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Density Stress has Minimal Impacts on the Barley or Maize Seedling Transcriptome

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Abstract

High planting density affects the morphology and productivity of many crop species. Our objectives were to examine the phenotypic and transcriptomic changes that occur during plant density stress in barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) seedlings. In maize and barley seedlings, density stress impacted several morphological traits. Gene expression profiles were examined in four barley and five maize genotypes grown at low and high plant densities. Only 221 barley and 35 maize genes exhibited differential expression in response to plant density stress. The majority of the gene expression changes were observed in a subset of the genotypes and reflected minor changes in the level of expression, indicating that the plant density stress imposed in this study did not result in major changes in gene expression. Also, little overlap was observed within barley or maize genotypes in gene expression during density stress, indicating that genotypic differences play a major role in the response to density stress. While it is clear that gene expression differences are involved in morphological changes induced by high plant densities, it is likely that many of these gene expression differences are subtle and restricted to particular tissues and developmental time.

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PLANT POPULATION DENSITY stress elicits a competitive response between neighboring plants for sunlight, water, and nutrients. Plants detect crowding by attempting to outgrow their neighbors by reallocating their resources into longer stems, longer leaves, and early flowering (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). 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; Widdicombe and Thelen, 2002). In maize (*Zea mays* L.), plant density has increased from 39,520 plants ha^{-1} in the 1960s to 66,690 plants ha^{-1} 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). The ability of plant breeders to select for this trait indicates that there is genetic variation in the response to high plant densities.

Several groups have assessed transcriptional changes that are induced by plant density stress and shade (a component of density stress). Several genes (2 out of 15) were found to exhibit allelic differences in transcript accumulation in

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Abbreviations: cDNA, complementary DNA; FDR, false discovery rate; LTP, lipid transfer protein; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; R:Fr, red to far red; RT-PCR, reverse transcription-polymerase chain reaction.

a maize hybrid grown at high and low densities (Guo et al., 2004). In a genome-wide survey, Guo et al. (2006) used GeneCalling messenger RNA (mRNA) profiling (described in Shimkets et al., 1999) to determine if a density-stressed environment alters gene expression patterns in immature ear tissue. As the plant density increased, the proportion of genes exhibiting the midparent expression value decreased and expression became biased toward the paternal allele. The percentage of genes with a midparent expression value varied in the two hybrids. Gene expression in *Arabidopsis thaliana* (L.) Heynh. for response to shade in 7-d-old seedlings was assayed 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 a low ratio of red to far red (R:Fr) light. Plants sense crowding by detecting a low R:Fr ratio (Ballare, 1999; Smith, 1982). Genes related to shading such as photosystem I, photosystem II, proline-rich protein, and six transcription factors that regulate the indole-3-acetic acid (IAA) biosynthetic pathway were detected.

In this study, we examined the phenotypic response and transcript accumulation changes in maize and barley (*Hordeum vulgare* L.) seedlings planted at low and high densities. We used five maize inbred lines and four barley varieties. The objectives of this study were (i) to evaluate the phenotypic response to plant density stress in barley and maize seedlings and (ii) to quantify the transcriptional response to plant density stress at early growth stages by identifying genes differentially expressed between low and high planting densities in seedlings of four barley genotypes and five maize genotypes.

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 the plant density experiments (Supplemental Table S1). Harrington and Baronesse exhibit two-row spike morphology and Steptoe and Morex exhibit six-row spike morphology. 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 low, medium, and high planting densities (Supplemental Tables S2 and S3). Plant height and stem diameter data were collected from 10 to 23 d after planting for maize and barley. Data on the number of tillers per plant was also recorded for barley. Based on these preliminary data and other data not shown, we designed maize and barley experiments to obtain additional phenotypic data and obtain transcript accumulation data in maize and barley grown in low and high densities.

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 per pot) and two pots at the high density (18 plants per pot) for each genotype. Five genotypes were used: B73, Mo17, P39, Oh43, and Hp301 (Supplemental Table S1). Plants were grown in 50:50 mixture of compost:Metromix200 (Sun Gro Horticulture CM Ltd., Vancouver, BC, Canada) in 24 cm diameter pots spaced 10 cm apart. Days were extended to 16 h with 1000-watt (530 to 710 $\mu mol^{-2}\ s^{-1}$) vapor lights and temperatures ranging from 22 to 32°C. Plants were watered thoroughly when needed. Phenotypic measurements were taken 12 d after planting on two plants per pot. Stalk diameter was taken 2 cm 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 per pot) and high density (21 plants per pot). Four genotypes were used: Steptoe, Morex, Harrington, and Baronesse (Supplemental Table S1). To ensure uniform germination, seeds were cold treated in complete darkness for 3 d at 4°C and then placed for 2 d at room temperature. Germinated seeds were transplanted into 10 by 10 cm pots with Metromix 200 and fertilized with Osmocote Slow Release fertilizer (14–14–14; Scott's Co., Marysville, OH). Pots were placed 8 cm 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 h with light intensity ranging from 280 to 300 $\mu mol^{-2}\ s^{-1}$. Phenotypic measurements were taken on two plants per pot 14 d after transplant. Stem diameter was taken approximately 1 cm 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 plant density for the five maize inbred lines at 12 d after planting and for the high and low density at 15 d after transplanting for the four barley genotypes (1 d 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 and genotype combination and directly frozen in liquid nitrogen and stored at −80°C. A total of 30 tissue samples for maize (five inbred lines, two treatments, and three replications) and 24 tissue samples for barley (four varieties, two treatments, and three replications) were collected. Collection times were between 0800 and 0900 h.

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) 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 RNA samples were further purified using a lithium chloride precipitation. For each RNA sample, 5 µg of RNA was sent to the Biomedical Genomics Center at the University of Minnesota (Minneapolis, MN) for RNA labeling, GeneChip hybridization to either the Maize (http://www.affymetrix.com/estore/ [verified 7 Feb. 2011]) or Barley1 Affymetrix microarray (Close et al., 2004), and GeneChip scanning. The Maize Genome Array consists of 17,555 probe sets and represents 13,339 genes. The Barley1 GeneChip contains 22,840 probe sets representing 21,439 genes (Close et al., 2004). The full complement of genes in the barley and maize genomes are not represented on the GeneChips.

GeneChip Data Analysis

The microarray data was normalized using GeneSpring GX 9.0 Software (Agilent Technologies. 2008) 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 between 0.989 and 0.997 for maize. Clustering analysis for both maize and barley was performed by implementing a two-way ANOVA for genotype × density using a cutoff p-value < 0.05 and no false discovery rate correction to obtain all expressed genes. Hierarchical clustering was then conducted on the expressed genes using a Euclidean distance metric.

To identify differentially expressed gene transcripts, a greater than or equal to twofold change and then 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 after the greater than or equal to twofold change was imposed, therefore a false-discovery rate was not calculated. All CEL files were uploaded to the Plant Expression database (PLEXdb, available at http://www.plexdb.org/ [verified 10 Feb. 2011]). The accession numbers for the experiments are ZM27 and BB90 for maize and barley, respectively.

Validation of Differentially Expressed Genes using Quantitative Reverse Transcription-Polymerase Chain Reaction

The barley microarray data was further validated and investigated by conducting another density stress experiment. The same procedures used for plant growth and 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 d after germination. Tissues collected were the crown region and all other above-ground biomass (stem and leaves). Ribonucleic acid was extracted from tissue as described above. Single strand complementary DNA (cDNA) was synthesized from 5 ng of RNA using Superscript III One-Step RT-PCR kit (Invitrogen Corp., Carlsbad, CA). Three primer pairs were developed for transcripts that showed differential accumulation between the low and high plant densities: contig14901_at (auxin-responsive protein), contig1689_at (lipid transfer protein [LTP]), and contig6484_at (NAC domain protein) and for contig baak4o13_s_at (Actin) to use as a positive control (Supplemental Table S4). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in an Applied Biosystems 7900HT Real-time RTPCR system (Applied Biosystems, Foster, CA) using the SYBRgreen PCR Master Mix Kit (Applied Biosystems, Foster, CA) and 5 ng of cDNA in a 20 ml reaction volume. The two-step qRT-PCR 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. 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

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

in which Ct is the cycle threshold, 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

Morphological Differences in Maize and Barley Seedlings due to Planting Density

Initial experiments were conducted to obtain an understanding of the parameters that play a role in the phenotypic effect of planting density on seedlings. For both maize and barley, high planting densities affected plant height and stem width after 2 to 3 wk of growth (Supplemental Tables S2 and S3). One of our objectives was to study the transcriptional changes induced by planting density. Maize seedlings were grown at 2 (low) or 18 (high) plants per pot for 12 d and barley seedlings were grown at 3 (low) or 21 (high) plants per pot for 14 d after germination. These time points reflect a period just before phenotypic changes in maize and coincident with the time of phenotypic changes in barley. There were minor phenotypic differences between the low- and high-density plantings of 12-d-old maize seedlings (Table 1). Several of the genotypes of 12-d-old maize seedlings exhibit significant variation in plant height or stem width. However, these were very small magnitude changes and the direction of the change (increase vs. decrease) was inconsistent among genotypes. The phenotypic changes observed between high- and low-density planted barley were more pronounced. Significant (p < 0.05) reductions were observed in stem width and number of tillers in the high density for all genotypes of 15-d-old barley (Table 1). 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 1). It is worth noting that the barley

Table 1. Seedling phenotypic responses to plant 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	

*Significance value using unpaired *t* test of low versus high plant density at the 0.05 probability level.

**Significance value using unpaired *t* test of low versus high plant density at the 0.01 probability level.

***Significance value using unpaired *t* test of low versus high plant density at the 0.001 probability level.

†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. Phenotypic measurements were taken 14 d after germination for barley and 12 d after planting for maize.

phenotypic responses in plants sampled for the expression profiling were more substantial than the maize phenotypic responses (Table 1).

Identification of Transcripts that are Differentially Expressed between Low and High Plant Densities

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 Barley1 Genome Array. Seedlings of five maize genotypes (B73, Mo17, Oh43, P39, and Hp301) were sampled 12 d after planting and seedlings of four barley genotypes (Steptoe, Morex, Harrington, and Baronesse) were sampled at 15 d after germination. Microarray data were obtained for three biological replicates of pooled tissue for each genotype by treatment combination.

Several lines of evidence support the quality of the microarray data. First, 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 relatively few major transcriptional changes induced by the density stress. Second, hierarchical clustering analyses (Supplemental Fig. S1) showed high levels of similarity for the expression profiles for each genotype but did not provide evidence for clustering by planting density. Third, as expected, it was possible to identify numerous examples of differential expression when contrasting any two genotypes of barley or maize (Table 2). 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.

We proceeded to search for genes that exhibit variable transcript levels in maize or barley seedlings grown at low compared to high planting density for each genotype. The use of standard approaches (unpaired *t* test with a false discovery rate (FDR) of 5% and then a greater than or equal to twofold change) did not identify any differentially expressed genes at low versus high density in barley or maize. This is in agreement with the clustering and correlation results noted above. Therefore, we proceeded to implement a less stringent approach in an attempt to identify plant density-stress responsive genes. In this approach, we first identified the subset of genes (fewer than 1% of all genes) that exhibit greater than or equal to twofold variation between low and high density in at least one genotype. Subsequently, we performed an unpaired *t* test with a *p*-value of <0.05. The implementation of this method identified 219 transcripts in barley and 35 transcripts in maize (Supplemental Tables S5 and S6; Fig. 1).

Characterization of Differentially Expressed Genes

In barley, there were some genes that were differentially expressed in a single genotype while other genes exhibited conserved responses in multiple genotypes. A total of 221 barley transcripts were identified as differentially expressed between the low and high densities (Supplemental Table S5; Fig. 1). Steptoe had 144, Baronesse had 31, Harrington had 74, and Morex had 33 genes differentially expressed. The four genotypes had seven genes in common (Supplemental Table S5; Fig. 1). However, many genes overlapped with at least one other genotype. 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 50 genes were differentially expressed between the low and high densities. Two of these transcripts (Contig3427_at and Contig18182_at) were only identified after pooling the data from all four genotypes (Supplemental Table S5). While the transcripts may not exhibit significant variation in all genotypes, we used the fold change (averaged for all genotypes) to describe the regulation for a gene transcript. We found that 15% of transcripts showed decreased accumulation whereas 24% showed increased accumulation. The remaining 61% of transcripts that exhibited differential expression in at least one genotype did not have all four genotypes with a fold change in the same direction. Interestingly, the consensus among barley genotypes was substantially improved by removing Morex, which indicates that Morex frequently displayed a different expression response to high plant density.

In maize, only 35 gene transcripts were differentially expressed between the low and high densities (Supplemental Table S6). Differential expression was only noted in single genotypes including 20 genes in B73, five genes in Hp301, four genes in Mo17, and three genes each in Oh43 and P39. The five genotypes had no genes in common and very few (6 out of 35) show consistency in the direction of expression changes in all genotypes.

Gene transcripts that exhibited differential accumulation in barley and maize seedlings between low and high plant density were annotated (Supplemental Tables S5 and S6). A few of the differentially expressed gene transcripts that were upregulated (from low to high density) in barley were annotated as photosystem II 10 kDa protein (one contig), NAC domain protein (one contig), proteins related to auxin (two contigs), zinc finger proteins (two contigs), phytochrome A (one contig), phytochrome B (one contig), and cysteine proteinase (three contigs). There was an abundance of histones (62 contigs) that were downregulated and identified primarily in Steptoe (Supplemental Table S5). The annotation of a couple of the differentially expressed transcripts that were upregulated in maize encoded an auxin family protein (one contig) and a zinc finger protein (one contig) (Supplemental Table S6).

The genes with differential expression in barley and maize were compared using tblastn. Only two contigs matched between maize and 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). None of the remaining genes identified in either barley or maize were identified in the other species.

Validation of Differentially Expressed Transcripts using Quantitative Reverse Transcription-Polymerase Chain Reaction

To further explore the tissue-specificity and developmental affects on altered transcript levels, we investigated transcript accumulation variation by conducting an independent experiment. The barley genotype Steptoe was chosen because it exhibited the largest number of differentially expressed genes. Steptoe was grown at low and high densities and tissue was collected at 11, 15 (same as microarray experiment), and 19 d after germination. The two tissues collected were the crown region (including the shoot apical 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 (LTP); and contig6484_at (NAC domain protein).

Quantitative RT-PCR analysis indicated that the microarray experiment and the qRT-PCR experiment are consistent for the three gene transcripts tested (Fig. 2). For contig6484_at (NAC domain protein), microarray analysis indicated an upregulation in high-density treatments at 15 d after germination. The qRT-PCR assay for above ground biomass tissue showed upregulation of this transcript in high-density treatments for all time points

Table 2. Number of transcripts exhibiting differential accumulation between genotypes.

		High density		Low density		High and low density	
Genotype vs. genotype		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 greater than or equal to twofold change.

‡Number of probe sets with a greater than or equal to twofold change and an unpaired *t* test ($p < 0.05$).

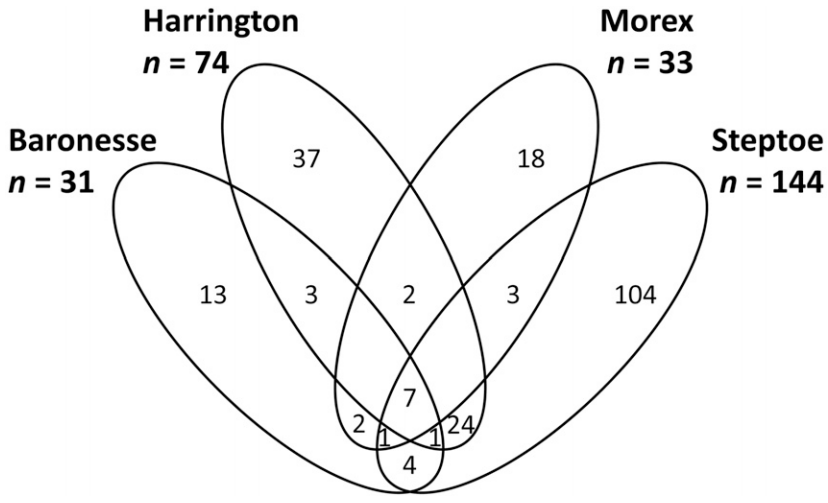

Figure 1. Venn diagram showing distribution of differentially expressed transcripts between two plant densities for four barley genotypes. The total number of unique genes differentially expressed is 221.

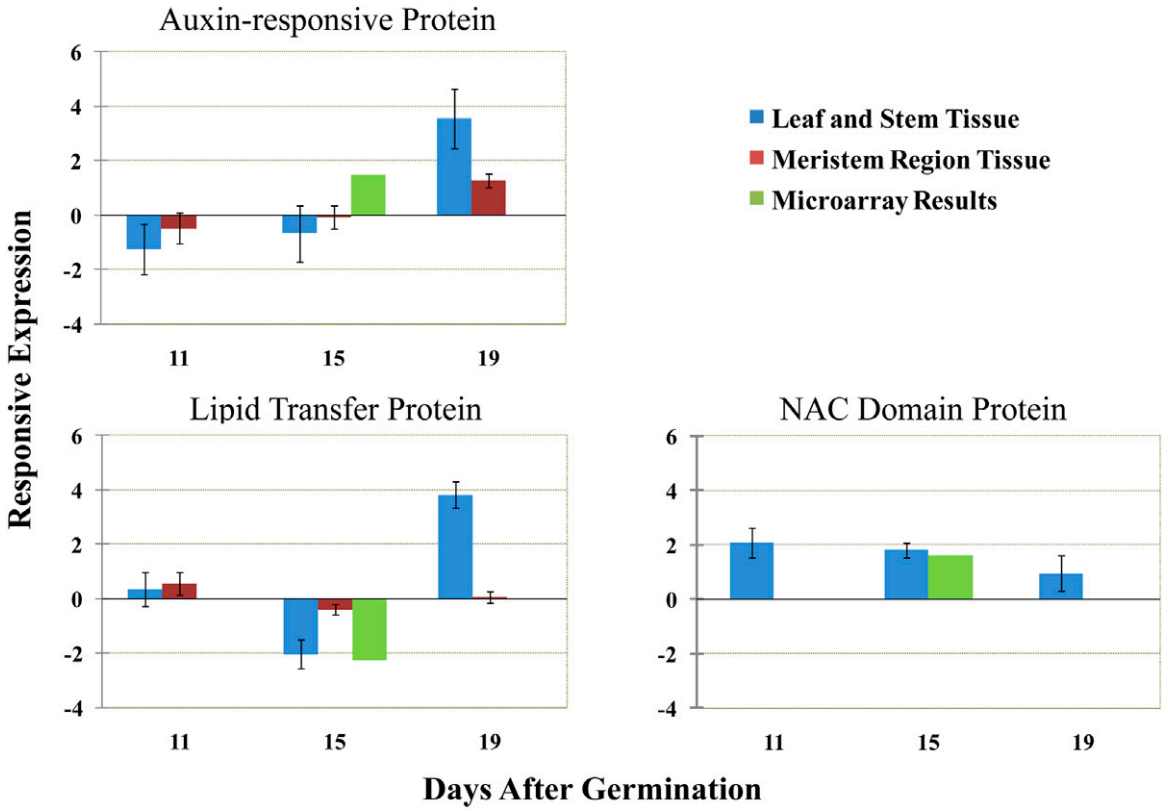

Figure 2. Real-time reverse transcription-polymerase chain reaction (RT-PCR) validation of three differentially accumulated barley transcripts related to density stress at three time points. Microarray expression data for Steptoe at 15 d after germination is also shown. Relative expression is displayed in log2 values. Positive numbers indicate upregulation from the low density to the high density, whereas negative numbers indicate downregulation 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).

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) was downregulated in stem and leaf tissue at 15 d after germination in both microarray and real-time analysis. However, the other time points (11 and 19 d 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), exhibited upregulation in the microarray data. Quantitative RT-PCR did not detect any difference in expression between the low and high densities at 11 and 15 d in either tissue, but gene expression was upregulated in both tissues at 19 d. 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 can be reproduced.

DISCUSSION

The ability of plants to maintain high levels of productivity even when planted at high density is a critical factor in contributing to high yield. In this study, we used microarrays to profile transcript levels in young maize and barley seedlings that were subjected to density stress. This study revealed two clear patterns. One, there are few genes that respond to density stress in barley and maize. This finding, despite the substantial differences in plant density, was quite surprising and will be discussed in more detail below. Two, there is little overlap for gene expression patterns among the genotypes, indicating that these genotypes may respond in very different ways and 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.

Relatively Few Genes are Affected by High Plant Densities in Barley and Maize Seedlings

The ability to identify many differentially expressed genes in different genotypes provides evidence that our microarray data is of high quality and that our analyses approaches could identify highly significant differential expression. However, despite the availability of high-quality data, we found very few genes exhibiting substantial differences in gene regulation between high and low planting densities. Even when using relaxed criteria, the genes that were identified often had relatively minor changes in expression level. There are several potential factors that could explain the identification of very few density-stress responsive transcripts.

It is likely that the timing of sampling limited the number of differentially expressed genes. The barley tissue was collected at the time when morphological changes were significant and the maize tissue was collected just before this point and we observed many more changes in barley than in maize. This suggests that if we had collected tissue at later time points we may have observed more genes with altered expression levels. However, sampling later developmental time points would likely result in differential expression of general stress-related genes.

The use of all aerial tissues for transcript profiling may have also contributed to the difficulties in identifying altered transcripts. It is likely that many of the transcriptional changes induced by high plant densities would initially be manifest only in some tissues such as leaf blade or meristem. By using a complex mixture of cell and tissue types the important transcriptional changes may have been "diluted" in the total transcriptome. The qRT-PCR analysis of several transcripts in different tissues provides evidence that there are tissue-specific differences in transcript levels in a contrast of high and low planting densities. In future experiments it would be worthwhile to focus on fewer genotypes but to include additional time points and to focus on more specific tissue types.

Characterization of Genes Affected by Density Stress

While we did not identify as many major transcriptional changes as expected, there were still a number of examples of genes with altered transcript levels between the high and low plant densities. Many of the genes identified as differentially expressed are related to changes in light intensity, which decreases when plants are grown at high densities compared to low densities. All transcripts coding for photosystem II 10 kDa protein were upregulated in the high density compared to low density. In addition, high density also decreases the ratio of R:Fr light that is available for the plants to detect (Ballare, 1999; Smith, 1982). 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 often associated with plant density stress. Auxins have been shown to be phytochrome mediated and alter cell elongation and leaf angle (Sawers et al., 2005) and inhibit lateral shoot growth (Tian and Reed, 2001). We found one transcript related to an 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. Tessadori et al. (2009) investigated how light intensity controls chromatin compaction and showed 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.

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 thaliana*, 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 β-amylase, cytochrome P450, lipid transfer protein, peroxidase, protein kinase, syntaxin, and zinc finger protein.

Overall, our results demonstrate that, for the barley and maize genotypes and the tissue and developmental time point and experimental conditions used, plant density stress does not elicit major changes in the transcriptome. Thus, one hypothesis is that density stress does not reprogram the transcriptome in a fundamental manner. Further efforts will be required to examine different developmental time points, tissues, and genotypes to fully understand the plant response to increased planting density.

Supplemental Information Available

Supplemental material is available free of charge at http://www.crops.org/publications/tpg.

Supplementary Figure 1. Cluster analysis of a subset of the maize and barley transcripts.

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

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

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

Supplementary Table 4. Primers designed for Real-Time RT-PCR in barley.

Supplementary Table 5. Differentially expressed genes in 15-day old barley seedlings during density stress.

Supplementary Table 6. Differentially expressed genes in 12-day old maize seedlings during density stress.

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