harrington	Steptoe	686	532	718	647	648	614
Morex	Steptoe	852	685	657	535	616	599
Maize							
B73	Hp301	1595	1402	1504	1378	1476	1451
B73	Mo17	1339	1157	1366	1192	1312	1276
B73	Oh43	1603	1484	1865	1716	1695	1678
B73	P39	1483	1361	1671	1671	1504	1485
Hp301	Mo17	1606	1433	1601	1354	1568	1524
Hp301	Oh43	1655	1517	1832	1690	1699	1665
Hp301	P39	1550	1412	1552	1423	1505	1489
Mo17	Oh43	1551	1391	1761	1542	1623	1580
Mo17	P39	1480	1293	1578	1370	1489	1452
Oh43	P39	1458	1367	1463	1368	1408	1402

[†] FC, Fold change, number of probe sets with a ≥2 fold change.
[‡] number of probe sets with a ≥2 fold change and an unpaired t-test ($p<0.05$).

Table 7. Differentially expressed genes comparing the low to the high plant density for each genotype in barley on seedlings 14 days after germination.

Probe Set Name	Annotation[†]	Fold Change[‡]				
		Baronesse	Harrington	Morex	Steptoe	All Genotypes
HVSMEg0017A08r2_at	Amino acid transport protein, putative	0.24[§]	0.13	-0.08	1.03*	0.44
HVSMEf0016E23r2_s_at	Anthranilate N-hydroxycinnamoyl	0.12	0.96	1.25*	0.77	0.61
Contig10104_at	Anti-silencing protein, putative	-0.19	-0.11	0.19	-1.39*	-0.60
Contig5994_s_at	Arginine decarboxylase	-0.58	-0.84	-1.11*	-1.11*	-0.85
HVSMEl0010A03r2_s_at	Asparagine synthetase, putative	-0.66	-0.05	-0.17	-1.30*	-0.53
Contig2783_s_at	Asparaginyl endopeptidase	0.46	1.47	1.13*	1.22*	1.07*
Contig3815_at	ATP-citrate synthase, putative	-0.25	-1.02*	-0.24	-0.09	-0.39
Contig1762_s_at	Auxin-repressed protein	0.85	0.99	1.17*	0.75	0.87
Contig14901_at	Auxin-response protein	1.34*	1.69*	1.46*	1.59*	1.52*
Contig1411_s_at	Beta-amylase	-2.02*	-0.99	-1.39*	-0.63	-1.26*
Contig24097_at	B-keto acyl reductase, putative	-0.30	0.09	-1.33*	-0.26	-0.15
Contig9852_at	Blue copper protein, putative	1.10*	0.04	0.34	0.13	0.26
Contig18032_at	Bowman-Birk type trypsin inhibitor	-0.70	-0.65	-0.14	-1.03*	-0.79
Contig2088_s_at	Bowman-Birk type trypsin inhibitor	-0.20	-0.26	0.12	-1.07*	-0.53
Contig21149_s_at	bZIP transcription factor ABI5, putative	0.33	1.03*	0.20	0.43	0.54
Contig20292_at	bZIP transcription factor RF2b, putative	0.24	-0.24	1.00	-1.34*	-0.34
HT06F11u_s_at	Catalase isozyme 2	0.87	1.24	1.05	1.00*	1.04*
Contig5066_at	Cell wall surface anchor family protein	-0.36	-0.55	-0.17	-1.12*	-0.67
Contig7441_at	Centromere/microtubule binding protein, putative	-0.60	-1.27*	0.68	-0.63	-0.63
rbags17k13_s_at	Cinnamoyl-CoA reductase, putative	0.00	0.00	-0.08	1.10*	0.34
Contig1326_s_at	Cold-regulated protein BLT14	0.78	1.92*	0.19	0.79	1.09
EBpi07_SQ002_E23_s_at	Cyclin-selective ubiquitin carrier, putative	-0.33	-0.14	-0.28	-1.28*	-0.68
Contig9601_s_at	Cyclopropane fatty acid synthase, putative	-0.16	-0.46	0.40	-1.10*	-0.57
Contig12498_at	Cystatin Hv-CPI9	-0.47	-0.15	-1.25*	-0.02	-0.24
Contig3900_at	Cysteine proteinase precursor	1.05*	1.64*	1.61*	1.89*	1.55*
Contig3901_s_at	Cysteine proteinase precursor	1.84*	2.52*	2.32*	2.63*	2.33*

rbaal21f05_s_at	Cysteine proteinase precursor	1.48*	2.22*	2.19*	2.19*	2.02*
HVSMEm0003G16r2_at	Cytochrome P450, putative	1.45*	-0.38	0.34	-0.15	0.34
Contig6933_s_at	Defensin precursor	-0.39	-0.17	0.25	-1.18*	-0.60
S69616_s_at	Dihydroflavonol-4-reductase	-1.24*	-0.49	-0.29	0.31	-0.59
Contig568_s_at	Endoplasmin homolog precursor	-1.36*	0.05	-0.32	-0.28	-0.26
Contig2716_s_at	Ferritin	0.94	1.30*	0.85	0.24	0.82
Contig3392_at	Fructan 6-fructosyltransferase	-0.89	-1.05	0.47	-1.22*	-1.06
Contig16702_s_at	G2/mitotic-specific cyclin-2	-0.55	-0.56	0.22	-1.41*	-0.74
Contig24832_at	Glucose-6-phosphate/phosphate-translocate, putative	-1.31*	-0.55	-0.51	-0.21	-0.65
HV11O04r_s_at	Glutamine-dependent asparagine synthetase	-0.60	-1.01	-0.09	-1.81*	-1.11
Contig9031_at	GRAB2 protein	0.62	1.32*	1.06	0.36	1.04
Contig6690_at	H/ACA ribonucleoprotein	-0.31	-0.57	0.55	-1.13*	-0.65
HV12N24u_s_at	H/ACA ribonucleoprotein	-0.44	-0.85	0.45	-1.25*	-0.88
Contig873_s_at	Heat shock protein	-1.24*	-0.36	-1.16	0.00	-0.54
Contig10029_at	Heat shock protein, putative	-1.46	-1.25*	-0.49	-0.19	-0.57
Contig3426_at	High molecular mass early light-inducible protein	-0.68	-1.15*	-0.13	-0.97	-0.97
Contig2258_at	Histone H1	-0.43	-1.18*	0.58	-2.17*	-1.08
Contig2260_at	Histone H1	-0.32	-0.60	-0.52	-1.15*	-0.65
Contig130_at	Histone H1, Predicted	-0.67	-1.14	0.54	-2.21*	-1.47
Contig186_x_at	Histone H1, Predicted	-0.31	-0.52	0.20	-1.12*	-0.61
Contig196_s_at	Histone H1, Predicted	-0.82	-2.06	1.21	-3.66*	-1.33*
Contig338_at	Histone H1, Predicted	-0.68	-1.45	0.70	-3.59*	-1.82
Contig338_s_at	Histone H1, Predicted	-1.06	-2.97	0.85	-4.93*	-2.03*
Contig349_s_at	Histone H1, Predicted	-0.96	-1.75	1.10	-4.42*	-1.51*
Contig657_s_at	Histone H1, Predicted	-0.87	-2.07	1.00	-3.49*	-1.36*
Contig660_s_at	Histone H1, Predicted	-0.45	-0.66	0.64	-1.42*	-0.82
Contig787_at	Histone H1, Predicted	-1.08	-2.00*	1.27	-4.31*	-1.53*
HA22C01r_s_at	Histone H1, Predicted	-0.45	-0.52	0.28	-1.01*	-0.64
Contig188_at	Histone H2A	-0.98	-2.07*	1.49	-5.25*	-1.70*
Contig3398_at	Histone H2A	-0.71	-0.70	0.21	-1.66*	-1.03
Contig412_x_at	Histone H2A	-0.46	-0.33	0.57	-1.92*	-0.90
Contig414_at	Histone H2A	-0.93	-1.57*	0.91	-3.81*	-1.35*

Contig414_x_at	Histone H2A	-1.01	-1.26	1.40	-4.17*	-2.11
Contig76_at	Histone H2A	-0.62	-1.39*	0.79	-3.54*	-1.19*
Contig96_s_at	Histone H2A	-0.58	-1.70	1.12	-3.11*	-1.07*
EBro01_SQ003_E14_s_at	Histone H2A	-0.98	-2.45*	1.03	-4.76*	-1.79*
HA12K13u_at	Histone H2A	-0.54	-1.65*	0.94	-2.75*	-1.00*
HA12K13u_s_at	Histone H2A	-0.56	-2.12*	0.75	-3.33*	-1.32*
Contig1122_s_at	Histone H2B	-0.64	0.12	0.66	-2.28*	-1.02
Contig1127_at	Histone H2B	-0.93	-1.47	0.79	-2.65*	-1.53
Contig1132_s_at	Histone H2B	-0.60	-1.13*	0.80	1.26	-0.15
Contig1138_at	Histone H2B	-0.68	-1.79	0.24	-1.41*	-1.24
Contig1140_at	Histone H2B	-0.27	-0.46	0.50	-1.98*	-0.88
Contig1141_s_at	Histone H2B	-0.37	-0.46	0.43	-1.21*	-0.77
Contig1142_at	Histone H2B	-0.14	0.30	0.73	-1.06*	-0.27
Contig1147_s_at	Histone H2B	-0.48	-0.71	0.43	-1.82*	-1.08
Contig1151_s_at	Histone H2B	-0.44	-0.15	0.43	-1.57*	-0.67
Contig1154_s_at	Histone H2B	-0.36	-0.61	0.70	-1.55*	-0.88
Contig1156_at	Histone H2B	-0.67	0.06	0.25	-1.14*	-0.78
Contig1161_at	Histone H2B	-0.58	-1.08*	0.44	-2.97*	-1.05*
Contig1162_at	Histone H2B	-0.31	-1.00	0.67	-1.94*	-0.96
Contig1167_s_at	Histone H2B	-0.38	-0.41	0.14	-1.09*	-0.61
Contig1169_x_at	Histone H2B	-1.14	-2.03	0.98	-3.53*	-1.43*
Contig1177_at	Histone H2B	-0.02	-1.20	-0.01	-3.15*	-1.38
Contig16_at	Histone H2A	-0.59	-0.15	0.09	-1.20*	-0.70
Contig350_x_at	Histone H2A	-0.58	-0.88	0.77	-2.54*	-1.16
Contig106_x_at	Histone H3	-0.58	-1.28*	1.00	-2.69*	-1.61
Contig971_at	Histone H3	-0.70	-2.31*	1.01	-3.88*	-1.47*
Contig135_at	Histone H3, Predicted	-0.57	-0.96	0.22	-1.01*	-0.85
Contig176_at	Histone H3, Predicted	-0.63	-1.46	0.71	-3.04*	-1.10*
Contig204_s_at	Histone H3, Predicted	-0.71	-1.39*	0.65	-3.04*	-1.12*
Contig669_at	Histone H3, Predicted	-1.67	-1.29	1.34	-4.89*	-1.63*
Contig684_s_at	Histone H3, Predicted	-0.65	-2.17*	0.93	-2.44*	-1.08*
Contig131_at	Histone H4	-0.20	-0.35	0.56	-1.29*	-0.63

Contig197_at	Histone H4	-0.91	-1.80	0.52	-3.43*	-1.40*
Contig197_s_at	Histone H4	-0.78	-1.35*	0.77	-2.91*	-1.07*
Contig250_at	Histone H4	-0.46	-0.97	0.60	-2.28*	-1.27
Contig30_s_at	Histone H4	-0.46	-1.10*	1.04	-2.31*	-1.25
Contig488_at	Histone H4	-0.43	-1.81*	0.60	-2.72*	-1.09*
Contig9_x_at	Histone H4	-0.21	-1.19	0.71	-2.96*	-1.33
EBpi01_SQ002_F17_s_at	Histone H4	-0.13	-1.15*	-0.02	-1.29*	-0.81
Contig175_at	Histone H4, predicted	-0.64	-0.23	0.62	-1.73*	-0.90
Contig175_x_at	Histone H4, predicted	-0.62	-0.01	1.06	-1.92*	-0.76
Contig434_s_at	Histone H4, predicted	-0.40	-0.96	0.53	-1.71*	-1.02
Contig576_s_at	Histone H4, predicted	-0.18	-0.50	0.37	-1.47*	-0.82
Contig695_s_at	Histone H4, predicted	-0.16	-0.25	0.82	-1.45*	-0.63
Contig700_s_at	Histone H4, predicted	-0.65	-1.13	0.19	-1.58*	-1.19
HA03I22u_s_at	Histone H4, predicted	-0.66	-1.11	0.22	-2.45*	-1.24
Contig5792_at	HMG-I/Y protein HMGa	-0.30	-0.58	0.38	-1.13*	-0.68
Contig25699_at	Integral membrane-like protein	0.58	1.36*	0.79	0.62	0.81
Contig1582_x_at	Leaf-specific thionin	-0.03	1.26	-4.30*	0.23	0.52
Contig1689_at	Lipid transfer protein	-0.37	-0.75	0.43	-2.25*	-1.14
Contig3777_at	Lipid transfer protein	-0.65	-0.17	-2.64*	0.02	-0.20
Contig3782_x_at	Lipid transfer protein	-0.07	0.08	-0.05	-2.03*	-0.63
HI02E21u_s_at	Lipoxygenase 2	-1.56*	-0.69	-0.17	-1.22*	-1.16
HY03N19u_s_at	Lipoxygenase 2	-0.96	-0.74	-0.06	-1.15*	-0.95
Contig13483_at	MATE efflux family protein	0.28	-1.02	0.82	-1.49*	-0.80
Contig4755_at	Meiosis 5	-0.21	-0.58	0.55	-1.46*	-0.77
Contig10700_at	Monothiol glutaredoxin-S2	0.74	1.23*	0.70	0.45	0.79
Contig6484_at	NAC domain protein NAC1, putative	1.33	1.75	1.03	1.63*	1.44*
HVSMEb0008I19r2_s_at	Nicotianamine aminotransferase A, putative	1.18*	0.54	0.34	0.59	0.65
Contig3756_at	Nucleolar protein Nop56, putative	-0.44	-1.06*	0.56	-0.75	-0.70
Contig3547_s_at	O-methyltransferase	0.81	2.48*	1.74*	1.41	1.61*
HVSMEf0005J23f_s_at	O-methyltransferase	-0.27	0.21	1.48*	0.38	0.01
HW02A11u_s_at	O-methyltransferase	-0.03	2.69*	0.09	0.11	0.85
Contig3156_s_at	Oxalate oxidase-like protein	0.06	1.14*	0.48	1.10*	0.72

Contig5345_s_at	Oxidoreductase, putative	-2.89*	-0.68	-2.65*	-2.75*	-2.24*
Contig2717_s_at	Peptidylprolyl isomerase	-1.35*	-0.65	-1.85*	-0.02	-0.57
Contig3239_at	Peroxidase 6	0.66	1.48*	0.71	0.38	0.85
Contig3243_x_at	Peroxidase 6	0.59	0.40	0.06	1.05*	0.70
Contig996_s_at	Photosystem II 10 kDa polypeptide	0.68	1.19*	0.91	0.86	0.90
HVSMEk0021N05r2_s_at	Phytochrome A	1.08*	1.19*	0.13	0.27	0.78
Contig12847_at	Phytochrome B	0.71	0.98	0.70	1.27*	0.87
Contig6099_at	Proliferating cell nuclear antigen	-0.43	-1.00	1.14	-2.42*	-1.20
Contig4072_at	Proline-and threonine-rich protein	-0.83	-0.68	-1.32*	-1.34*	-1.04*
Contig10368_at	Protease inhibitor	0.10	-0.15	0.89	-1.04*	-0.37
Contig3427_at	Protein early light-induced protein	1.2	1.44	1.31	0.98	-1.23*
Contig18182_at	Protein flowering promoting, putative	0.84	1.8	0.74	1.65	1.26*
Contig5924_s_at	Protein kinase	0.82	0.73	1.02*	-0.38	0.53
Contig5926_at	Protein kinase	0.86	0.52	1.46*	0.30	0.57
Contig2764_s_at	Protochlorophyllide reductase A	1.52	1.86	1.07	1.10*	1.39*
Contig7366_s_at	Receptor serine/threonine kinase, putative	-0.08	1.24*	-0.07	0.19	0.46
Contig5185_at	RNase S-like protein	-1.07	-0.35	-1.92*	-0.76	-0.73
baak13l10_s_at	Stripe rust resistance protein Yr10, putative	-0.12	-0.43	0.38	-1.81*	-0.80
Contig8488_at	Syntaxin 6, putative	1.30*	0.26	-0.05	0.07	0.36
Contig24856_at	Syringomycin biosynthesis enzyme	-1.17*	-0.51	-0.62	-0.49	-0.73
Contig15771_at	Thiamin pyrophosphokinase 1, putative	-0.05	0.04	-1.05*	0.09	-0.01
Contig7007_s_at	Thionin Osthi1, putative	-0.19	-0.58	0.20	-1.32*	-0.68
Contig15378_at	Transcription factor RF2b	0.07	-1.31*	0.05	-0.25	-0.43
Contig18951_at	Transcriptional coactivator p15 (PC4)	0.03	-0.88	0.49	-1.06*	-0.57
rbah19o01_s_at	Two-component response regulator	0.72	0.73	0.53	1.06*	0.82
Contig6708_at	Ureide permease 2, Putative	0.38	1.02*	0.09	0.15	0.47
Contig9917_at	WIR1A protein	-2.07	1.54*	0.65	0.07	-0.24
Contig5688_at	Zinc finger protein	0.71	1.15*	0.43	0.54	0.81
Contig12472_at	Zinc finger protein, putative	0.87	1.00*	0.40	1.51*	1.17
Contig10552_at	Unknown	0.96	1.58*	0.86	1.35*	1.19*
Contig11401_at	Unknown	0.83	1.24*	0.86	0.14	0.71
Contig117_at	Unknown	-0.53	-0.78	0.67	-2.37*	-1.04

Contig11825_at	Unknown	-0.32	-0.43	0.73	-1.13*	-0.62
Contig11884_at	Unknown	0.74	1.17*	0.94	2.09	1.23*
Contig11919_at	Unknown	1.11*	0.32	0.54	0.93	0.77
Contig12272_s_at	Unknown	-0.34	-1.11*	0.28	-0.94	-0.78
Contig12444_at	Unknown	0.66	1.24*	0.62	0.57	0.65
Contig12484_at	Unknown	2.94	2.23	2.09*	2.75	2.50*
Contig12574_at	Unknown	0.50	0.81	0.27	1.28*	0.81
Contig137_x_at	Unknown	-0.51	-1.39*	0.87	-2.34*	-1.66
Contig13769_at	Unknown	-0.07	-1.14*	0.29	-0.42	-0.50
Contig13799_at	Unknown	0.40	1.22*	0.32	0.53	0.74
Contig145_x_at	Unknown	-0.37	-0.29	0.38	-1.03*	-0.50
Contig14515_at	Unknown	-0.33	-0.71	0.23	-1.21*	-0.76
Contig14804_s_at	Unknown	0.73	0.92	0.55	1.22*	0.93
Contig15186_at	Unknown	1.02	1.05	0.00	1.37*	1.03
Contig154_at	Unknown	-0.62	-0.91	1.38	-3.24*	-1.58
Contig158_s_at	Unknown	-0.32	-0.75	0.50	-1.25*	-0.81
Contig163_x_at	Unknown	-0.42	-0.20	0.16	-1.36*	-0.57
Contig17468_at	Unknown	0.03	-0.39	1.07	-1.69*	-0.62
Contig17600_at	Unknown	-0.37	-1.10*	-0.14	-0.02	-0.39
Contig1788_at	Unknown	0.21	-0.28	-1.56*	-0.16	-0.06
Contig18347_at	Unknown	-0.30	-0.21	-0.54	-1.33*	-0.52
Contig1881_s_at	Unknown	-0.43	-0.71	0.19	-1.09*	-0.74
Contig19861_at	Unknown	-0.47	-1.40*	-0.83	-0.69	-0.77
Contig20427_at	Unknown	1.30*	1.50	0.09	0.15	0.82
Contig21245_at	Unknown	-0.94	-1.77*	-1.52*	-1.29	-1.38*
Contig22600_at	Unknown	-1.80*	-0.85	-0.74	-0.56	-1.15
Contig239_s_at	Unknown	-0.62	-2.13*	1.53	-3.28*	-1.13*
Contig25479_at	Unknown	0.38	1.47*	0.02	0.43	0.58
Contig2586_at	Unknown	-0.31	-0.52	0.41	-1.59*	-0.76
Contig286_s_at	Unknown	-0.98	-3.06*	1.15	-5.01*	-1.98*
Contig2957_at	Unknown	-0.40	-1.16*	0.11	-0.96	-0.75
Contig30_at	Unknown	-0.62	-0.34	-0.51	-3.34*	-1.59

Contig3185_s_at	Unknown	-1.27*	-1.77*	0.68	-2.37*	-1.18*
Contig3198_x_at	Unknown	0.69	1.41	0.29	1.35*	1.19
Contig3812_at	Unknown	0.99	1.45*	0.74	0.63	0.96
Contig3814_at	Unknown	-0.53	-0.08	-1.03*	-0.86	-0.54
Contig383_at	Unknown	2.52*	2.47*	2.65*	1.58*	2.31*
Contig4424_s_at	Unknown	-0.49	0.23	0.14	-1.09*	-0.48
Contig4948_s_at	Unknown	1.25*	1.30*	1.20*	1.49*	1.31*
Contig571_at	Unknown	-1.28*	-0.78	-0.08	-2.67*	-1.2*
Contig5759_at	Unknown	-0.44	-0.59	0.00	-1.72*	-0.91
Contig6119_at	Unknown	-0.31	-0.32	0.43	-1.18*	-0.60
Contig659_at	Unknown	-1.44*	-0.92	0.14	-3.14*	-1.34*
Contig6706_at	Unknown	-1.15	1.42*	-0.30	0.13	0.20
Contig7004_at	Unknown	-0.51	1.09*	0.44	0.33	0.33
Contig709_at	Unknown	-0.32	-0.68	0.68	-2.24*	-0.78
Contig7427_at	Unknown	1.37*	1.56*	0.73	0.30	1.05
Contig7895_at	Unknown	-0.87	-0.80	-1.07*	-0.76	-0.82
Contig9863_at	Unknown	0.77	1.25*	0.65	0.88	0.94
EBpi01_SQ005_I12_x_at	Unknown	-0.80	-1.99*	0.94	-3.91*	-1.44*
EBpi03_SQ003_P13_at	Unknown	-0.63	-0.96	-0.42	-1.14*	-0.87
HA01J24u_x_at	Unknown	-0.55	-1.21*	0.31	-1.07	-0.89
HB28C15r_at	Unknown	0.13	-0.21	-1.10*	-0.06	0.02
HK04G05r_at	Unknown	0.16	-0.67	1.04*	-0.60	-0.38
HO14H22S_s_at	Unknown	1.16*	1.35*	0.56	0.90	1.12
HS06C04u_s_at	Unknown	1.16*	0.59	-0.63	0.74	0.88
HU14G14r_s_at	Unknown	1.63*	2.40*	3.26*	1.97*	2.31*
HV12O17u_x_at	Unknown	-0.38	-0.56	0.49	-1.67*	-0.88
HVSMEb0012I21r2_at	Unknown	-0.09	-0.02	1.82*	0.19	0.03
HVSMEc0015E05f_at	Unknown	0.11	-1.07*	0.03	-0.11	-0.28
HVSMEg0018F23r2_at	Unknown	0.36	-0.38	1.18	-1.51*	-0.51
HVSMEh0088I08r2_s_at	Unknown	-0.38	-0.87	0.63	-1.08*	-0.77
HVSMEn0008D16r2_at	Unknown	0.33	0.67	0.45	1.11*	0.50
HW01K06u_s_at	Unknown	-0.13	-0.07	0.17	-1.09*	-0.53

HW02D01u_at	Unknown	-0.08	1.62*	0.27	0.74	0.38
rbaal5k15_s_at	Unknown	1.24*	0.97	0.63	1.58*	1.11*
rbaal9h21_s_at	Unknown	0.69	1.39	0.78	1.36*	1.06*

† Rice annotations were obtained using Harvest (harvest.ucr.edu). All E-Score values are less than 3E-11.
‡ Fold change is displayed in log2 values. Positive numbers indicate up regulation from the low density to the high density whereas negative numbers indicate down regulation from the low density to the high density.
§ Genes were identified as differentially expressed with a ≥2 fold change and an unpaired t-test with Benjamini-Hochberg false-discovery rate correction ($p<0.05$).
* Fold change greater than two and a p-value less than 0.05 for an unpaired t-test of low versus high plant population density.

Table 8. Differentially expressed genes in 12 day old maize seedlings when comparing low and high plant density for each genotype.

		Fold Change[‡]					
Probe Set Name	Annotation[†]	B73	Hp301	Mo17	Oh43	P39	All Genotypes
Zm.9486.1.A1_at	3-hydroxyisobutyryl-coenzyme A hydrolase	0.43[§]	-0.75	-1.06*	-0.06	0.69	0.15
Zm.7611.1.A1_a_at	AUX/IAA family	0.37	1.15*	-0.19	0.21	-0.09	0.29
Zm.6151.1.A1_at	Carbonic anhydrase, putative	-0.62	-0.01	0.01	0.00	1.29*	0.13
Zm.369.1.A1_at	Hsp20/alpha crystallin family, putative	0.24	-0.11	-0.38	-2.05*	-0.14	0.49
Zm.14103.2.A1_at	Leucine-rich repeat protein	0.28	-0.35	-1.21*	-0.09	0.12	0.25
Zm.1315.1.A1_at	Oxalate oxidase-like protein	-1.97*	-0.01	-0.02	-0.02	0.10	0.38
Zm.11809.1.A1_at	Phosphate-induced protein 1 conserved region	1.10*	0.71	0.46	0.83	0.32	0.68
Zm.3038.1.A1_x_at	Plant invertase/pectin methylesterase inhibitor	-0.14	1.20*	0.30	-0.17	0.13	0.26
Zm.10553.1.A1_at	Sucrose/H+ symporter	1.27*	0.10	0.57	-0.09	-0.05	0.36
Zm.14591.3.S1_a_at	Triosephosphate isomerase	-0.38	-0.12	0.22	-1.28*	0.00	0.31
Zm.5005.1.A1_at	Ubiquinol-cytochrome c reductase complex	-0.03	-1.02*	0.10	-0.09	-0.07	0.22
Zm.17580.1.S1_at	Zinc finger protien, putative	0.36	1.15*	0.18	0.00	0.46	0.43
Zm.1053.1.A1_at	Unknown	-0.40	-0.95	-0.19	-1.31*	-0.12	0.59
Zm.9508.1.A1_at	Unknown	-1.04*	0.00	-0.05	-0.01	0.02	0.22
Zm.17987.1.A1_at	Unknown	-1.24*	0.39	0.00	-0.16	-0.02	0.21
Zm.5466.1.A1_at	Unknown	1.03*	-0.05	-0.03	-0.07	0.00	0.18
Zm.5663.1.A1_at	Unknown	1.29*	0.01	-0.07	0.20	0.10	0.31
ZmAffx.767.1.S1_a_at	Unknown	0.62	-0.08	1.11*	0.04	0.51	0.44
Zm.4026.1.A1_at	Unknown	-1.02*	-0.05	-0.41	0.11	-0.04	0.28
Zm.9528.1.A1_at	Unknown	-1.29*	0.18	0.02	-0.12	0.05	0.23
ZmAffx.1439.1.S1_at	Unknown	2.11	-1.61	-1.16	0.82	-2.13*	0.39
Zm.2522.1.A1_at	Unknown	1.71*	-0.04	-0.02	-0.12	0.03	0.31
Zm.6190.1.A1_at	Unknown	-1.01*	-0.06	-0.22	-0.10	-0.04	0.28
ZmAffx.1220.1.S1_at	Unknown	0.58	-1.89	-2.52	-0.29	-1.25*	1.07*
ZmAffx.1489.1.S1_at	Unknown	1.01*	-0.61	-0.31	-0.21	-0.46	0.11
Zm.10659.1.A1_at	Unknown	1.32*	0.09	0.28	0.70	-0.15	0.44

Zm.8578.3.S1_s_at	Unknown	0.61	0.76	1.11*	0.62	0.03	0.62
Zm.5794.1.S1_at	Unknown	1.22*	0.05	-0.33	-0.25	-1.67	0.20
Zm.16524.3.A1_at	Unknown	1.08*	-0.03	-0.02	-0.13	0.03	0.19
Zm.7899.1.A1_at	Unknown	-1.23*	-0.06	-0.04	-0.17	0.02	0.30
Zm.9059.1.A1_at	Unknown	1.38*	-0.01	-0.01	-0.05	0.03	0.27
Zm.4648.1.S1_at	Unknown	-0.49	1.15*	0.15	0.31	0.21	0.27
Zm.1128.1.S1_s_at	Unknown	-1.40*	-0.20	-0.39	-0.09	0.03	0.41
Zm.125.1.S1_at	Unknown	1.59*	-0.13	-0.91	-0.14	0.03	0.09
Zm.18459.1.S1_at	Unknown	-1.02*	0.17	0.21	0.11	0.04	0.10

†Rice annotations are referenced in Makarevitch et al. (2007). All E-Score values are less than 1E-25.
‡Fold change is displayed in log2 values. Positive numbers indicate up regulation from the low density to the high density whereas negative numbers indicate down regulation from the low density to the high density.
§Genes were identified as differentially expressed by implementing unpaired t-tests for each genotype and then identifying genes that had significant t-tests in two or more genotypes.
* Significant values using unpaired t-test with Benjamini-Hochberg false-discovery rate correction ($p<0.05$).

Figure 1. Clustering analysis of a subset of the maize and barley transcripts. A two-way ANOVA was implemented for each genotype x density with a p-value <0.05 and no FDR correction to obtain all expressed genes. Hierarchical clustering analysis shows that there is a high level of similarity for each genotype between the low (L) and high (H) plant densities. Abbreviations for barley genotypes are: Bar (Baronesse), Step (Steptoe) and Har (Harrington).

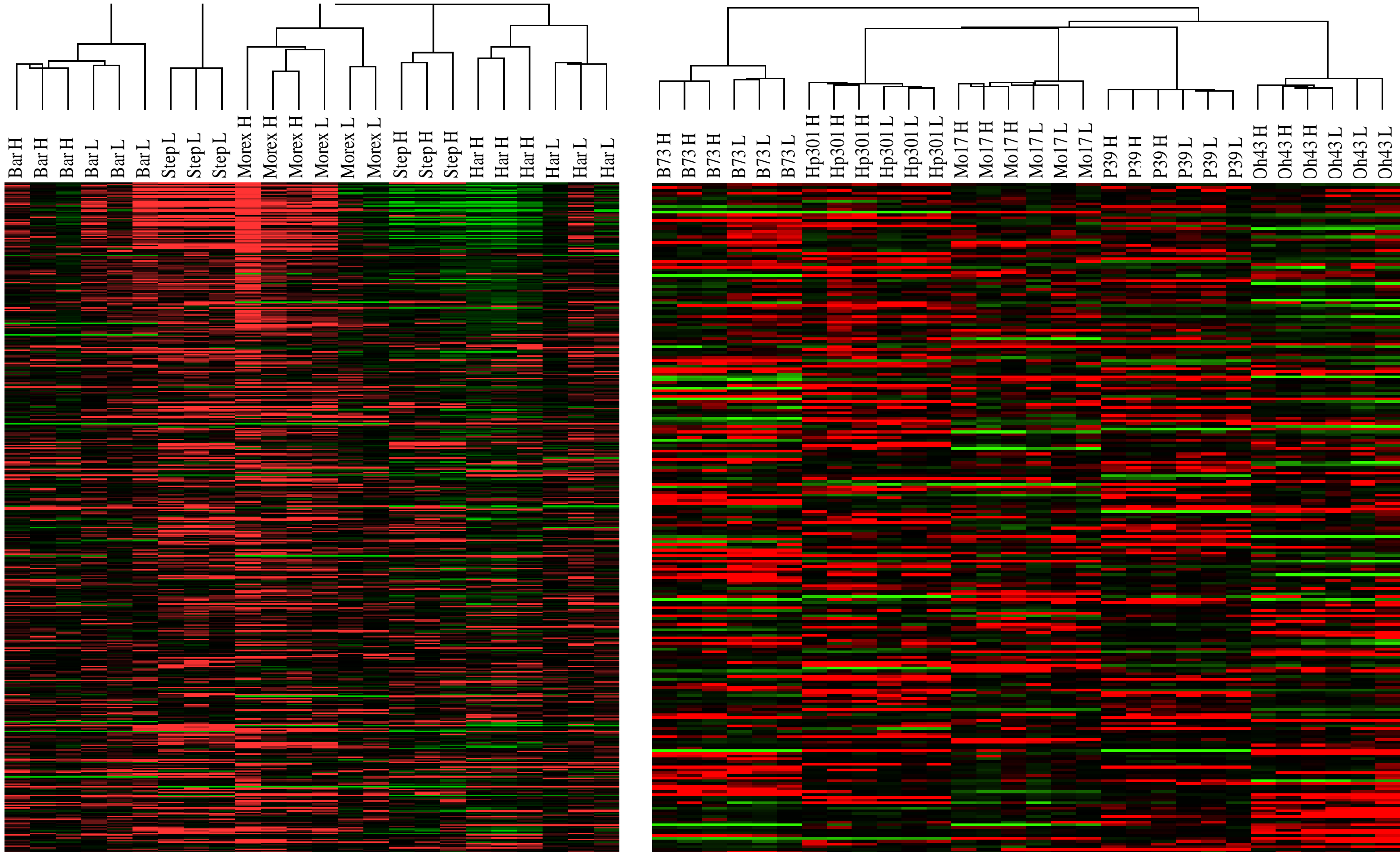


Figure 2. Venn diagram showing distribution of differentially expressed transcripts between two plant densities for four barley genotypes: Baronesse, Harrington, Morex and Steptoe. Total number of unique genes differentially expressed is 219.

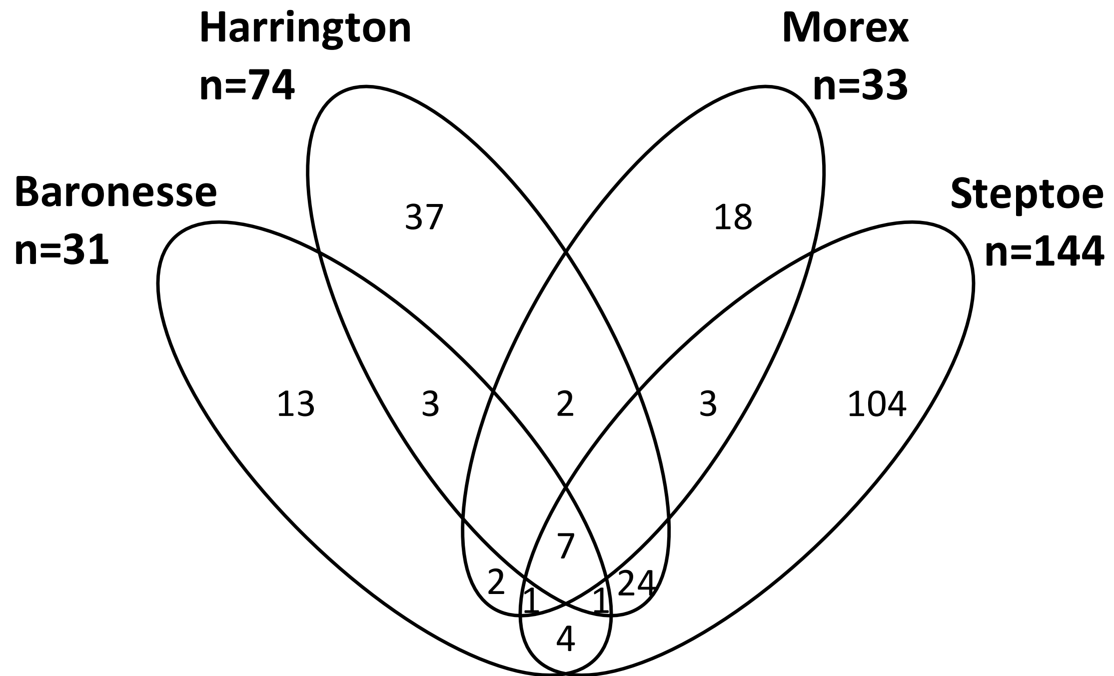
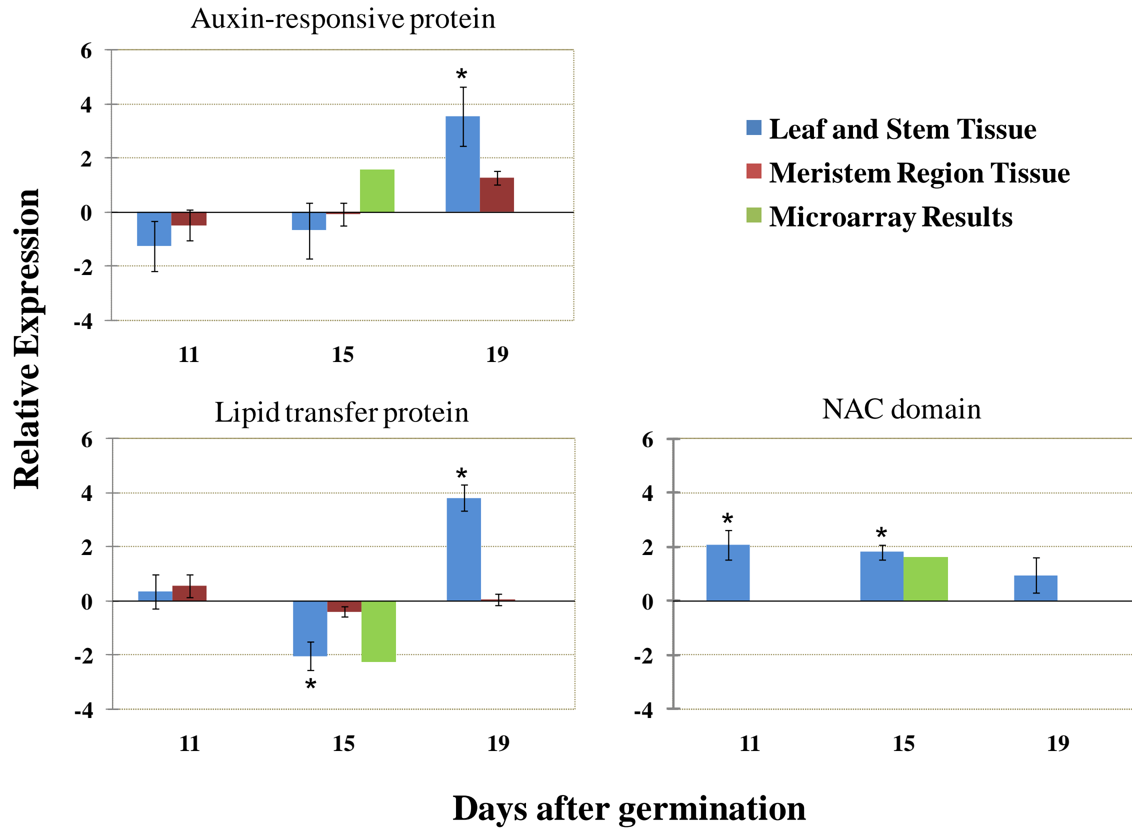


Figure 3. Real-Time RTPCR validation of three differentially accumulated transcripts related to plant density stress at three different time points. Microarray expression data for Steptoe at 15 days after germination is also shown. Relative expression is displayed in log2 values. Positive numbers indicate up regulation from the low density to the high density, whereas negative numbers indicate down regulation from the low density to the high density. Transcripts analyzed are contig14901_at (auxin-responsive protein); contig1689_at (Lipid transfer protein (LTP)); and contig6484_at (NAC domain protein). Bars with asterisk (*) indicate significantly (p-value<0.05) different than zero.



Chapter Three:

Field Evaluations for Biomass and Grain Yield Due to Plant Density Stress

Introduction

Total biomass per plant and grain per plant is reduced during density stress and exhibit a negative response with a high correlation with plant density (Borras et al., 2002; Sarlangue et al., 2007). However, increasing plant population density can increase biomass and grain yield on a per area unit basis (Duncan, 1958; Hashemi et al., 2005; Silva et al., 2007; Tollenaar, 1989). This relationship is curvilinear where eventually a high plant density will overwhelm the tolerance to density stress and yield per unit area will also decrease (Cox, 1996; Hashemi et al., 2005; Kirby, 1967; Sarlangue et al., 2007; Silva et al., 2007; Spink et al., 2000). Because of this quadratic relationship, there is an optimum plant density with the maximum yield per unit area. However, the optimum plant density varies depending on the genotype (Hashemi et al., 2005; Sarlangue et al., 2007). Plant breeders have understood this relationship and have been breeding for more density tolerant plants to increase the optimum plant density and further increase grain yield per unit area (Cox, 1996; Hashemi et al., 2005; Sarlangue et al., 2007; Tollenaar and Wu, 1999; Widdicome and Thelen, 2002). However, with the potential role of lignocellulosic ethanol it is important to develop a better understanding of the relationship between plant density and biomass yield at high planting densities.

In maize, plant density has increased from 39,520 seeds ha^{-1} in the 1960's to 66,690 seeds ha^{-1} in 2005 by increasing planting rates and reducing row spacing resulting in an increase of 21% in grain yield per hectare (Cardwell, 1982; Elmore and Abendroth, 2006). Semi-dwarf wheat varieties can tolerate higher densities than tall wheat. When grown at optimum density, grain yield was approximately 200 more seeds m^{-2} in two

semi-dwarf varieties compared to a tall variety (Faris and DePauw, 1981). Planting date also plays a strong role in planting rates for both wheat and barley. Planting barley three weeks later than the initial planting increased the optimum plant density for barley by 15 plants m^{-2} (Wiersma, 2002).

Plant morphology impacts density stress tolerance. Modern maize hybrids have been selected to intercept and utilize photosynthetically-active radiation more efficiently than older hybrids by having a more compact canopy structure (Subedi et al., 2006), a higher leaf area index after silking (Tollenaar and Aguilera, 1992) and higher leaf photosynthesis rates (Dwyer et al., 1991). Leaf and root angles have also been shown to play an important role in plant density stress tolerance (Duvick and Cassman, 1999; Fellner et al., 2003; Hammer et al., 2009). Other traits are altered by plant density stress. Stem diameter, leaf width and leaf length have been shown to decrease as plant density increases (Smith and Whitelam, 1997). There is also a reduction in tiller development related to plant density stress in wheat (Casal, 1988; Spink et al., 2000) and barley (Davis and Simmons, 1994; Kirby and Faris, 1972). Winter wheat grown at higher densities has a lower concentration of potassium and nitrogen in the tillers increasing the risk of frost damage (Sato et al., 1993). In maize, high plant densities reduces the quality of corn silage by decreasing the nutrition content (Graybill et al., 1991), increasing neutral detergent fiber (NDF) and decreasing crude protein (CP) concentrations (Cox and Cherney, 2001).

The majority of previous studies employed plant populations relatively close to the normal range to examine grain and biomass yield (i.e. 3 to 12 plants m^{-2} for maize,

200 to 640 for wheat and barley (Conry, 1998; Geleta et al., 2002; Spink et al., 2000; Turk et al., 2003; Whaley et al., 2000; Wiersma, 2002)). However, a study increasing plant density at 20 plants m^{-2} found the optimum biomass and grain yield to be at the highest plant density used in maize (Edwards et al., 2005). In addition, two spring wheat cultivars were found to have their highest grain yield at 675 seeds m^{-2} (Faris and DePauw, 1981). In these two studies, the full potential of these genotypes was not seen due to insufficient increases in plant population density.

In maize, biomass production related to plant density has been studied for silage (Cox 1996; Cox and Cherney, 2001), however to our knowledge no study has looked at biomass production related to increasing plant density in wheat and barley. Increasing plant density per unit area could increase biomass production used for lignocellulosic ethanol. Harvesting biomass from conventional grass crops such as barley, wheat and maize can be quickly implemented with very little change to current farming practices. Added benefits to using these crops is that the requirements to grow these crops are already known and the production of biomass would be done in tandem with grain production. In 2005, 75.1 million acres of maize, 50.1 million acres of wheat and 3.3 million acres of barley were grown in the United States (USDA, 2006). With current practices and combining corn, wheat and barley stover, it has been proposed that the sustainable availability of biomass (40 to 50% of crop residue) that can be removed without having an environmental impact would be approximately 78 million dry tons per year (Perlack et al., 2005). Although this biomass will assist with the production of biofuels, there still needs to be an increase in biomass production.

In this study, phenotypic responses to plant density stress were examined for barley, wheat and maize genotypes in the field at plant populations up to three times the normal planting rate. The objectives of this study were to 1) determine whether cultivars differ in responses to varying plant population densities; 2) determine the optimum planting density for each genotype for grain and biomass yield per hectare; and 3) examine the relationship between the biomass and grain yield during plant density stress.

Materials and Methods

Site and Crop management.

Field experiments were conducted in 2008 and 2009 for barley on the St. Paul campus of the University of Minnesota (44˚59'N, 93˚10'W; Waukegan silt loam) and Crookston Northwest Research and Outreach Center (47˚46'N, 96˚36'W; Wheatville fine sandy loam); in 2008 for wheat at St. Paul and Crookston; and for maize in 2008 at St. Paul and Rosemount Research Center (44˚43'N, 93˚03'W; Waukegan silt loam) and in 2009 at Rosemount.

Planting dates for barley were 29 April 2008 and 6 May 2009 in St. Paul and 6 May 2008 and 19 May 2009 in Crookston. Wheat planting dates were 6 May and 17 April in St. Paul and Crookston, respectively, in 2008. Maize planting dates were 6 May 2008 in St. Paul and 9 May 2008 and 6 May 2009 in Rosemount. Barley and wheat followed soybeans at all locations and years while maize followed soybeans in Rosemount and maize in St. Paul. Soil fertility levels were amended so nutrients were not a limiting factor. Post emergence herbicides and hand weeding were used to keep plots adequately weed free. Barley and wheat were not irrigated. Maize was not irrigated in Rosemount, but was irrigated in St. Paul.

Plant Material and Experimental Design.

For all field experiments, treatments were arranged in a randomized complete block design. Genotypes used represent a range of genetic diversity in all three species (Table 1).

In 2008, the barley experiment consisted of fourteen varieties (Baronesse, Chevron, Frederickson, Harrington, Lacey, M69, Morex, Rasmusson, Robust, Royal, Stander, Stellar-ND, Steptoe, and Tradition). In 2009, only Rasmusson and M69 were selected to be grown. Varieties were grown at three plant densities: 300 (low), 600 (medium) and 900 (high) plants m^{-2}, with 300 plants m^{-2} being the normal plant density grown in Minnesota. In 2008, each subplot consisted of one row 1.8 meters long and spaced 30.5 cm apart from other subplots and data were collected on 1m subsection in the row. In 2009, each subplot consisted of four rows 1.8m long and data collection was taken from a subsection 0.5m long from each of the two middle rows.

Sixteen wheat varieties (Ada (MN95229-A), Alsen, Briggs, Faller (ND 805), Glenn, Granger, Blade, Kelby, Steele-ND, Marshall, Oklee, RB07 (MN99436-6), Rush, Tom (MN01311-A-1), Traverse (SD3687) and Vantage) were grown at three plant densities: 300 (low), 600 (medium) and 900 (high) plants m^{-2}, with 300 plants m^{-2} being the normal plant density grown in Minnesota. Each subplot consisted of seven rows spaced 15.25cm apart and length of 2.4 meters. Data collection was taken from a subsection 0.5m long from each of the three middle rows.

In 2008, the maize experiment consisted of five inbreds (B73, I205, Mo17, MS71, and Oh43) and four hybrids (B73xMo17, B73xOh43, hp301xMo17, and Oh43xMo17). In 2009, three inbreds (B73, Mo17 and Oh43) and two hybrids (B73xMo17 and Oh43xMo17) were selected. Genotypes were grown at three planting densities: 7.7 (low); 12.6 (medium); and 17.5 (high) plants m^{-2} with 7.7 being the normal plant density grown in Minnesota. In 2008, each subplot consisted of one row 6.1 meters long and

spaced 30.5 cm apart from the other sub-plots and data collection was taken from six plants in each row. In 2009, each subplot consisted of three rows 1.5m long and data collection was taken from three plants in the middle row.

Phenotypic Measurements.

For barley and wheat, approximately 2 weeks after planting seedlings were counted to obtain the actual plant density. Data for morphological traits collected for both species during the growing stages were heading date, plant height, and length of spike. Heading date for barley was noted when 50 percent of the spikes were 50 percent out of the boot. Heading date for wheat was when 50 percent of the spikes in the row have completely emerged from the boot. Plant height for both species was measured from the ground to the base of the spike and length of spike was measured from the base of the spike to the tip of the last kernel.

Subplots were hand harvested ~3cm above the ground (biomass and grain) and bundled. The sample bundles were placed in paper bags and dried down to obtain a steady weight. The dried bundles were weighed and then threshed to obtain the grain weight. The grain weight was then subtracted from the bundle weight to obtain the dry biomass weight. Biomass and grain yield per plant were calculated by dividing the biomass and grain yield per subplot by the actual plant density for the subplot. Kernel size was also measured. For barley, seeds were sifted through a 6/64 screen using a Strand Sizer Shaker (Seedburo Equipment Co., Chicago, IL). Seeds that did not sift

through (plump seeds) were weighed and the percent plump seeds were calculated. For wheat, 1000 kernels were counted and weighed from each sample.

For maize, after the V6 stage plants (Ritchie et al., 1996) were counted in each subplot to obtain the actual plant density. Data for morphological traits was collected on six randomly chosen plants per subplot. Vegetative phenotypic data (plant height, stalk diameter and leaf architecture) were taken after ear emergence. Plant height was measured from the ground to the tip of the tassel and stalk diameter was taken 2cm above the first ear. Leaf architecture (width, length and angle) was taken using the first leaf above the ear. Leaf width was taken from the widest part of the leaf and leaf length was measured from the stalk to the tip of the leaf following the midrib. Leaf angle was noted as the angle of the leaf in relation to the stalk. Reproductive data was also recorded. Date of anthesis was taken when anthers were first visibly showing. Length of tassel and number of branches on each tassel were documented.

After physiological maturity, six plants per sub-plot were hand-harvested and pooled together. All above ground biomass was harvested and ears were removed. Stalk biomass wet weight was recorded and the stalks were shredded to create a sub-sample (~1/3 of the stalk biomass). The shredded biomass sub-samples and ears were dried down to obtain a steady weight. The shredded biomass sub-sample dry weight was recorded and used to calculate biomass per plant. Dry grain weight was measured and calculated per plant. Biomass yield m^{-2} and grain yield m^{-2} was calculated using biomass/plant and grain/plant multiplied by the actual plant density. Biomass sub-samples were further analyzed for stover composition. Cellulose, hemi-cellulose, neutral

fiber (NDF), acid detergent fiber (ADF), in vitro true digestibility (IVTD) concentrations are presented as g kg^{-2}.

Data Analysis.

Although there is variation among the actual plant population densities for all three species, the actual densities are close to the target plant density and there were significant differences among the densities (Table 2). Only maize genotype Oh43 had poor germination and no data was taken for the high density. Experiments were designed differently between the two years for both barley and maize. However, the trends were similar in both years and we combined the data.

Phenotypic data for all three species were evaluated with SAS (SAS Institute, 2003) using PROC GLM. Effects are considered significant with a p-value <0.05. Traits that showed a significant interaction between plant density and genotype were further analyzed on each individual genotype. Traits that had a non-significant plant density by genotype interaction indicated no difference among genotypes and no further analysis was conducted. Because we only wanted to know plant density effects for each morphological trait, means at each density for morphological traits were calculated and the relationship (either positive or negative) between each trait and plant density was recorded. Only traits related to biomass and grain yield were further analyzed by conducting a mean separation between plant densities and genotypes using Tukey's Least Significant Difference Test (P=0.05).

Phenotypic correlations using all genotypes and data points for each species were calculated with Pearson Correlation. Because of the large number of entries in the calculation, correlations were considered significant with a p-value <0.05 and an r>0.50 or r<0.50.

Results

Phenotypic trait differences at plant densities.

The levels of significance of main effects of location, genotype, density, and genotype*density interaction for various traits in barley, wheat and maize are presented in Table 3 and Table 4 as well as the relationship between each trait and plant density. Genotypes selected for this study offer a wide range of genetic diversity (Table 1) and genotypic effects were significant with all traits except for grain protein concentration in barley. Interestingly, there is only significant genotype by density interaction for plant height and leaf length for wheat and maize, respectively, indicating that for most traits, genotypes acted in a similar fashion in different planting densities.

For barley and wheat, plant density effects were significant for all morphological traits measured except for grain protein content (Table 3). Plant density had a decreasing effect on heading date, spike length, percent plump, and harvest index (Table 3). While plant density had a significant effect on plant height, plant height decreased in barley whereas plant height direction varied on the genotype in wheat (Table 3).

In maize, stalk diameter, leaf width, leaf length, tassel height, cob weight, and harvest index significantly decreased as plant density increased (Table 4). Plant density had a significant increasing effect on days to anthesis. In maize, plant density had no effect on plant height, tassel branches and leaf angle. Our results found that plant density had no effect on hemi-cellulose, NDF concentrations and IVTD concentrations; however, we found an increase for ADF and cellulose. These results show that while genotypes

may act in a similar fashion to plant density stress all three species, many phenotypes are effected by plant density stress.

Effect of density on biomass and grain traits.

In all three species, there was a significant negative effect of plant density for biomass/plant and grain/plant (Table 5, Figures 1 and 2). In barley and wheat, an increase from low plant density to high plant density resulted in a decrease of 30-40% for both biomass/plant and grain/plant and mean separation between plant densities indicated significant differences in each density (Figure 1). Maize had a decrease of 79 and 66% for biomass/plant and grain/plant, respectively, and there was only a significant difference between the low plant density and the other two densities (Figure 2). In all three species, the reduction between low plant density and medium plant density is greater than between medium plant density and high plant density for both biomass/plant and grain/plant.

Plant density had a significant effect for biomass yield m^{-2} for all three species (Table 5). Combining all genotypes, mean separation shows that the high plant density had the greatest biomass yield m^{-2} for wheat and maize while barley had no significant difference among the three densities for biomass yield m^{-2} (Figures 1 and 2). For grain yield m^{-2}, plant density had a significant effect for barley and maize, but not for wheat (Table 5). Mean separation shows that for barley the high plant density had the lowest grain yield m^{-2} and the low plant density and medium plant density yielded the same (Figure 1). In maize, the high plant density had the greatest grain yield m^{-2} (Figure 2).

These results indicate that the high plant density may still have been too low to identify the optimum plant density in maize and wheat.

The complete model did not find significant genotype*density interaction. However, there were differences among genotypes on how they responded to density stress for biomass yield m^{-2} and grain yield m^{-2} (Table 6). In barley, Steptoe, Royal, and Fredrickson had a significant decrease in biomass yield m^{-2} in the higher plant densities (Table 6). Harrington and Steptoe significantly decreased in grain yield m^{-2}, whereas Tradition significantly increased as plant density increased. Although not all were significant, varieties that had consistent trends for both biomass yield m^{-2} and grain yield m^{-2} were Tradition and M69 with an increase and Steptoe, Fredrickson, Morex, Robust, Stellar with a decrease in biomass and grain yield m^{-2} as plant density increased.

In wheat, Ada, Alsen and Marshall had a significant biomass yield m^{-2} increase in the high density compared to the low density (Table 6). All other wheat varieties displayed a trend of increasing biomass yield m^{-2} as plant density increases. No varieties had significant differences among the densities for grain yield m^{-2}, however Briggs, Granger and Traverse showed the greatest decrease in the high density compared to the low density.

The trend for all genotypes, except MS71, in maize showed increased biomass yield m^{-2} and grain yield m^{-2} at either medium or high density compared to the low density. B73, B73xOh43, and Hp301xMo17 had significant increases in the high density compared to the low density (Table 6). There were significant differences between the

medium and low density for Oh43. For grain yield m^{-2}, MS71 decreased as plant density increased.

Traits correlated with biomass and grain yield.

For both barley and wheat, grain yield m^{-2} and biomass yield m^{-2} were significantly correlated to one another. Percent plump was significantly correlated to grain yield m^{-2} and biomass yield m^{-2} in both species. Harvest index was correlated to grain in both wheat and barley and grain for barley. In wheat, heading date was correlated to both grain and biomass. Only plant height was correlated with biomass in barley (Tables 7 and 8).

For maize, grain yield m^{-2} and biomass yield m^{-2} were significantly correlated to one another. Plant height was correlated to both grain yield m^{-2} and biomass yield m^{-2}. Grain yield m^{-2} was also correlated with leaf width, tassel height, cob weight, harvest index, NDF, ADF, Cellulose, and Hemi-cellulose (Table 9).

Discussion

Our results show that, for the tested high planting densities, biomass yield m^{-2} and grain yield m^{-2} for maize and biomass yield m^{-2} for wheat continued to increase. The majority of previous studies related to plant density and optimum density only tested genotypes planted near the normal plant population density (i.e. 3 to 12 plants m^{-2} for maize, 200 to 640 for wheat and barley (Conry, 1998; Geleta et al., 2002; Spink et al., 2000; Turk et al., 2003; Whaley et al., 2000; Wiersma, 2002)). In this range there is a curvilinear relationship with plant density. Biomass and grain yield per area increases until a high plant density will overwhelm the tolerance to density stress and yield per unit area begins to decrease. The optimum density (ymax) can then be calculated (Duncan, 1958; Sarlangue et al., 2007; Tollenaar, 1989). Because we used a plant density out of the normal range, we could not use this method. Instead, we can only identify the highest yielding density for the three densities. However, a study in maize increased plant density to 20 plants m^{-2} found the optimum biomass yield and grain yield to be at the highest plant density used (Edwards et al., 2005). In addition, two spring wheat cultivars planted at 75, 150, 300, 450, 675 and 1350 seeds m^{-2} were found to have their highest grain yield at 675 seeds m^{-2} (Faris and DePauw, 1981). However, the full potential of these two cultivars was not seen in this study since the next seeding rate was 1350 seeds m^{-2} and both cultivars significantly decreased in grain yield.

As expected, biomass/plant and grain/plant decreased as plant density increased for all three species (Figures 1 and 2). The reduction between low plant density and medium plant density is greater than between medium plant density and high plant

density for both biomass/plant and grain/plant. In our study, maize had no significant difference between the two densities. A previous study for grain/plant in maize found a reduction of 5.7-6.8% from 3 to 9 plants m^{-2} and a smaller reduction of 0-5.3% from 9 to 12 plants m^{-2} (Borras et al., 2002). Similar results have also been reported in another study for biomass/plant and grain/plant in maize (Sarlangue et al., 2007). This indicates that the rate of decrease from increasing plant density becomes closer to zero as plants continue to be stressed. Therefore, if the amount of biomass or grain yield per plant remains the same as plant density increases, adding more plants per m^{-2} could actually increase yield m^{-2}. This is illustrated in the wheat and maize experiments conducted in this paper. When combining all genotypes, the high plant density had the greatest biomass yield m^{-2} for wheat and maize, and high plant density had the greatest grain yield m^{-2} in maize.

An objective of this experiment was to identify genotypes that exhibit tolerance to plant density stress. By looking at individual genotypes, we were able to increase biomass and grain yield m^{-2} as plant density increased, indicating tolerance to plant density stress in barley (Tradition and M69), wheat (Ada, Alsen and Marshall), and maize (B73, I205 and Mo17) increased biomass yield m^{-2} and grain yield m^{-2}. Genotypes with less tolerance to plant density stress were also identified for barley (Steptoe, Fredrickson, Morex, and Robust), wheat (Brigss, Granger, and Traverse) and maize (MS71). Although not all of these genotypes showed statistically different means among plant densities, there were trends for either the increase or decrease for both biomass yield m^{-2} and grain yield m^{-2}.

Our results showed consistencies with previous studies. In our study, for wheat and barley, heading date, spike length, percent plump, and harvest index decreased as plant density increased (Table 3). These results are consistent with other studies. An increase in seeding rates decreased heading date for spring wheat (Geleta et. al, 2002) and decreased spike length in barley (Turk et al., 2003). Additionally, lower number of kernels per spike, which results in a shorter spike length, has been identified (Conry, 1998; Spink et al., 2000). Significant decreases for thousand grain weight has also be found in winter wheat (Spink et al., 2000; Whaley et al., 2000), however Conry (1998) did not find significant differences in spring malting barley. Plant height decreased in barley whereas plant height varied on the genotype in wheat (Table 3). Plant height has been found to decrease as plant density increases in winter barley and spring wheat (Faris and DePauw, 1981; Turk et al., 2003) whereas seeding rate did not affect plant height in winter wheat (Geleta et al., 2002).

In maize, stalk diameter, leaf width, leaf length, tassel height, cob weight, and harvest index significantly decreased as plant density increased (Table 4). Other research has found increasing plant density decreases in stalk diameter, leaf width and leaf length (Silva et al., 2007; Smith and Whitelam, 1997), tassel height (Otegui, 1997), and size of cobs (Bavec and Bavec., 2002). However, we found a significant decrease for harvest index while there has been no difference previously found (Cox, 1996; Graybill et al., 1991).

Plant density had a significant increasing effect on days to anthesis, which has been previously found to increase (Modarres et al., 1998). In maize, plant density had no

effect on plant height. Based on previous experiments this is not surprising. Silva et al. (2007) found no difference in height whereas other studies have found an increase (Modarres et al., 1998) and decrease (Silva et al., 2003) in plant height. We found no effect for number of tassel branches whereas Silva et al. (2007) found a decrease in tiller branching as plant density increased. Plant density had no effect on leaf angle.

Our results found that plant density had no effect on NDF concentrations. Plant density has been found to either increase (Cox and Cherney, 2001) or have little effect (Graybill et al., 1991) for NDF concentrations. There was no effect on hemi-cellulose in this study and this is consistent with a previous study (Carmi et al., 2006). There were some inconsistencies between our study and previous studies for chemical concentrations. We found an increase for ADF and cellulose whereas previous studies found little or no effect for each of these traits (Carmi et al., 2006; Graybill et al., 1991). Plant density had no effect for IVTD and it has been previously found that IVTD concentrations are decreased as plant density increases (Cox and Cherney 2001).

Conclusions

Our study involved genotypes in maize, wheat and barley planted at a range of plant population densities that are not normally used in identifying optimum density. We were able to demonstrate that biomass yield m^{-2} and grain yield m^{-2} can continue to increase at extremely high plant densities in genotypes in all three species. These results indicate that for some genotypes, the plant densities used in this experiment were still too low to identify the optimum plant density. Eventually resources such as light, water and nutrients would become too scarce and yield m^{-2} would decrease. Future experiments increasing plant density even further for these genotypes would determine at what plant density would biomass yield m^{-2} and grain yield m^{-2} decrease.

Table 1. Description of maize, wheat, and barley genotypes used in the plant density experiments.

Genotype	Description	Publication
Maize		
B73	Dent, Iowa Stiff Stalk Synthetic (ISSS)	Russell, W.A. 1972. Registration of B70 and B73 Parental Lines of Maize (Reg. Nos. PL16 and PL17). Crop Sci. 12:721.
I205	Popcorn, Iowa Experiment Station Reid Yellow Dent	Developed by Merle T. Jenkins and released in 1937 (Troyer, A.F. 1999. Background of US hybrid corn. Crop Sci. 39:601-626.)
Mo17	Dent, Missouri Lancaster Sure Crop	Zuber, M.S. 1973. Registration of 20 Maize Parental Lines (Reg. Numbers PL 18 to 37). Crop Sci. 13:779a-780a.
MS71	CG-Lancaster	Lee, E.A., R. Chakravarty, B. Good, M.J. Ash and L.W. Kannenberg. 2006. Registration of 38 maize (*Zea mays* L.) breeding populations adapted to short-season environments. Crop Sci. 46:2728-2733.
Oh43	Dent, Lancaster Sure Crop Illinois	Bergquist, R.R. 1982. Registration of Maize Inbred LP Oh43 Rp_1^{Td} Germplasm (Reg. No. GP 93). Crop Sci. 22:451-452.
Hp301xMo17	Hp301: Popcorn, Indiana	USDA, ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)*. [Online Database] National Germplasm Resources Laboratory, Beltsville, Maryland. Available: http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1084096 (23 December 2009).
B73xOh43		
B73xMo17		
Oh43xMo17		
Wheat		
Ada (MN95229-A)	hard red spring	Anderson, J.A., R.H. Busch, D.V. McVey, J.A. Kolmer, Y. Jin, G.L. Linkert, J.V. Wiersma, R. Dill-Macky, J.J. Wiersma, and G.A. Hareland. 2007. Registration of 'Ada' wheat. Crop Sci. 47:434.
Alsen	hard red spring	Mergoum, M., R.C. Frohberg, R.W. Stack, T. Olson, and J.D. Miller. 2006. Registration of 'Alsen' wheat. Crop Sci. 46:2311.
Briggs	hard red spring	Glover, K., R. Devkota, J. Rudd, Y Jin, R.G. Hall, and G.A. Hareland. 2007. Registration of 'Briggs' wheat. Crop Sci. 47:432.
Faller (ND 805)	hard red spring	Mergoum, M., R.C. Frohberg, R.W. Stack, J.W. Rasmussen, and T.L. Friesen. 2008. Registration of 'Faller' spring wheat. Journ. Plant Reg. 2:224-229.
Glenn	hard red spring	Mergoum, M., R. Frohberg, R. Stack, T. Olson, T. Friesen, and J. Rasmussen. 2006. Registration of 'Glenn' wheat. Crop Sci. 46(1):473.
Granger	hard red spring	Glover, K., J. Rudd, R. Devkota, R. Hall, Y. Jin, and J. Rickertsen. 2006. Registration of

		‘Granger’ wheat. Crop Sci. 46:1390.
Blade	hard red spring	Developed by WestBred, LLC, Bozeman, MT. [http://www.westbred.com/] Cited 6 January 2010.
Kelby	hard red spring	Developed by AgriPro, Berthoud, CO. (Ransom, J.K., M. Mergoum, S. Simsek. 2009. North Dakota hard red spring wheat variety trial results for 2009 and selection guide. [http://www.ag.ndsu.edu/pubs/plantsci/smgrains/a574.pdf]. Cited 6 January 2010.)
Steele-ND	hard red spring	Mergoum, M., R. C. Frohberg, J. D. Miller, and R. W. Stack. 2005. Registration of ‘Steele-ND’ wheat. Crop Sci. 45:1163-1164.
Marshall	hard red spring	Busch, R., D. McVey, V. Youngs, R. Heiner, and F. Elsayed. 1983. Registration of ‘Marshall’ wheat. Crop Sci. 23:187.
Oklee	hard red spring	Anderson, J.A., R.H. Busch, D.V. Mcvey, J.A. Kolmer, G.L. Linkert, J.V. Wiersma, R. Dill-Macky, J.J. Wiersma, and G.A. Hareland. 2005. Registration of Oklee wheat. Crop Sci. 45:784-785.
RB07 (MN99436-6)	hard red spring	Anderson, J. A., G.L. Linkert, R.H. Busch, J.J. Wiersma, J.A. Kolmer, Y. Jin, R. Dill-Macky, J.V. Wiersma, G.A. Hareland, and D.V. McVey. 2009. Registration of ‘RB07’ wheat. Journ. Plant Reg. 3:175-180.
Rush	hard red spring	Developed by WestBred, LLC, Bozeman, MT. [http://www.westbred.com/] Cited 6 January 2010.
Tom (MN01311-A-1)	hard red spring	United States Department of Agriculture. 2008. Wheat breeding and genetics. [http://www.reeis.usda.gov/web/crisprojectpages/7347.html]. Cited 6 January 2010.
Traverse (SD3687)	hard red spring	Developed by South Dakota State University, Brookings, South Dakota. [http://plantsci.sdstate.edu/wheat/pi.cfm?piName=Dr.%20Karl%20Glover] Cited 6 January 2010.
Vantage	hard red spring	Developed by WestBred, LLC, Bozeman, MT. [http://www.westbred.com/] Cited 6 January 2010.

Barley

Baronesse	2 Row Feed	Developed in Nordsaat, Germany and marketed in U.S. by WestBred, LLC (Bozeman, MT).
Chevron	6 Row Feed	Shands, R.G. 1939. Chevron, a barley variety resistant to stem rust and other diseases. Phytopathology 29:209-211.
Frederickson	2 Row Feed	Developed in Japan (Mesfin, A., K.P. Smith, R. Dill-Macky, C.K. Evans, R. Waugh, C.D. Gustus, and G.J. Muehlbauer. 2003. Quantitative trait loci for fusarium head blight resistance in barley detected in a two-rowed by six-rowed population. Crop Sci. 43:307-318.)
Harrington	2 Row Malt	Williams, K.J., J. Cheong, K. Willsmore, M.Cakir, and H.A. Wallwork. 2003. A comparison of the genetics of seedling and adult plant resistance to the spot form of net blotch (*Pyrenophora teres* f. *maculata*) Aust. J. Agric. Res. 54:1387-1394.
Lacey	6 Row Malt	Rasmusson D.C., Smith K.P., Dill-Macky R., Schiefelbein E.L., and J.V. Wiersma. 2001.

		Registration of ‘Lacey’ Barley. Crop Sci 41:1991.
Morex	6 Row Malt	Rasmusson, D.C. and R.W. Wilcoxson. 1979. Registration of ‘Morex’ barley. Crop Sci. 19:293.
M69	6 Row Feed	University of Minnesota breeding line
Rasmusson	6 Row Malt	University of Minnesota variety release
Robust	6 Row Malt	Rasmusson, D.C., and R.D. Wilcoxson. 1983. Registration of ‘Robust’ barley. Crop Sci. 23:1216.
Royal	6 Row Feed	Rasmusson, D.C., C.C. Sheaffer, S.R. Simmons, and E. Schiefelbein. 1994. Registration of ‘Royal’ barley. Crop Sci. 34:1412.
Stander	6 Row Malt	Rasmusson, D.C., R.D. Wilcoxson, and J.V. Wiersma. 1993. Registration of ‘Stander’ barley. Crop Sci. 33:1403.
Stellar-ND	6 Row Malt	Horsley, R., J.D. Franckowiak, P.B. Schwarz, and S.M. Neate. 2006. Registration of ‘Stellar-ND’ barley. Crop Sci. 46:980.
Steptoe	6 Row Feed	Muir, C.E. and R.A. Nilan. 1973. Registration of ‘Steptoe’ barley. Crop Sci. 13:770.
Tradition	6 Row Malt	Government of Alberta. 2007. [http://www1.agric.gov.ab.ca/general/cropvart.nsf/Varieties/Tradition?OpenDocument]. Cited 6 January 2010.

Table 2. Target and actual plant population densities (plants m^{-2}).

	Target	Actual[†]		Target	Actual
Density	**Barley & Wheat**	**Barley**	**Wheat**	**Maize**	
Normal	300	284[a‡]	301[a]	7.7	7.8[a]
Medium	600	563[b]	579[b]	12.6	12.2[b]
High	900	840[c]	854[c]	17.5	16.6 [c]

[†] Actual plant densities are means for all genotypes and years.

[‡] Means followed with different letters are significantly different ($p<0.05$).

Table 3. Analysis of variance of barley and wheat phenotype data. Trait levels of significance and degrees of freedom (df) for the main effects and interaction between location, genotype, density and genotype*density along with the relationship (positive, negative or both) between each trait and plant density.

Crop	Source	df	Heading Date	Plant Height	Spike Length	Percent Plump	Protein Content	Harvest Index
Barley	Location	3	<0.001	<0.001	<0.001	<0.001	0.810	<0.001
	Rep(Location)	8	<0.001	<0.001	0.012	0.007	0.505	0.230
	Genotype	13	<0.001	<0.001	<0.001	<0.001	0.078	<0.001
	Density	2	<0.001	<0.001	<0.001	<0.001	0.203	0.003
	Genotype*Density	26	0.081	0.493	0.227	0.787	0.032	0.238
	Residuals	270						
	Relationship		negative	negative	negative	negative	n/s	negative
Wheat	Location	1	<0.001	<0.001	<0.001	<0.001	n/a	<0.001
	Rep(Location)	4	0.299	0.156	0.091	0.597	n/a	0.278
	Genotype	15	<0.001	<0.001	<0.001	<0.001	n/a	<0.001
	Density	2	<0.001	<0.001	<0.001	<0.001	n/a	<0.001
	Genotype*Density	30	0.965	0.030	0.300	0.507	n/a	0.442
	Residuals	235						
	Relationship		negative	both	negative	negative	n/a	negative

Table 4. Analysis of variance of maize phenotype data. Trait levels of significance and degrees of freedom (df) for the main effects and interaction between location, genotype, density and genotype*density along with the relationship (positive, negative or both) between each trait and plant density.

Source	df	Days to Anthesis	Plant Height	Stalk Diameter	Leaf Width	Leaf Length	Leaf Angle	Tassel Height	Tassel Branch	Cob Weight	Harvest Index
Location	2	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
Rep(Location)	2	0.073	0.955	0.073	0.104	0.330	0.739	0.147	0.997	<0.001	0.008
Genotype	8	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Density	2	0.001	0.449	<0.001	<0.001	0.002	0.206	<0.001	0.103	<0.001	0.017
Genotype*Density	15	0.295	0.237	0.708	0.122	0.047	0.352	0.360	<0.001	0.324	0.702
Residuals	113										
Correlation		positive	n/s	negative	negative	negative	n/s	negative	n/s	negative	negative

		NDF	ADF	Cellulose	Hemi-Cellulose	IVTD
Location	1	0.403	<0.001	0.051	0.555	<0.001
Rep(Location)	2	0.006	0.030	0.030	0.004	0.033
Genotype	7	<0.001	<0.001	<0.001	<0.001	<0.001
Density	2	0.183	0.033	0.035	0.394	0.450
Genotype*Density	13	0.601	0.238	0.308	0.806	0.342
Residuals	67					
Correlation		n/s	positive	positive	n/s	n/s

Table 5. Analysis of variance of barley, wheat and maize yield data. Trait levels of significance and degrees of freedom (df) for the main effects and interaction between location, genotype, density and genotype*density.

Crop	Source	df	Biomass/ Plant	Grain/ Plant	Biomass Yield m^{-2}	Grain Yield m^{-2}
Barley	Location	3	<0.001	<0.001	<0.001	<0.001
	Rep(Location)	8	0.150	0.454	<0.001	0.011
	Genotype	13	<0.001	<0.001	<0.001	<0.001
	Density	2	<0.001	<0.001	0.030	<0.001
	Genotype*Density	26	0.972	0.518	0.051	0.101
	Residuals	270				
Wheat	Location	1	<0.001	<0.001	<0.001	<0.001
	Rep(Location)	4	0.031	0.545	0.156	0.156
	Genotype	15	<0.001	<0.001	<0.001	<0.001
	Density	2	<0.001	<0.001	<0.001	0.206
	Genotype*Density	30	0.051	0.608	0.678	0.191
	Residuals	235				
Maize	Location	2	<0.001	0.013	<0.001	0.244
	Rep(Location)	2	0.394	<0.001	0.228	0.002
	Genotype	8	<0.001	<0.001	<0.001	<0.001
	Density	2	<0.001	<0.001	<0.001	0.049
	Genotype*Density	15	0.205	0.081	0.227	0.949
	Residuals	113				

Table 6. Plant density effect on biomass and grain traits for each genotype averaged across years, locations and replications for barley, wheat, and maize.

	Biomass m^{-2}			Grain m^{-2}		
Genotype	**L**†	**M**	**H**	**L**	**M**	**H**
Barley						
Baronesse	**569**^{a‡}	447^{a}	498^{a}	478^{a}	391^{a}	471^{a}
Chevron	504^{a}	488^{a}	561^{a}	305^{a}	278^{a}	242^{a}
Fredrickson	**506**^{a}	479^{ab}	435^{a}	314^{a}	329^{a}	275^{a}
Harrington	585^{a}	597^{a}	528^{a}	**436**^{a}	**444**^{a}	342^{b}
Lacey	524^{a}	547^{a}	479^{a}	511^{a}	603^{a}	514^{a}
M69	541^{a}	555^{a}	598^{a}	429^{a}	433^{a}	487^{a}
Morex	520^{a}	498^{a}	461^{a}	497^{a}	476^{a}	372^{a}
Rasmusson	528^{a}	526^{a}	550^{a}	474^{a}	448^{a}	421^{a}
Robust	579^{a}	519^{a}	512^{a}	553^{a}	507^{a}	430^{a}
Royal	**425**^{a}	288^{b}	**438**^{a}	359^{a}	356^{a}	414^{a}
Stander	497^{a}	552^{a}	498^{a}	529^{a}	560^{a}	482^{a}
Stellar	562^{a}	523^{a}	543^{a}	584^{a}	566^{a}	569^{a}
Steptoe	**505**^{a}	**467**^{ab}	386^{b}	**533**^{a}	**477**^{ab}	405^{b}
Tradition	522^{a}	525^{a}	564^{a}	512^{a}	576^{b}	**554**^{c}
Wheat						
Ada	520^{a}	528^{a}	**597**^{b}	388^{a}	348^{a}	394^{a}
Alsen	466^{a}	494^{a}	**562**^{b}	370^{a}	368^{a}	373^{a}
Blade	484^{a}	509^{a}	523^{a}	360^{a}	360^{a}	345^{a}
Briggs	567^{a}	498^{a}	577^{a}	413^{a}	363^{a}	351^{a}
Faller	529^{a}	538^{a}	580^{a}	406^{a}	415^{a}	406^{a}
Glenn	518^{a}	543^{a}	516^{a}	368^{a}	363^{a}	319^{a}
Granger	524^{a}	520^{a}	544^{a}	362^{a}	363^{a}	300^{a}
Kelby	437^{a}	536^{a}	565^{a}	337^{a}	437^{a}	445^{a}
Marshall	421^{a}	**504**^{b}	**487**^{b}	292^{a}	343^{a}	308^{a}
Oklee	489^{a}	552^{a}	593^{a}	413^{a}	408^{a}	384^{a}

RB07	534[a]	593[a]	573[a]	414[a]	424[a]	420[a]
Rush	549[a]	557[a]	549[a]	372[a]	367[a]	325[a]
Steele	556[a]	534[a]	564[a]	390[a]	370[a]	368[a]
Tom	471[a]	470[a]	497[a]	399[a]	384[a]	422[a]
Traverse	477[a]	518[a]	540[a]	395[a]	355[a]	330[a]
Vantage	529[a]	594[a]	649[a]	338[a]	344[a]	367[a]
Maize						
B73	960[a]	1135[ab]	**1428[b]**	503[a]	592[a]	908[a]
B73XMo17	1117[a]	1235[a]	1225[a]	1398[a]	1356[a]	1401[a]
B73xOh43	969[a]	1192[ab]	**1641[b]**	1300[a]	1039[a]	1805[a]
hp301xMo17	922[a]	1496[ab]	**1762[b]**	1531[a]	1288[a]	1816[a]
I205	459[a]	799[a]	1284[a]	270[a]	233[a]	775[a]
Mo17	753[a]	1006[a]	1048[a]	636[a]	720[a]	604[a]
MS71	537[a]	746[a]	546[a]	**299[a]**	**375[a]**	79[b]
Oh43	473[a]	**644[b]**	----	500[a]	546[a]	----
Oh43xMo17	937[a]	975[a]	1043[a]	1291[a]	1223[a]	1275[a]

[†] Plant population: The barley experiment was planted at 300 (L), 600 (M) and 900 (H) plants m^{-2}. The maize experiment was planted at 7.7 (L), 12.6 (M) and 17.5 (H) plants m^{-2}.

[‡] Means followed with different letters are significantly different ($p<0.05$) and type in bold indicates the highest yield m^{-2}.

Table 7. Barley phenotypic correlations between plant traits over three plant densities.

Trait	Heading Date	Plant Height	Spike Length	Biomass/ Plant	Grain/ Plant	Biomass Yield m^{-2}	Grain Yield m^{-2}	Percent Plump	Harvest Index
Plant Height	-0.11*								
Spike Length	0.15*	0.10							
Biomass/ Plant	0.31***	0.22***	0.30***						
Grain/ Plant	0.17*	0.29***	0.26***	0.89***					
Biomass Yield m^{-2}	0.40***	0.49***	0.26***	0.41***	0.40***				
Grain Yield m^{-2}	0.04	0.48***	0.15**	0.26***	0.53***	0.71***			
Percent Plump	0.29***	0.50***	0.27***	0.28***	0.043***	0.70***	0.79***		
Harvest Index	-0.26***	0.18***	-0.03	-0.03	0.36***	0.03	0.69***	0.43***	
Protein Content	0.22	0.30	0.41*	0.55**	0.38	0.36	0.02	0.04	-0.37

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

Table 8. Wheat phenotypic correlations between plant traits over three plant densities.

Trait	Heading Date	Plant Height	Spike Length	Biomass/ Plant	Grain/ Plant	Biomass Yield m^{-2}	Grain Yield m^{-2}	Percent Plump
Plant Height	0.26***							
Spike Length	0.26***	0.13*						
Biomass/ Plant	0.39***	0.27***	0.40***					
Grain/ Plant	0.59***	0.30***	0.37***	0.92***				
Biomass Yield m^{-2}	0.82***	0.30***	0.14*	0.37***	0.48***			
Grain Yield m^{-2}	0.93***	0.29***	0.22***	0.45***	0.67***	0.87***		
Percent Plump	0.83***	0.40***	0.35***	0.50***	0.68***	0.68***	0.84***	
Harvest Index	0.82***	0.23***	0.26***	0.39***	0.66***	0.55***	0.88***	0.82***

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

Table 9. Maize phenotypic correlations between plant traits over three plant densities.

Trait	Days to Anthesis	Plant Height	Stalk Diameter	Leaf Width	Leaf Length	Leaf Angle	Tassel Height	Tassel Branch	Cob Weight
Plant Height	0.26**								
Stalk Diameter	-0.83***	-0.11							
Leaf Width	0.12	0.56***	0.00						
Leaf Length	-0.20*	0.56***	0.29***	0.47***					
Leaf Angle	-0.27**	0.27***	0.09	0.37***	0.24**				
Tassel Height	0.08	0.67***	-0.02	0.85***	0.44***	0.45***			
Tassel Branch	0.33***	0.55***	-0.27**	0.42***	0.37***	0.30***	0.59***		
Cob Weight	0.42***	0.54***	-0.15	0.53***	0.20*	0.05	0.53***	0.40***	
Biomass/ Plant	0.26**	0.48***	0.03	0.42***	0.15	0.07	0.42***	0.29***	0.78***
Grain/ Plant	0.10	0.60***	0.12	0.72***	0.39***	0.26**	0.72***	0.38***	0.83***
Biomass Yield m^{-2}	0.21*	0.56***	-0.11	0.06	0.13	0.01	0.16	0.26**	0.40***
Grain Yield m^{-2}	0.10	0.72***	0.00	0.52***	0.41***	0.25**	0.62***	0.41***	0.62***
Harvest Index	-0.14	0.44***	0.19*	0.60***	0.39***	0.30***	0.63***	0.22**	0.42***
NDF	-0.42***	0.63***	0.20*	0.46***	0.36***	0.28**	0.56***	0.36***	0.46***
ADF	-0.23*	0.67***	0.17	0.32**	0.30**	0.21*	0.44***	0.27**	0.42***
Cellulose	-0.34***	0.69***	0.18	0.41***	0.37***	0.24*	0.52***	0.33**	0.40***
Hemi-Cellulose	-0.52***	0.44***	0.16	0.49***	0.28**	0.37**	0.54***	0.33**	0.40***
IVTD	-0.05	-0.45***	-0.16	-0.13	0.00	-0.18	-0.24*	-0.02	-0.55***

Table 9 (continued). Maize phenotypic correlations between plant traits over three plant densities.

	Biomass/ Plant	Grain/ Plant	Biomass Yield m^{-2}	Grain Yield m^{-2}	Harvest Index	NDF	ADF	Cellulose	Hemi- Cellulose
Plant Height									
Stalk Diameter									
Leaf Width									
Leaf Length									
Leaf Angle									
Tassel Height									
Tassel Branch									
Cob Weight									
Biomass/ Plant									
Grain/ Plant	0.71***								
Biomass Yield m^{-2}	0.63***	0.34***							
Grain Yield m^{-2}	0.52***	0.80***	0.65***						
Harvest Index	0.12	0.72***	-0.01	0.69***					
NDF	0.23*	0.57***	0.37**	0.70***	0.77***				
ADF	0.27**	0.44***	0.43***	0.63***	0.59***	0.92***			
Cellulose	0.23*	0.48***	0.39***	0.67***	0.67***	0.95***	0.98***		
Hemi-Cellulose	0.17	0.56***	0.17	0.60***	0.77***	0.92***	0.72***	0.78***	
IVTD	-0.42***	-0.42***	-0.41***	-0.48***	-0.38***	-0.72***	-0.82***	-0.73***	-0.57***

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

Figure 1. Yield per plant and yield m^{-2} means for barley and wheat at three densities 300 (1x), 600 (2x) and 900 (3x) plants m^{-2}. Different letters indicate means are significantly different at P< 0.05, and the error bars are LSD<0.05.

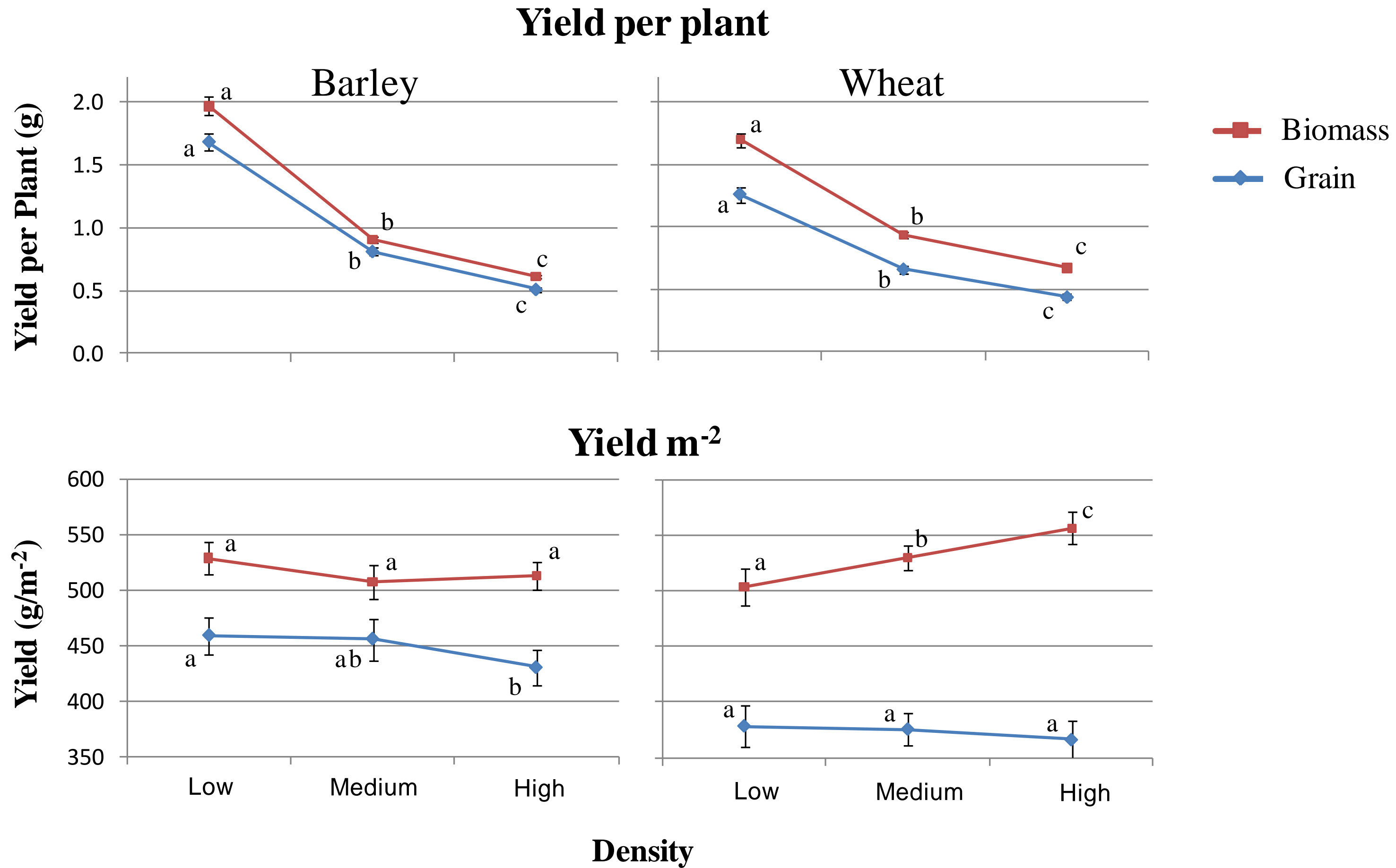
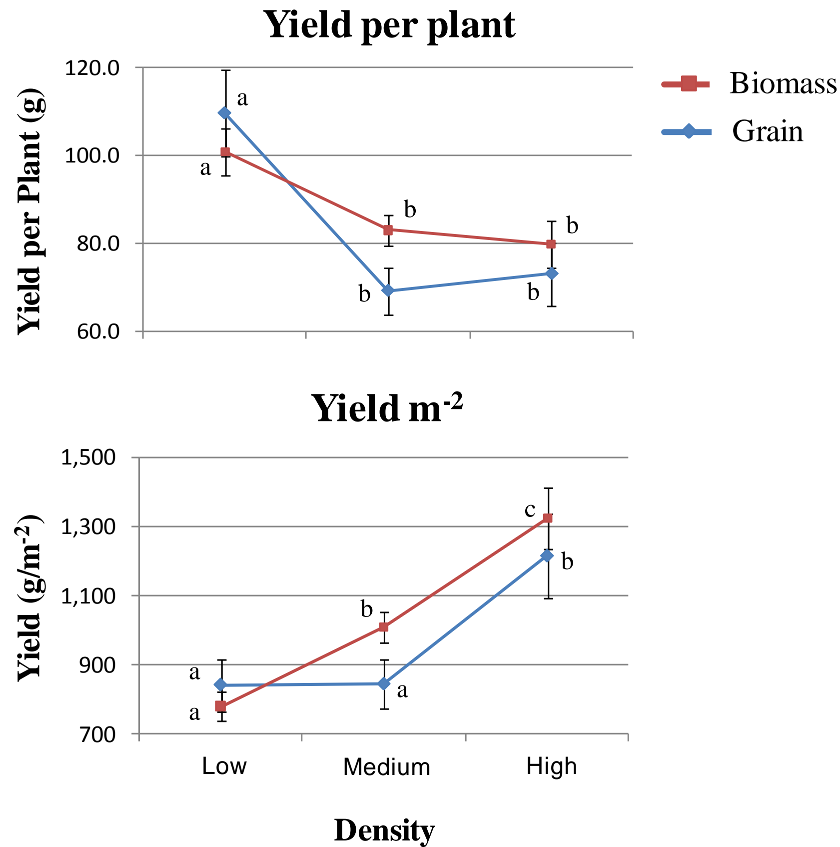


Figure 2. Yield per plant and yield m^{-2} means for maize at three densities 7.7 (1x), 12.6 (2x) and 17.5 (3x) plants m^{-2}. Different letters indicate means are significantly different at P< 0.05, and the error bars are LSD<0.05.



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